GENE MODIFICATION AND BIOTECHNOLOGICAL APPLICATIONS OF MESENCHYMAL STEM CELLS

by

PABLO BOSCH

(Under the Direction of Steven L. Stice)

ABSTRACT

Mesenchymal stem cells (MSCs) are pluripotent cells that reside primarily in the mammalian bone marrow. They possess self-renewal capacity and the ability to differentiate in vivo and in vitro into a number of mesodermal derivatives, including fat, bone and cartilage. These features along with their vast proliferative potential make MSCs an excellent tool for cell-based gene therapy strategies. Development of methods for improved gene delivery in MSCs and appropriate large animal models for testing various therapeutic protocols are needed. Therefore, we have designed experiments to: a) isolate pig MSCs (pMSCs) from bone marrow and identify appropriate in vitro culture conditions for these cells; b) investigate non-viral and viral approaches for transient and stable gene delivery into pMSCs; c) develop methods for optimal gene delivery in pMSCs using adenoviral vectors complexed with GeneJammer, a commercial polyamine-based transfection reagent; and d) study the effect of GeneJammer on adenoviral transduction of human cell lines. The results demonstrate that MSCs can be readily obtained from the bone marrow of pigs. Porcine MSCs grew well in a basic media and their proliferation was enhanced when cultured in low oxygen atmosphere. Transient and

stable genetic modification of pMSCs was obtained by non-viral and viral vectors. Presence of GeneJammer during adenoviral vector transduction enhanced vector-encoded transgene expression in pMSCs. Interestingly, GeneJammer transduced cells retained multipotential differentiation capability in vitro. Similarly, GeneJammer improved adenoviral gene delivery efficiency in human MSCs, human mononuclear peripheral blood cells, and rodent cells lines. Human and murine cell lines infected with adenovectors carrying the gene for bone morphogenetic protein 2 (BMP2) in presence of GeneJammer achieved higher levels of BMP2 expression in vitro and in vivo. In conclusion, we have been able to established adult pMSC lines from live animals using a minimally invasive BM aspiration technique. These adult stem cells can undergo transient and stable genetic modification with non-viral and viral vectors. The use of GeneJammer as described in these studies leads to high transgene expression levels in porcine and human MSCs and rodent cells lines. These results will facilitate future use of adenoviral vectors in MSC-mediated gene therapy models and therapies.

INDEX WORDS: Mesenchymal stem cells, differentiation, nuclear transfer, transgenic, livestock, adenovirus vectors, transduction, gene delivery, cell-based therapy, animal models

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CHAPTER 1

INTRODUCTION

It has been long recognized the existence of stem cells in several adult tissues where these precursor cells can replenish differentiated cells lost during physiologic turnover or tissue damage [1-5]. Classic examples of organs with ability to regenerate from tissue specific stem cells are the skin, liver, intestine and bone marrow. However, more recently cells with properties of stem cells have been identified in the adult nervous system [6] and heart [7] suggesting that even these postmitotic tissues retain at least some regenerative potential. Hallmark features shared by adult and embryonic stem cells are extensive self-renewal and the ability to differentiate to cells with distinct phenotype from that of the precursor. Although it was originally thought that the fate of adult stem cells is restricted to originate cell phenotypes of the source tissue, mounting experimental evidence suggests larger plasticity, i.e. they can differentiate to phenotypes beyond their tissue of origin [8]. Self-renewal, plasticity, broad distribution and the possibility to be harvested from patients make adult stem cells a natural candidate for many therapeutic applications in tissue repair and regenerative medicine.

Mesenchymal stem cells (MSCs) are one of the most extensively studied adult stem cells. These cells are believed to reside in tissues of mesenchymal origin particularly in the bone marrow [9-11]. Both in vivo and in vitro, these cells can differentiate down

the adipogenic, chondrogenic, osteogenic [9, 12-14]. MSCs cells also play an important role in hematopoiesis since they form part of the bone marrow microenvironment that promote hematopoietic stem cell proliferation and differentiation [15-18]. In addition to the accepted orthodox plasticity of MSCs, several laboratories have reported a broader differentiation spectrum for MSCs including cell phenotypes from other embryonic germ layers such as neuron-like cells [19, 20]. MSC populations are selected based on their plastic adherence properties and cultured in basic medium with 10-20% fetal bovine serum which provides the growth factors required for cell proliferation [21]. Due to their stem cell nature, MSCs exhibit extensive proliferative potential in vitro (~40 cell doublings) while retaining multipotential differentiation capacity [9, 22]. An important feature of MSCs in the context of future clinical use is that these cells are readily available from the patient's own bone marrow through a minimally invasive aspiration procedure opening the possibility for autologous cell transplantation avoiding immunological rejection associated with allogeneic grafts. Therefore, MSCs hold promise as a potential cell source for development of autologous cell-based therapeutic approaches for treatment of various human diseases. Local administration of ex vivo expanded MSCs alone or in matrices has proven to be useful for treatment of bone and cartilage defects in a number of animal models [23, 24] and humans [25]. MSC-based treatment of genetic skeletal diseases like osteogenic imperfecta has been attempted. In one study with children affected by a severe form of osteogenesis imperfecta some clinical improvements were observed after systemic transplantation of normal allogeneic MSCs [26]. Another approach envisions the use of genetically modified MSCs as in vivo mini-pumps for delivery of various therapeutic factors. For instance, MSC delivery

system that can produce high levels of active bone morphogenetic protein 2 (BMP2) and induce bone formation in vivo [27, 28] may prove to be of use for bone regeneration and repair. Based on the same principle, MSCs may serve as an effective platform for delivery of anti-tumor agents [29, 30]. Initial experimentation demonstrated that MSCs genetically modified to produce the anti-tumoral molecule interferon beta homed preferentially to tumor tissue, suppressed the growth of metastasis and extended life span of treated mice [29]. These data provide “proof of principle” that MSCs can effectively function as carriers for targeted delivery of therapeutic agents.

The successful clinical applications of these and others MSC-based gene therapy approaches will depend greatly on our ability to efficiently deliver the gene of interest into MSCs. Moreover, development of large animal models in which study cell distribution, patterns of transgene expression and cell doses to achieve a therapeutic effect will be required before these ex vivo gene therapy strategies move to human clinical trials. Striking morphologic and physiologic similarities between humans and pigs make them an appropriate large animal model in which to test biosafety concerns associated with gene therapy protocols.

The studies that comprise this dissertation were designed to test the following hypotheses: 1) MSCs can be isolated from pig bone marrow and grown in vitro for an extended period of time; 2) porcine MSCs are amenable to transient and stable genetic manipulation by non-viral and viral vectors; 3) MSC nuclei can be reprogrammed when transferred to enucleated metaphase II oocytes to drive development of reconstructed cloned embryos to the blastocyst stage; and 4) adenoviral transduction efficiency of porcine and human MSCs is enhanced by complexing adenovectors with a commercial

polyamine-based transfection reagent (GeneJammer®, Stratagene, CA, USA). The following specific aims were pursued:

a. Isolate pig MSCs (pMSCs) from bone marrow and identify appropriate in vitro culture conditions for proliferation of these cells.

b. Investigate non-viral and viral approaches to achieve transient and stable gene modification of pMSCs.

c. To investigate the ability of pMSCs to drive embryo development when used as karyoplasts in nuclear transfer procedures.

d. Develop methods for optimal gene delivery into pMSCs using human adenoviral vectors complexed with GeneJammer, a commercial polyamine-based transfection reagent.

e. Study the effect of GeneJammer on adenoviral transduction of human MSCs, human mononuclear peripheral blood cells and rodent cell lines.

References

1. Weissman IL. Translating stem and progenitor cell biology to the clinic: barriers and opportunities. Science 2000; 287: 1442-1446.
2. Gage FH. Mammalian neural stem cells. Science 2000; 287: 1433-1438.
3. Potten CS. Stem cells in gastrointestinal epithelium: numbers, characteristics and death. Philos Trans R Soc Lond B Biol Sci 1998; 353: 821-830.
4. Alison M, Sarraf C. Hepatic stem cells. J Hepatol 1998; 29: 676-682.
5. Watt FM. Epidermal stem cells: markers, patterning and the control of stem cell fate. Philos Trans R Soc Lond B Biol Sci 1998; 353: 831-837.

6. Gage FH, Ray J, Fisher LJ. Isolation, characterization, and use of stem cells from the CNS. Annu Rev Neurosci 1995; 18: 159-192.

7. Hierlihy AM, Seale P, Lobe CG, Rudnicki MA, Megeney LA. The post-natal heart contains a myocardial stem cell population. FEBS Lett 2002; 530: 239-243.

8. Lakshmipathy U, Verfaillie C. Stem cell plasticity. Blood Rev 2005; 19: 29-38.

9. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science 1999; 284: 143-147.

10. Kuznetsov SA, Mankani MH, Gronthos S, Satomura K, Bianco P, Robey PG. Circulating skeletal stem cells. J Cell Biol 2001; 153: 1133-1140.

11. Tholpady SS, Katz AJ, Ogle RC. Mesenchymal stem cells from rat visceral fat exhibit multipotential differentiation in vitro. Anat Rec A Discov Mol Cell Evol Biol 2003; 272: 398-402.

12. Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. J Cell Biochem 1997; 64: 295-312.

13. Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp Cell Res 1998; 238: 265-272.

14. Justesen J, Stenderup K, Eriksen EF, Kassem M. Maintenance of osteoblastic and adipocytic differentiation potential with age and osteoporosis in human marrow stromal cell cultures. Calcif Tissue Int 2002; 71: 36-44.

15. Moreau I, Duvert V, Caux C, Galmiche MC, Charbord P, Banchereau J, Saeland S. Myofibroblastic stromal cells isolated from human bone marrow induce the proliferation of both early myeloid and B-lymphoid cells. Blood 1993; 82: 2396-2405.

16. Dexter TM. Stromal cell associated haemopoiesis. J Cell Physiol Suppl 1982; 1: 87-94.

17. Owen M. Lineage of osteogenic cells and their relationship to the stromal system. In: Peck WA (ed.) Bone and mineral research, vol. 3. New York: Elsevier; 1985: 1-25.

18. Cherry, Yasumizu R, Toki J, Asou H, Nishino T, Komatsu Y, Ikehara S. Production of hematopoietic stem cell-chemotactic factor by bone marrow stromal cells. Blood 1994; 83: 964-971.

19. Kohyama J, Abe H, Shimazaki T, Koizumi A, Nakashima K, Gojo S, Taga T, Okano H, Hata J, Umezawa A. Brain from bone: efficient "meta-differentiation" of marrow stroma-derived mature osteoblasts to neurons with Noggin or a demethylating agent. Differentiation 2001; 68: 235-244.

20. Woodbury D, Schwarz EJ, Prockop DJ, Black IB. Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res 2000; 61: 364-370.

21. Barry FP. Biology and clinical applications of mesenchymal stem cells. Birth Defects Res C Embryo Today 2003; 69: 250-256.

22. Muraglia A, Cancedda R, Quarto R. Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. J Cell Sci 2000; 113 (Pt 7): 1161-1166.

23. Mankani MH, Kuznetsov SA, Fowler B, Kingman A, Robey PG. In vivo bone formation by human bone marrow stromal cells: effect of carrier particle size and shape. Biotechnol Bioeng 2001; 72: 96-107.

24. Tsuchida H, Hashimoto J, Crawford E, Manske P, Lou J. Engineered allogeneic mesenchymal stem cells repair femoral segmental defect in rats. J Orthop Res 2003; 21: 44-53.

25. Quarto R, Mastrogiacomo M, Cancedda R, Kutepov SM, Mukhachev V, Lavroukov A, Kon E, Marcacci M. Repair of large bone defects with the use of autologous bone marrow stromal cells. N Engl J Med 2001; 344: 385-386.

26. Horwitz EM, Prockop DJ, Fitzpatrick LA, Koo WW, Gordon PL, Neel M, Sussman M, Orchard P, Marx JC, Pyeritz RE, Brenner MK. Transplantability and

therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. Nat Med 1999; 5: 309-313.

27. Olmsted-Davis EA, Gugala Z, Gannon FH, Yotnda P, McAlhany RE, Lindsey RW, Davis AR. Use of a chimeric adenovirus vector enhances BMP2 production and bone formation. Hum Gene Ther 2002; 13: 1337-1347.

28. Cheng SL, Lou J, Wright NM, Lai CF, Avioli LV, Riew KD. In vitro and in vivo induction of bone formation using a recombinant adenoviral vector carrying the human BMP-2 gene. Calcif Tissue Int 2001; 68: 87-94.

29. Studeny M, Marini FC, Champlin RE, Zompetta C, Fidler IJ, Andreeff M. Bone marrow-derived mesenchymal stem cells as vehicles for interferon-beta delivery into tumors. Cancer Res 2002; 62: 3603-3608.

30. Nakamizo A, Marini F, Amano T, Khan A, Studeny M, Gumin J, Chen J, Hentschel S, Vecil G, Dembinski J, Andreeff M, Lang FF. Human bone marrow-derived mesenchymal stem cells in the treatment of gliomas. Cancer Res 2005; 65: 3307-3318.

CHAPTER 2

LITERATURE REVIEW

Mesenchymal stem cells: biology and applications in cell and gene therapy

Introduction

In addition to hematopoietic stem cells, it has been long recognized that the post-natal bone marrow in mammals is populated by a distinct stem cell population known as mesenchymal stem cells (MSCs) also referred as marrow somatic cells or colony forming unit fibroblastic cells. In vivo, MSCs and other non-hematopoietic cells (e.g. macrophages, reticular cells, endothelial cells, smooth muscle cells, adipocytes) in combination with the extra cellular matrix form what is known as “hematopoietic inductive microenvironment”. This complex bone marrow microenvironment provides support for hematopoiesis, i.e., process of formation of new blood cells, through cell-to-cell interactions and soluble clues. In addition to promote proliferation and differentiation of hematopoietic cells, MSCs are considered pluripotent cells with capacity to differentiate into mesodermal lineages, such as adipocytes, osteoblasts and chondrocytes both in vivo and in vitro. Several studies have demonstrated that these pluripotent stem cells derived from the marrow stroma can be easily isolated from bone marrow specimens, proliferate extensively ex vivo to originate relatively homogeneous cell

populations [1, 2] and are endowed with the capacity to differentiate into mesodermal [2] and non-mesodermal [3, 4] cell types. Due to their multipotentiality and extensive self-renewal capacity, MSCs hold great promise as source of cells for many cell-based strategies for the treatment of human diseases. The aim of the present review is to present an in-depth description of the biology, isolation, characterization and potential therapeutic applications of MSCs.

Isolation and culture of mesenchymal stem cells

Cells with features of mesenchymal precursors have been isolated from the bone marrow of many mammals including laboratory rodents [5, 6], humans [2], monkeys [7], cats [8], dogs [9] and pigs [10]. In humans, MSCs are normally isolated from bone marrow aspirates collected from the superior iliac crest of the pelvis [2, 11], tibial or femoral compartment [12, 13] or thoracic and lumbar vertebra [14]. In non-rodent animals bone marrow can be harvested from the iliac crest, sternum or head of long bones like the humerus using similar aspiration techniques adapted from humans [15-17]. Alternatively, in sacrificed animals, bone marrow can be harvested from spongy bone present in the epiphysis of long bones [10]. Unlike in large animals, in laboratory rodents bone marrow is flushed out from the mid-diaphysis of the tibia or femur [18-20].

Bone marrow specimens (aspirates or biopsies) are composed of a mixture of hematopoietic and stromal components from the bone marrow plus a variable amount of contaminating blood. It has been estimated that approximately 1 in every 10,000 nucleated cells present bone marrow is a MSC [2]. Therefore, in order to eliminate the bulk of unwanted cells, including erythrocytes and platelets, a common practice during isolation of MSCs is to subject the bone marrow sample to fractionation in a density

gradient such as Percoll. Mononuclear cells are recovered from the interface and plated on plastic dishes or flasks in a basal medium such as Dulbecco's modified Eagle's medium with 10-20% fetal bovine serum (FBS). MSCs are selected from other cells present in the mixture (e.g., macrophages, endothelial cells, lymphocytes and smooth muscle cells) based on their strong adherence to the plastic surface. Within 24-48 h from plating, MSCs have attached to plastic and non-adherent cells are removed with culture medium exchanges. Discrete colonies of adherent fibroblastic cells can be observed as early as 4-5 days of culture. This colonies originated from a single cells eventually coalesce to form an near-confluent culture by day 14 when cells can be trypsinized and expanded by sequential passages to confluence. Theoretically, if the right culture conditions are met, stem cells would have unlimited proliferative capacity in vitro. Despite their longer lifespan in vitro, MSCs would eventually senesce during in vitro culture [21]. For instance, multi-colony derived (non-clonal) MSC lines cultured under optimal conditions can undergo about 25 passages representing more than 50 cell doublings before senescence [22]. In another study, human MSC clonal lines completely stopped growing at about 22 cell doublings after approximately 80 days in culture [1], showing a significant, although limited life span in vitro. Species specific differences in life span of MSCs exist since senescence in rat MSCs has not been observed [19]. It is plausible that by adjusting some environmental variables during MSC in vitro culture, such as oxygen tension or growth factors, it might be possible to promote self renewal capacity of these adult stem cells. For instance, higher populations doublings (>50 PDs) have been achieved by incorporation of fibroblast growth factor 2 (FGF-2) to the basal

culture medium [22]. However, the absolute self-renewal capacity of MSCs in vivo and in vitro remains an open question that needs to be experimentally addressed.

An important property of MSCs is the clonal expansion capability when they are plated at low densities or sorted as single cells. This colony-forming feature of MSCs was first described by the pioneering work of Friedenstein et al. [23, 24] who reported for the first time the isolation of bone marrow stromal fibroblastic cells capable of osteogenic differentiation. The number and size of individual MSC colonies can be objectively studied when cells are plated at low densities in the colony forming unit fibroblastic (CFU-F) assay providing a mean to estimate the frequency of MSCs in bone marrow aspirates and investigate the effect of different culture conditions on MSCs. Analysis of colonies originated from the CFU-F assay has revealed a great heterogeneity in terms of cell morphology (size and shape) and differentiation potential among colonies derived from the same bone marrow specimen [11, 25, 26]. It has been clearly establish through the analysis of the progeny originated from a single cell that in a given MSC population there are cells with different differentiation potential and expansion capacity [11]. A small proportion of cells has tripotential differentiation capacity (adipogenic, chondrogenic and osteogenic) while most of the cells possess only bi or even unipotential capacity [11]. Furthermore, Muraglia et al. [1] have documented a gradual loss of differentiation potential with passaging. In conjunction, these data could be interpreted to as MSC populations isolated by culture of adherent cells as described in this review are in fact mixture of stem cells and progenitors with different degree of commitment to specific lineages supporting a hierarchal model in MSC cultures [27] similar to that described for hematopoietic cells.

Isolation and ex vivo expansion of MSCs depend entirely on the growth factors present in FBS used as supplement of the basal medium [28]. Development of serum-free defined culture system that support ex vivo expansion of MSCs while retaining the pluripotential capacity would be highly desirable not only to study the effect of particular molecules (e.g., growth factors, cytokines, etc.) on MSC function but also for future therapy strategies which will require cells derived from xeno-free culture conditions. It is currently known that proliferation of MSCs is enhanced by several mitogenic factors including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), transforming growth factor beta (TGF-β) and insulin-like growth factor one (IGF-I) [29]. Addition of FGF-2 to the culture medium of MSCs has been associated with increased life span and increased telomere length indicating that this growth factor may select for cell populations with extend proliferation capacity [22]. In addition to cell proliferation and cell longevity, growth factors may influence different aspects of in vitro and in vivo differentiation pathways in MSCs. For instance FGF-2 added to the culture medium of MSCs led to enhanced osteogenic [30] and chondrogenic [31] potential in cultured human MSCs. More research in this area will provide the tools for the development of defined growth media for MSCs to achieve more reproducible culture protocols and safety in future clinical use.

Despite the fact that MSCs have been primarily derived from bone marrow, other tissue sources of MSCs have been identified. Using methodologies similar to that originally described by Friedenstein et al. to isolate stromal fibroblastic cells from rat bone marrow [32], cells with analogous properties to MSCs have been isolated from compact bone, adipose tissue, cord blood, amniotic fluid, fetal tissues and blood [33-43].

It is not known at this point however, whether the stromal precursors isolated from those tissues are truly the same MSCs originally isolated from bone marrow or the use of similar techniques for isolation and culture make them appear the same cell type in vitro. A major limitation in these studies has been the lack of specific MSC surface markers to prospectively identify mesenchymal cells.

A long-lasting hypothesis has been that bone marrow derived MSCs are a source of undifferentiated cells for maintenance and regeneration of normal or injured peripheral tissues. Central to this hypothesis is the transit of MSCs from the bone marrow to mesenchymal tissues through circulation. Several studies have been carried out in human and animals in search of circulating MSCs which resulted in successful isolation of circulating mesenchymal progenitor cells from fetal and adult peripheral blood [35, 36, 44-48]. However, Lazarus et al. [49] and Wexler et al. [50] were unable to reproduce these results in similar studies. Differences in the mobilization procedures and cell preparation may account for these conflicting data. Interestingly, large numbers of MSC-like cells are present in the fetal circulation from the 7^{th} to approximately 12^{th} week of gestation [35]. These cells are phenotypically similar to the adult MSCs and as expected they have greater proliferative potential and a broader differentiation capacity compared with the adult counterparts [35]. This finding suggest a surge of circulating stromal pluripotent cells during the early fetal life aimed to populate the stromal compartment of hematopoietic and other tissues. Whether these pluripotent stromal cells present during the fetal life persist throughout life and whether they represent the ancestors of the well-characterized MSC from bone marrow is currently unknown. Another piece of evidence that support the hypothesis of MSC involvement in tissue repair and regeneration is the

engraftment of systemically infused MSCs in multiple mesenchymal tissues [51, 52]. After infusion, donor-derived MSCs have been detected in peripheral tissues at very low frequency (usually detected by PCR) [53] and definitive evidence that the grafted cells differentiate in participatory cells of the host tissue has not been definitely provided. Furthermore, engraftment of systemically administered MSCs does not imply that marrow-derived MSCs naturally mobilize in response to tissue injury. The complete understanding of the role of MSCs in peripheric tissue maintenance and repair will certainly depend on the development of methodologies to study MSC function in vivo.

Another cell type that is believed to be distinct from, but somehow related to bone marrow MSCs is the multipotent adult progenitor cell (MAPC) more recently isolated and characterized Verfaillie et al. [54, 55]. MAPCs have been isolated from human, rat and mouse bone marrow as a subpopulation of CD45/glycophorin-A depleted bone marrow-derived mononuclear cells that selectively attach to laminin-coated plates and grow in serum-low or serum-free conditions with EGF and PDGF-BB. MAPCs, which have been isolated from muscle and brain, are much more plastic than the "classic" bone marrow MSC since they can differentiate not only in mesenchymal derivatives (osteoblasts, chondrocytes, adipocytes and fibroblasts) but also in almost all mesodermal cell lineages [56, 57]. There is also evidence that MAPCs can be induced to differentiate into neuroectodermal and endodermal lineages [58]. When injected as single cell into mouse blastocysts, MAPCs contributed to the formation of all tissues, including all cell types of the central nervous system [56]. Furthermore MAPCs possess vast proliferative capacity in vitro (>100 cell doubling) without evidence of replicative senescence or loss of differentiation potential. Although it has been suggested that MAPCs represent a more

primitive pluripotent progenitor of MSCs, the relationship between these two cell types has not been establish.

Phenotypic characterization of mesenchymal stem cells

Considerable effort has been invested to characterize the antigenic profile of cultured MSCs. Identification of appropriate cell markers for selection, isolation and testing of MSCs would be of utmost importance to study MSC biology and future practical applications. However, none of the several markers described so far, alone or in combination, have been useful to unequivocally identify MSC populations. There is consensus among different reports that MSCs are devoid of the following hematopoietic surface markers: CD45, CD14, CD31, CD133 and CD11b [2]. Species specific differences have been observed for the hematopoietic marker CD34 which is not expressed in human and rat MSCs but it is variably expressed in murine MSCs [19, 59, 60]. MSCs express a number of cell adhesion molecules such as CD44, CD49e, CD62 and several integrins which are certainly very important in hematopoietic-stroma cell interactions (reviewed by Verfaillie [61]). Different laboratories have reported variable expression of CD90 (Thy 1.1), CD117 (c-kit), CD105 (endoglin), and CD73. These discrepancies are probably originated in differences in isolation method, culture conditions and origin of MSCs.

Several monoclonal antibodies have been raised against antigenic determinants present on the cell membrane of MSCs. Stro 1 is a monoclonal antibody which is expressed at high levels in MSCs [62], though some populations of hematopoietic cells express low levels of Stro 1 [63]. Gronthos et al. [64] have reported the use Stro 1 to isolate a fairly pure population of non-cycling bone marrow stromal progenitor cell

populations which exhibited telomerase activity and multilineage potential. SH-2 antibody described by Haynesworth et al. [65], which recognizes an epitope present on the TGF-β receptor, has been used to immunomagnetically select populations of MSCs [66]. SH-3 and SH-4 are two distinct monoclonal antibodies that apparently react with epitopes on the membrane-bound ecto-5'-nucleotidase (CD73) present on the surface of MSCs [67]. There is however no agreement on which of these monoclonal antibodies is most useful for characterization and isolation of bone marrow MSCs.

Differentiation potential of MSCs

The ability of cultured expanded MSC populations from different species to differentiate into mesenchymal tissues like bone, cartilage and fat (in vivo and in vitro) has been described and extensively characterized. In vivo grafting followed by demonstration of differentiation has been used as the gold standard to establish pluripotency of MSCs. In addition, several studies have reported differentiation of MSCs into other pathways including differentiation into cell types from unrelated tissues such as neurons [4, 68, 69]. Most of these studies claim differentiation of MSCs base on morphologic, gene expression and/or phenotypic data. However, in most of these reports the functional criterion has not been satisfied. Until these data are confirmed and the functional criteria are satisfied, differentiation into non-mesodermal cell types cannot be considered a hallmark characteristic of MSCs.

Osteogenic differentiation: In vitro bone formation is normally induced by exposing MSC monolayers to serum-containing medium supplemented with β-glycerol-phosphate, ascorbic acid-2-phosphate and dexamethasone [2, 19]. MSCs cultured under these conditions acquired an osteoblastic morphology, expressed osteogenic genes and

deposited mineralized extracellular matrix. Assessment of differentiation is accomplished by histochemical stains, patterns of gene expression and/or phenotypic characteristic. Accumulation of phosphates and carbonates, indicative of osteogenic differentiation or calcium deposits are commonly demonstrated by the von Kossa silver reduction method [2] or alizarin red method respectively. Along with histochemical stains, upregulation of osteogenic genes like ostocalcin, osteopontin and osteonectin have been used as indication of osteogenic induction. Alkaline phosphatase activity is also upregulated in MSCs undergoing osteogenesis. Friedenstein et al. [23, 70] were the first to demonstrate that in vitro expanded stromal precursor transplanted in closed systems (diffusion chambers) or open systems (under the renal capsule, or subcutaneously) not only could reconstitute the hematopoietic supporting stroma but also originate bone tissue. This initial demonstration was later confirmed by numerous reports of MSC osteogenic differentiation upon grafting in appropriate animal models [15, 71, 72] and set the stage for the use of cultured expanded MSCs in future therapeutic applications for skeletal repair.

Chondrogenic differentiation: The following in vitro culture conditions are normally required to induce cartilage formation in MSC cultures: 1) a three dimensional culture system (e.g. micromass culture); 2) a serum free environment; and 3) incorporation of a member of the TGF-β super-family [2, 73]. Under these conditions, a profound change of morphology of fibroblastic MSCs is accompanied by secretion of a number of cartilage-specific extracellular matrix components such as glycosaminoglycan [74]. Interestingly, the patterns of sulfation of chondroitin sulfate during in vitro cartilage formation are similar to those observed in maturation of human articular cartilage [75].

Chondrogenic differentiation is seldom observed in monolayer cultures of MSCs; some kind of three-dimensional culture system is required. By far the most common approach utilizes micromass, a culture system in which MSCs are pelleted on the bottom of a tube where they form a round mass of cells within ~24 h of culture. It is believed that this system provide low oxygen tension permissive for chondrogenesis. In addition, in vitro cartilage formation by MSCs is induced by TGF-β1, TGF-β2 or TGF-β3. However, TGF-β2 and TGF-β3 seem to be more effective than TGF-β1 in inducing differentiation of MSC cultures down the chondrogenic pathway [74]. The chondrogenic potential of MSCs has been also demonstrated in vivo by transplantation of MSCs loaded in diffusion chambers [76] or fibronectin-coated hydroxyapatite cubes [77].

Adipogenic differentiation: A variable proportion of MSCs growing in monolayers can differentiate into adipocytes when exposed to substances that induce elevation of intracellular cyclic AMP. Isobutylmethylxanthine alone or in combination with dbcAMP has been reported to elicit differentiation of MSCs in adipocyte-like cells with large lipid-filed vacuoles which stain positive with lipid-specific dyes like oil O red or Nile red. In addition to morphological changes, induced cells upregulate genes involved in lipogenesis including peroxisome proliferator-activated receptor-gamma (PPAR-γ) and fatty acid synthetase. Release of leptin into the culture medium has been used as an indicator of lipogenic differentiation of MSCs [78, 79].

Therapeutic applications of MSCs

Due to the ability of MSCs to proliferate extensively ex vivo while maintaining their pluripotent differentiation capabilities (in vivo and in vitro), they are regarded as a particularly attractive cell type for many cell-based therapies in humans. For these

applications, autologous or allogenic ex vivo expanded MSCs can be locally or systemically delivered into patients (Figure 2.1). This setting opens the possibility of genetic manipulation (transient or stable) of MSCs during in vitro expansion previous to transplantation. Another feature of MSCs that enhance their therapeutic appeal is the alleged capacity of these cells to suppress immune responses and their particular immunophenotype that could render them hypoimmunogenic or nonimmunogenic upon allotransplantation. In vitro studies indicated that MSCs can suppress T-lymphocyte proliferation induced by irradiated allogeneic blood lymphocytes, dendritic cells, or phytohemaglutinin [80]. Le Blanc [81] has reported that MSCs can inhibit the formation of cytotoxic lymphocytes and can escape lysis by cytotoxic lymphocytes and natural killing cells. Cultures MSCs express HLA Class I antigen but are negative for HLA Class II, CD40, CD80, and CD86 [82, 83]. This immunophenotype is considered as nonimmunogenic and suggests that MSCs could induce immunotolerance after transplantation to HLA-mismatched individuals. In line with these in vitro findings, systemic administration of HLA-mismatched MSCs prolonged the survival of skin allografts in immunocompetent baboons [84]. Another study showed that mouse tumoral cells injected subcutaneously in allogeneic hosts formed tumors only when coinjected with MSCs [85]. Furthermore, transplantation of allogeneic MSCs in human trials has resulted in good tolerance to grafted cells with no evidence of graft-versus-host disease [86-88]. Immunomodulatory properties of MSCs make them a strong candidate cell for many therapeutic applications, especially acute conditions that require immediate cell-based treatment. Based on these alleged immunologic properties, MSCs could be used

"off the shelf" for conditions like myocardial infarction in which cellular therapy needs to be performed before scar tissue develops.

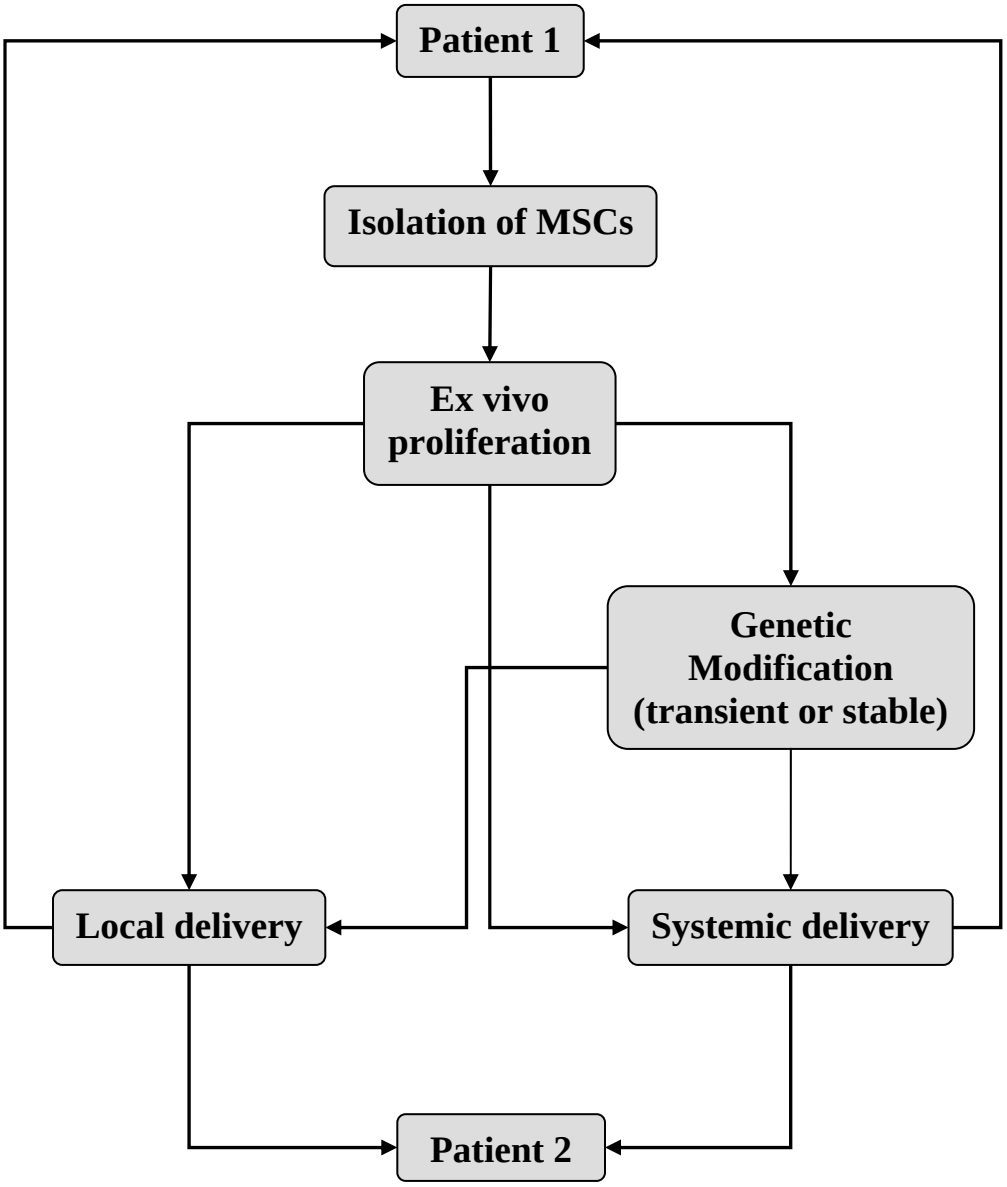

Figure 2.1. Flowchart showing different therapeutic alternatives of MSCs. Cells are harvested from a donor (Patient 1) and expanded in vitro. Intact or genetically manipulated cells are locally or systemically grafted into the same patient 1 (autologous transplantation) or different patient (patient 2; allogeneic transplantation)

Local transplantation

In this approach the cells are directly placed in the damaged tissue, avoiding cell losses associated with the systemic route. Local administration of cells alone or loaded in different matrices has been the method of choice for the treatment of bone and cartilage defects [89-91]. Preclinical studies conducted in animal models have unequivocally demonstrated the benefits of using MSC grafts for orthopedic purposes [15, 92-95]. Quarto et al. [96] have reported successful treatment of long bone defects in patients with defective fracture healing by local injection of autologous, in vitro expanded MSCs. Site-directed administration of MSCs has been also used to repair focal defects in aurticular cartilage [97, 98] and tendon [99]. In a caprine model of osteoarthritis, autologous MSCs injected in the knee joint were capable of engraftment and repair of the damaged tissues [16]. MSCs engineered to produce growth factors such as those involved in bone and cartilage formation hold great promise in the area of orthopedic medicine [100, 101]. Despite positive results, much work lays ahead in order to optimize these procedures. For instance, determination of the ideal culture conditions for ex vivo expansion of MSCs, optimal composition and structure of the cell carriers and appropriate cell doses to achieve bone/cartilage formation are some areas that will need further study.

Systemic transplantation

Infusion of MSCs into the general circulation has been attempted in the context of bone marrow transplantation (BMT) or for the treatment of generalized skeletal diseases in which local delivery would not be practical. Since during oncological or hematological BMT the stroma (containing MSC populations) is not commonly transplanted along with hematopoietic tissue [102, 103], it has been proposed that cotransplantation of MSCs

during BMT might hasten hematopoietic recovery [104, 105]. A second rationale for the inclusion of MSCs in BMT is based on the immunological properties of MSCs which might favor engraftment and reduce the risk of graft-versus-host disease [104]. Based on these unproven but intuitive concepts, several clinical trials aimed to assess the effects of MSCs cotransplantation with heamtopoietic cells have been carried out. In general these studies have reported well tolerance of transplanted cells but low engraftment of donor MSCs and short-term persistence of donor stromal cells [106-109]. However, in other study cotransplantation of human ex vivo expanded MSCs with hematopoietic stem cells ($CD34^+$) into NOD-SCID mice induced a 10-20 fold increase in engraftment compared with that in animals receiving $CD34^+$ cells alone [110]. Therefore, the benefits and extent of stromal cell or MSC engraftment following standard myeloablative BMT is still source of controversy.

The ability of MSCs infused into circulation to colonize other tissues has been the focus of many studies. It is clear that infusion of large numbers of MSCs systemically results in non specific sequestration of cells in capillary beads of different organs specially lung [53, 111, 112]. Low numbers of systemically infused MSCs are found distributed in different tissues providing evidence for donor cell survival but stringent criteria to determine engraftment has not been normally provided. Convincing clinical improvements were seen after allogeneic BMT in patients with osteogenesis imperfecta despite the low engraftment observed (only 1.5-2% of osteoblasts were from donor origin) [87, 113]. On the other hand, there is some experimental evidence that suggests specific homing of infused MSCs to sites of injury or tumoral tissue [114]. The mechanisms that guide implanted MSCs to wounded areas or tumors are not clear. One

study indicated that inflammatory chemotactic agents and cytokines released during cerebral ischemic damage are responsible for selective migration of infused MSCs into the injured brain [115]. Additional evidence for MSC homing has been provided by Rombouts and Ploemacher [116] in a syngeneic mouse model. The authors reported that homing and engraftment of MSCs to bone marrow in sublethally irradiated mice was much higher than that in unirradiated counterparts. This study also demonstrated that in vitro culture of MSCs previous to transplantation hinders their ability to home and engraft in the bone marrow. The capacity of MSCs infused intravenously to seek out the site of tissue damage has been also demonstrated in animal models of myocardial infarction [117, 118]. A better understanding of the mechanisms governing homing of MSCs will certainly lead to improved MSC-based therapeutic protocols.

Myocardial repair

Much attention has attracted the application of systemic or site-directed cell-based therapies for future treatment of myocardial damage. Using animal models of acute myocardial infarction, improvements in myocardial function have been reported after administration of cells from a number of sources: fibroblasts, skeletal myoblasts, cardiomyoblasts, unprocessed bone marrow cells, hematopoietic stem cells, and MSCs [117, 119-131]. Several studies have documented that enriched populations of MSCs when placed in the hearts of adult laboratory rodents engraft in the myocardium and undergo cardiomyocyte differentiation [117, 128, 132]. Despite the fact that MSCs usually integrate at low rates, they exhibited a cardiomyocyte phenotype as evidenced by expression of sarcomeric myosin heavy chain, cardiac troponin I, desmin, and α-actin. In addition to cardiomyocyte replacement, alternative mechanisms to explain improved

cardiac and hemodynamic function in these animal models have been postulated, including induction of angiogenesis, inhibition of apoptosis, and increased collagen production. Which cell type will be ultimately superior or more practical than the others for cell-based myocardial regeneration remains to be established.

Central nervous system and spinal injury

Central nervous system (CNS) and spinal injuries are other promising experimental targets for MSC-based therapy strategies. Several laboratories have reported induction of MSC differentiation along the neurogenic pathway under different in vitro culture conditions [4, 68, 69, 133]. Following the demonstration of in vitro neurogenic plasticity, the beneficial effect of local administration of MSCs in animal models of CNS and spinal damage has been documented [134-136]. In addition to replacing cellular components, it is believed that transplanted MSCs could release trophic factors which in turn might improve the neurologic outcome.

Genetically modified MSCs for gene therapy

Bone marrow-derived MSCs are emerging as an attractive cell type for many cell-based gene therapy strategies. These adult stem cells possess characteristics that make them ideal vehicles for gene delivery: they are relatively easy to obtain from bone marrow aspirates, can be expanded extensively in culture while maintaining their pluripotent differentiation capabilities [2] and are amenable to genetic manipulation to elicit efficient transgene expression [137].

Depending on the final objective, transient or permanent gene modification of cultured MSCs is the desired outcome. When a short-lived effect is necessary, such as for

skeletal regeneration, transient transduction can be achieved using DNA plasmids in combination with electroporation or chemical methods like lipofection or calcium phosphate.

An alternative method to obtain transient transfection is the use of adenovirus vectors which can carry double stranded linear DNA of up to ~36 Kb in length in the last generation vectors [138, 139]. Adenoviruses are particularly attractive vectors for ex vivo gene transfer due to their ability to infect a wide range of cell types including quiescent cells, accommodate large pieces of exogenous DNA, and the possibility of production of stocks with high viral titers [140]. Since adenovirus very rarely integrates into the host genome by non-homologous recombination, replication-defective recombinant adenoviral vectors are used as efficient expression vectors particularly for those applications in which high transgene expression for a limited period of time are required.

In replication deficient adenovirus vectors some or all of the viral genes can be removed (reviewed in [141]). In the first generation adenoviral vectors, the E1 and/or E3 viral genes were deleted making space for the introduction of up to 6.5 Kb of foreign DNA, usually under the control of a heterologous promoter. In second generation adenovectors, some or all of the E2 genes were removed [142, 143], leading to vectors without the capacity to replicate their DNA and to produce replication-competent adenovirus. In a later generation of adenovirus vectors (third generation) more viral genes were removed [144]. In the latest versions, nearly the entire viral DNA but all the inverted terminal repeats (ITRs) and packaging sequences were excised ("gutless" vectors) [138, 139], making enough space to accommodate transgenes of up ~36 Kb in length.

Efficiency of adenovirus-mediated gene deliver is largely limited by the availability of specific viral receptors on target cells. Adenovirus serotype 5-based vectors cell entry is mediated through a receptor mediated biphasic process. First the vector recognizes the target cells through attachment of the knob domain of the viral fiber capsid protein to the cellular coxsackie and adenovirus receptor (CAR) [145]. The second step involves the internalization of the vector particle via interaction of the caspid penton protein with $\alpha_V\beta$ integrins present in target cells [146]. Therefore, infectivity of a particular cell type depends largely on the density of CAR and integrins [147, 148]. Despite the broad adenoviral tropism, many cell types are refractory to adenoviral infection due the lack of or low expression of adenovirus specific receptors. For instance, adenoviral receptors are expressed at low levels in primitive hematopoietic stem cells [149, 150] and human MSCs [151, 152] leading to poor transduction efficiency by type 5 adenovirus vectors. Consequently, several approaches have been undertaken in order to circumvent this problem. The use of fiber-modified adenovectors or Ad5 vectors possessing fiber proteins from a different adenovirus serotype has become an increasingly popular approach to achieve infection of cells via CAR-independent mechanisms. One of such chimeric vectors is the Ad5/F35 in which the Ad5 fiber has been replaced by the fiber of Ad35 serotype. In this way, the tropism of Ad5 can be retargeted to that of Ad35 which utilizes cofactor protein CD46 as cellular receptor [153] and thereby can infect CAR-deficient target cells such as human primitive hematopoietic cells [154] and human MSCs [151]. However, CD46 appear to be preferentially expressed in eyes and testes in rodent species [155-157] limiting the versatility of chimeric Ad5/F35 vectors in these species.

In addition to methods that abrogate CAR tropism, combination of various compounds with adenovirus has been reported to enhance transduction of target cells likely via a receptor independent pathway. Polycations and cationic lipids form complexes with adenoviral particles facilitating in vitro transduction of refractory tumor cell lines [158, 159] and primary and established cell lines [160-163]. Similarly, adenoviral infection of primitive human hematopoietic cells can be strongly enhanced by several cationic lipids [164]. Therefore, by using adenovirus vector complexed with polycations it is possible to combine the advantages of each system to achieve high transduction efficiencies: excellent cellular uptake contributed by the polycation [165] with endosomal escape and nuclear targeting function provided by the vector [166].

One promising application is the autografting or allografting of genetically modified MSCs as vehicles for locally delivery of therapeutic gene products [114, 151, 167, 168]. For instance, MSC delivery system that can produce high levels of active bone morphogenetic protein 2 (BMP2) and induce bone formation in vivo [100, 101, 151, 167, 169] may prove to be of use for treatment of several skeletal injuries and diseases. Another particularly interesting application is the use of genetically engineered MSCs as platform for delivery of chemotherapeutics into tumors. The feasibility of this approach has been recently demonstrated in a xenogeneic mouse model [114, 168, 170]. Systemic administration of human MSCs engineered to express human interferon-β (IFN-β), a biological agent with known antiproliferative activity, preferentially homed to tumors where they induced tumor regression presumably through local delivery of IFN-β [114, 170]. These promissory data obtained in a xenogeneic model (i.e., human MSCs, human

tumors grown in immunodeficient mice) if successfully replicated in an allogeneic system might prove to be a novel strategy for cancer therapy.

For diseases in which a biological activity is impaired or absent, long-lasting or stable genetic modification of MSCs is usually required for effective treatment. To achieve permanent genetic changes in target cells, virus vectors with intrinsic ability to intercalate in the host genome are chosen. These include: retrovirus, lentivirus, adeno-associated virus and a number of hybrid vectors between adenovirus (non-integrating) with retrovirus, adeno-associated or Epstein-Barr virus. Each of the individual vectors has advantages and disadvantages; the election of a particular one ultimately depends on the specific application. Many studies have documented in vitro transduction of MSCs with different viral vectors (retrovirus, adeno-associated or lentivirus vectors) or a combination of ex vivo viral transduction followed by transplantation into animal models to investigate in vivo gene expression [171-188]. Generation of genetically engineered MSCs directing the synthesis of reporter molecules, interleukin 3, erythropoietin, coagulation factor VIII, tyrosine hydroxylase, or 3,4-dihydroxyphenylalanine (L-DOPA) has been reported [171, 173, 179-182, 184, 188]. An interesting alternative to the systemic or local delivery of transduced cells is the implantation of a matrix (e.g. Matrigel) in which genetically engineered MSCs have been embedded. These MSC-matrix masses placed in subcutaneous spaces are neovascularized to form an “organoid” which releases transgenic protein into the bloodstream [181]. These studies demonstrate the great therapeutic potential of these strategies, but at the same time they underscore the hurdles to overcome before this technology is available for clinical use. One obstacle is the development of methods to achieve optimal ex vivo transduction of MSCs. The

second problem relates to the relatively short-lived protein expression in the recipient (no more than 4 months) [176]. Loss of transplanted cells and methylation-dependent gene silencing were implicated as major factors responsible for the transitory in vivo transgene expression [173, 179]. Finally, short and long term safety concerns associated with the use of vector transduced cells (e.g., neoplastic transformation) must be addressed in appropriate animal models before these technologies can move to the clinical setting.

References

1. Muraglia A, Cancedda R, Quarto R. Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. J Cell Sci 2000; 113 (Pt 7): 1161-1166.

2. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science 1999; 284: 143-147.

3. Kohyama J, Abe H, Shimazaki T, Koizumi A, Nakashima K, Gojo S, Taga T, Okano H, Hata J, Umezawa A. Brain from bone: efficient "meta-differentiation" of marrow stroma-derived mature osteoblasts to neurons with Noggin or a demethylating agent. Differentiation 2001; 68: 235-244.

4. Woodbury D, Schwarz EJ, Prockop DJ, Black IB. Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res 2000; 61: 364-370.

5. Friedenstein AJ, Gorskaja JF, Kulagina NN. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp Hematol 1976; 4: 267-274.

6. Simmons DJ, Seitz P, Kidder L, Klein GL, Waeltz M, Gundberg CM, Tabuchi C, Yang C, Zhang RW. Partial characterization of rat marrow stromal cells. Calcif Tissue Int 1991; 48: 326-334.

7. Mendelow B, Grobicki D, de la Hunt M, Katz J, Metz J. Characterization of bone marrow stromal cells in suspension and monolayer cultures. Br J Haematol 1980; 46: 15-22.

8. Martin DR, Cox NR, Hathcock TL, Niemeyer GP, Baker HJ. Isolation and characterization of multipotential mesenchymal stem cells from feline bone marrow. Exp Hematol 2002; 30: 879-886.

9. Huss R, Hoy CA, Deeg HJ. Contact- and growth factor-dependent survival in a canine marrow-derived stromal cell line. Blood 1995; 85: 2414-2421.

10. Ringe J, Kaps C, Schmitt B, Buscher K, Bartel J, Smolian H, Schultz O, Burmester GR, Haupl T, Sittinger M. Porcine mesenchymal stem cells. Induction of distinct mesenchymal cell lineages. Cell Tissue Res 2002; 307: 321-327.

11. Digirolamo CM, Stokes D, Colter D, Phinney DG, Class R, Prockop DJ. Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. Br J Haematol 1999; 107: 275-281.

12. Murphy JM, Dixon K, Beck S, Fabian D, Feldman A, Barry F. Reduced chondrogenic and adipogenic activity of mesenchymal stem cells from patients with advanced osteoarthritis. Arthritis Rheum 2002; 46: 704-713.

13. Oreffo RO, Bord S, Triffitt JT. Skeletal progenitor cells and ageing human populations. Clin Sci (Lond) 1998; 94: 549-555.

14. D'Ippolito G, Schiller PC, Ricordi C, Roos BA, Howard GA. Age-related osteogenic potential of mesenchymal stromal stem cells from human vertebral bone marrow. J Bone Miner Res 1999; 14: 1115-1122.

15. Kadiyala S, Young RG, Thiede MA, Bruder SP. Culture expanded canine mesenchymal stem cells possess osteochondrogenic potential in vivo and in vitro. Cell Transplant 1997; 6: 125-134.

16. Murphy JM, Fink DJ, Hunziker EB, Barry FP. Stem cell therapy in a caprine model of osteoarthritis. Arthritis Rheum 2003; 48: 3464-3474.

17. Shake JG, Gruber PJ, Baumgartner WA, Senechal G, Meyers J, Redmond JM, Pittenger MF, Martin BJ. Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects. Ann Thorac Surg 2002; 73: 1919-1925; discussion 1926.

18. Kuznetsov SA, Friedenstein AJ, Robey PG. Factors required for bone marrow stromal fibroblast colony formation in vitro. Br J Haematol 1997; 97: 561-570.

19. Javazon EH, Colter DC, Schwarz EJ, Prockop DJ. Rat marrow stromal cells are more sensitive to plating density and expand more rapidly from single-cell-derived colonies than human marrow stromal cells. Stem Cells 2001; 19: 219-225.

20. Phinney DG, Kopen G, Isaacson RL, Prockop DJ. Plastic adherent stromal cells from the bone marrow of commonly used strains of inbred mice: variations in yield, growth, and differentiation. J Cell Biochem 1999; 72: 570-585.

21. Banfi A, Bianchi G, Notaro R, Luzzatto L, Cancedda R, Quarto R. Replicative aging and gene expression in long-term cultures of human bone marrow stromal cells. Tissue Eng 2002; 8: 901-910.

22. Bianchi G, Banfi A, Mastrogiacomo M, Notaro R, Luzzatto L, Cancedda R, Quarto R. Ex vivo enrichment of mesenchymal cell progenitors by fibroblast growth factor 2. Exp Cell Res 2003; 287: 98-105.

23. Friedenstein AJ, Chailakhjan RK, Lalykina KS. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet 1970; 3: 393-403.

24. Friedenstein AJ, Deriglasova UF, Kulagina NN, Panasuk AF, Rudakowa SF, Luria EA, Ruadkow IA. Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp Hematol 1974; 2: 83-92.

25. Owen M, Friedenstein AJ. Stromal stem cells: marrow-derived osteogenic precursors. Ciba Found Symp 1988; 136: 42-60.

26. Kuznetsov SA, Krebsbach PH, Satomura K, Kerr J, Riminucci M, Benayahu D, Robey PG. Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation in vivo. J Bone Miner Res 1997; 12: 1335-1347.

27. Minguell JJ, Erices A, Conget P. Mesenchymal stem cells. Exp Biol Med (Maywood) 2001; 226: 507-520.

28. Castro-Malaspina H, Gay RE, Resnick G, Kapoor N, Meyers P, Chiarieri D, McKenzie S, Broxmeyer HE, Moore MA. Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny. Blood 1980; 56: 289-301.

29. Gronthos S, Simmons PJ. The growth factor requirements of STRO-1-positive human bone marrow stromal precursors under serum-deprived conditions in vitro. Blood 1995; 85: 929-940.

30. Martin I, Muraglia A, Campanile G, Cancedda R, Quarto R. Fibroblast growth factor-2 supports ex vivo expansion and maintenance of osteogenic precursors from human bone marrow. Endocrinology 1997; 138: 4456-4462.

31. Solchaga LA, Penick K, Porter JD, Goldberg VM, Caplan AI, Welter JF. FGF-2 enhances the mitotic and chondrogenic potentials of human adult bone marrow-derived mesenchymal stem cells. J Cell Physiol 2005; 203: 398-409.

32. Friedenstein AJ, Chailakhyan RK, Latsinik NV, Panasyuk AF, Keiliss-Borok IV. Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. Transplantation 1974; 17: 331-340.

33. Roufosse CA, Direkze NC, Otto WR, Wright NA. Circulating mesenchymal stem cells. Int J Biochem Cell Biol 2004; 36: 585-597.

34. Charbord P, Oostendorp R, Pang W, Herault O, Noel F, Tsuji T, Dzierzak E, Peault B. Comparative study of stromal cell lines derived from embryonic, fetal, and postnatal mouse blood-forming tissues. Exp Hematol 2002; 30: 1202-1210.

35. Campagnoli C, Roberts IA, Kumar S, Bennett PR, Bellantuono I, Fisk NM. Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. Blood 2001; 98: 2396-2402.

36. Kuznetsov SA, Mankani MH, Gronthos S, Satomura K, Bianco P, Robey PG. Circulating skeletal stem cells. J Cell Biol 2001; 153: 1133-1140.

37. Gronthos S, Franklin DM, Leddy HA, Robey PG, Storms RW, Gimble JM. Surface protein characterization of human adipose tissue-derived stromal cells. J Cell Physiol 2001; 189: 54-63.

38. in 't Anker PS, Noort WA, Scherjon SA, Kleijburg-van der Keur C, Kruisselbrink AB, van Bezooijen RL, Beekhuizen W, Willemze R, Kanhai HH, Fibbe WE. Mesenchymal stem cells in human second-trimester bone marrow, liver, lung, and spleen exhibit a similar immunophenotype but a heterogeneous multilineage differentiation potential. Haematologica 2003; 88: 845-852.

39. In 't Anker PS, Scherjon SA, Kleijburg-van der Keur C, Noort WA, Claas FH, Willemze R, Fibbe WE, Kanhai HH. Amniotic fluid as a novel source of mesenchymal stem cells for therapeutic transplantation. Blood 2003; 102: 1548-1549.

40. Nakahara H, Dennis JE, Bruder SP, Haynesworth SE, Lennon DP, Caplan AI. In vitro differentiation of bone and hypertrophic cartilage from periosteal-derived cells. Exp Cell Res 1991; 195: 492-503.

41. O'Donoghue K, Choolani M, Chan J, de la Fuente J, Kumar S, Campagnoli C, Bennett PR, Roberts IA, Fisk NM. Identification of fetal mesenchymal stem cells in maternal blood: implications for non-invasive prenatal diagnosis. Mol Hum Reprod 2003; 9: 497-502.

42. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 2002; 13: 4279-4295.

43. Luria EA, Panasyuk AF, Friedenstein AY. Fibroblast colony formation from monolayer cultures of blood cells. Transfusion 1971; 11: 345-349.

44. Zvaifler NJ, Marinova-Mutafchieva L, Adams G, Edwards CJ, Moss J, Burger JA, Maini RN. Mesenchymal precursor cells in the blood of normal individuals. Arthritis Res 2000; 2: 477-488.

45. Fernandez M, Simon V, Herrera G, Cao C, Del Favero H, Minguell JJ. Detection of stromal cells in peripheral blood progenitor cell collections from breast cancer patients. Bone Marrow Transplant 1997; 20: 265-271.

46. Huss R, Lange C, Weissinger EM, Kolb HJ, Thalmeier K. Evidence of peripheral blood-derived, plastic-adherent CD34(-/low) hematopoietic stem cell clones with mesenchymal stem cell characteristics. Stem Cells 2000; 18: 252-260.

47. Wu GD, Nolta JA, Jin YS, Barr ML, Yu H, Starnes VA, Cramer DV. Migration of mesenchymal stem cells to heart allografts during chronic rejection. Transplantation 2003; 75: 679-685.

48. Erices A, Conget P, Minguell JJ. Mesenchymal progenitor cells in human umbilical cord blood. Br J Haematol 2000; 109: 235-242.

49. Lazarus HM, Haynesworth SE, Gerson SL, Caplan AI. Human bone marrow-derived mesenchymal (stromal) progenitor cells (MPCs) cannot be recovered from peripheral blood progenitor cell collections. J Hematother 1997; 6: 447-455.

50. Wexler SA, Donaldson C, Denning-Kendall P, Rice C, Bradley B, Hows JM. Adult bone marrow is a rich source of human mesenchymal 'stem' cells but umbilical cord and mobilized adult blood are not. Br J Haematol 2003; 121: 368-374.

51. Pereira RF, Halford KW, O'Hara MD, Leeper DB, Sokolov BP, Pollard MD, Bagasra O, Prockop DJ. Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. Proc Natl Acad Sci U S A 1995; 92: 4857-4861.

52. Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 1997; 276: 71-74.

53. Gao J, Dennis JE, Muzic RF, Lundberg M, Caplan AI. The dynamic in vivo distribution of bone marrow-derived mesenchymal stem cells after infusion. Cells Tissues Organs 2001; 169: 12-20.

54. Reyes M, Lund T, Lenvik T, Aguiar D, Koodie L, Verfaillie CM. Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. Blood 2001; 98: 2615-2625.

55. Reyes M, Verfaillie CM. Characterization of multipotent adult progenitor cells, a subpopulation of mesenchymal stem cells. Ann N Y Acad Sci 2001; 938: 231-233; discussion 233-235.

56. Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 2002; 418: 41-49.

57. Schwartz RE, Reyes M, Koodie L, Jiang Y, Blackstad M, Lund T, Lenvik T, Johnson S, Hu WS, Verfaillie CM. Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. J Clin Invest 2002; 109: 1291-1302.

58. Reyes M, Dudek A, Jahagirdar B, Koodie L, Marker PH, Verfaillie CM. Origin of endothelial progenitors in human postnatal bone marrow. J Clin Invest 2002; 109: 337-346.

59. Peister A, Mellad JA, Larson BL, Hall BM, Gibson LF, Prockop DJ. Adult stem cells from bone marrow (MSCs) isolated from different strains of inbred mice vary in surface epitopes, rates of proliferation, and differentiation potential. Blood 2004; 103: 1662-1668.

60. Colter DC, Class R, DiGirolamo CM, Prockop DJ. Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. Proc Natl Acad Sci U S A 2000; 97: 3213-3218.

61. Verfaillie CM. Adhesion receptors as regulators of the hematopoietic process. Blood 1998; 92: 2609-2612.

62. Simmons PJ, Torok-Storb B. Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. Blood 1991; 78: 55-62.

63. Gronthos S, Graves SE, Ohta S, Simmons PJ. The STRO-1+ fraction of adult human bone marrow contains the osteogenic precursors. Blood 1994; 84: 4164-4173.

64. Gronthos S, Zannettino AC, Hay SJ, Shi S, Graves SE, Kortesidis A, Simmons PJ. Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. J Cell Sci 2003; 116: 1827-1835.

65. Haynesworth SE, Baber MA, Caplan AI. Cell surface antigens on human marrow-derived mesenchymal cells are detected by monoclonal antibodies. Bone 1992; 13: 69-80.

66. Barry FP, Boynton RE, Haynesworth S, Murphy JM, Zaia J. The monoclonal antibody SH-2, raised against human mesenchymal stem cells, recognizes an epitope on endoglin (CD105). Biochem Biophys Res Commun 1999; 265: 134-139.

67. Barry F, Boynton R, Murphy M, Haynesworth S, Zaia J. The SH-3 and SH-4 antibodies recognize distinct epitopes on CD73 from human mesenchymal stem cells. Biochem Biophys Res Commun 2001; 289: 519-524.

68. Deng W, Obrocka M, Fischer I, Prockop DJ. In vitro differentiation of human marrow stromal cells into early progenitors of neural cells by conditions that increase intracellular cyclic AMP. Biochem Biophys Res Commun 2001; 282: 148-152.

69. Sanchez-Ramos J, Song S, Cardozo-Pelaez F, Hazzi C, Stedeford T, Willing A, Freeman TB, Saporta S, Janssen W, Patel N, Cooper DR, Sanberg PR. Adult bone marrow stromal cells differentiate into neural cells in vitro. Exp Neurol 2000; 164: 247-256.

70. Friedenstein AJ, Piatetzky S, II, Petrakova KV. Osteogenesis in transplants of bone marrow cells. J Embryol Exp Morphol 1966; 16: 381-390.

71. Goshima J, Goldberg VM, Caplan AI. The origin of bone formed in composite grafts of porous calcium phosphate ceramic loaded with marrow cells. Clin Orthop Relat Res 1991: 274-283.

72. Krebsbach PH, Kuznetsov SA, Satomura K, Emmons RV, Rowe DW, Robey PG. Bone formation in vivo: comparison of osteogenesis by transplanted mouse and human marrow stromal fibroblasts. Transplantation 1997; 63: 1059-1069.

73. Mackay AM, Beck SC, Murphy JM, Barry FP, Chichester CO, Pittenger MF. Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. Tissue Eng 1998; 4: 415-428.

74. Barry F, Boynton RE, Liu B, Murphy JM. Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components. Exp Cell Res 2001; 268: 189-200.

75. Bayliss MT. Proteoglycan structure and metabolism during maturation and ageing of human articular cartilage. Biochem Soc Trans 1990; 18: 799-802.

76. Ashton BA, Allen TD, Howlett CR, Eaglesom CC, Hattori A, Owen M. Formation of bone and cartilage by marrow stromal cells in diffusion chambers in vivo. Clin Orthop Relat Res 1980: 294-307.

77. Barry FP, Murphy JM. Mesenchymal stem cells: clinical applications and biological characterization. Int J Biochem Cell Biol 2004; 36: 568-584.

78. Ryden M, Dicker A, Gotherstrom C, Astrom G, Tammik C, Arner P, Le Blanc K. Functional characterization of human mesenchymal stem cell-derived adipocytes. Biochem Biophys Res Commun 2003; 311: 391-397.

79. Janderova L, McNeil M, Murrell AN, Mynatt RL, Smith SR. Human mesenchymal stem cells as an in vitro model for human adipogenesis. Obes Res 2003; 11: 65-74.

80. Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, Grisanti S, Gianni AM. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood 2002; 99: 3838-3843.

81. Le Blanc K. Immunomodulatory effects of fetal and adult mesenchymal stem cells. Cytotherapy 2003; 5: 485-489.

82. Tse WT, Pendleton JD, Beyer WM, Egalka MC, Guinan EC. Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. Transplantation 2003; 75: 389-397.

83. Majumdar MK, Keane-Moore M, Buyaner D, Hardy WB, Moorman MA, McIntosh KR, Mosca JD. Characterization and functionality of cell surface molecules on human mesenchymal stem cells. J Biomed Sci 2003; 10: 228-241.

84. Bartholomew A, Sturgeon C, Siatskas M, Ferrer K, McIntosh K, Patil S, Hardy W, Devine S, Ucker D, Deans R, Moseley A, Hoffman R. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. Exp Hematol 2002; 30: 42-48.

85. Djouad F, Plence P, Bony C, Tropel P, Apparailly F, Sany J, Noel D, Jorgensen C. Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. Blood 2003; 102: 3837-3844.

86. Koc ON, Day J, Nieder M, Gerson SL, Lazarus HM, Krivit W. Allogeneic mesenchymal stem cell infusion for treatment of metachromatic leukodystrophy (MLD) and Hurler syndrome (MPS-IH). Bone Marrow Transplant 2002; 30: 215-222.

87. Horwitz EM, Prockop DJ, Fitzpatrick LA, Koo WW, Gordon PL, Neel M, Sussman M, Orchard P, Marx JC, Pyeritz RE, Brenner MK. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. Nat Med 1999; 5: 309-313.

88. Fouillard L, Bensidhoum M, Bories D, Bonte H, Lopez M, Moseley AM, Smith A, Lesage S, Beaujean F, Thierry D, Gourmelon P, Najman A, Gorin NC. Engraftment of allogeneic mesenchymal stem cells in the bone marrow of a patient with severe idiopathic aplastic anemia improves stroma. Leukemia 2003; 17: 474-476.

89. Diduch DR, Jordan LC, Mierisch CM, Balian G. Marrow stromal cells embedded in alginate for repair of osteochondral defects. Arthroscopy 2000; 16: 571-577.

90. Mankani MH, Kuznetsov SA, Fowler B, Kingman A, Robey PG. In vivo bone formation by human bone marrow stromal cells: effect of carrier particle size and shape. Biotechnol Bioeng 2001; 72: 96-107.

91. Tsuchida H, Hashimoto J, Crawford E, Manske P, Lou J. Engineered allogeneic mesenchymal stem cells repair femoral segmental defect in rats. J Orthop Res 2003; 21: 44-53.

92. Bruder SP, Kraus KH, Goldberg VM, Kadiyala S. The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. J Bone Joint Surg Am 1998; 80: 985-996.

93. Bruder SP, Fink DJ, Caplan AI. Mesenchymal stem cells in bone development, bone repair, and skeletal regeneration therapy. J Cell Biochem 1994; 56: 283-294.

94. Kon E, Muraglia A, Corsi A, Bianco P, Marcacci M, Martin I, Boyde A, Ruspantini I, Chistolini P, Rocca M, Giardino R, Cancedda R, Quarto R. Autologous bone marrow stromal cells loaded onto porous hydroxyapatite ceramic accelerate bone repair in critical-size defects of sheep long bones. J Biomed Mater Res 2000; 49: 328-337.

95. Ohgushi H, Goldberg VM, Caplan AI. Repair of bone defects with marrow cells and porous ceramic. Experiments in rats. Acta Orthop Scand 1989; 60: 334-339.

96. Quarto R, Mastrogiacomo M, Cancedda R, Kutepov SM, Mukhachev V, Lavroukov A, Kon E, Marcacci M. Repair of large bone defects with the use of autologous bone marrow stromal cells. N Engl J Med 2001; 344: 385-386.

97. Ponticiello MS, Schinagl RM, Kadiyala S, Barry FP. Gelatin-based resorbable sponge as a carrier matrix for human mesenchymal stem cells in cartilage regeneration therapy. J Biomed Mater Res 2000; 52: 246-255.

98. Solchaga LA, Gao J, Dennis JE, Awadallah A, Lundberg M, Caplan AI, Goldberg VM. Treatment of osteochondral defects with autologous bone marrow in a hyaluronan-based delivery vehicle. Tissue Eng 2002; 8: 333-347.

99. Young RG, Butler DL, Weber W, Caplan AI, Gordon SL, Fink DJ. Use of mesenchymal stem cells in a collagen matrix for Achilles tendon repair. J Orthop Res 1998; 16: 406-413.

100. Gazit D, Turgeman G, Kelley P, Wang E, Jalenak M, Zilberman Y, Moutsatsos I. Engineered pluripotent mesenchymal cells integrate and differentiate in regenerating bone: a novel cell-mediated gene therapy. J Gene Med 1999; 1: 121-133.

101. Riew KD, Wright NM, Cheng S, Avioli LV, Lou J. Induction of bone formation using a recombinant adenoviral vector carrying the human BMP-2 gene in a rabbit spinal fusion model. Calcif Tissue Int 1998; 63: 357-360.

102. Ma DD, Da WM, Purvis-Smith S, Biggs JC. Chromosomal analysis of bone marrow stromal fibroblasts in allogeneic HLA compatible sibling bone marrow transplantations. Leuk Res 1987; 11: 661-663.

103. Laver J, Jhanwar SC, O'Reilly RJ, Castro-Malaspina H. Host origin of the human hematopoietic microenvironment following allogeneic bone marrow transplantation. Blood 1987; 70: 1966-1968.

104. Deans RJ, Moseley AB. Mesenchymal stem cells: biology and potential clinical uses. Exp Hematol 2000; 28: 875-884.

105. Fan TX, Hisha H, Jin TN, Yu CZ, Lian ZX, Guo SB, Cui YZ, Feng B, Yang GX, Li Q, Ikehara S. Successful allogeneic bone marrow transplantation (BMT) by injection of bone marrow cells via portal vein: stromal cells as BMT-facilitating cells. Stem Cells 2001; 19: 144-150.

106. Stute N, Fehse B, Schroder J, Arps S, Adamietz P, Held KR, Zander AR. Human mesenchymal stem cells are not of donor origin in patients with severe aplastic anemia who underwent sex-mismatched allogeneic bone marrow transplant. J Hematother Stem Cell Res 2002; 11: 977-984.

107. Awaya N, Rupert K, Bryant E, Torok-Storb B. Failure of adult marrow-derived stem cells to generate marrow stroma after successful hematopoietic stem cell transplantation. Exp Hematol 2002; 30: 937-942.

108. Simmons PJ, Przepiorka D, Thomas ED, Torok-Storb B. Host origin of marrow stromal cells following allogeneic bone marrow transplantation. Nature 1987; 328: 429-432.

109. Agematsu K, Nakahori Y. Recipient origin of bone marrow-derived fibroblastic stromal cells during all periods following bone marrow transplantation in humans. Br J Haematol 1991; 79: 359-365.

110. Brandt JE, Galy AH, Luens KM, Travis M, Young J, Tong J, Chen S, Davis TA, Lee KP, Chen BP, Tushinski R, Hoffman R. Bone marrow repopulation by human

marrow stem cells after long-term expansion culture on a porcine endothelial cell line. Exp Hematol 1998; 26: 950-961.

111. Hou Z, Nguyen Q, Frenkel B, Nilsson SK, Milne M, van Wijnen AJ, Stein JL, Quesenberry P, Lian JB, Stein GS. Osteoblast-specific gene expression after transplantation of marrow cells: implications for skeletal gene therapy. Proc Natl Acad Sci U S A 1999; 96: 7294-7299.

112. Pereira RF, O'Hara MD, Laptev AV, Halford KW, Pollard MD, Class R, Simon D, Livezey K, Prockop DJ. Marrow stromal cells as a source of progenitor cells for nonhematopoietic tissues in transgenic mice with a phenotype of osteogenesis imperfecta. Proc Natl Acad Sci U S A 1998; 95: 1142-1147.

113. Horwitz EM, Prockop DJ, Gordon PL, Koo WW, Fitzpatrick LA, Neel MD, McCarville ME, Orchard PJ, Pyeritz RE, Brenner MK. Clinical responses to bone marrow transplantation in children with severe osteogenesis imperfecta. Blood 2001; 97: 1227-1231.

114. Studeny M, Marini FC, Champlin RE, Zompetta C, Fidler IJ, Andreeff M. Bone marrow-derived mesenchymal stem cells as vehicles for interferon-beta delivery into tumors. Cancer Res 2002; 62: 3603-3608.

115. Wang L, Li Y, Chen X, Chen J, Gautam SC, Xu Y, Chopp M. MCP-1, MIP-1, IL-8 and ischemic cerebral tissue enhance human bone marrow stromal cell migration in interface culture. Hematology 2002; 7: 113-117.

116. Rombouts WJ, Ploemacher RE. Primary murine MSC show highly efficient homing to the bone marrow but lose homing ability following culture. Leukemia 2003; 17: 160-170.

117. Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation 2002; 105: 93-98.

118. Nagaya N, Fujii T, Iwase T, Ohgushi H, Itoh T, Uematsu M, Yamagishi M, Mori H, Kangawa K, Kitamura S. Intravenous administration of mesenchymal stem cells improves cardiac function in rats with acute myocardial infarction through angiogenesis and myogenesis. Am J Physiol Heart Circ Physiol 2004; 287: H2670-2676.

119. Barbash IM, Chouraqui P, Baron J, Feinberg MS, Etzion S, Tessone A, Miller L, Guetta E, Zipori D, Kedes LH, Kloner RA, Leor J. Systemic delivery of bone marrow-derived mesenchymal stem cells to the infarcted myocardium: feasibility, cell migration, and body distribution. Circulation 2003; 108: 863-868.

120. Saito T, Kuang JQ, Lin CC, Chiu RC. Transcoronary implantation of bone marrow stromal cells ameliorates cardiac function after myocardial infarction. J Thorac Cardiovasc Surg 2003; 126: 114-123.

121. Orlic D, Kajstura J, Chimenti S, Bodine DM, Leri A, Anversa P. Bone marrow stem cells regenerate infarcted myocardium. Pediatr Transplant 2003; 7 Suppl 3: 86-88.

122. Orlic D, Kajstura J, Chimenti S, Bodine DM, Leri A, Anversa P. Transplanted adult bone marrow cells repair myocardial infarcts in mice. Ann N Y Acad Sci 2001; 938: 221-229; discussion 229-230.

123. Gandy KL, Weissman IL. Tolerance of allogeneic heart grafts in mice simultaneously reconstituted with purified allogeneic hematopoietic stem cells. Transplantation 1998; 65: 295-304.

124. Thompson RB, Emani SM, Davis BH, van den Bos EJ, Morimoto Y, Craig D, Glower D, Taylor DA. Comparison of intracardiac cell transplantation: autologous skeletal myoblasts versus bone marrow cells. Circulation 2003; 108 Suppl 1: II264-271.

125. Tambara K, Sakakibara Y, Sakaguchi G, Lu F, Premaratne GU, Lin X, Nishimura K, Komeda M. Transplanted skeletal myoblasts can fully replace the infarcted

myocardium when they survive in the host in large numbers. Circulation 2003; 108 Suppl 1: II259-263.

126. Min JY, Sullivan MF, Yang Y, Zhang JP, Converso KL, Morgan JP, Xiao YF. Significant improvement of heart function by cotransplantation of human mesenchymal stem cells and fetal cardiomyocytes in postinfarcted pigs. Ann Thorac Surg 2002; 74: 1568-1575.

127. Leor J, Patterson M, Quinones MJ, Kedes LH, Kloner RA. Transplantation of fetal myocardial tissue into the infarcted myocardium of rat. A potential method for repair of infarcted myocardium? Circulation 1996; 94: II332-336.

128. Tomita S, Li RK, Weisel RD, Mickle DA, Kim EJ, Sakai T, Jia ZQ. Autologous transplantation of bone marrow cells improves damaged heart function. Circulation 1999; 100: II247-256.

129. Sakai T, Li RK, Weisel RD, Mickle DA, Jia ZQ, Tomita S, Kim EJ, Yau TM. Fetal cell transplantation: a comparison of three cell types. J Thorac Cardiovasc Surg 1999; 118: 715-724.

130. Jackson KA, Majka SM, Wang H, Pocius J, Hartley CJ, Majesky MW, Entman ML, Michael LH, Hirschi KK, Goodell MA. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. J Clin Invest 2001; 107: 1395-1402.

131. Mangi AA, Noiseux N, Kong D, He H, Rezvani M, Ingwall JS, Dzau VJ. Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. Nat Med 2003; 9: 1195-1201.

132. Wang JS, Shum-Tim D, Galipeau J, Chedrawy E, Eliopoulos N, Chiu RC. Marrow stromal cells for cellular cardiomyoplasty: feasibility and potential clinical advantages. J Thorac Cardiovasc Surg 2000; 120: 999-1005.

133. Black IB, Woodbury D. Adult rat and human bone marrow stromal stem cells differentiate into neurons. Blood Cells Mol Dis 2001; 27: 632-636.

134. Hofstetter CP, Schwarz EJ, Hess D, Widenfalk J, El Manira A, Prockop DJ, Olson L. Marrow stromal cells form guiding strands in the injured spinal cord and promote recovery. Proc Natl Acad Sci U S A 2002; 99: 2199-2204.

135. Wu S, Suzuki Y, Ejiri Y, Noda T, Bai H, Kitada M, Kataoka K, Ohta M, Chou H, Ide C. Bone marrow stromal cells enhance differentiation of cocultured neurosphere cells and promote regeneration of injured spinal cord. J Neurosci Res 2003; 72: 343-351.

136. Lu D, Li Y, Wang L, Chen J, Mahmood A, Chopp M. Intraarterial administration of marrow stromal cells in a rat model of traumatic brain injury. J Neurotrauma 2001; 18: 813-819.

137. Hung SC, Lu CY, Shyue SK, Liu HC, Ho LL. Lineage differentiation-associated loss of adenoviral susceptibility and Coxsackie-adenovirus receptor expression in human mesenchymal stem cells. Stem Cells 2004; 22: 1321-1329.

138. Morsy MA, Gu M, Motzel S, Zhao J, Lin J, Su Q, Allen H, Franlin L, Parks RJ, Graham FL, Kochanek S, Bett AJ, Caskey CT. An adenoviral vector deleted for all viral coding sequences results in enhanced safety and extended expression of a leptin transgene. Proc Natl Acad Sci U S A 1998; 95: 7866-7871.

139. Steinwaerder DS, Carlson CA, Lieber A. Generation of adenovirus vectors devoid of all viral genes by recombination between inverted repeats. J Virol 1999; 73: 9303-9313.

140. Amalfitano A. Utilization of adenovirus vectors for multiple gene transfer applications. Methods 2004; 33: 173-178.

141. Russell WC. Update on adenovirus and its vectors. J Gen Virol 2000; 81: 2573-2604.

142. Lusky M, Grave L, Dieterle A, Dreyer D, Christ M, Ziller C, Furstenberger P, Kintz J, Hadji DA, Pavirani A, Mehtali M. Regulation of adenovirus-mediated

transgene expression by the viral E4 gene products: requirement for E4 ORF3. J Virol 1999; 73: 8308-8319.

143. Moorhead JW, Clayton GH, Smith RL, Schaack J. A replication-incompetent adenovirus vector with the preterminal protein gene deleted efficiently transduces mouse ears. J Virol 1999; 73: 1046-1053.

144. Amalfitano A, Hauser MA, Hu H, Serra D, Begy CR, Chamberlain JS. Production and characterization of improved adenovirus vectors with the E1, E2b, and E3 genes deleted. J Virol 1998; 72: 926-933.

145. Bergelson JM, Cunningham JA, Droguett G, Kurt-Jones EA, Krithivas A, Hong JS, Horwitz MS, Crowell RL, Finberg RW. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science 1997; 275: 1320-1323.

146. Wickham TJ, Mathias P, Cheresh DA, Nemerow GR. Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. Cell 1993; 73: 309-319.

147. Wickham TJ, Roelvink PW, Brough DE, Kovesdi I. Adenovirus targeted to heparan-containing receptors increases its gene delivery efficiency to multiple cell types. Nat Biotechnol 1996; 14: 1570-1573.

148. Goldman M, Su Q, Wilson JM. Gradient of RGD-dependent entry of adenoviral vector in nasal and intrapulmonary epithelia: implications for gene therapy of cystic fibrosis. Gene Ther 1996; 3: 811-818.

149. Thoma SJ, Lamping CP, Ziegler BL. Phenotype analysis of hematopoietic CD34+ cell populations derived from human umbilical cord blood using flow cytometry and cDNA-polymerase chain reaction. Blood 1994; 83: 2103-2114.

150. Rebel VI, Hartnett S, Denham J, Chan M, Finberg R, Sieff CA. Maturation and lineage-specific expression of the coxsackie and adenovirus receptor in hematopoietic cells. Stem Cells 2000; 18: 176-182.

151. Olmsted-Davis EA, Gugala Z, Gannon FH, Yotnda P, McAlhany RE, Lindsey RW, Davis AR. Use of a chimeric adenovirus vector enhances BMP2 production and bone formation. Hum Gene Ther 2002; 13: 1337-1347.

152. Conget PA, Minguell JJ. Adenoviral-mediated gene transfer into ex vivo expanded human bone marrow mesenchymal progenitor cells. Exp Hematol 2000; 28: 382-390.

153. Gaggar A, Shayakhmetov DM, Lieber A. CD46 is a cellular receptor for group B adenoviruses. Nat Med 2003; 9: 1408-1412.

154. Yotnda P, Onishi H, Heslop HE, Shayakhmetov D, Lieber A, Brenner M, Davis A. Efficient infection of primitive hematopoietic stem cells by modified adenovirus. Gene Ther 2001; 8: 930-937.

155. Tsujimura A, Shida K, Kitamura M, Nomura M, Takeda J, Tanaka H, Matsumoto M, Matsumiya K, Okuyama A, Nishimune Y, Okabe M, Seya T. Molecular cloning of a murine homologue of membrane cofactor protein (CD46): preferential expression in testicular germ cells. Biochem J 1998; 330 (Pt 1): 163-168.

156. Miwa T, Nonaka M, Okada N, Wakana S, Shiroishi T, Okada H. Molecular cloning of rat and mouse membrane cofactor protein (MCP, CD46): preferential expression in testis and close linkage between the mouse Mcp and Cr2 genes on distal chromosome 1. Immunogenetics 1998; 48: 363-371.

157. Sohn JH, Kaplan HJ, Suk HJ, Bora PS, Bora NS. Chronic low level complement activation within the eye is controlled by intraocular complement regulatory proteins. Invest Ophthalmol Vis Sci 2000; 41: 3492-3502.

158. Clark PR, Stopeck AT, Brailey JL, Wang Q, McArthur J, Finer MH, Hersh EM. Polycations and cationic lipids enhance adenovirus transduction and transgene expression in tumor cells. Cancer Gene Ther 1999; 6: 437-446.

159. Lee EM, Hong SH, Lee YJ, Kang YH, Choi KC, Choi SH, Kim IH, Lim SJ. Liposome-complexed adenoviral gene transfer in cancer cells expressing various levels of coxsackievirus and adenovirus receptor. J Cancer Res Clin Oncol 2004; 130: 169-177.

160. Dodds E, Piper TA, Murphy SJ, Dickson G. Cationic lipids and polymers are able to enhance adenoviral infection of cultured mouse myotubes. J Neurochem 1999; 72: 2105-2112.

161. Fasbender A, Zabner J, Chillon M, Moninger TO, Puga AP, Davidson BL, Welsh MJ. Complexes of adenovirus with polycationic polymers and cationic lipids increase the efficiency of gene transfer in vitro and in vivo. J Biol Chem 1997; 272: 6479-6489.

162. Arcasoy SM, Latoche JD, Gondor M, Pitt BR, Pilewski JM. Polycations increase the efficiency of adenovirus-mediated gene transfer to epithelial and endothelial cells in vitro. Gene Ther 1997; 4: 32-38.

163. Qiu C, De Young MB, Finn A, Dichek DA. Cationic liposomes enhance adenovirus entry via a pathway independent of the fiber receptor and alpha(v)-integrins. Hum Gene Ther 1998; 9: 507-520.

164. Byk T, Haddada H, Vainchenker W, Louache F. Lipofectamine and related cationic lipids strongly improve adenoviral infection efficiency of primitive human hematopoietic cells. Hum Gene Ther 1998; 9: 2493-2502.

165. Zabner J, Fasbender AJ, Moninger T, Poellinger KA, Welsh MJ. Cellular and molecular barriers to gene transfer by a cationic lipid. J Biol Chem 1995; 270: 18997-19007.

166. Seth P. Mechanism of adenovirus-mediated endosome lysis: role of the intact adenovirus capsid structure. Biochem Biophys Res Commun 1994; 205: 1318-1324.

167. Cheng SL, Lou J, Wright NM, Lai CF, Avioli LV, Riew KD. In vitro and in vivo induction of bone formation using a recombinant adenoviral vector carrying the human BMP-2 gene. Calcif Tissue Int 2001; 68: 87-94.

168. Nakamizo A, Marini F, Amano T, Khan A, Studeny M, Gumin J, Chen J, Hentschel S, Vecil G, Dembinski J, Andreeff M, Lang FF. Human bone marrow-derived mesenchymal stem cells in the treatment of gliomas. Cancer Res 2005; 65: 3307-3318.

169. Chang SC, Wei FC, Chuang H, Chen YR, Chen JK, Lee KC, Chen PK, Tai CL, Lou J. Ex vivo gene therapy in autologous critical-size craniofacial bone regeneration. Plast Reconstr Surg 2003; 112: 1841-1850.

170. Studeny M, Marini FC, Dembinski JL, Zompetta C, Cabreira-Hansen M, Bekele BN, Champlin RE, Andreeff M. Mesenchymal stem cells: potential precursors for tumor stroma and targeted-delivery vehicles for anticancer agents. J Natl Cancer Inst 2004; 96: 1593-1603.

171. Schwarz EJ, Alexander GM, Prockop DJ, Azizi SA. Multipotential marrow stromal cells transduced to produce L-DOPA: engraftment in a rat model of Parkinson disease. Hum Gene Ther 1999; 10: 2539-2549.

172. Li KJ, Dilber MS, Abedi MR, Bjorkstrand B, Smith CI, Garoff H, Gahrton G, Xanthopoulos KG. Retroviral-mediated gene transfer into human bone marrow stromal cells: studies of efficiency and in vivo survival in SCID mice. Eur J Haematol 1995; 55: 302-306.

173. Allay JA, Dennis JE, Haynesworth SE, Majumdar MK, Clapp DW, Shultz LD, Caplan AI, Gerson SL. LacZ and interleukin-3 expression in vivo after retroviral transduction of marrow-derived human osteogenic mesenchymal progenitors. Hum Gene Ther 1997; 8: 1417-1427.

174. Gordon EM, Skotzko M, Kundu RK, Han B, Andrades J, Nimni M, Anderson WF, Hall FL. Capture and expansion of bone marrow-derived mesenchymal

progenitor cells with a transforming growth factor-beta1-von Willebrand's factor fusion protein for retrovirus-mediated delivery of coagulation factor IX. Hum Gene Ther 1997; 8: 1385-1394.

175. Bulabois CE, Yerly-Motta V, Mortensen BT, Fixe P, Remy-Martin JP, Herve P, Tiberghien P, Charbord P. Retroviral-mediated marker gene transfer in hematopoiesis-supportive marrow stromal cells. J Hematother 1998; 7: 225-239.

176. Ding L, Lu S, Batchu R, Iii RS, Munshi N. Bone marrow stromal cells as a vehicle for gene transfer. Gene Ther 1999; 6: 1611-1616.

177. Oyama M, Tatlock A, Fukuta S, Kavalkovich K, Nishimura K, Johnstone B, Robbins PD, Evans CH, Niyibizi C. Retrovirally transduced bone marrow stromal cells isolated from a mouse model of human osteogenesis imperfecta (oim) persist in bone and retain the ability to form cartilage and bone after extended passaging. Gene Ther 1999; 6: 321-329.

178. Brouard N, Chapel A, Thierry D, Charbord P, Peault B. Transplantation of gene-modified human bone marrow stromal cells into mouse-human bone chimeras. J Hematother Stem Cell Res 2000; 9: 175-181.

179. Chuah MK, Van Damme A, Zwinnen H, Goovaerts I, Vanslembrouck V, Collen D, Vandendriessche T. Long-term persistence of human bone marrow stromal cells transduced with factor VIII-retroviral vectors and transient production of therapeutic levels of human factor VIII in nonmyeloablated immunodeficient mice. Hum Gene Ther 2000; 11: 729-738.

180. Bartholomew A, Patil S, Mackay A, Nelson M, Buyaner D, Hardy W, Mosca J, Sturgeon C, Siatskas M, Mahmud N, Ferrer K, Deans R, Moseley A, Hoffman R, Devine SM. Baboon mesenchymal stem cells can be genetically modified to secrete human erythropoietin in vivo. Hum Gene Ther 2001; 12: 1527-1541.

181. Eliopoulos N, Al-Khaldi A, Crosato M, Lachapelle K, Galipeau J. A neovascularized organoid derived from retrovirally engineered bone marrow

stroma leads to prolonged in vivo systemic delivery of erythropoietin in nonmyeloablated, immunocompetent mice. Gene Ther 2003; 10: 478-489.

182. Lu L, Zhao C, Liu Y, Sun X, Duan C, Ji M, Zhao H, Xu Q, Yang H. Therapeutic benefit of TH-engineered mesenchymal stem cells for Parkinson's disease. Brain Res Brain Res Protoc 2005; 15: 46-51.

183. Kumar S, Mahendra G, Nagy TR, Ponnazhagan S. Osteogenic differentiation of recombinant adeno-associated virus 2-transduced murine mesenchymal stem cells and development of an immunocompetent mouse model for ex vivo osteoporosis gene therapy. Hum Gene Ther 2004; 15: 1197-1206.

184. Chan J, O'Donoghue K, de la Fuente J, Roberts IA, Kumar S, Morgan JE, Fisk NM. Human fetal mesenchymal stem cells as vehicles for gene delivery. Stem Cells 2005; 23: 93-102.

185. Anjos-Afonso F, Siapati EK, Bonnet D. In vivo contribution of murine mesenchymal stem cells into multiple cell-types under minimal damage conditions. J Cell Sci 2004; 117: 5655-5664.

186. Lee CI, Kohn DB, Ekert JE, Tarantal AF. Morphological analysis and lentiviral transduction of fetal monkey bone marrow-derived mesenchymal stem cells. Mol Ther 2004; 9: 112-123.

187. Totsugawa T, Kobayashi N, Okitsu T, Noguchi H, Watanabe T, Matsumura T, Maruyama M, Fujiwara T, Sakaguchi M, Tanaka N. Lentiviral transfer of the LacZ gene into human endothelial cells and human bone marrow mesenchymal stem cells. Cell Transplant 2002; 11: 481-488.

188. Zhang XY, La Russa VF, Bao L, Kolls J, Schwarzenberger P, Reiser J. Lentiviral vectors for sustained transgene expression in human bone marrow-derived stromal cells. Mol Ther 2002; 5: 555-565.

CHAPTER 3

ISOLATION, CHARACTERIZATION, GENE MODIFICATION AND NUCLEAR REPROGRAMMING OF PORCINE MESENCHYMAL STEM CELLS[1]

[1] Pablo Bosch, Scott L. Pratt, and Steven L. Stice, Submitted to *Biology of Reproduction*, 06/30/2005

ABSTRACT

Bone marrow mesenchymal stem cells (MSC) are adult pluripotent cells considered an important resource for human cell-based therapies. Understanding MSC clinical potential may require their use in preclinical large animal models such as pigs. The objectives of this study were: 1) to establish porcine MSC (pMSC) cultures; 2) to optimize in vitro pMSC culture conditions 3) to investigate whether pMSCs are amenable to genetic manipulation; and 4) to determine pMSC reprogramming potential using somatic cell nuclear transfer (SCNT). pMSCs isolated from bone marrow grew, attached to plastic with a fibroblast-like morphology, and expressed the mesenchymal surface marker CD90 but not the hematopoietic marker CD11b. Furthermore, pMSCs underwent lipogenic, chondrogenic and osteogenic differentiation when exposed to specific inducing conditions. Porcine MSCs grew well in a variety of media and proliferative capacity was enhanced by culture under low oxygen atmosphere. Transient transduction of pMSCs and isogenic skin fibroblasts (SF) with a human adenovirus carrying the gene for green fluorescent protein (GFP) (Ad5-F35eGFP) resulted in more pMSCs expressing GFP compared with SFs. Cell lines with stable genetic modifications and extended expression of transgene were obtained when pMSCs were transfected with a plasmid containing the GFP gene. Infection of pMSC and SF cell lines by an adeno-associated virus resulted in ~12% transformed cells, which formed transgenic clonal lines after propagation as single cells. pMSCs can be expanded in vitro and used as nuclear donors to produce SCNT embryos. Thus, pMSCs are an attractive cell type for large animal autologous and allogenic cell therapy models and SCNT transgenesis.

INTRODUCTION

Mesenchymal stem cells (MSCs) are pluripotent precursor cells that localize to the stromal compartment of the bone marrow where they support hematopoiesis and differentiate into mesenchymal lineages. The potential of MSCs to form bone, cartilage and adipose tissues both in vivo [1-3] and in vitro [4] has been well documented. However, their plasticity is not limited to those mesenchymal derivatives; recent reports have shown that MSCs can differentiate into neurons [5, 6], myoblasts [7], and cardiomyocytes [8]. Cells with features of mesenchymal precursors have been isolated from the bone marrow of many mammals including laboratory rodents [9, 10], humans [4], cats [11], dogs [12] and pigs [13]. MSCs from all species studied proliferate in vitro as adherent fibroblastic cells, a feature that has been exploited in order to enrich MSCs from hematopoietic cells that normally remain in suspension. In humans, pluripotent stem cells derived from marrow stroma proliferate ex vivo to form a phenotypically homogeneous population of cells that express several surface markers, such as CD90, CD44, and CD71, but do not express the hematopoietic markers CD45 and CD11b [4]. Like MSCs from other species, porcine MSCs (pMSC) were capable of growing and attaching to plastic with a fibroblast-like morphology and then differentiating into adipose, bone and cartilage tissues in vitro [13]. However, surface marker expression and culture requirements for ex vivo expansion of MSCs in this species have not been yet defined.

Due to the ability of MSCs to proliferate extensively ex vivo while maintaining their pluripotent differentiation capabilities (in vivo and in vitro), they are regarded as a particularly attractive cell type for cell-based therapies in humans. Of particular interest is

the use of intact or genetically engineered MSCs for the treatment of skeletal disorders like osteogenesis imperfecta [14, 15]. Moreover, MSCs have attracted much attention as tools for targeted delivery of anticancer agents into tumors [16, 17]. Before human clinical trials are approved, scaled up cell production and delivery into a large animal model in which cell doses (number of cells) comparable to those that will be used in human trials is often required in order to satisfy regulatory safety concerns. Beyond safety issues, the reprogramming of pMSCs via somatic cell nuclear transfer (SCNT) lays the foundation for future isogenic comparisons between adult pMSCs and reprogrammed embryonic cell sources (therapeutic cloning) in porcine disease models. Kato et al. [18] have recently reported the birth of the first calf originated from a bovine MSC, demonstrating bovine MSCs can be reprogrammed to drive term development after SCNT.

Development of SCNT has provided a new and faster way to create transgenic animals. It is now possible to introduce genetic modifications in cultured cells that can be later used as donor cells to produce cloned animals bearing the genetic transformation (reviewed in [19, 20]). Genetic manipulations of cultured cells can range from simple random integration of the gene of interest to targeted homologous recombination to abolish (knockout) or modulate gene function. DNA plasmids in combination with electroporation or a particular transfection compound have been used to transform cultured cells for use in nuclear transfer (NT) [21, 22], and transgenic animals have been subsequently generated with these genetically transformed cells [23, 24]. In addition, transient expression of proteins in donor cells could open new opportunities from basic

studies of donor cell physiology and nuclear reprogramming to more applied studies aimed to improve cloning efficiencies by conditioning donor cells before NT.

The use of viruses as vectors has emerged as a promising alternative to the classic mechanical methods of gene delivery. The ability of retroviruses to randomly integrate into the host genome has been exploited to stably introduce the green fluorescent protein (GFP) reporter gene in pig cell lines that were later used to produce embryos [25] and transgenic cloned pigs by SCNT [26, 27]. Lentivirus, which is a complex retrovirus, is considered a promising alternative to the original oncogenic retroviral vectors due to their ability to infect non-dividing mammalian cells and to resist methylation-dependent gene silencing. Lentiviral infection of bovine fibroblasts followed by SCNT has resulted in the production of transgenic animals [28].

Adeno-associated virus (AAV) is an integrating, non-pathogenic human virus which requires coinfection with a helper virus such as adenovirus or herpesvirus for productive infection. In absence of a helper virus, AAV integrates in a site-specific manner in the host genome where it remains as latent infection. Vectors derived from AAV are attractive candidates for transgenesis by virtue of their nonpathogenicity, integration capability, infectivity of dividing and non-dividing cells and ability to infect a wide variety of cell types. AAV vectors have been used to insert small (<20 bp) and large transgenes (>1Kb) by homologous recombination in human cells in culture [29]. More recently, Hirata et al. [30] demonstrated that AAV vectors can efficiently disrupt one allele of the PRNP gene in cultured bovine fibroblasts; expanding the use of AAV vectors to animal transgenesis.

Targeted homologous recombination has also been accomplished in mammalian cells with adenovirus vectors [31]. Since adenovirus very rarely integrates into the host genome by non-homologous recombination, replication-defective recombinant adenoviral vectors are used as efficient expression vectors. Transient expression of endogenous or even foreign proteins in cultured cells by adenovirus vectors would represent a potential tool to manipulate donor cells in culture. Furthermore, silencing of endogenous genes by adenovirus-mediated expression of small interfering RNA is now a reality [32]. These novel adenovirus-based approaches could open new possibilities for controlling cell processes such as cycle progression, DNA methylation or apoptosis in SCNT donor cells.

In the present study we have isolated and established adult pMSC lines from live animals using a minimally invasive bone marrow (BM) aspiration technique. The mesenchymal identity of isolated cells was determined by expression of surface markers and multilineage differentiation potential. We then designed experiments aimed to: a) optimize in vitro culture conditions of pMSCs; b) compare transfection/transduction efficiencies of pMSCs and isogenic skin fibroblasts (SF) exposed to integrating and non-integrating vectors; and c) examine the ability of pMSCs to drive development of SCNT embryos to blastocyst stage. We have shown that adult pMSC can be genetically modified and be used to produce SCNT embryos. This is significant in that it prepares us for future large animal autologous cell/gene therapy modeling comparing the adult cells to embryonic cells derived through SCNT.

MATERIALS AND METHODS

Bone marrow and skin collection

BM aspirates were obtained from anesthetized young adult female pigs (~6 month-old). General anesthesia was induced with a combination of Ketamine, 10 mg/Kg BW, intramuscularly (IM) and xylazine, 2 mg/kg BW, IM and maintained with inhalation anesthesia (halothane). Aspirates of bone marrow (approximately 20 ml) were collected from the humeral head with an 11 g biopsy-aspiration needle (Medical Device Technologies, Inc., Gainesville, FL, USA) attached to a heparinized syringe. An ear skin sample was obtained from the same animal by punch biopsy. Bone marrow and skin samples were immediately transported to the laboratory for further processing. All animal procedures were approved by the Institutional Animal Care and Use Committee at The University of Georgia.

Isolation of bone marrow pMSCs and skin fibroblasts

Mononuclear cells were separated by centrifugation of BM aspirates through a solution of polysucrose and sodium diatrizoate (Histopaque®, density: 1.077; Sigma Chemical, St Louis, MO) as indicated by the manufacturer. Briefly, 5 ml of PBS were added to 3 ml of marrow aspirate and mixed in a 15 ml centrifuge tube. The cell suspension was deposited over 3 ml of Histopaque, and centrifuged at 400 x g for 30 minutes at room temperature. Mononuclear cells were recovered with a Pasteur pipette from the opaque interface and washed 2X with D-PBS. For SF isolation, cartilage tissue was removed from the ear skin sample followed by scratching the surface of it with a scalpel to partially remove the dermis. Then, specimens were finely chopped with a scalpel blade and digested by treatment with 10 ml of digestion solution containing 0.2%

trypsin (Cat Number T4799, Sigma, St. Louis. MO, USA) and 0.2% collagenase (Cat. Number C9263, Sigma, St. Louis. MO, USA) in D-PBS containing 5 mg BSA per ml at 37˚C with agitation. At 10 min intervals the supernatant containing cells was removed and replaced by fresh digestion solution. Approximately 30 min were required to digest the tissue corresponding to a skin sample. Cells recovered from one skin sample were washed and plated in ~8 75 cm^2 flasks in MEM alpha medium (Invitrogen Corporation, Cat No 12000-022) supplemented with 10% fetal bovine serum (FBS).

Culture of pMSC

After washing, mononuclear cells were resuspended in MEM alpha medium (Invitrogen Corporation, Cat No 12000-022) supplemented with 10% FBS and plated on plastic flasks at a density of ~500,000 cells/cm^2. After 24 h, unattached cells were washed off the flask during medium exchange. Adherent fibroblast-like cells were allowed to grow for 10-14 days with media replacement every 3rd day. Cells were passaged at 80-90% confluence by trypsinization (0.25% trypsin-EDTA solution, Sigma, St. Louis. MO, USA), and reseeded at a density of 5,000-6,000 cells/cm^2 in plastic flasks.

Expression of surface markers

Expression of surface markers in MSC cultures for phenotypic characterization was performed by indirect immunofluorescence and flow cytometric analysis. For immunocytochemistry, cells grown in glass chamber slides were fixed with 2% formaldehyde for 5 min, washed and blocked with 3% goat serum in D-PBS for 30 min. Then, cultures were incubated with either 1:500 dilution of the primary antibody (anti-CD90 or anti-CD11b), or isotype control (Mouse IgG_1, clone: MOPC-31C, BD Biosciences, Pharmingen, San Jose, CA, USA), or D-PBS (negative control) for 1:15 h.

Primary antibody used was anti-human CD-90 monoclonal antibody that cross-react with pig antigens (Clone 5E10, BD Biosciences, Pharmingen, San Jose, CA, USA) or anti-pig CD11b monoclonal antibody (Clone 2F4/11, BD Biosciences, Pharmingen, San Jose, CA, USA). After washing in D-PBS, cell monolayers were incubated with Alexa Fluor® 488 goat anti-mouse IgG (1:100 dilution; Molecular Probes, Eugene, OR, USA) for 1 h. Cells were washed, stained with DAPI (1µg/ml; Calbiochem, San Diego, CA, USA) and mounted with Vectashild mounting medium (Vector Laboratories, Burlingame, CA). Specimens were examined under an epifluorescent inverted microscope (Nikon Eclipse TE2000-S, Nikon Corporation, Tokyo, Japan) equipped with a digital camera (Qimaging Ratiga 1300, Qimaging, Burnaby, BC, Canada).

The same basic staining procedure described for immunocytochemistry was used to prepare the cells for flow cytometric analysis with minor modifications. Cell cultures were trypsinized, washed in D-PBS and fixed with 2% formaldehyde solution for 3 minutes. Non-specific binding was prevented by incubating the cells in 3% goat serum for 30 min. Cells were incubated in 15 µg/ml of the primary antibody (anti-CD90 or anti-CD11b) or isotype control for 45 min at room temperature. After washing, cells were incubated with a 1:500 dilution of Alexa Fluor® 488 goat anti-mouse IgG. Fluorescent cell analysis was performed with FACSCalibur cytometer (Becton Dickinson Immunocytometry System, San Jose, CA, USA) and data analyzed by FlowJo software (Tree Star, Inc, Ashland, OR, USA).

Lineage differentiation of MSC

pMSC cultures were exposed to chondrogenic, lipogenic or osteogenic conditions for 14-20 days to determine multipotency. Lipogenic and osteogenic induction was

applied to cells growing in monolayers. Chondrogenic and osteogenic differentiation was induced on cell masses as previously described in human [33] and porcine MSC [13]. Briefly, aliquots of 200,000 cells were distributed in 15-ml conical tubes and centrifuged 5 min at 600 x g. Sedimented cells were cultured in the tubes with loosened caps to allow gas exchange. Cells formed a spherical mass on the bottom of the tube by 24 h of culture. Composition of differentiation media is shown in Table 3.1. Differentiation media were replaced every 3-4 days. For lipogenic differentiation, cells were first exposed to induction medium for 2-3 days and then cultured in maintenance medium (Table 1) for another 2-3 days. This alternating treatment was repeated 3-4 times to achieve full lipogenic differentiation.

Histochemical stains were used to assess cell differentiation into specific lineages in adherent cell cultures and histological cryosections of cell masses. Cell masses were embedded in a water soluble embedding medium, frozen in liquid N_2 and sectioned (10 μm thickness) with a Leica CM3050 cryostat (Leica, Nussloch, Germany). Accumulation of phosphates and carbonates, indicative of osteogenic differentiation was demonstrated by the von Kossa silver reduction method [4]. Cultures or cryosections were fixed with 4% formaldehyde, exposed to 5% silver nitrate solution and immediately exposed to direct UV light for 45-60 minutes. Specimens were then washed and incubated for 2-3 min in 5% sodium thiosulfate solution. Expression of alkaline phosphatase was assessed by a commercial kit (Vector Red Alkaline Phosphatase Substrate Kit I, Vector Laboratories, Burlingame, CA). Intracellular accumulation of neutral lipids was demonstrated by Oil Red O staining [4]. For this assay monolayers were fixed and stained with Oil Red O working solution for 1 h. The working solution was made fresh

each time by mixing 1 part distilled water with 1.5 parts of a saturated Oil Red O solution (0.5% w/v Oil Red O in 99% isopropyl alcohol). Acidic mucopolysaccharides present in cartilage tissue were stained with alcian blue 8GX (Sigma, St. Louis. MO, USA). Briefly, cryosections were fixed with 3% acetic acid and stained with alcian blue solution (1% w/v alcian blue in 3% acetic acid, pH 2.5) for 30 min. After washing, slides were mounted with 90% glycerol and inspected with a transmitted light microscope. Photographs were taken with a digital camera (Qimaging Ratiga 1300, Qimaging, Burnaby, BC, Canada) mounted on the microscope.

Optimization of culture conditions for pMSC

Colony forming unit fibroblast (CFU-F) assay. Passage 2-3 MSCs growing in flasks were trypsinized and plated at density of 10 cells/cm^2 in 100 x 20 mm dishes. Cells were cultured for 14 days under different experimental conditions. Medium was replaced every 3rd-4th day. Cell colonies were washed with D-PBS and stained with 3% Crystal Violet in methanol for 15-20 min and the number and size of colonies recorded for each experimental group. Number of colonies and major and minor axes of each colony were measured with the aid of an ocular micrometer. The averages of the major and minor axes are reported as colony diameter. For each experimental condition, the best treatment is the one that induces the highest number of colonies with the larger diameter.

Cell proliferation assay (CyQUANT assay). Passage 2-3 MSCs growing in flasks were trypsinized and plated at a density of 800 cells/well of 96 well assay plates (black plate with clear bottom; Corning Incorporated, Corning, NY, USA). Cells were grown under different experimental conditions for 4 days. Media were removed and the plates stored at -80 until the CyQUANT proliferation assay was performed following the

manufacturer instructions (Molecular Probes, Eugene, OR, USA). Fluorescence in the samples was measure with a fluorescence microplate reader (SPECTRAmax GEMINI, Molecular Devices Corporation, Sunnyvale, CA, USA) with filters appropriate for 485 nm excitation and 538 nm emission.

CyQUANT proliferation assay kit was also used to investigate cell adhesion efficiency. Cells were plated in two plates at a density of 13,000 cells per well and incubated under different experimental conditions at 37°C in 5% CO_2 in air. After 5-hour incubation one plate was centrifuged to pellet the cells and the media were carefully removed. This plate was used to determine total cell numbers. Media from the second plate were removed and wells washed 3 times with D-PBS. All plates were stored at -80˚C until the CyQUANT proliferation assay was performed according to manufacturer instructions.

Experiment 1: Effect of FBS concentration on proliferation of pMSC. The objective of this experiment was to study the effect of media containing increasing concentrations of FBS (2.5, 5, 10, 20, or 30%) on growing characteristics of pMSC in the CFU-F assay.

Experiment 2: Effect of media and oxygen tension on proliferation of MSC. The ability of different media to support pMSC grow in vitro under low or high oxygen concentration atmosphere was investigated using the CFU-F and CyQUANT proliferation assays. pMSC were cultured in either alpha MEM medium (Invitrogen Corporation, Cat No 12000-022), low glucose DMEM containing 2.2 g/L of sodium bicarbonate, low glucose DMEM containing 3.7 g/L sodium bicarbonate (Invitrogen Corporation, Cat No 31600-034) or DMEM/F12 (Sigma Cat No D0547). All media were

supplemented with 10% FBS. Culture was carried out in low oxygen concentration (5% O_2, 5% CO_2 and 90% N_2) or high oxygen concentration (5% CO_2 in air). In the CyQUANT proliferation experiment cells were seeded in 96-well plates in alpha MEM media with 10% FBS and allowed to attach for 12 h. After this, media were removed and treatments applied. The design of the CFU-F experiment was slightly different, since cells were directly seeded in 100 mm dishes in the treatment media.

Experiment 3: Effect of ascorbic acid supplementation and oxygen tension on proliferation of pMSC. The potential stimulatory effect of increasing concentrations of ascorbic acid 2-phosphate (control, 5, 50, 500 or 5,000 μg/ml; A8960, Sigma Chemical, St Louis, MO) added to the culture medium (low glucose DMEM) on proliferation of pMSC was investigated. The design also included the effect of culture under an atmosphere with low or high oxygen concentration on proliferation of pMSC across all ascorbic acid treatments.

Transient genetic modification

Transduction with a human adenovirus. A chimeric adenovirus type 5 that contains the adenovirus type 35 fiber and carries the GFP gene (Ad5F35-eGFP; 5 x 10^{12} particles/ml, 3.45 x 10^{10} pfu/ml) was obtained from the Vector Development Laboratory at Baylor College of Medicine, Huston, TX.

Passage 2-3 pMSC and matching isogenic SF cell lines were seeded at a density of 43,000 cells/cm^2 in 12-well plates (150,500 cells/well). Twenty-four hours after plating, cultures were infected with 100 MOI in 500 μL of alpha MEM with 10% FBS. The percentage of GFP positive cells, relative fluorescence intensity (RFI) of the GFP positive cell population and cell viability by exclusion of propidium iodide (50 μ/ml;

Roche Applied Science, Indianapolis, IN, USA) was determined 24 h after viral exposure by flow cytometric analysis using a FACSCalibur cytometer and FlowJo software.

Transfection with GFP plasmid. Early passage (2-3) pMSCs and isogenic SFs were plated in 12-well plates (120,000 cells/well) and 20 h later were transfected with a plasmid containing EGFP gene under control of cytomegalovirus (CMV) promoter and neomycin-resistant gene under control of an SV40 promoter which allows selection using geneticin (EGFP-N1, Clontech Laboratories, Palo Alto, CA, USA). Transfection was carried out in presence of a polyamine-based transfection reagent (GeneJammer, Stratagene, La Jolla, CA, USA) according to manufacturer recommendations (2 μg plasmid DNA per well). Transfected cells were sorted based on GFP fluorescence 72 h after transfection using a MoFlow FACS set to sort 1 cell per well of 96-well plates (3 plates per cell line). Cell were cultured in alpha MEM with 15% FBS for 14 days and development of GFP expressing colonies was determined at this point by inspection under a microscope equipped with UV light.

Stable genetic transformation

Transfection with GFP plasmid. Passage 2-3 MSCs and isogenic SFs were plated in 4 100x20 mm plastic dishes per cell line at 1.2 x 10^6 cells/dish. Cultures were transfected with EGFP-N1 plasmid (12 μg plasmid DNA/dish) using GeneJammer Transfection Reagent according to manufacturer specifications. Selection for transgenic cells was initiated in 3 dishes per cell line 72 h after transfection by culturing the cells in medium containing Geneticin (250 μg/ml, G418, Sigma). Number of GFP expressing colonies was determined 14 days after transfection. The remaining 100 mm dish was passaged and propagated in alpha MEM with 15% FBS. After 8-9 days, cells were

trypsinized and stained with 50 µg/ml propidium iodide (Roche Applied Science, Indianapolis, IN, USA). Viable GFP expressing cells were sorted with a MoFlo instrument (DakoCytomation, Ft. Collins, CO, USA) as single cells in 96-well plates (3 plates per cell line) containing culture medium supplemented with 20% FBS and colonies were allowed to grow for 14 days (with media change at day 7). At the end of the culture period, colonies were graded according their development as follows: category 1, colony covering all or almost all the surface of the well; category 2, colony covering approximately half of the well; and category 3, colony covering one forth of the well. Colonies were also graded as GFP positive (high, medium or low florescence intensity) or negative.

Adeno-associated viral transduction. Human adeno-associated virus vector carrying the GFP gene was kindly provided by Vector Development Laboratory at Baylor College of Medicine, Huston, TX. pMSC and SF cultures (passage 2-3) were seeded in 4-well plates (40,000 cells/well). Cell cultures were transduced 24 h after plating with 3 x 10^8 viral particles per well in alpha MEM with 2% FBS. Serum concentration was adjusted to 10% by adding FBS 3.5 h after transduction. Transduced cells were passaged and expanded for 9-10 days in alpha MEM supplemented with 15% FBS before sorting viable GFP positive cells in 96-well plates (1 cell per well; 3 plates per cell line) using a FACS cytometer (MoFlo, DakoCytomation, Ft. Collins, CO, USA). Cells were cultured for 14 days in alpha MEM containing 15% FBS (replaced at Day 7 of culture), and colony development was evaluated as described above.

Somatic cell nuclear transfer

Confluent (passage # 2) MSC and SF cultures exposed to roscovitine (15 μM; Sigma) during the last 24 h of culture [34] were used as karyoplasts to produce NT embryos. In vitro matured oocytes were enucleated and a single cell (MSC or SF) was transferred into the perivitelline space. Cell-oocyte couplets were fused in Zimmerman's medium with a single electric pulse (250 V/mm for 20 μsec) delivered through a needle-type electrode. NT units were electrically activated (2 pulses of 75 V/mm for 60 μsec separated by 5 sec) in a chamber 1 h after fusion and transferred to drops of NCSU-23 medium. Embryos were examined for cleavage and blastocyst formation at 2 and 7 days after NT respectively.

Statistical analysis

CFU-F data from Experiment 1 were analyzed by one-way analysis of variance (ANOVA) using the general linear models (GLM) procedure of the Statistical Analysis System [35], followed by protected LSD. CFU-F and proliferation data from Experiments 2 and 3 were analyzed by two-way ANOVA using GLM procedure of SAS under a completely randomized factorial design. The model included variation due to treatment (media in Experiment 2 or ascorbic acid in Experiment 3), oxygen tension (high or low) and their interaction. When a significant effect was detected with the ANOVAs, treatment means were compared by protected LSD. Student's *t*-test was used for comparing data from two groups, i.e., pMSC versus SF. All values are mean ± SEM from at least 3 replicates. Differences were considered to be significant at $P<0.05$.

RESULTS

Isolation of cell lines

Mesenchymal stem cell lines were successfully established from bone marrow collected from 10 anesthetized gilts (n=10). The number of mononuclear cells per BM aspirate (~20 ml) recovered from the density gradient was $2.33 \pm 0.5 \times 10^8$ mononuclear cells, enough to plate ~6 75 cm^2 flasks. Most of the non-adherent cells were removed during the first media change at 24-48 h. Discrete colonies of fibroblast-like cells attached to the plastic were evident at Day 4-5 after initial seeding. Most cell lines were composed of cells with a characteristic spindle shape while others had cells with polygonal morphology. The number and size of the colonies increased progressively to reach 80% confluency by Day 14-15 (Fig. 3.1, A and B) after seeding.

Expression of surface markers

Immunocytochemistry revealed that fibroblast-like cells from pig bone marrow were positive for the cell surface marker CD90 (Fig. 3.2A) and negative for CD11b (Fig. 3.2B). Flow cytometric analysis confirmed that $99.4 \pm 0.20\%$ of the cells expressed CD90 antigen (Fig 3.2C) and virtually the entire population was negative for CD11b (Fig. 3.2D).

Lineage differentiation of MSC

Results indicated that BM mesenchymal cells acquire morphological and histochemical characteristics of adipose, cartilage or bone tissues when exposed to specific differentiation inducing conditions (Fig. 3.3). Conversely, isogenic SFs exposed to identical induction conditions failed to differentiate (Fig. 3.3). Cells with discrete,

although small lipid droplets were present as early as Day 4-5 of culture. The number of cells with lipid accumulation and the size of the lipid droplets increased until Day 8-9 and plateaued until the end of culture period (Day 12-14). The percentage of cells undergoing lipogenic differentiation was highly variable among cell lines, ranging from ~1 to 15%. Oil Red O confirmed the presence of neutral lipid accumulation in differentiated pMSCs. (Fig. 3.3A). Lipogenesis was not evident in pMSCs maintained in culture medium alone (Control; Fig. 3.3B) or isogenic SFs exposed to lipogenic medium (Fig. 3.3C). Alcian blue staining revealed presence of acidic mucopolosaccharides in sections of pMSC masses cultured in chondrognic medium for 14-17 days (Fig. 3.3D). Cell morphology was also compatible with cartilage tissue. pMSC controls and SFs cultured in differentiation media were negative for alcian blue stain (Fig. 3.3, E and F). Extensive osteogenic differentiation, as evidenced by black deposits with von Kossa stain (Fig. 3.3G) was only noticeable in pMSCs exposed to osteogenic conditions; pMSC controls and SFs cultured in osteogenic differentiation media were both negative for von Kossa stain (Fig. 3.3, H and I)

Optimization of culture conditions for pMSC

Experiment 1: Effect of FBS concentration on proliferation of pMSC. Percentage of FBS in the culture medium greatly influenced both the number of colonies per dish and the mean colony diameter. The number of colonies per dish increased with increasing FBS concentrations up to 10% when a plateau was reached (Fig. 3.4A). A similar positive effect of FBS on colony diameter was also evident, however, a plateau was not reached with 30 % FBS (Fig. 3.4B).

Experiment 2: Effect of media and oxygen tension on proliferation of MSC. The effect of media and oxygen tension on CFU-F assay was replicated with 4 cell lines (obtained from 4 different animals). In 3 out of 4 cell lines the number and diameter of colonies were markedly smaller in DMEM/F12 medium compared with the other media studied, while in the remaining cell line the response to DMEM/F12 medium was similar to the other treatments. Since the inclusion of data from this cell line in the statistical analysis would mask the negative effect observed for DMEM/F12 medium in 3 out of 4 cell lines, we decided to exclude it from the statistical analysis. The number and diameter of colonies was not different for MSCs grown in alpha MEM, DMEM 2.2 or DMEM 3.7 (Fig. 3.4, C and E). Cells cultured in DMEM/F12 responded with fewer and smaller colonies compared with the other media studied (Fig. 3.4, C and E). Oxygen concentration affected neither the number nor the mean diameter of colonies (Fig. 3.4G) in the CFU-F assay.

The effect of media type on proliferation of pMSCs in two different oxygen tensions (low or high) was investigated with QyQUANT proliferation assay. Significant effect of medium type ($P<0.0001$) and oxygen tension ($P<0.0001$) but not interaction ($P=0.49$) between these variables was observed. Relative fluorescence units (RFU), which is correlated to the amount of DNA, was higher in DMEM/F12 treated cells compared with the other media types studied (Fig. 3.4D). Low oxygen tension had a positive effect on cell proliferation as evidenced by a higher RFU compared with cells maintained in high oxygen atmosphere (Fig. 3.4H).

The ability of alpha MEM and DMEM/F12 to induce adhesion of pMSCs to plastic was compared with CyQUANT assay. Alpha MEM had a better ability to induce

pMSC attachment than DMEM/F12 medium (189.33 ± 10.05 versus 164.66 ± 2.96 RFU in alpha MEM and DMEM/F12 respectively; P<0.05).

Experiment 3: Effect of ascorbic acid supplementation and oxygen tension on proliferation of pMSCs. Significant effects of ascorbic acid concentration (P<0.0001) and oxygen tension (P<0.0001) but not interaction (P=0.56) between these variables were observed. Supplementation of the culture medium with 5 to 500 µg of ascorbic acid per ml did not affect cell proliferation compared with the control (Fig. 3.4F). However, addition of 5000 µg of ascorbic acid/ml impaired pMSC proliferation (Fig. 3.4F). Coinciding with results from Experiment 2, low oxygen tension significantly improved (P<0.05) pMSC proliferation rate.

Transient genetic modification of SFs and pMSCs

Transduction with a human adenovirus. Microscopic inspection of cultures under UV light 24 h after infection revealed a superior transduction efficiency in pMSCs compared to SFs (Fig. 3.5, E and F), a finding that was later confirmed by flow cytometry. The percentage of cells expressing GFP was ~15% higher in pMSCs than that in isogenic SFs (70.25 ±5.45 vs. 55.31 ± 6.83 % of GFP+ in pMSCs and SFs respectively; P=0.02; Fig. 3.5, A-C). Relative fluorescence intensity was also higher in pMSCs compared with that in SFs (959.66 ± 73.25 vs. 585.75 ± 19.32 RFI, in pMSCs and SFs respectively; P=0.005; Fig. 3.5A). Percentage of PI positive cells was higher in pMSCs (5.35 ± 0.38%) compared with that in SFs (3.45 ± 0.24%; p=0.01; Fig. 3.5A).

Transfection with GFP plasmid. There was no difference in the proportions of SFs and pMSCs expressing GFP (SFs: 3.99 ± 0.95%, pMSC: 8.44 ± 2.33%, P=0.22; Fig. 3.5D). Viability was also similar between the two experimental groups (Fig. 3.5D). GFP

positive cells were individually sorted in 3 96-well plates per cell line and checked for GFP expressing colonies after 14 days of culture. Only 7 out of 648 colonies that developed in SF and pMSC plates were GFP positive.

Stable genetic transformation

We used two different vectors, namely a GFP plasmid and AAV to obtain cell populations displaying extended expression of the transgene, which is normally associated with integration of the transgene into the host DNA. Comparison of proportions of cells that remained GFP+ after transfection/transduction and proliferation in vitro (8-10 days) revealed a higher proportion of GFP expressing cells in the AAV than in the GFP plasmid transfected group, irrespective of cell line (Fig 3.6A). There was no difference in transfection/transduction efficiency between SF and pMSC (Fig. 3.6A).

Transfection with GFP plasmid. The mean number of colonies per 100 mm dish expressing GFP after 14 days of G418 selection was 74.32 ± 8.34 in SF and 74.38 ± 4.58 in pMSC ($P>0.05$).

We implemented an approach to reduce the number of cells with transient expression of GFP from episomes which consisted in propagation of transfected cell lines for 10 days and then sorting individual cells in 96-well plates based on GFP fluorescence. Two weeks after plating, approximately 35% of plated cells formed a colony (Fig. 3.6D). Irrespective of cell line (SF or pMSC), the vast majority of these colonies (~92%) did not express GFP. Colony development was similar between SF and pMSC (Fig. 3.6E). There was a higher percentage of high fluorescence SF colonies compared with the same category pMSC (Fig. 3.6E; $P>0.05$). A pMSC colony originated from one cell transfected

with the GFP plasmid and positive for GFP after 14 days of culture is shown in Figure 3.6B.

Adeno-associated viral transduction. The proportion of plated cells that formed a colony was not different between cell lines ranging from 26.39 ± 1.31 in pMSC to 40.04 ± 7.79% in SF. Contrasting with results obtained with GFP plasmid transfected cells, 90.2% of SF colonies and 96.2% of pMSC colonies expressed GFP (compare Fig. 3.6D with Fig. 3.6F). There was no difference in the proportion of colonies category 1, 2 or 3 between cell lines (Fig. 3.6G). pMSC had a larger proportion of high fluorescence colonies and lower percentage of low fluorescence intensity colonies compared to SF ($P<0.05$; Fig. 3.6G). A pMSC colony positive for GFP after 14 days of culture is shown in Figure 3.6C.

Somatic cell nuclear transfer

pMSC and SF synchronized with roscovitine were used in two replicates as nuclear donors to produce cloned porcine embryos. Cleavage rates were 44.5% (65/146) for MSC and 53.1% (60/113) for SF NT embryos. Development to blastocyst stage was 4.1% (6/146) in the MSC group and 1.77% (2/113) in SF group. The number of cells per blastocyst ranged from 7-23 cells per blastocyst. Low percentages of embryo development obtained precluded statistical analysis of NT data.

DISCUSSION

Since first identified by the pioneering work of Freidman et al. in the early 70s [36, 37], MSC, also known as marrow somatic cells or colony forming unit fibroblastic cells, have been the subject of numerous studies aimed to decipher the roles of these stem cells in the complex marrow physiology. MSCs are considered non-hematopoietic

precursor cells that support hematopoiesis and can differentiate down the lipogenic, chondrogenic, osteogenic and tenogenic pathways (reviewed in [38]). The broad differentiation potential along with extensive ex vivo proliferative capacity makes these stromal precursors attractive candidates for autologous and allogeneic cell therapy and potentially for SCNT transgenesis. We have isolated and characterized the growing properties of pMSC under different culture conditions. Then we examined the ability of pMSC and isogenic SF to undergo transient and stable genetic modifications using a combination of GFP-plasmid with a transfection reagent and viral vectors. Finally, our studies suggest that pMSC can undergo nuclear reprogramming to generate cloned blastocysts.

We were able to establish a primary MSC line from each of 10 individual animal bone marrow aspirations. Two approaches were undertaken in order to determine the MSC identity of fibroblastic cells isolated from pig BM: (a) expression of surface markers by immunocytochemistry and flow cytometry analysis, and (b) pluripotency as determined by multilineage differentiation in vitro. Imunocytochemistry revealed that most cells expressed the mesenchymal marker CD90. These results were later confirmed by flow cytometric analysis of pMSCs which demonstrated that ~99% of the cells expressed the antigen. Using the same techniques, it was establish that bone marrow fibroblastic cell did not express CD11b, a hematopoietic marker present in granulocytes, monocytes, NK cells, subsets of T cells, and subsets of B cells [39]. Cell surface marker expression in bone marrow-derived cells isolated in the present study supports the mesenchymal origin of these cells and agrees with previous work in human and rat MSCs, which consistently express CD90 and lack CD11b expression [4, 6]. Furthermore,

analysis of forward and scatter light data from pMSCs revealed homogenous cell populations (size and granularity; data not shown) coinciding with flow cytometry results from human MSCs [4]. MSCs but not fibroblasts are capable of differentiating down mesenchymal lineages [4, 10, 11, 13], and we used this to confirm that isolated cells from pig bone marrow were truly MSCs. Only pMSCs differentiated along the lipogenic, chondrogenic and osteogenic lineages when exposed to specific induction media; isogenic SFs failed to do so (Fig. 3.3). Morphology, surface antigen profile and pluripotency characteristics provide convincing evidence that the bone marrow cells isolated in the present study are pMSCs.

Since little is known about culture conditions that support pMSC proliferation in vitro, we first sought to characterize the growth properties of pMSCs under different culture conditions. The CFU-F assay was used to investigate the effect of FBS on number and diameter of pMSC colonies. Development of MSC colonies depended entirely on the growth factors present in FBS (Fig. 3.4, A and B). No colonies were present when the FBS was omitted and a clear positive dose-response relationship was observed between colony numbers and FBS concentrations. A similar response to FBS was reported for human MSCs [40]. A positive effect of FBS was also evident on the mean colony diameter; however the plateau was not reached with 30% FBS. Overall, it is apparent that addition of 10-20% of serum to pMSC culture medium provides adequate support for pMSC expansion. The CFU-F assay was used to investigate growing properties of pMSCs in different media resulting in a cell line dependent response. In 3 out of 4 cell lines, the number and diameter of colonies was not different for pMSCs cultured in alpha MEM, DMEM 2.2 and DMEM 3.7, but significantly lower for cells growing in

DMEM/F12. Unexpectedly, DMEM/F12 medium enhanced proliferation of pMSCs in the CyQUANT assay (Fig. 3.4D), contrasting with results obtained in the CFU-F assay. This inconsistent response may have arisen from inherent differences between the assays. For instance, cells in the CFU-F assay were plated in treatment media, whilst in the proliferation assay cells were allowed to adhere for 12 h in alpha MEM and then exposed to different treatment media. Therefore, CFU-F assay measured both plating efficiency and proliferation capacity of media whereas CyQUANT assay only measured proliferation capability. We hypothesized that DMEM/F12 low plating efficiency could explain, at least partially, the contrasting results. To test this we compared plating efficiency of alpha MEM and DMEM/F12 and showed that DMEM/F12 had comparatively lower ability to promote cell attachment in the CyQUANT plating assay. Different cell concentrations between CFU-F (very low cell density) and CyQUANT (high cell density) assays might have also contributed to the differential outcomes observed.

The effect of oxygen tension during culture on colony formation was investigated in the CFU-F assay. Despite the fact that oxygen tension did not affect number and size of the colonies (Fig. 3.4G), we observed darker colonies in cultures maintained under low oxygen tension (5%) suggesting higher number of cells per colony. These results prompted us to study the combined effect of media type and oxygen tension on pMSC proliferation. Since an interaction was not detected, each factor (medium type and oxygen tension) was analyzed separately. pMSCs growing in an atmosphere low in oxygen (5%) proliferated faster than cells cultured in higher oxygen tension (21%), which is consistent with our previous observation of darker pMSC colonies in cultures grown under low

oxygen environment. Culture of cells in reduced oxygen tension has been reported to cause inhibition [41, 42] or stimulation of cell growth in vitro [43-47]. Our results demonstrated increased proliferation rate of pMSCs in an oxygen concentration that more closely mimic the in vivo conditions. Therefore, exogenous control of oxygen tension may have important implications for in vitro propagation of pMSC and possibly differentiation [47, 48].

Ascorbic acid or vitamin C, a primary antioxidant for cells, has been associated with enhancement of cell proliferation [49-51], and it contributes to collagen synthesis in mesenchymal cells [52-54]. One difficulty associated with supplying ascorbate to cultured cells is the instability of this vitamin under standard culture conditions (neutral pH, 37°C, and aerobic environment) resulting from its autoxidation [55]. To overcome this problem we used an esterified ascorbate (ascorbic acid 2-phosphate) which is more resistant to autoxidation [50] and therefore more stable in aqueous solutions. Addition of ascorbic acid to a vitamin C-free medium over a wide range of concentrations (5-500 μg/ml) did not affect proliferation rate of pMSCs. The causes for the lack of effect of ascorbic acid supplementation on pMSC proliferation in our experiment are not readily evident. The amount of free ascorbate available for the cells is dependent upon the rate of conversion of ascorbate 2-phosphate to ascorbate in a given cell type and culture condition. We are unaware of the rate of conversion of ascorbate from its esterified precursor in our culture system; thus, the amount of ascorbic acid readily available for the cells might not have been the optimal. Additionally, we cannot rule out the possibility that the method used to estimate cell numbers in our study was not sensitive enough to detect subtle effects of ascorbic acid on cell proliferation. Impairment of cell proliferation

observed when ascorbate was added at 5,000 μg/ml of medium is consistent with the idea that at high concentrations, ascorbate favors the generation of free radicals, promoting in this way a pro-oxidative rather than an antioxidative state [56].

The recent development of somatic cell nuclear transfer to produce cloned animals has provided a new method to create transgenic livestock that clearly advances previously available technologies like pronuclear microinjection [20]. A particular cell type must satisfy four basic donor cell criteria in order to be considered for SCNT transgenesis, including easily cultured, clonal propagation, amenable to genetic manipulation and nuclear reprogramming. To establish whether pMSCs can be genetically manipulated we performed a series of experiments to address this and compare transfection/transduction efficiencies between pMSCs and isogenic SFs using viral and non-viral methods. Here we have used a recombinant adenoviral vector carrying the eGFP gene to transiently transduce pMSC and SF in combination with flow cytometry analysis to determine expression of the reporter gene. We have shown that both cell types can be infected with the non-integrating human adenovirus vector. Interestingly, a higher proportion of pMSCs expressed GFP compared with isogenic SFs (Fig. 3.5). Likewise, RFI revealed a shift in GFP intensity to the right in pMSCs indicating that a larger number of adenoviral particles entered into these cells compared with that in SFs (Fig. 3.5A). Since adenoviral entry into the host cells is mediated through membrane receptors, particularly CD46 for Ad5F35 [57], it is likely that pMSCs possess higher density of adenoviral receptors, which would explain the higher transduction efficiency. We also observed a higher proportion of non-viable pMSCs (Fig. 3.5A), which could be the result of viral cytopathic effects associated with the higher viral

infection achieved in this group. Nonetheless, overall cell viability was very high (> 95%) in both cell lines. When pMSCs and SFs were transfected with a plasmid containing the eGFP gene, the proportion of cells transiently expressing the transgene at 72 h was not different between cell lines and it was 9-fold lower than the proportions of GFP positive cells achieved with the adenovirus vector. Transient gene transfer into cultured cells with subsequent expression of the transgene has become a valuable tool for physiological studies, functional genomics and gene therapy. Furthermore, ectopic expression of signaling molecules and transcription factors has proven useful to manipulate the differentiation fate of stem cells [58, 59] and might prove valuable to manipulate donor cell function.

For animal transgenesis, stable integration of the gene in the host genome is required. We designed a series of experiments to investigate stable transgene expression in pMSC and SF using a GFP plasmid and a viral vector (AAV). Plasmid integration was confirmed by selection of transformants with the antibiotic G418; approximately 74 GFP positive colonies developed in each 100 mm dish. Since 1.2×10^6 cells per dish were transfected, then approximately 1 in every ~16,000 treated cells integrated the transgene. To further study extended expression of transgenes delivered by a plasmid or AAV vector, transfected/transduced cells were allowed to propagate in vitro; we then used FACS for clonal propagation of GFP positive cells in 96-well plates. At sorting, the percentage of GFP positive cells transduced with AAV vector was double that of cells transfected with the plasmid (Fig. 3.6A). GFP expressing cells from these populations were sorted as single cells in multi-well plates and the total number of colonies and GFP expressing colonies determined after 14 days. Contrasting results in terms of percentage

of GFP positive colonies were obtained between plasmid transfected and AAV transduced cells irrespective of cell line (pMSC or SF). Approximately 8% of colonies were GFP positive in the plasmid transfected group versus ~93% in the AAV vector transduced group. To determine how long these GFP+ cells could maintain transgene expression as a measure of integration, some GFP positive colonies originating from cells transfected with either GFP plasmid or AAV vector were expanded in vitro up to ~ 1 x 10^6 cells without losing GFP expression (~ 21 cell doublings; data not shown). These data along with the well known integrative capacity of AAV vectors indicates stable transgene integration. We demonstrate here that pMSCs can be genetically manipulated, clonally propagated and sustain transgene expression. Furthermore, the AAV vector used in the present study was clearly superior to a conventional GFP plasmid to produce stable integration. Viral vectors have previously been used to create transgenic cell lines that were later used to generate SCNT transgenic embryos [25] and animals [26, 27]. Recently, Hofmann et al. [28] obtained high transduction rates of bovine fibroblasts in culture with a lentivirus vector, and these transgenic cells were able to drive development to term. Therefore the use of integrating viral vectors like lentivirus and AAV is emerging as highly effective alternative method to deliver DNA into cells.

There is some experimental evidence that supports the idea that the nucleus of less differentiated cells may have a configuration that is more amenable to reprogramming upon nuclear transfer into the recipient oocyte (reviewed in [60]). Some studies have reported improved post-implantation survival of clones originated from mouse embryonic stem cells [61, 62] and enhanced in vitro development of preimplantation pig embryos reconstructed with fetal skin-derived stem cells [63].

Bovine MSCs were able to undergo nuclear reprogramming after SCNT and supported development to term [18]; unfortunately embryos reconstructed with other somatic cell types were not included in this study for comparison purposes. Nuclear transfer results from the present study suggest that pMSC can support blastocyst development after being transferred to enucleated MII oocytes. Although both the pMSCs and SFs produced low cleavage and blastocyst rates, this limited study does indicate that pMSCs can undergo nuclear reprogramming at least to support development to the blastocyst stage. Additional replicates will be necessary in order to establish any potential differences in reprogramming ability between cell lines and types. Having shown that pMSC-donor cells can develop into initial SCNT blastocyst stage embryos, future studies might investigate the differentiation potential or differences between pMSC and embryonic cells derived from the same donor cells. In addition, this work can lead to studies that determine the effects of stable (AAV) or transient (Ad5-F35 vector) expression of certain exogenous genes and their effect on nuclear reprogramming.

In conclusion, we have been able to establish adult pMSC lines from live animals using a minimally invasive BM aspiration technique. These adult stem cells can proliferate extensively in vitro (~ 30 cell doublings until senescence; data not shown) and undergo transient and stable genetic modification with non-viral and viral vectors. Of particular interest is the highly efficient transduction of MSCs with a non-integrating human adenovirus and AAV vectors. All these characteristics along with favorable clonal cell propagation properties make pMSCs an attractive source of cells for large animal preclinical testing. These significant findings will lead to future autologous cell/gene therapy studies comparing easily cultured genetically modified adult pMSC to isogenic

embryonic cells derived via SCNT, thus addressing cell rejection issues in non murine models for disease and tissue repair.

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REFERENCES

1. Friedenstein AJ, Chailakhyan RK, Latsinik NV, Panasyuk AF, Keiliss-Borok IV. Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. Transplantation 1974; 17: 331-340.
2. Beresford JN. Osteogenic stem cells and the stromal system of bone and marrow. Clin Orthop Relat Res 1989: 270-280.
3. Owen M. Marrow stromal stem cells. J Cell Sci Suppl 1988; 10: 63-76.
4. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science 1999; 284: 143-147.
5. Kohyama J, Abe H, Shimazaki T, Koizumi A, Nakashima K, Gojo S, Taga T, Okano H, Hata J, Umezawa A. Brain from bone: efficient "meta-differentiation" of marrow stroma-derived mature osteoblasts to neurons with Noggin or a demethylating agent. Differentiation 2001; 68: 235-244.
6. Woodbury D, Schwarz EJ, Prockop DJ, Black IB. Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res 2000; 61: 364-370.

7. Wakitani S, Saito T, Caplan AI. Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. Muscle Nerve 1995; 18: 1417-1426.

8. Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, Sano M, Takahashi T, Hori S, Abe H, Hata J, Umezawa A, Ogawa S. Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest 1999; 103: 697-705.

9. Friedenstein AJ, Gorskaja JF, Kulagina NN. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp Hematol 1976; 4: 267-274.

10. Simmons DJ, Seitz P, Kidder L, Klein GL, Waeltz M, Gundberg CM, Tabuchi C, Yang C, Zhang RW. Partial characterization of rat marrow stromal cells. Calcif Tissue Int 1991; 48: 326-334.

11. Martin DR, Cox NR, Hathcock TL, Niemeyer GP, Baker HJ. Isolation and characterization of multipotential mesenchymal stem cells from feline bone marrow. Exp Hematol 2002; 30: 879-886.

12. Huss R, Hoy CA, Deeg HJ. Contact- and growth factor-dependent survival in a canine marrow-derived stromal cell line. Blood 1995; 85: 2414-2421.

13. Ringe J, Kaps C, Schmitt B, Buscher K, Bartel J, Smolian H, Schultz O, Burmester GR, Haupl T, Sittinger M. Porcine mesenchymal stem cells. Induction of distinct mesenchymal cell lineages. Cell Tissue Res 2002; 307: 321-327.

14. Chamberlain JR, Schwarze U, Wang PR, Hirata RK, Hankenson KD, Pace JM, Underwood RA, Song KM, Sussman M, Byers PH, Russell DW. Gene targeting in stem cells from individuals with osteogenesis imperfecta. Science 2004; 303: 1198-1201.

15. Horwitz EM, Prockop DJ, Fitzpatrick LA, Koo WW, Gordon PL, Neel M, Sussman M, Orchard P, Marx JC, Pyeritz RE, Brenner MK. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. Nat Med 1999; 5: 309-313.

16. Studeny M, Marini FC, Champlin RE, Zompetta C, Fidler IJ, Andreeff M. Bone marrow-derived mesenchymal stem cells as vehicles for interferon-beta delivery into tumors. Cancer Res 2002; 62: 3603-3608.

17. Nakamizo A, Marini F, Amano T, Khan A, Studeny M, Gumin J, Chen J, Hentschel S, Vecil G, Dembinski J, Andreeff M, Lang FF. Human bone marrow-derived mesenchymal stem cells in the treatment of gliomas. Cancer Res 2005; 65: 3307-3318.

18. Kato Y, Imabayashi H, Mori T, Tani T, Taniguchi M, Higashi M, Matsumoto M, Umezawa A, Tsunoda Y. Nuclear transfer of adult bone marrow mesenchymal stem cells: developmental totipotency of tissue-specific stem cells from an adult mammal. Biol Reprod 2004; 70: 415-418.

19. Denning C, Priddle H. New frontiers in gene targeting and cloning: success, application and challenges in domestic animals and human embryonic stem cells. Reproduction 2003; 126: 1-11.

20. Bosch P, Hodges CA, Stice SL. Generation of transgenic livestock by somatic cell nuclear transfer. Biotecnologia Aplicada 2004; 21: 128-136.

21. Arat S, Gibbons J, Rzucidlo SJ, Respess DS, Tumlin M, Stice SL. In vitro development of bovine nuclear transfer embryos from transgenic clonal lines of adult and fetal fibroblast cells of the same genotype. Biol Reprod 2002; 66: 1768-1774.

22. Arat S, Rzucidlo SJ, Gibbons J, Miyoshi K, Stice SL. Production of transgenic bovine embryos by transfer of transfected granulosa cells into enucleated oocytes. Mol Reprod Dev 2001; 60: 20-26.

23. Lee GS, Kim HS, Hyun SH, Lee SH, Jeon HY, Nam DH, Jeong YW, Kim S, Kim JH, Han JY, Ahn C, Kang SK, Lee BC, Hwang WS. Production of transgenic cloned piglets from genetically transformed fetal fibroblasts selected by green fluorescent protein. Theriogenology 2005; 63: 973-991.

24. Gong G, Dai Y, Fan B, Zhu H, Zhu S, Wang H, Wang L, Tang B, Li R, Wan R, Liu Y, Huang Y, Zhang L, Sun X, Li N. Birth of calves expressing the enhanced

green fluorescent protein after transfer of fresh or vitrified/thawed blastocysts produced by somatic cell nuclear transfer. Mol Reprod Dev 2004; 69: 278-288.

25. Uhm SJ, Kim NH, Kim T, Chung HM, Chung KH, Lee HT, Chung KS. Expression of enhanced green fluorescent protein (EGFP) and neomycin resistant (Neo(R)) genes in porcine embryos following nuclear transfer with porcine fetal fibroblasts transfected by retrovirus vector. Mol Reprod Dev 2000; 57: 331-337.

26. Park KW, Cheong HT, Lai L, Im GS, Kuhholzer B, Bonk A, Samuel M, Rieke A, Day BN, Murphy CN, Carter DB, Prather RS. Production of nuclear transfer-derived swine that express the enhanced green fluorescent protein. Anim Biotechnol 2001; 12: 173-181.

27. Lai L, Park KW, Cheong HT, Kuhholzer B, Samuel M, Bonk A, Im GS, Rieke A, Day BN, Murphy CN, Carter DB, Prather RS. Transgenic pig expressing the enhanced green fluorescent protein produced by nuclear transfer using colchicine-treated fibroblasts as donor cells. Mol Reprod Dev 2002; 62: 300-306.

28. Hofmann A, Zakhartchenko V, Weppert M, Sebald H, Wenigerkind H, Brem G, Wolf E, Pfeifer A. Generation of transgenic cattle by lentiviral gene transfer into oocytes. Biol Reprod 2004; 71: 405-409.

29. Hirata R, Chamberlain J, Dong R, Russell DW. Targeted transgene insertion into human chromosomes by adeno-associated virus vectors. Nat Biotechnol 2002; 20: 735-738.

30. Hirata RK, Xu C, Dong R, Miller DG, Ferguson S, Russell DW. Efficient PRNP gene targeting in bovine fibroblasts by adeno-associated virus vectors. Cloning Stem Cells 2004; 6: 31-36.

31. Mitani K, Wakamiya M, Hasty P, Graham FL, Bradley A, Caskey CT. Gene targeting in mouse embryonic stem cells with an adenoviral vector. Somat Cell Mol Genet 1995; 21: 221-231.

32. Xia H, Mao Q, Paulson HL, Davidson BL. siRNA-mediated gene silencing in vitro and in vivo. Nat Biotechnol 2002; 20: 1006-1010.

33. Mackay AM, Beck SC, Murphy JM, Barry FP, Chichester CO, Pittenger MF. Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. Tissue Eng 1998; 4: 415-428.

34. Gibbons J, Arat S, Rzucidlo J, Miyoshi K, Waltenburg R, Respess D, Venable A, Stice S. Enhanced survivability of cloned calves derived from roscovitine- treated adult somatic cells. Biol Reprod 2002; 66: 895-900.

35. SAS. SAS User's Guide: Statistics. Cary, NC: SAS Inst. Inc.; 1985.

36. Friedenstein AJ, Deriglasova UF, Kulagina NN, Panasuk AF, Rudakowa SF, Luria EA, Ruadkow IA. Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp Hematol 1974; 2: 83-92.

37. Friedenstein AJ, Chailakhjan RK, Lalykina KS. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet 1970; 3: 393-403.

38. Kassem M. Mesenchymal stem cells: biological characteristics and potential clinical applications. Cloning Stem Cells 2004; 6: 369-374.

39. Mazzone A, Ricevuti G. Leukocyte CD11/CD18 integrins: biological and clinical relevance. Haematologica 1995; 80: 161-175.

40. Castro-Malaspina H, Gay RE, Resnick G, Kapoor N, Meyers P, Chiarieri D, McKenzie S, Broxmeyer HE, Moore MA. Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny. Blood 1980; 56: 289-301.

41. Rempel D, Abrahamsson SO. The effects of reduced oxygen tension on cell proliferation and matrix synthesis in synovium and tendon explants from the rabbit carpal tunnel: an experimental study in vitro. J Orthop Res 2001; 19: 143-148.

42. Wang DW, Fermor B, Gimble JM, Awad HA, Guilak F. Influence of oxygen on the proliferation and metabolism of adipose derived adult stem cells. J Cell Physiol 2005; 204: 184-191.

43. Studer L, Csete M, Lee SH, Kabbani N, Walikonis J, Wold B, McKay R. Enhanced proliferation, survival, and dopaminergic differentiation of CNS precursors in lowered oxygen. J Neurosci 2000; 20: 7377-7383.

44. Bradley TR, Hodgson GS, Rosendaal M. The effect of oxygen tension on haemopoietic and fibroblast cell proliferation in vitro. J Cell Physiol 1978; 97: 517-522.

45. Taylor WG, Richter A, Evans VJ, Sanford KK. Influence of oxygen and pH on plating efficiency and colony development of WI-38 and Vero cells. Exp Cell Res 1974; 86: 152-156.

46. Nevo Z, Beit-Or A, Eilam Y. Slowing down aging of cultured embryonal chick chondrocytes by maintenance under lowered oxygen tension. Mech Ageing Dev 1988; 45: 157-165.

47. Lennon DP, Edmison JM, Caplan AI. Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on in vitro and in vivo osteochondrogenesis. J Cell Physiol 2001; 187: 345-355.

48. Fink T, Abildtrup L, Fogd K, Abdallah BM, Kassem M, Ebbesen P, Zachar V. Induction of adipocyte-like phenotype in human mesenchymal stem cells by hypoxia. Stem Cells 2004; 22: 1346-1355.

49. Ivanov VO, Ivanova SV, Niedzwiecki A. Ascorbate affects proliferation of guinea-pig vascular smooth muscle cells by direct and extracellular matrix-mediated effects. J Mol Cell Cardiol 1997; 29: 3293-3303.

50. Hata R, Senoo H. L-ascorbic acid 2-phosphate stimulates collagen accumulation, cell proliferation, and formation of a three-dimensional tissuelike substance by skin fibroblasts. J Cell Physiol 1989; 138: 8-16.

51. Chepda T, Cadau M, Girin P, Frey J, Chamson A. Monitoring of ascorbate at a constant rate in cell culture: effect on cell growth. In Vitro Cell Dev Biol Anim 2001; 37: 26-30.

52. Kurata S, Senoo H, Hata R. Transcriptional activation of type I collagen genes by ascorbic acid 2-phosphate in human skin fibroblasts and its failure in cells from a

patient with alpha 2(I)-chain-defective Ehlers-Danlos syndrome. Exp Cell Res 1993; 206: 63-71.

53. Phillips CL, Tajima S, Pinnell SR. Ascorbic acid and transforming growth factor-beta 1 increase collagen biosynthesis via different mechanisms: coordinate regulation of pro alpha 1(I) and Pro alpha 1(III) collagens. Arch Biochem Biophys 1992; 295: 397-403.

54. Ono M, Aratani Y, Kitagawa I, Kitagawa Y. Ascorbic acid phosphate stimulates type IV collagen synthesis and accelerates adipose conversion of 3T3-L1 cells. Exp Cell Res 1990; 187: 309-314.

55. Buettner GR. Ascorbate autoxidation in the presence of iron and copper chelates. Free Radic Res Commun 1986; 1: 349-353.

56. Buettner GR, Jurkiewicz BA. Catalytic metals, ascorbate and free radicals: combinations to avoid. Radiat Res 1996; 145: 532-541.

57. Gaggar A, Shayakhmetov DM, Lieber A. CD46 is a cellular receptor for group B adenoviruses. Nat Med 2003; 9: 1408-1412.

58. Ishizaka S, Shiroi A, Kanda S, Yoshikawa M, Tsujinoue H, Kuriyama S, Hasuma T, Nakatani K, Takahashi K. Development of hepatocytes from ES cells after transfection with the HNF-3beta gene. Faseb J 2002; 16: 1444-1446.

59. Gratsch TE, O'Shea KS. Noggin and chordin have distinct activities in promoting lineage commitment of mouse embryonic stem (ES) cells. Dev Biol 2002; 245: 83-94.

60. Rideout WM, 3rd, Eggan K, Jaenisch R. Nuclear cloning and epigenetic reprogramming of the genome. Science 2001; 293: 1093-1098.

61. Eggan K, Akutsu H, Rideout III W, Wakayama T, Wutz A, Jackson-Grusby L, Yanagimachi R, Jaenisch R. Nuclear transfer in the laboratory mouse: parameters affecting long term survival of clones. Biol Reprod 2000; 62: 84.

62. Rideout WM, 3rd, Wakayama T, Wutz A, Eggan K, Jackson-Grusby L, Dausman J, Yanagimachi R, Jaenisch R. Generation of mice from wild-type and targeted ES cells by nuclear cloning. Nat Genet 2000; 24: 109-110.

63. Zhu H, Craig JA, Dyce PW, Sunnen N, Li J. Embryos derived from porcine skin-derived stem cells exhibit enhanced preimplantation development. Biol Reprod 2004; 71: 1890-1897.

TABLE 3.1. Composition of differentiation media and methods used to assess lineage differentiation.

Lineage	Differentiation medium composition	Assessment of differentiation
Lipogenic	Induction: High glucose alpha MEM supplemented with ITS+1 (Sigma), sodium pyruvate (10 mM), methyl isobutylxanthine (0.5 mM) and dexamethasone (1μM). Maintenance: High glucose alpha MEM supplemented with ITS+1 (Sigma) and sodium piruvate (10 mM)	Oil Red O stain: lipid accumulation
Chondrogenic	Alpha MEM supplemented with pTGF-β1 (10 ng/ml), dexamethasone (100 nM), ascorbic acid 2-phosphate (50 μg/ml), thyroxine (50 ng/ml) and ITS+1 (Sigma)	Alcian blue stain: acidic mucopolysaccharides
Osteogenic	Alpha MEM supplemented with 10% FBS, dexamethasone (10^{-8}M), ascorbic acid (50 μg/ml), and β-glycerophosphate (10 mM).	von Kossa stain: phosphates and carbonates. Alkaline phosphatase (AP) activity

FIG 3.1. Morphology of adherent fibroblast-like cells, later identified as pMSCs, isolated from pig bone marrow after 14 days from initial plating (A). Same cell line at higher magnification showing detailed fibroblastic morphology of pMSCs (B). Bar = 200 μm (A) and 100 μm (B).

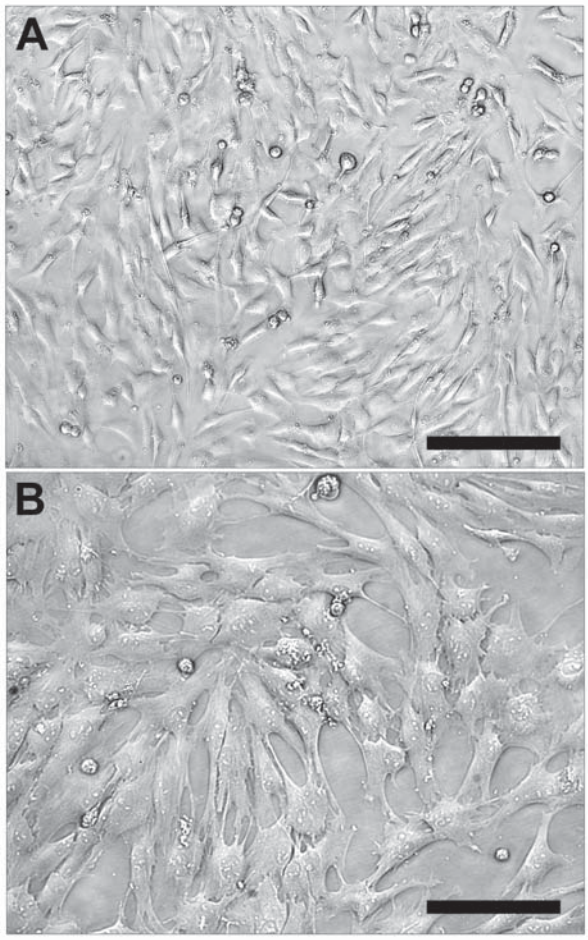

A
B

FIG 3.2. Immunofluorescence and flow cytometry for the surface markers CD90 and CD11b in pMSCs. Specific imunoreactivity for CD90 was observed in pMSCs growing on chamber slides (A). CD11b immunoreactivity was absent in pMSC monolayers (B). (Green: Immunoreactive CD90; Blue: DAPI). These results were later confirmed by flow cytometry (C, D). pMSC suspension was fixed and immunostainied for CD90 (C) or CD11b expression (D). Histograms show frequency distribution and fluorescence intensity data. Blue curves represent the distribution of cells incubated with anti-CD90 or CD11d primary antibody, whereas red curves represent the distribution of cells incubated with the immunoglobulin isotype control. In histogram (C), from a representative pMSC line, 99.6% of total cells were positive for CD90. In (D) CD11b (blue) and isotype (red) curves overlap, indicative of absence of imunoreactive sites for CD11b on pMSC. Bar = 100 μm.

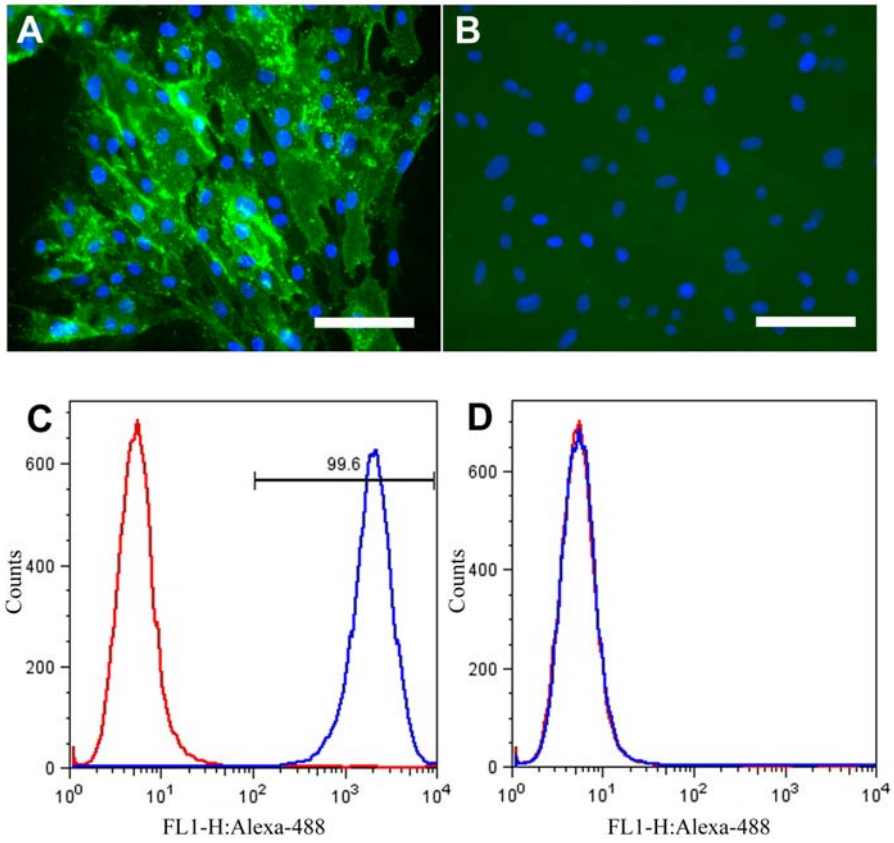

A
B
C
D
99.6
600
400
200
0
Counts
10^0
10^1
10^2
10^3
10^4
FL1-H:Alexa-488

FIG 3.3. Histochemical stains of SFs and pMSCs exposed to lipogenic, chondrogenic, osteogenic or control media. pMSCs underwent lipogenic (A), chondrogenic (D) and osteogenic differentiation when exposed to specific induction media. pMSCs cultured in control medium (B, E and H) and isogenic SFs (C, F and I) exposed to differentiation conditions failed to differentiate. Bar = 100 μm (A-C) and 0.5 mm (D-I).

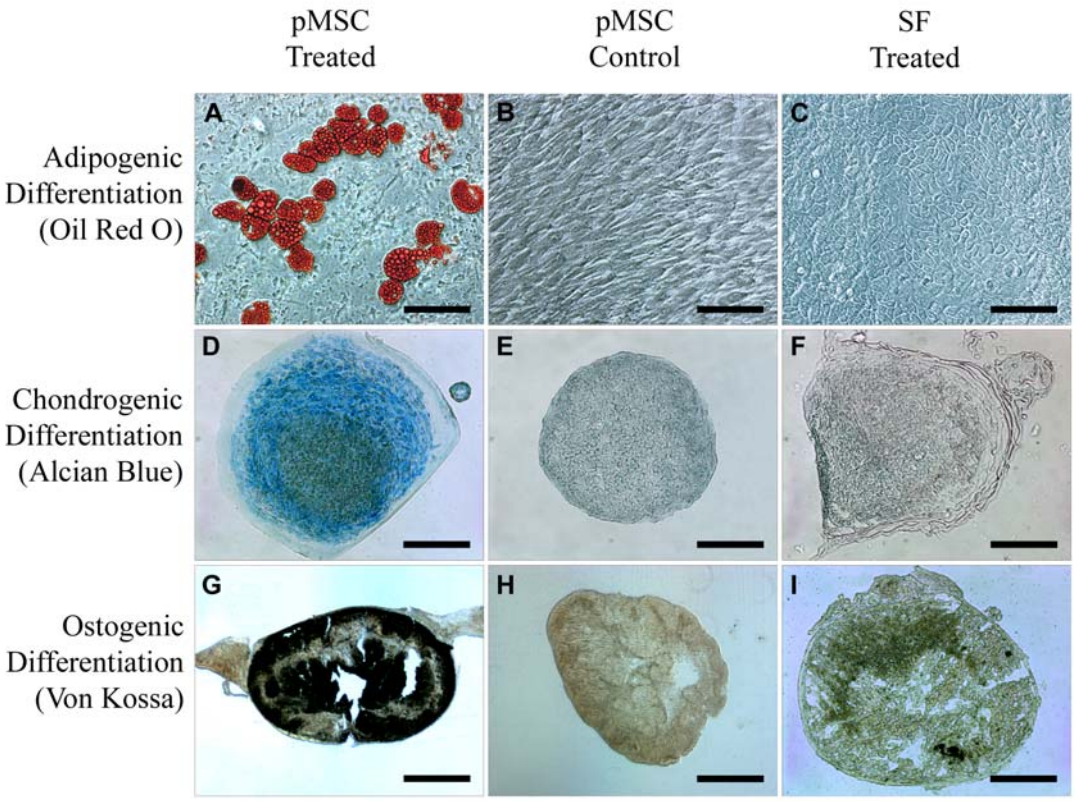

pMSC
Treated
pMSC
Control
SF
Treated
Adipogenic
Differentiation
(Oil Red O)
Chondrogenic
Differentiation
(Alcian Blue)
Ostogenic
Differentiation
(Von Kossa)
A
B
C
D
E
F
G
H
I

FIG 3.4. Effect of culture conditions on proliferation of pMSCs as determined by CFU-F or CyQUANT assays. Effect of supplementation of culture media with different FBS concentrations on the number of colonies (A) and colony diameter (B). Response of pMSCs to medium type in the CFU-F (C, E) and CyQUANT proliferation assays (D). Effect of increasing concentrations of ascorbic acid on proliferation of pMSCs in the CyQUANT assay (F). Effect of low or high oxygen tension on colony formation in the CFU-F assay (G) and proliferation in the CyQUANT assay (H). Values are the mean ± SEM of at least 4 independent replicates. Bars with different letters are statistically different at $P<0.05$; ANOVA followed by LSD.

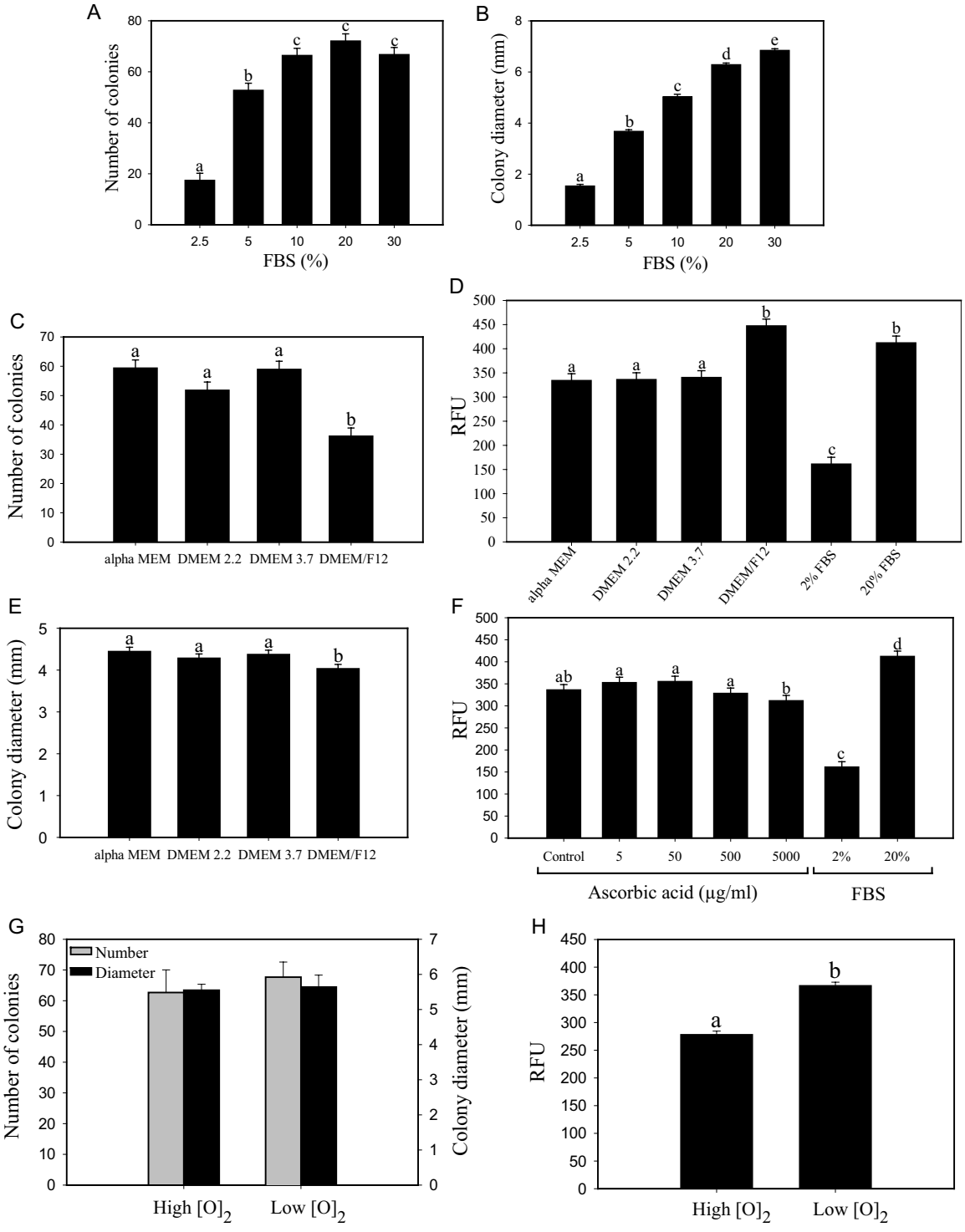

A
Number of colonies
FBS (%)
2.5
5
10
20
30
B
Colony diameter (mm)
FBS (%)
C
Number of colonies
alpha MEM
DMEM 2.2
DMEM 3.7
DMEM/F12
D
RFU
alpha MEM
DMEM 2.2
DMEM 3.7
DMEM/F12
2% FBS
20% FBS
E
Colony diameter (mm)
alpha MEM
DMEM 2.2
DMEM 3.7
DMEM/F12
F
RFU
Control
5
50
500
5000
2%
20%
Ascorbic acid (µg/ml)
FBS
G
Number
Diameter
Number of colonies
Colony diameter (mm)
High [O]2
Low [O]2
H
RFU
High [O]2
Low [O]2

FIG 3.5. Transient transduction/transfection of SFs and pMSCs with a human adenovirus (Ad5F35-eGFP) or a plasmid carrying the EGFP gene. SFs and pMSCs were transfected with Ad5F35-eGFP and after 24 h characterized by flow cytometry. Percentage of GFP positive, mean fluorescence intensity and proportions of viable cells (A) were estimated from the flow cytometry data. Representative dot plots of transduced isogenic SFs (B) and pMSCs (C) showing distribution of cell populations based on GFP intensity (X axis) and PI staining (Y axis). Very low proportions of cells are non-viable (top left quadrants in B and C). A larger percentage of cells are viable and GFP positive in pMSCs (74.6%, bottom right quadrant in C) compared with that in SFs (44.1%, bottom right quadrant in B). SFs and pMSCs were transfected with GFP plasmid and characterized by flow cytometry 72 h after transfection (D). Percentage of GFP positive, non-viable cells were estimated from the flow cytometry data. Photomicrographs of SFs (E) and pMSCs (F) 24 h after transduction with Ad5F35-eGFP. Different symbols within each variable denotes significant difference at $P<0.05$; Student *t*-test. Bar = 200 μm.

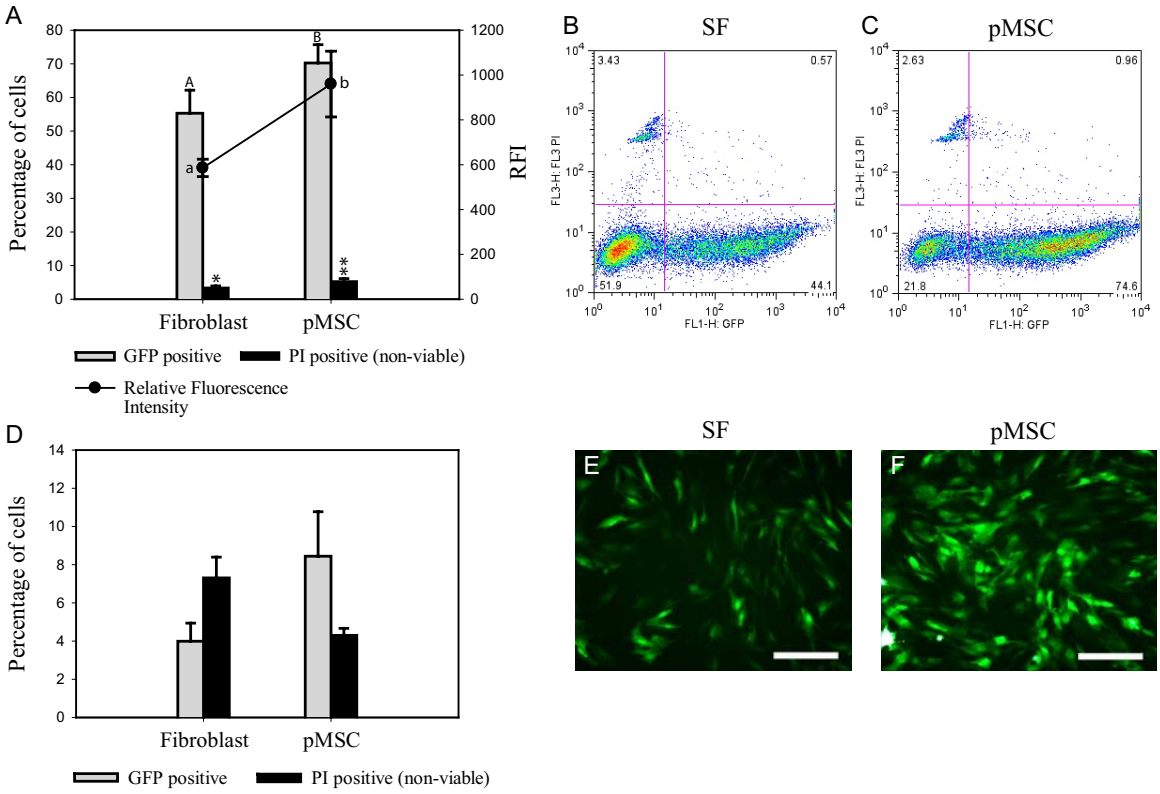
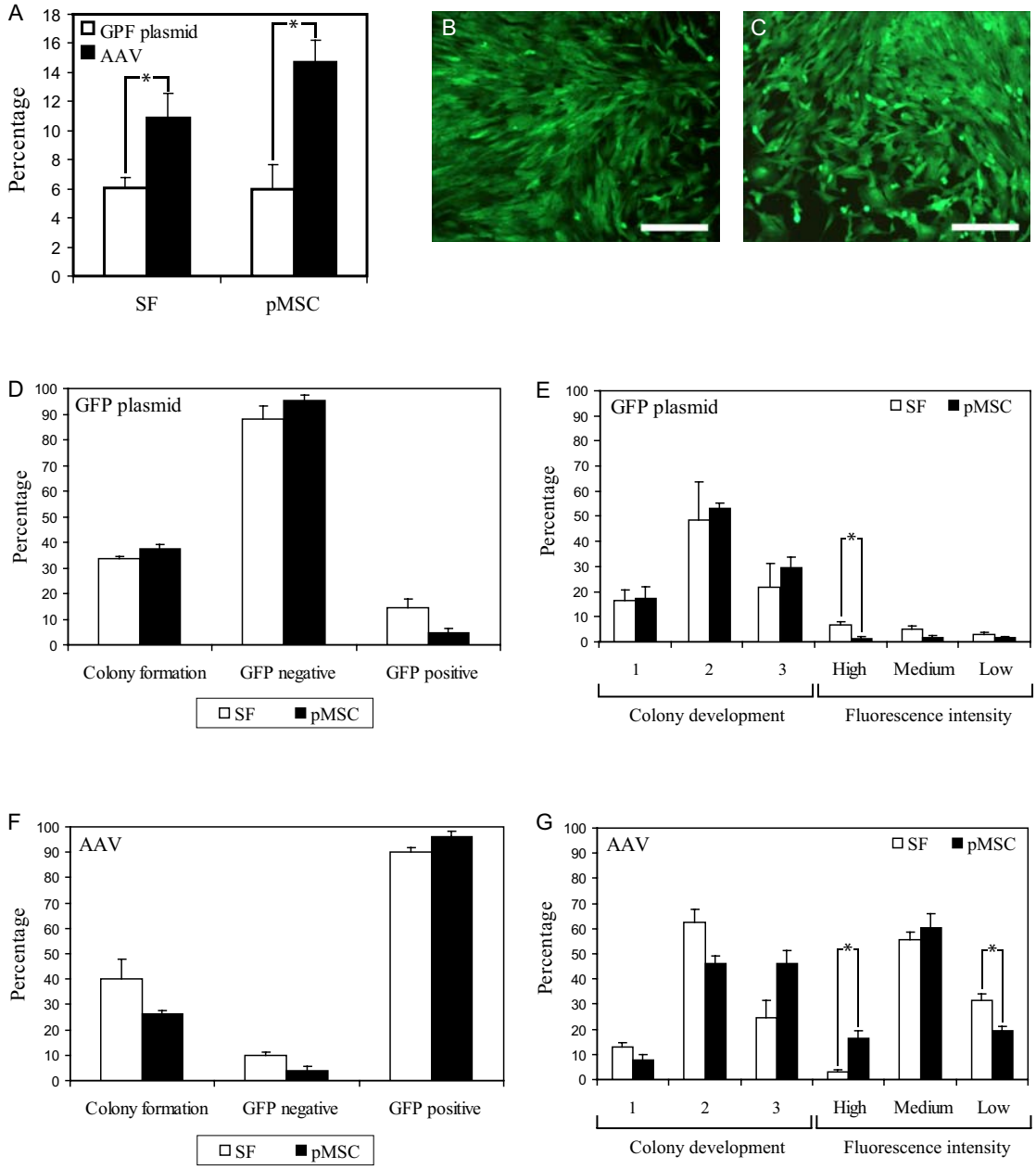

A
Percentage of cells
RFI
Fibroblast
pMSC
GFP positive
PI positive (non-viable)
Relative Fluorescence Intensity
B
SF
C
pMSC
FL3-H: FL3 PI
FL1-H: GFP
3.43
0.57
51.9
44.1
2.63
0.96
21.8
74.6
D
Percentage of cells
Fibroblast
pMSC
GFP positive
PI positive (non-viable)
SF
pMSC
E
F

FIG. 3.6. Stable transformation of SFs and pMSCs with a GFP plasmid or an AAV vector. SFs and pMSCs were transfected with a GFP plasmid or AAV, expanded in vitro for 9-10 days, and sorted as single GFP positive cells by flow cytometry. Percentage of GFP positive SF and pMSC transfected/transduced cells after propagation in vitro (A). GFP expressing cells sorted in 96-well plates were cultured in vitro for 14 days and the percentage of colonies, GFP negative and GFP positive colonies were determined for cells transfected with GFP plasmid (D) or AAV (F). Colonies were classified according to degree of development (1, 2 or 3) and fluorescence intensity (high, medium or low) (E, G). Photomicrographs of GFP positive pMSC colonies generated from a single cell transfected either with GFP plasmid (B) or AAV (C). *$P<0.05$; Student *t*-test. Bar = 200 μm.


A
GPF plasmid
AAV
Percentage
SF
pMSC
B
C
D
GFP plasmid
Percentage
Colony formation
GFP negative
GFP positive
SF
pMSC
E
GFP plasmid
SF
pMSC
Percentage
1
2
3
High
Medium
Low
Colony development
Fluorescence intensity
F
AAV
Percentage
Colony formation
GFP negative
GFP positive
SF
pMSC
G
AAV
SF
pMSC
Percentage
1
2
3
High
Medium
Low
Colony development
Fluorescence intensity

CHAPTER 4

EFFICIENT ADENOVIRAL MEDIATED GENE DELIVERY INTO PORCINE MESENCHYMAL STEM CELLS[1]

[1] Pablo Bosch, Christine Fouletier-Dilling, Elizabeth A. Olmsted-Davis, Alan R. Davis and Steven L. Stice, To be submitted to *Experimental Hematology*

ABSTRACT

Mesenchymal stem cell-mediated gene therapy research has been predominantly conducted on rodent disease and injury models. Appropriate scaled up large animal models may provide additional safety and efficacy information prior to human clinical trials. Here we report for the first time efficient transduction of porcine bone marrow derived mesenchymal stem cells (MSCs) (~90% of the cells expressing the transgene) while retaining their ability to differentiate in vitro into mesodermal lineages. Porcine MSCs (pMSC) were infected under different experimental conditions with replication-defective adenoviral vectors carrying the GFP gene and later transgene expression analyzed by flow cytometry. There was a 5.5-fold increase in the percentage of GFP expressing cells when we delivered adenovirus type 5 that contains an adenovirus type 35 fiber (Ad5F35eGFP) in presence of GeneJammer. Transduction of pMSC with 1,500-2,000 vp/cells in presence of GeneJammer yielded the highest percentage of GFP expressing cells (~90%) without affecting cell viability. Transduction enhancement mediated by GeneJammer was not dependent on adenovirus type 35 fiber protein, since a similar positive effect was detected when pMSCs were infected with an Ad5 vector. Presence of fetal bovine serum (FBS) during adenoviral transduction enhanced vector-encoded transgene expression in both GeneJammer-treated and control groups. Complexing Ad5F35 vector with the cationic lipid Lipofectamine augmented transgene expression at levels similar to GeneJammer; however, Lipofectamine-transduced cells exhibited increased side-scatter intensity values suggesting cellular toxicity. MSCs transduced with adenovirus vector in presence of GeneJammer underwent lipogenic, chondrogenic and osteogenic differentiation. Thus, addition of GeneJammer during

adenoviral infection of pMSCs can revert the poor transduction efficiency of pMSCs while retaining their pluripotent differentiation capacity. GeneJammer enhanced transduction will facilitate the use of adenoviral vectors in MSC-mediated gene therapy models and therapies.

INTRODUCTION

Several studies have demonstrated that bone marrow mesenchymal stem cells (MSC), also referred as marrow stromal cells or colony forming unit fibroblastic cells, can be easily isolated and cultured in vitro, proliferate extensively ex vivo to originate large cell populations [1,2] and are endowed with the capacity to differentiate into mesodermal [2] and non-mesodermal [3,4] cell types. All of these characteristics make these stromal precursors attractive candidates for development of autologous cell-based therapeutic approaches for treatment of various human diseases. Of particular interest is the use of intact or genetically engineered MSCs for the treatment of skeletal disorders like osteogenesis imperfecta [5,6]. One approach envisions the use of genetically modified MSCs as targeted delivery vehicles for various therapeutic molecules. For instance, a MSC delivery system that can produce high levels of active bone morphogenetic protein 2 (BMP2) and induce bone formation in vivo [7-10] may prove to be of use for treatment of several skeletal injuries and diseases. A therapeutic strategy that have attracted much attention is the use of ex vivo expanded, genetically modified MSCs as cellular vehicles to locally deliver therapeutic gene products such as anticancer agents in tumors [11,12]. Ideally, MSCs for these applications would be readily obtainable from the patient own bone marrow which would avoid immunological rejection associated with allogeneic transplantation. The clinical application of gene

therapy approaches will depend greatly on efficient delivery of the gene of interest into the cells. Therefore, a species independent approach of gene delivery will be useful for development of animal models. Furthermore, a scaled up cell production and delivery into a large animal model in which cell doses could be 100X greater than that used in the mouse, will undoubtedly more closely mimic what will be used in human trials. In this context, pigs are regarded as a particularly suited animal model due to their similar organ physiology, size, and genomic organization [13,14] with humans.

Adenovirus are particularly attractive vectors for ex vivo gene transfer due to their ability to infect a wide range of cell types including quiescent cells, accommodate large pieces of exogenous DNA, and the possibility of production of stocks with high viral titers [15]. Since adenovirus very rarely integrates into the host genome by non-homologous recombination, replication-defective recombinant adenoviral vectors are efficient transient expression vectors.

A drawback to adenovirus-mediated gene deliver is the low transduction efficiency in some cell types, due in part to limited availability of specific viral receptors on these cells. Adenovirus serotype 5-based vectors cell entry is mediated through a receptor mediated biphasic process. First the vector recognizes the target cells through attachment of the knob domain of the viral fiber capsid protein to the cellular coxsackie and adenovirus receptor (CAR) [16]. The second step involves the internalization of the vector particle via interaction of the caspid penton protein whit $\alpha_V\beta$ integrins present in target cells [17]. Despite the broad adenoviral tropism, many cell types are refractory to adenoviral infection due the lack or low expression adenovirus specific receptors. Therefore, infectivity of a particular cell type depends largely on the density of CAR and

integrins [18,19]. For instance, adenoviral receptors are expressed at low levels in primitive hematopoietic stem cells [20,21] and human MSCs [7,22] leading to poor transduction efficiency by type 5 adenovirus vectors. Consequently, several approaches have been undertaken in order to circumvent this problem. The use of fiber-modified adenovectors or Ad5 vectors possessing fiber proteins from a different adenovirus serotype has become an increasingly popular approach to achieve infection of cells. One of such chimeric vectors is the Ad5/F35 in which the Ad5 fiber has been replaced by the fiber of Ad35 serotype. In this way, the tropism of Ad5 can be retargeted to that of Ad35 which utilized cofactor protein CD46 as cellular receptor [23] and thereby can infect target cells such as human primitive hematopoietic cells [24] and human MSCs [7]. However, CD46 appear to be preferentially expressed in eyes and testes in rodent species [25-27] limiting the versatility of chimeric Ad5/F35 vectors in this species.

Species specificity of receptor-mediated adenoviral vector gene therapy may pose problems in translating results from one animal model to the next and eventually to humans. A receptor independent mechanism of delivery might eliminate this source of variability. Combining various compounds with adenovirus has been reported to enhance transduction of target cells likely via a receptor independent pathway. Polycations and cationic lipids form complexes with adenoviral particles facilitating in vitro transduction of refractory tumor cell lines [28,29] and primary and established cell lines [30-33]. There is one report of adenoviral infection of primitive human hematopoietic cells using cationic lipids [34].

Here we demonstrate for the first time that the polyamine-based transfection reagent GeneJammer (Stratagene, La Jolla, CA, USA) significantly improves Ad5F35

and Ad5 vector-mediated gene transduction in cultured pMSCs (~90% infected cells) without a significant reduction of cell viability in contrast to reduced cell viability observed when a cationic lipid was used. We further demonstrate that in vitro multipotential differentiation capability of adenoviral transduced pMSCs is not compromised by the treatment.

MATERIALS AND METHODS

Isolation and culture of pMSCs

Mesenchymal stem cells were isolated from bone marrow (BM) aspirates collected from anesthetized gilts. Mononuclear fraction was obtained by centrifugation of BM aspirates through a solution of polysucrose and sodium diatrizoate (Histopaque®, density: 1.077; Sigma Chemical, St Louis, MO). Mononuclear cells were plated on plastic flasks at a density of 300,000-600,000 cells/cm^2 in αMEM medium with 10% fetal bovine serum (FBS). After 24 h, unattached cells were washed off the flask during medium exchange. Colonies of adherent cells with a fibroblastic morphology were evident by day 4-5 after initial plating. Adherent fibroblast-like cells were allowed to grow for 10-14 days with media replacement every 3rd day. Cells were passaged at 80-90% confluence by trypsinization (0.25% trypsin-EDTA solution, Sigma, St. Louis. MO, USA), and reseeded at a density of 5,000-6,000 cells/cm^2 in plastic flasks. The MSC identity of adherent cells isolated from pig BM was established by cell surface markers (CD90 positive and CD11b negative) and the ability of these cells to undergo osteogenic, lipogenic or chondrogenic differentiation when exposed to specific media (data not shown).

Expression of CD46 in pMSC by flow cytometry

Expression of CD46 was performed by immunostaining of pMSC followed by flow cytometry analysis. Passage 2-3 pMSC cultures were trypsinized, washed in D-PBS and fixed with 2% formaldehyde solution for 3 minutes. Non-specific antibody binding was prevented by incubating the cells in 3% goat serum for 30 min. Then, cells were incubated in 15 μg/ml of the primary antibody anti-CD46 (Clone JM6C11, BD Biosciences, Pharmingen, San Jose, CA, USA) or isotype control (Mouse IgG_1, clone: MOPC-31C, BD Biosciences, Pharmingen, San Jose, CA, USA) for 45 min at room temperature. After washing, cells were incubated with a 1:300 dilution of Alexa Fluor® 488 goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA). Fluorescent cell analysis was performed with FACSCalibur cytometer (Becton Dickinson Immunocytometry System, San Jose, CA, USA) and data analyzed by FlowJo software (Tree Star, Inc, Ashland, OR, USA).

Adenovirus vectors

Replication defective E1-E3 deleted first generation human type 5 adenovirus (Ad5) and/or modified forms in which the normal fiber protein has been substituted for the human adenovirus type 35 fiber (Ad5F35) were constructed to contain the GFP gene in the E1 region of the virus [35] under the control of the immediate-early promoter from cytomegalovirus (CMV). The virus particles to plaque forming units are 5×10^{12} particles/mL to 3.45×10^{10} pfu/mL for Ad5F35eGFP and 5×10^{12} particles/mL to 3×10^{10} pfu/mL for Ad5eGFP. Viruses were negative for replication competent adenovirus.

Viral transduction

Passage 2-3 BM pMSCs were seeded at a density of 43,000 cells/cm^2 in 12-well plates (150,500 cells/well). Twenty-four hours after plating, MSC cultures were incubated for 5.30 h in 500 μL of medium containing adenovirus under different experimental conditions as described in Experimental Design.

Flow cytometry

Percentage of GFP positive cells, relative fluorescence intensity (RFI) of the GFP positive cell population and cell viability by exclusion of Propidium Iodide (50 μ/mL; Roche) was determined 24 h after viral exposure by flow cytometric analysis using a FACSCalibur cytometer (Becton Dickinson, San Jose, CA) and FlowJo software.

In vitro differentiation of pMSCs

pMSC cultures were induced to differentiate to lipogenic, chondrogenic or osteogenic lineages as previously described [2]. pMSCs were cultured under specific differentiation conditions for 14-20 days and differentiation assessed by histochemical stains. Lipogenic and osteogenic induction was applied to cells growing in monolayers. Chondrogenic and osteogenic differentiation was induced on cells masses as previously described in human [36] and porcine MSC [37]. Briefly, aliquots of 200,000 cells were distributed in 15-ml conical tubes and centrifuged 5 min at 600g. Sedimented cells were cultured in the tubes with loosened caps to allow gas exchange. Cells formed a spherical mass on the bottom of the tube by 24 h of culture. Composition of chondrogenic differentiation media was: Alpha MEM supplemented with pTGF-β1 (10 ng/mL), dexamethasone (100 nM), ascorbic acid 2-phosphate (50 μg/mL), thyroxine (50 ng/mL) and ITS+1 (Sigma). Differentiation media were replaced every 3-4 days. Lipogenesis was

induced by a biphasic treatment with induction medium (high glucose alpha MEM supplemented with ITS+1 (Sigma), 10 mM sodium pyruvate, 0.5 mM methyl isobutylxanthine and 1 μM dexamethasone) and maintenance medium (high glucose alpha MEM supplemented with ITS+1 (Sigma) and 10 mM sodium pyruvate). pMSC cultures were first exposed to induction medium for 2-3 days and then cultured in maintenance medium for another 2-3 days. This alternating treatment was repeated 3-4 times to achieve full lipogenic differentiation. Osteogenic differentiation media was: alpha MEM supplemented with 10% FBS, dexamethasone (10^{-8}M), ascorbic acid (50 μg/mL) and β-glycerophosphate (10 mM). Histochemical stains were used to assess cell differentiation into specific lineages in adherent cell cultures and histological cryosections of cell masses. Cell masses were embedded in a water soluble embedding medium, frozen in liquid N_2 and sectioned (10 μm thickness) with a Leica CM3050 cryiostat (Leica, Nussloch, Germany). Accumulation of phosphates and carbonates, indicative of osteogenic differentiation was demonstrated by the von Kossa silver reduction method [2]. Cultures or cryosections were fixed with 4% formaldehyde, exposed to 5% silver nitrate solution and immediately exposed to direct UV light for 45-60 minutes. Specimens were then washed and incubated for 2-3 min in 5% sodium thiosulfate solution. Intracellular accumulation of neutral lipids was demonstrated by Oil Red O staining [2]. For this, monolayers were fixed and stained with Oil Red O working solution for 1 h. The working solution was made fresh each time by mixing 1 part of distilled water with 1.5 parts of a saturated Oil Red O solution (0.5% w/v Oil Red O in 99% isopropyl alcohol). Acidic mucopolysaccharides present in cartilage tissue were stained with alcian blue 8GX (Sigma, St. Louis. MO, USA). Briefly, cryosections were

fixed with 3% acetic acid and stained with alcian blue solution (1% w/v alcian blue in 3% acetic acid, pH 2.5) for 30 min. After washing, slides were mounted with 90% of glycerol and inspected with a transmitted light microscope. Photographs were taken with a digital camera (Qimaging Ratiga 1300, Qimaging, Burnaby, BC, Canada) mounted on the microscope.

Experimental design

Experiment 1: Transduction of pMSCs with Ad5eGFP or AdF35eGFP: pMSC cultures were incubated with increasing concentrations of Ad5eGFP or Ad5F35eGFP. Cells were incubated for 5 h in the following multiplicity of infection (MOI, defined as pfu/cell) of Ad5eGFP or Ad5F35eGFP: 1, 10, 100, 500, 1,000 or 2,000 MOI.

Experiment 2: Effect of GeneJammer on transduction efficiency of pMSCs with Ad5F35eGFP: pMSC cultures were transduced with 2,000 vp/cell in presence of increasing concentrations of GeneJammer® Transfection Reagent (Statagene, La Jolla, CA). Virus-GeneJammer suspension was prepared as follow: GeneJammer® (0, 2, 4, 8, 16, 32 or 64 µL) was suspended in 200 µL of serum-free antibiotic-free αMEM and incubated 10 min at room temperature. Then, stock viral suspension was added to the diluted GeneJammer® transfection reagent and incubated at room temperature for 10 min. Medium in each well was replaced by 300 µL of fresh αMEM with 3.3% FBS and the GeneJammer®-virus mixture (200 µL) was added drop wise for a final volume of 500 µL per well (Therefore, final concentrations of GeneJammer were: 0.4, 0.8, 1.6, 3.2, 6.4 or 12.5 µL/100µL of medium). After incubation for 5.30 h at 37ºC in 5% CO_2 in air, 500 µL of αMEM with 18% FBS were added in each well (final volume 1 mL/well).

Since GeneJammer active components are resusupended in an 80% ethanol solution and this may affect per se transduction efficiency, we conducted a vehicle control study to investigate this possibility. pMSC cultures were transfected with increasing doses of ethanol 80% (0, 1.2, 2.4 or 4.8 μL/100μL of medium) following the procedures described above for GeneJammer.

Experiment 3: Effect of Ad5F35eGFP dose on transduction efficiency of pMSCs: the aim of this experiment was to determine the dose of Ad5F35eGFP to achieve optimal transduction efficiency. pMSC cultures were infected with increasing doses of adenovirus (100-100,000 vp/cell) and transduced without or with GeneJammer at 1.6% as indicated in Experiment 1.

Experiment 4: Effect of FBS on transduction efficiency of pMSCs with Ad5F35eGFP: this experiment was conducted to investigate the effect of FBS on Ad5F35 transduction efficiency. pMSCs were transduced with Ad5F35eGFP without or with GeneJammer (1.6%) under different FBS concentrations (0, 2, 10 or 20%).

Experiment 5: Effect of GeneJammer on transduction of pMSCs with Ad5eGFP: to determine the effect of GeneJammer on transduction efficiency of pMSCs by Ad5eGFP vector, cultures were transduced with 1,500 vp/cell in absence (control) or presence of 1.6% GeneJammer.

Experiment 6: Transduction efficiency of pMSCs infected with Ad5F35eGFP in presence of Lipofectamine: this experiment was designed to compare transduction efficiency of pMSCs transduced in presence of GeneJammer or various doses of Lipofectamine (Invitrogen Corporation, Carlsbad, California), an extensively used transfection reagent. Liofectamine was prepared as per manufacturer's instructions.

Briefly, for each well of a 12-well plate 1, 2 or 4 µL of Lipofectamine were diluted in 25 µL of serum-free DMEM/F12 medium and combined with 25 µL of media containing 2.25 x 10^8 Ad5F35eGFP vp (150,500 cells x 1,500 vp/cell). Transfection reagent-adenovirus mixture was incubated at room temperature for 20 min and 150 µL of DMEM/F12 were added. Medium in each well was replaced by 300 µL of fresh αMEM with 10% FBS and the Lipofectamine-virus mixture (200 µL) was added drop wise for a final volume of 500 µL per well. After incubation for 5 h at 37ºC in 5% CO_2 in air, 500 µL of αMEM containing 18% FBS were added in each well (final volume 1 mL/well). In addition to Lipofectamine treatments, a GeneJammer treated group (1.6%) and a control without any of the transfection reagents were included.

Experiment 7: Differentiation of Ad5F35eGFP transduced pMSCs: pMSC cultures were transduced with Ad5FeGFP vector (1,500 vp/cell) as described in Experiment 1. Transduced cells and non-transduced controls were exposed to lipogenic, chondrogenic or osteogenic differentiation conditions. After 14-20 d, cultures were inspected under UV light and photographed with a digital camera (Qimaging Ratiga 1300, Qimaging, Burnaby, BC, Canada) mounted in an inverted microscope (Nikon Eclipse TE2000-S, Nikon Corporation, Tokyo, Japan). Specimens were then subjected to histochemical stains as described above. The same area of the well photographed under UV light was identified and photographed with transmitted light.

Statistical analysis

Data from Experiment 2, 6 and 7 were analyzed by one-way analysis of variance (ANOVA) using the General Linear Models (GLM) of the Statistical Analysis System (SAS). Treatment means were compared by Tukey Test (HSD) in Experiment 2 and 6,

and Dunnet Test was applied in Experiment 7 to compare each treatment with the control. Data from Experiments 1, 3 and 4 were analyzed by two-way analysis of variance under a completely randomized 2 x 6 (Experiment 1), 2 x 8 (Experiment 3) or 2 x 4 (Experiment 4) design followed by least significant differences (LSD) when appropriate. Whenever a significant main effect and interaction between factors were detected, simple main effects of the interaction were compared using the SLICE option of the LSMEANS in GLM procedure of SAS. This analysis allowed us to look at differences between the cell means for one factor (vector type or GeneJammer) at each of the levels of the other (viral concentration in Experiments 1 and 3 and FBS concentration in Experiment 4). Means from one factor (adenovector type, GeneJammer or control) were compared across adenoviral concentration (Experiment 1 and 3) or FBS concentrations (Experiment 4) as simple effects with de PDIFF option of the LSMEANS in PROC GLM of SAS. Since relative fluorescence intensity data in Experiment 3 was not normally distributed, the Kruskal-Wallis test (nonparametric ANOVA) followed by Dunn's Multiple Comparisons Test was used. Unpaired, two tailed Student's *t* test was used to determine differences between the means of two groups (Experiment 5). In all experiments, data analysis was blocked by replicate in order to account for the experimental error contributed by cell lines. Values are presented as mean ± SEM from 2 independent replicates for Experiment 6; 3 independent replicates for Experiments 1, 2, 3, 4 and 5; and 4 replicates for Experiment 7. Differences were considered statistically significant at $P \leq 0.05$.

RESULTS

Experiment 1: Transduction of pMSCs with Ad5eGFP or AdF35eGFP:

Percentage of transduced cells increased rapidly between MOI 1-500 in both viral vectors

(Fig. 4.1A). Percentage of GFP positive cells reached a maximum at MOI 500 for Ad5F35eGFP and MOI 1,000 for Ad5eGFP to reach a plateau at higher MOI (Fig. 4.1A). The effect of adenovirus vector type on efficiency of infection at each MOI revealed that Ad5F35 infected more cells than Ad5eGFP at MOI 10 (17.37 ± 1.68% vs. 22.62 ± 3.24% for Ad5eGFP and Ad5F35eGFP respectively; P=0.02) and 100 (62.83% ± 3.99 vs. 74.84 ± 3.68% for Ad5eGFP and Ad5F35eGFP respectively; P<0.0001) (Fig. 4.1A). Increasing viral concentrations were associated with increments in RFI in cells transfected with either virus (Fig 4.1B). There was no difference in RFI between cells transduced with either adenovector at MOI 1, 10, 100 and 500; however, cells infected with Ad5F35eGFP exhibited higher (P<0.001) RFI than those transduced with Ad5eGFP at MOI 1,000 and 2,000 (Fig. 4.1B). Irrespective of vector type, transfection of cells with high viral doses (at MOI 1,000 and 2,000) caused a rise in the proportions of PI positive cells (Fig. 4.1C). Flow cytometry analysis revealed that 99.6 ± 0.11% (n=3) of pMSC were positive for the membrane cofactor protein (CD46) (Fig. 4.2).

Experiment 2: Effect of GeneJammer on transduction efficiency of pMSCs with Ad5F35eGFP: Addition of GeneJammer Transfection Reagent markedly increased the transfection efficiency of Ad5F35 at all doses studied (Fig. 4.3A). A similar enhancement in the percentage of GFP positive cells by GeneJammer was observed for doses between 0.4 to 3.2% (e.g., 89.18 ± 0.79% vs. 16.15 ± 1.71 % in GeneJammer 1.6% and control respectively; P<0.05); however, a reduction was evident at GeneJammer 6.4% (Fig. 4.3A). Relative fluorescence intensity variable exhibited a bell-shape response to increasing concentrations of GeneJammer with maximal values reached with GeneJammer 0.8%-3.2 (Fig 4.3A). This enhancement in RFI indicates that a higher

number of viruses entered each cell. Exposure of pMSC to GeneJammer at 12.8% killed most of the cells precluding further cytometric analysis and suggesting a toxic effect of GeneJammer at this high concentration. The positive effect of GeneJammer on transduction efficiency was evident by simple inspection of cell cultures (Fig 4.3C, control and Fig 4.3D, GeneJammer 1.6%) Cell viability measured by exclusion of PI was not affected by GeneJammer treatment (Fig. 4.3 B). Figure 4.3 E shows a representative distribution of GFP positive/negative events in non-treated (red curve) and GeneJammer treated cells (blue curve). Based on results from this experiment, GeneJammer concentrations between 0.8-3.2 rendered optimal transfection efficiency with no evident toxic effect. We have selected GeneJammer 1.6%, which induced a 5.5-fold increase in the proportion of GFP+ cells compared with the control, as the dose to use throughout this study.

Addition of 1.2 or 2.4% of ethanol 80% to the transfection medium did not affect the percentage of pMSCs expressing GFP. A small, although significant increase in transfection efficiency was observed when 4.8% ethanol 80% was added to the transduction medium.

Experiment 3: Effect of Ad5F35eGFP dose on transduction efficiency of pMSCs: In both control and GeneJammer treated groups the percentage of cells expressing the transgene increased with increasing viral dose (Fig. 4.4A). However, the characteristics of the responses were rather different. In GeneJammer treated cells a steep increase in the percentage of GFP positive cells was evident at low viral doses (100 to 1000 vp/cell) followed small increments and a plateau (Fig 4.4A). When GeneJammer was omitted, a steady increase in the percentage of GFP+ cells was observed across treatments.

Furthermore, comparison of the mean percentage of GFP positive cells between GeneJammer-treated and control groups at each viral dose revealed a significantly higher percentage of cells expressing the transgene in cells transduced in presence of GeneJammer (Fig. 4.4A). For instance, in cells transduced with 1,500 vp/cell with GeneJammer 88.92 ± 4.58% were GFP+ compared with 21.59 ± 7.66% in the control without GeneJammer ($P<0.05$) (Fig 4.4A). Increasing adenoviral dose was also associated with increased RFI in both non-treated and GeneJammer-treated groups (Fig. 4.4B). Percentage of non-viable cells was not affected by GeneJammer, therefore the data were pooled (Fig. 4.4C). Percentage of PI positive cells remained unchanged at viral doses ranging from 100 to 2,000 vp/cell; further increments of viral concentration induced a significant rise in the percentage of PI+ cells (Fig. 4.4C).

Experiment 4: Effect of FBS on transduction efficiency of pMSCs with Ad5F35eGFP: Medium containing fetal bovine serum (FBS) improved transfection efficiency in both GeneJammer and control groups (Fig. 4.5A). The maximal transduction efficiency in GeneJammer treated group was obtained when the medium was supplemented with 10% FBS (89.21 ± 0.74% of GFP+ cells), whereas in the control, 20% FBS had the highest percentage of GFP+ cells (37.72 ± 2.98% of GFP+ cells). Furthermore, comparison of the mean percentage of GFP expressing cells between GeneJammer-treated and control groups at each FBS concentration revealed a significantly higher percentage of cells expressing the transgene in cells transduced in presence of GeneJammer (Fig 4.5A). Relative fluorescence intensity was not affected by FBS concentration; however, GeneJammer enhanced RFI at all FBS concentrations studied (53.16 ± 2.28% in GeneJammer-treated groups vs. 30.03 ± 0.58 in the control

groups; P<0.05) (Fig. 4.5A). No statistical differences were detected for percentage of non-viable cells (Fig. 4.5B).

Experiment 5: Effect of GeneJammer on transduction of pMSCs with Ad5eGFP: Inclusion of GeneJammer (1.6%) in the Ad5eGFP transduction medium elicited a 3-fold (P=0.0003) and 2.5-fold (P=0.0036) increase in the percentage of GFP positive cells and RFI respectively (Fig. 4.6A), compared with that in controls transduced without GeneJammer. Enhanced transduction efficiency induce by the treatment was clearly evident upon visual inspection (Fig. 4.6 B and C). Representative dot plots of control and GeneJammer treated groups showing distribution of cell populations based on GFP intensity and PI staining are depicted in Fig 4.6D and E. Cell viability was not affected by the treatment (Fig 4.6A, D and E).

Experiment 6: Transduction efficiency of pMSCs infected with Ad5F35eGFP in presence of Lipofectamine: In this experiment we studied the effect of GeneJammer (1.6%) or Lipofectamine (1, 2 or 4 μl) on transfection efficiency (percentage of GFP+ and RFI), percentage of non-viable cells and side scatter (SSC) values. GeneJammer and Lipofectamine 2 μl induced a similar increase in the percentage of GFP+ cells compared with the control (Fig. 4.7A). Lipofectamine 1 μl also enhanced the proportion of GFP+ cells compared with the control, but it was statistically lower than GeneJammer and Lipofectamine 2 μL (P<0.05) (Fig. 4.7A). Similar results were obtained for RFI. Relative fluorescence intensity was highest in GeneJammer and Lipofectamine 2 μl treatments and lower in the control. Proportions of non-viable cells were below 6% for all treatments; however, transduction with Lipofectamine 4 μL, significantly increased the proportions of PI+ cells compared with GeneJammer and control treatments (P<0.05) (Fig 4.7A).

SSC-height of cells transduced in presence of GeneJammer was higher than that in control cells (Fig. 4.7C) and SSC-height in Lipofectamine treated cells higher than that in GeneJammer group. Increments in Lipofectamine concentration was associated with increased SSC-height (Fig. 4.7B). Representative histograms of the frequency distribution and SSC-height in control, GeneJammer and Lipofectamine 4 μL showing a shift to the right especially in Lipofectamine group are illustrated in Fig. 4.7C.

Experiment 7: Differentiation of Ad5F35eGFP transduced pMSCs: Non-transduced controls and transduced cells were exposed to induction media and differentiation assessed by histochemical stains. Both non-transduced and transduced cells underwent lipogenic, chondrogenic or osteogenic differentiation. Cells with characteristic lipid droplets fluoresced under UV light and stained red with ORO (Fig. 4.8A and B). Although most of the cells that acquired an adipocyte-like morphology expressed GFP (Fig. 4.8B), some differentiated cells did not fluoresce (Fig. 4.8 C, D and E). Similarly, non-transduced and transduced cell masses differentiate into cartilage and bone tissues. GFP fluorescence colocalized with alcian blue positive (Fig. 4.8F and G) and von Kossa positive (black; Fig. 4.8H and I) cryosections in chondrogenic and osteogenic differentiated cultures respectively.

DISCUSSION

Bone marrow-derived MSCs are an attractive cell type for cell-based gene therapy strategies. These cells can be injected directly into damaged organs or tissue and systemic intravenous injections of MSCs can home to multiple tissue types or sites of tissue damage [11,12,38-41]. Further, genetically modified MSCs, secreting biologicals can serve as vehicles for locally delivery of therapeutic gene products. In addition, MSCs are

ideal for ex vivo gene delivery systems because they are relatively easy to obtain from bone marrow aspirates and can be expanded extensively in culture [2]. However, development of efficient transfection/transductions systems for the delivery and expression of genes of interest in MSCs remains a significant challenge to overcome for future clinical use. Here for the first time we have demonstrated the relatively simple and efficient method to infect pMSCs using two adenoviral vectors, human adenovirus vector type 5 (Ad5) and a chimeric Ad5 bearing the fiber protein from Ad35 (Ad5F35).

Replication-deficient adenoviral vectors are particularly suitable for adult stem cell-based therapies because they can elicit high-level gene expression for a limited period of time [15]. To date most of this research has been conducted on rodent models. The development of large animal models will facilitate the study of cell distribution, patterns of gene expression and required cell doses to achieve a therapeutic effect in animals that are similar to humans in size and physiology. Striking morphologic and physiologic similarities between humans and pigs make them an appropriate large animal model for safety and efficacy preclinical studies. Despite the fact that methods for isolation and culture of porcine MSCs are available [37], adenoviral-mediated gene transfer into pig cells have not been explored.

We first sought to study the ability of human adenovectors to deliver the GFP gene into cultured pMSC. A clear positive dose-response relationship was observed between vector concentrations ranging from MOI 1-500 and percentage of GFP positive cells irrespective of adenovirus type. No further increments in proportions of cells expressing the transgene were observed beyond MOI 500 for cells transduced with Ad5F35 and MOI 1,000 for cells infected with Ad5 vector. Comparison of transduction

efficiency between adenovirus vector type at each viral MOI, revealed that Ad5F35 infected ~5% (P=0.02) and ~12% (P<0.0001) more cells than Ad5eGFP at MOI 10 and 100 respectively. These results may reflect differences in tropism between Ad5 and Ad5F35. Cell entrance in vectors derived from adenovirus type 5 is mediated by CAR and integrins [16,17], whilst the chimeric Ad5F35 appears to use a CAR-independent mechanism via CD46 as cellular receptor [23]. A high percentage of pMSCs (>99%) express CD46 (Fig. 4.2), and thus we expected better transduction efficiencies in pMSCs transduced with the Ad5F35 vector. In comparison, cell lines expressing adenoviral receptors such as human embryonic kidney-293 (HEK-293) low doses (~10 MOI) are enough to achieve transfection efficiencies close to 100% [24]. In our study, even in pMSC cultures infected with Ad5F35 at MOI of 100 only ~70% of pMSCs expressed the transgene and MOI of 500 was required to transduce the entire population. Although we cannot rule out that adenoviral vector entrance into pMSCs was mediated by CAR or CD46 receptors, transduction efficiencies observed in the present study are compatible with those observed in cells lacking adenoviral receptors in which large viral doses are required to achieve high infection rates. The relatively low homology between human and porcine CD46 (42% amino acid identity) [42] may have hindered the ability of human Ad5F35 to recognize and interact with porcine CD46 receptor. Even assuming that human Ad5F35-porcine CD46 recognition is preserved, there is evidence that CD46 is required [23] but probably not sufficient to mediate adenoviral intake; other cell membrane molecules, such us integrins, not expressed in pMSCs could be required for vector internalization. The variable adenoviral infection efficiencies of porcine cells

could hinder pig translational research if a more efficient and uniform system for transducing cells is not available.

We also observed a positive relationship between RFI and adenoviral vector concentration, however a plateau was not reached for this variable (Fig. 4.1B). It is assumed that RFI reflects the amount of GFP produced and this depends on the number of viral particles that entered each particular cell. Therefore RFI data from this study indicate that increments in adenoviral concentration results in larger number of viral particles entering each cell; even when virtually the entire population was transduced (at MOI 1,000 and 2,000). At high viral concentrations (MOI of 1,000 and 2,000) pMSCs transduced with Ad5F35 vector exhibited enhanced RFI. This differential response is difficult to explain. It is plausible that distinct capsid features of Ad5F35 favor cellular intake only at high viral doses. Along with increase RFI, cell death was also augmented at high viral doses (i.e., MOI 1,000 and 2,000) irrespective of vector type, likely reflecting adenovector cytopathogenic effects and/or cell damage due to excessive transgene expression.

Our results indicate that efficient adenoviral transduction of pMSCs is contingent on high adenoviral MOI; therefore, development of methods to achieve comparable adenoviral gene delivery with substantially lower viral doses is highly desirable for future MSC animal modeling. Here we found that transduction of pMSCs could be greatly enhanced by incorporation of the commercial polyamine-based transfection reagent GeneJammer into the adenovirus vector transfection medium. A significant increase in both the total number of transduced cells and level of transgene expression was detected at low MOI (2,000 vp/cell or MOI ~14) when the transduction was carried out in the

presence of GeneJammer (Fig. 4.2A). GeneJammer at 1.6% which induced a 5.5 and 12 fold-increase in the proportion of GFP+ cells and RFI respectively, was selected to investigate the effect of viral dose on transduction efficiency. The lowest viral concentration to achieve the best level of transduction was 1,500-2,000 vp/cell (MOI 10-14). At these viral doses and level of transgene expression no adverse effect on cell viability was evident (Fig. 4.3C).

To determine if the virus entry into the cells via the polyamine was specific to the adenovirus type 35 capsid, we infected pMSCs with Ad5eGFP with or without GenJammer. The enhancement of adenoviral transduction efficiency by GeneJammer was similar to that obtained with the Ad35 vector, suggesting a similar mechanism that is not dependent on adenovirus type 35 fiber. Other studies have reported enhanced viral uptake and subsequent transgene expression by cationic polymers and lipids [28-33]. The precise mechanism of polycation-enhanced adenoviral cell entrance remains unclear. Polycations may complex with negatively charged viral particles and reduce the electrostatic repulsion with the negatively charged cell plasma membrane [32]. Alternatively, viral aggregates induced by polycations [30,31] may facilitate viral access to the cell membrane and lead to greater viral uptake. The experimental evidence supports the idea that these polycationic molecules stimulate viral uptake by pathways independent of fiber receptor or cellular integrins [33]. Similarly, GeneJammer was able to enhance adenoviral entry in CAR- and CD46-deficient cell lines (Chinese hamster ovary cells and human MSCs), demonstrating that GeneJammer positive effect is mediated through an unknown alternative pathway independent of the classical adenoviral receptors (our unpublished results).

Lipofectamine, a cationic lipid-based transfection compound, has been extensively used to deliver DNA into cultured cells and shown to enhance adenoviral transduction of cultured primary [28,30,34] and established cell lines [28]. In agreement with these results, transduction of pMSCs in presence or Lipofectamine strongly enhanced both the percentage of transduced cells and gene expression (RFI) at levels comparable to those in GeneJammer-transduced cells. However, SSC intensity values collected during flow cytometric analysis in the Lipofectamine-treated groups were significantly higher than those in GeneJammer-treated group and control. Side scatter values reveal light-refractive and light reflective properties of the cells associated with optical inhomogeneity of cell structures such as condensation of cytoplasm or nucleus and granularity. Changes in light scattering during apoptosis have been well characterized [43]. In some cell systems, during early stages of apoptosis increased SSC intensity has been reported; probably reflecting chromatin and cytoplasm condensation and nuclear fragmentation which are followed by decreased SSC intensity at later stages of apoptosis. The increased SSC intensity found in pMSCs might reflect changes associated with initial apoptosis induced by the cationic lipid. However, since a change in light scatter is not a specific marker of apoptosis, other measurements like DNA fragmentation needs to be performed in future studies for a more definite identification of apoptosis.

Neutralizing antibodies or charged components in serum that may inhibit adenovirus uptake has favored the use of low serum concentration (~2%) in the adenoviral transduction media. It is also known the profound effects of low serum exposure on cell physiology, especially in stem cells in which culture in such conditions may trigger cell differentiation [44]. Results from this study indicate that incorporation of

FBS (up to 20%) in the transduction medium of pMSCs substantially increases the percentage of cells expressing the transgene, but not the RFI. Thus, the use of GeneJammer provides the versatility of achieving high adenoviral vector transfection over a broad range of serum concentrations.

One of the hallmark characteristics of MSCs that make these cells of particular value in areas of cell therapy and regenerative and reconstructive medicine is their capacity to differentiate into diverse mesodermal lineages [2,37]. Therefore, preservation of the differentiation potential of MSCs after in vitro manipulation is of major interest in MSC ex vivo therapeutic protocols aimed at regeneration of mesenchymal tissues after MSC transplantation. One study reported that the transfected MSC subpopulation was not prone to differentiate into adipocytic lineages [22]. From this study it was not clear whether transduction led to the loss of adipogenic differentiation potential or the transduced subpopulation had a restrictive differentiation potential. In order to test whether pMSCs transduced using the GeneJammer protocol described here, would retain the capacity to differentiate into adipogenic, chondrogenic and osteogenic lineages, transduced and non-transduced MSCs were cultured in specific differentiation media. Porcine MSCs transduced in presence of GeneJammer were able to form adipose, cartilage and bone tissues. In agreement with the results presented here, human MSCs transduced with adenovirus vectors preserved the differentiation potential to adipogenic [45,46] and osteogenic [22,46] lineages.

In summary, we have developed a highly effective adenoviral transduction system for cultured pMSCs which are normally poorly infected by human adenovectors and these cells should now serve as a valuable translational large animal cell-based gene

therapy model. The use of GeneJammer as described in this study leads to high transgene expression levels in pMSCs while minimizing adverse reactions and retaining differentiation potential. In light of the multiple applications of MSCs in areas of gene therapy and targeted delivery of therapeutics, results from this study will facilitate addressing scalability, multiple biological questions and safety concerns in a large animal model.

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REFERENCES

1. Muraglia A, Cancedda R, Quarto R (2000) Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. J Cell Sci 113 (Pt 7):1161
2. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284:143
3. Kohyama J, Abe H, Shimazaki T, Koizumi A, Nakashima K, Gojo S, Taga T, Okano H, Hata J, Umezawa A (2001) Brain from bone: efficient "meta-differentiation" of marrow stroma-derived mature osteoblasts to neurons with Noggin or a demethylating agent. Differentiation 68:235
4. Woodbury D, Schwarz EJ, Prockop DJ, Black IB (2000) Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res 61:364
5. Chamberlain JR, Schwarze U, Wang PR, Hirata RK, Hankenson KD, Pace JM, Underwood RA, Song KM, Sussman M, Byers PH, Russell DW (2004) Gene

targeting in stem cells from individuals with osteogenesis imperfecta. Science 303:1198

6. Horwitz EM, Prockop DJ, Fitzpatrick LA, Koo WW, Gordon PL, Neel M, Sussman M, Orchard P, Marx JC, Pyeritz RE, Brenner MK (1999) Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. Nat Med 5:309

7. Olmsted-Davis EA, Gugala Z, Gannon FH, Yotnda P, McAlhany RE, Lindsey RW, Davis AR (2002) Use of a chimeric adenovirus vector enhances BMP2 production and bone formation. Hum Gene Ther 13:1337

8. Cheng SL, Lou J, Wright NM, Lai CF, Avioli LV, Riew KD (2001) In vitro and in vivo induction of bone formation using a recombinant adenoviral vector carrying the human BMP-2 gene. Calcif Tissue Int 68:87

9. Gazit D, Turgeman G, Kelley P, Wang E, Jalenak M, Zilberman Y, Moutsatsos I (1999) Engineered pluripotent mesenchymal cells integrate and differentiate in regenerating bone: a novel cell-mediated gene therapy. J Gene Med 1:121

10. Tsuchida H, Hashimoto J, Crawford E, Manske P, Lou J (2003) Engineered allogeneic mesenchymal stem cells repair femoral segmental defect in rats. J Orthop Res 21:44

11. Studeny M, Marini FC, Champlin RE, Zompetta C, Fidler IJ, Andreeff M (2002) Bone marrow-derived mesenchymal stem cells as vehicles for interferon-beta delivery into tumors. Cancer Res 62:3603

12. Nakamizo A, Marini F, Amano T, Khan A, Studeny M, Gumin J, Chen J, Hentschel S, Vecil G, Dembinski J, Andreeff M, Lang FF (2005) Human bone marrow-derived mesenchymal stem cells in the treatment of gliomas. Cancer Res 65:3307

13. Kappes SM (1999) Utilization of gene mapping information in livestock animals. Theriogenology 51:135

14. Band MR, Larson JH, Rebeiz M, Green CA, Heyen DW, Donovan J, Windish R, Steining C, Mahyuddin P, Womack JE, Lewin HA (2000) An ordered comparative map of the cattle and human genomes. Genome Res 10:1359

15. Amalfitano A (2004) Utilization of adenovirus vectors for multiple gene transfer applications. Methods 33:173

16. Bergelson JM, Cunningham JA, Droguett G, Kurt-Jones EA, Krithivas A, Hong JS, Horwitz MS, Crowell RL, Finberg RW (1997) Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science 275:1320

17. Wickham TJ, Mathias P, Cheresh DA, Nemerow GR (1993) Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. Cell 73:309

18. Wickham TJ, Roelvink PW, Brough DE, Kovesdi I (1996) Adenovirus targeted to heparan-containing receptors increases its gene delivery efficiency to multiple cell types. Nat Biotechnol 14:1570

19. Goldman M, Su Q, Wilson JM (1996) Gradient of RGD-dependent entry of adenoviral vector in nasal and intrapulmonary epithelia: implications for gene therapy of cystic fibrosis. Gene Ther 3:811

20. Thoma SJ, Lamping CP, Ziegler BL (1994) Phenotype analysis of hematopoietic CD34+ cell populations derived from human umbilical cord blood using flow cytometry and cDNA-polymerase chain reaction. Blood 83:2103

21. Rebel VI, Hartnett S, Denham J, Chan M, Finberg R, Sieff CA (2000) Maturation and lineage-specific expression of the coxsackie and adenovirus receptor in hematopoietic cells. Stem Cells 18:176

22. Conget PA, Minguell JJ (2000) Adenoviral-mediated gene transfer into ex vivo expanded human bone marrow mesenchymal progenitor cells. Exp Hematol 28:382

23. Gaggar A, Shayakhmetov DM, Lieber A (2003) CD46 is a cellular receptor for group B adenoviruses. Nat Med 9:1408

24. Yotnda P, Onishi H, Heslop HE, Shayakhmetov D, Lieber A, Brenner M, Davis A (2001) Efficient infection of primitive hematopoietic stem cells by modified adenovirus. Gene Ther 8:930

25. Tsujimura A, Shida K, Kitamura M, Nomura M, Takeda J, Tanaka H, Matsumoto M, Matsumiya K, Okuyama A, Nishimune Y, Okabe M, Seya T (1998) Molecular cloning of a murine homologue of membrane cofactor protein (CD46): preferential expression in testicular germ cells. Biochem J 330 (Pt 1):163

26. Miwa T, Nonaka M, Okada N, Wakana S, Shiroishi T, Okada H (1998) Molecular cloning of rat and mouse membrane cofactor protein (MCP, CD46): preferential expression in testis and close linkage between the mouse Mcp and Cr2 genes on distal chromosome 1. Immunogenetics 48:363

27. Sohn JH, Kaplan HJ, Suk HJ, Bora PS, Bora NS (2000) Chronic low level complement activation within the eye is controlled by intraocular complement regulatory proteins. Invest Ophthalmol Vis Sci 41:3492

28. Clark PR, Stopeck AT, Brailey JL, Wang Q, McArthur J, Finer MH, Hersh EM (1999) Polycations and cationic lipids enhance adenovirus transduction and transgene expression in tumor cells. Cancer Gene Ther 6:437

29. Lee EM, Hong SH, Lee YJ, Kang YH, Choi KC, Choi SH, Kim IH, Lim SJ (2004) Liposome-complexed adenoviral gene transfer in cancer cells expressing various levels of coxsackievirus and adenovirus receptor. J Cancer Res Clin Oncol 130:169

30. Dodds E, Piper TA, Murphy SJ, Dickson G (1999) Cationic lipids and polymers are able to enhance adenoviral infection of cultured mouse myotubes. J Neurochem 72:2105

31. Fasbender A, Zabner J, Chillon M, Moninger TO, Puga AP, Davidson BL, Welsh MJ (1997) Complexes of adenovirus with polycationic polymers and cationic lipids increase the efficiency of gene transfer in vitro and in vivo. J Biol Chem 272:6479

32. Arcasoy SM, Latoche JD, Gondor M, Pitt BR, Pilewski JM (1997) Polycations increase the efficiency of adenovirus-mediated gene transfer to epithelial and endothelial cells in vitro. Gene Ther 4:32

33. Qiu C, De Young MB, Finn A, Dichek DA (1998) Cationic liposomes enhance adenovirus entry via a pathway independent of the fiber receptor and alpha(v)-integrins. Hum Gene Ther 9:507

34. Byk T, Haddada H, Vainchenker W, Louache F (1998) Lipofectamine and related cationic lipids strongly improve adenoviral infection efficiency of primitive human hematopoietic cells. Hum Gene Ther 9:2493

35. Olmsted EA, Blum JS, Rill D, Yotnda P, Gugala Z, Lindsey RW, Davis AR (2001) Adenovirus-mediated BMP2 expression in human bone marrow stromal cells. J Cell Biochem 82:11

36. Mackay AM, Beck SC, Murphy JM, Barry FP, Chichester CO, Pittenger MF (1998) Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. Tissue Eng 4:415

37. Ringe J, Kaps C, Schmitt B, Buscher K, Bartel J, Smolian H, Schultz O, Burmester GR, Haupl T, Sittinger M (2002) Porcine mesenchymal stem cells. Induction of distinct mesenchymal cell lineages. Cell Tissue Res 307:321

38. Diduch DR, Jordan LC, Mierisch CM, Balian G (2000) Marrow stromal cells embedded in alginate for repair of osteochondral defects. Arthroscopy 16:571

39. Gao J, Dennis JE, Muzic RF, Lundberg M, Caplan AI (2001) The dynamic in vivo distribution of bone marrow-derived mesenchymal stem cells after infusion. Cells Tissues Organs 169:12

40. Wang L, Li Y, Chen X, Chen J, Gautam SC, Xu Y, Chopp M (2002) MCP-1, MIP-1, IL-8 and ischemic cerebral tissue enhance human bone marrow stromal cell migration in interface culture. Hematology 7:113

41. Pereira RF, Halford KW, O'Hara MD, Leeper DB, Sokolov BP, Pollard MD, Bagasra O, Prockop DJ (1995) Cultured adherent cells from marrow can serve as

long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. Proc Natl Acad Sci U S A 92:4857

42. Toyomura K, Fujimura T, Murakami H, Natsume T, Shigehisa T, Inoue N, Takeda J, Kinoshita T (1997) Molecular cloning of a pig homologue of membrane cofactor protein (CD46). Int Immunol 9:869

43. Ormerod MG (1998) The study of apoptotic cells by flow cytometry. Leukemia 12:1013

44. Hattori T, Ide H (1984) Limb bud chondrogenesis in cell culture, with particular reference to serum concentration in the culture medium. Exp Cell Res 150:338

45. Mizuguchi H, Sasaki T, Kawabata K, Sakurai F, Hayakawa T (2005) Fiber-modified adenovirus vectors mediate efficient gene transfer into undifferentiated and adipogenic-differentiated human mesenchymal stem cells. Biochem Biophys Res Commun 332:1101

46. Hung SC, Lu CY, Shyue SK, Liu HC, Ho LL (2004) Lineage differentiation-associated loss of adenoviral susceptibility and Coxsackie-adenovirus receptor expression in human mesenchymal stem cells. Stem Cells 22:1321

Figure 4.1. Transduction of pMSCs with increasing concentrations of Ad5eGFP or Ad5F35eGFP. Cells were incubated with adenovectors (1, 10, 100, 500, 1,000 or 2,000 MOI) for 5 h. Twenty-four house posttransfection the percentage of GFP positive cells (A), relative fluorescence intensity (B) and percentage of non-viable cells (C) were determined by flow cytometry. (* and ** indicate significant differences between Ad5eGFP vs. Ad5F35eGFP treated cells at each adenovector concentration (* P=0.02 in A, P=0.0003 in B; ** P<0.0001). ^{a,b,c,} Bars with different letters denote significant differences at P<0.05 (ANOVA followed by LSD). Results are expressed as mean ± SEM from 3 independent replicates.

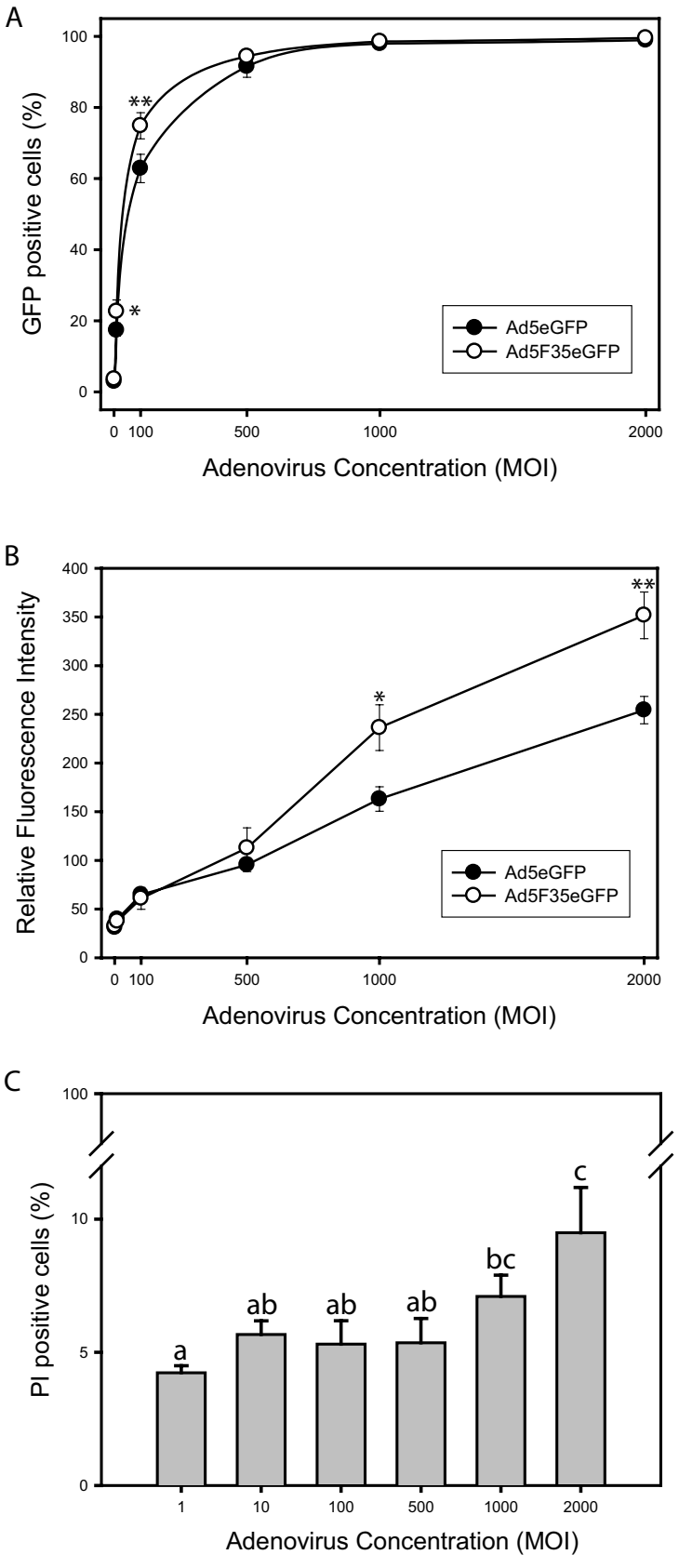

A
GFP positive cells (%)
100
80
60
40
20
0
**
*
Ad5eGFP
Ad5F35eGFP
0 100 500 1000 2000
Adenovirus Concentration (MOI)
B
Relative Fluorescence Intensity
400
350
300
250
200
150
100
50
0
**
*
Ad5eGFP
Ad5F35eGFP
0 100 500 1000 2000
Adenovirus Concentration (MOI)
C
PI positive cells (%)
100
10
5
0
a
ab
ab
ab
bc
c
1 10 100 500 1000 2000
Adenovirus Concentration (MOI)

Figure 4.2. Histogram showing frequency distribution and fluorescence intensity data from a representative pMSC line stained to demonstrate immunoreactive sites for CD-46. The blue curve represents the distribution of cells incubated with anti-CD46 primary antibody, whereas red curve represents the distribution of cells incubated with the immunoglobulin isotype control. In this particular cell line 99.4% of total cells were positive for CD46.

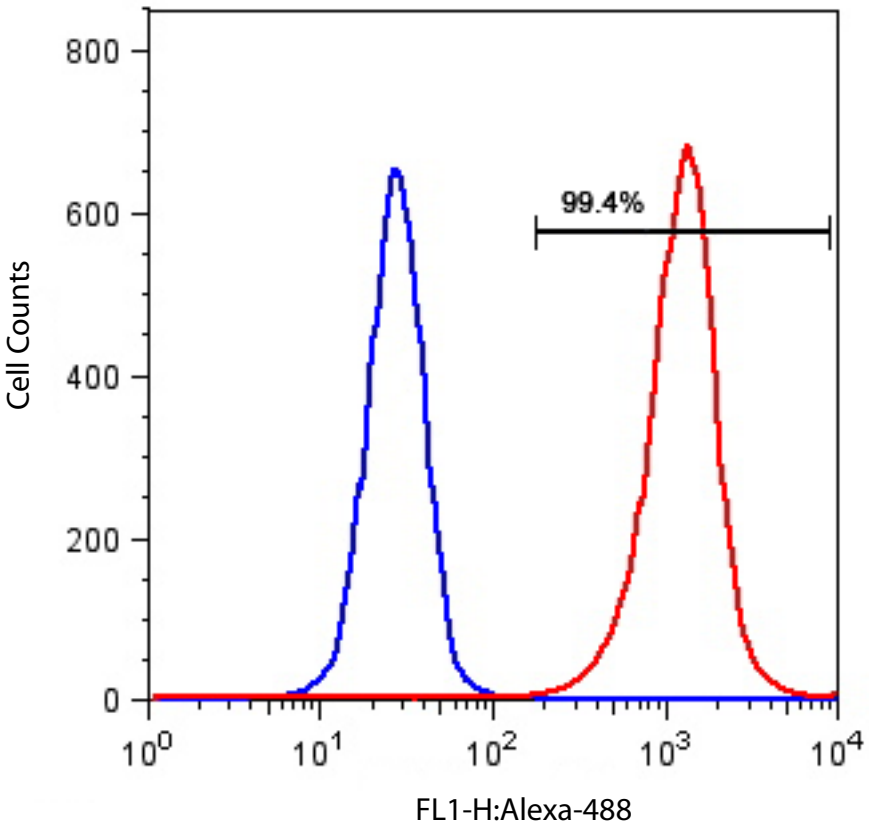

800
600
400
200
0
Cell Counts
99.4%
10^0
10^1
10^2
10^3
10^4
FL1-H:Alexa-488

Figure 4.3. Effect of GeneJammer on transduction efficiency of pMSCs with Ad5F35eGFP (2,000 vp/vell). pMSC cultures were transduced without (control) or with increasing concentrations of GeneJammer. Twenty-four hours after infection, percentage of pMSCs expressing GFP and relative fluorescence intensity (RFI) in pMSCs was analyzed by flow cytometry (A). GeneJammer treatment did not affect the percentage of non-viable cells as indicated by the rate of PI positive cells (B). Photomicrographs of pMSC cultures 24 h after transduction in absence (C) or presence (D) of GeneJammer (1.6%). Representative distribution of GFP negative/positive cells from non-treated (red curve; 14.0% of positive cells) and GeneJammer treated cells (1.6 µL/100 µL; blue curve, 90.1% of positive cells) (E). Effect of increasing concentrations of ethanol 80% (GeneJammer vehicle) during transduction on proportions of GFP+ cells (F). $^{a,b,c,d \text{ and } e,f,g}$ Bars with different letters denote significant differences ($P<0.05$; ANOVA) within each variable studied. Results are expressed as mean ± SEM from 3 independent replicates. (Bar=200 µm).

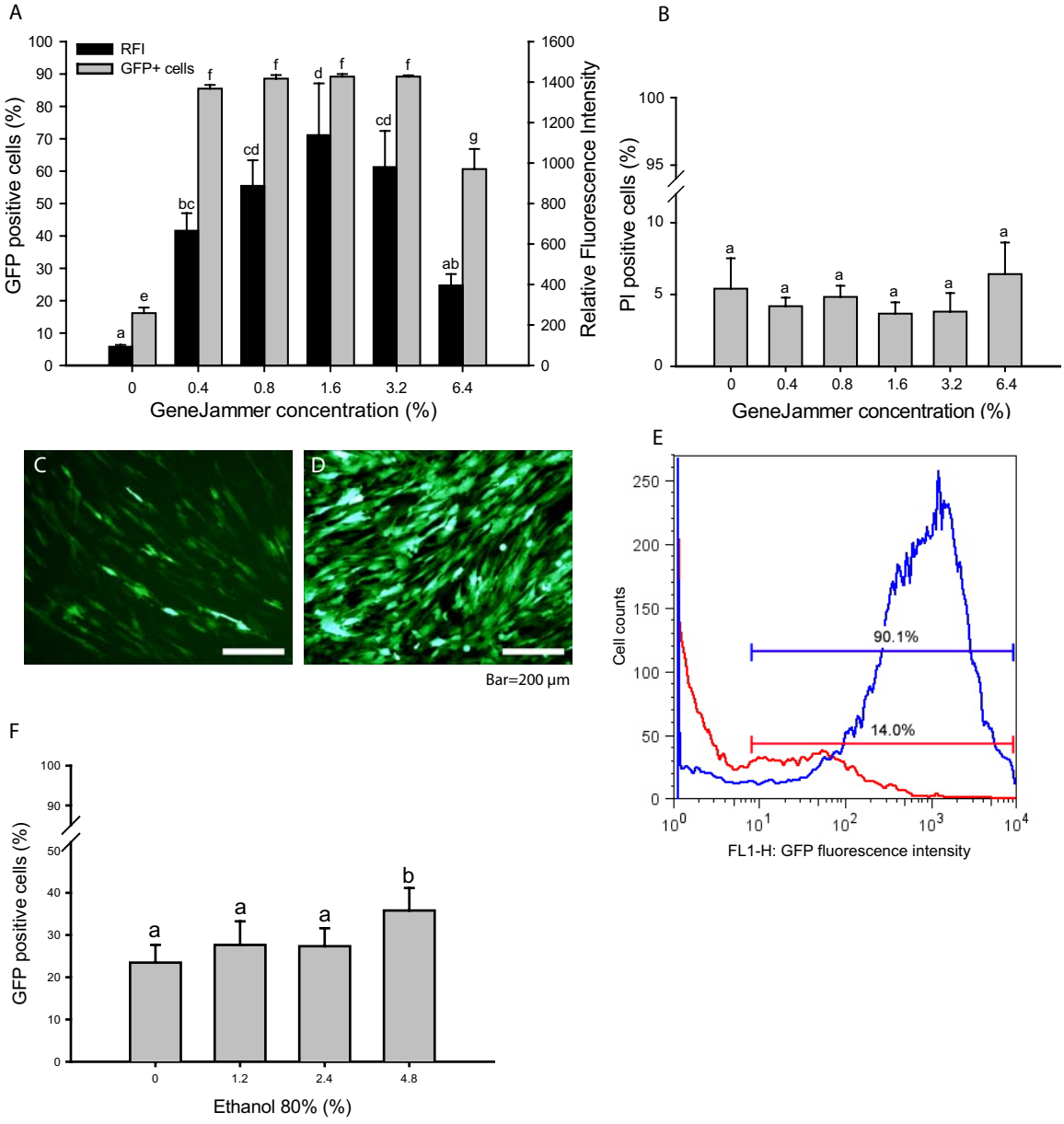

A
RFI
GFP+ cells
GFP positive cells (%)
Relative Fluorescence Intensity
GeneJammer concentration (%)
B
PI positive cells (%)
GeneJammer concentration (%)
C
D
Bar=200 μm
E
Cell counts
90.1%
14.0%
FL1-H: GFP fluorescence intensity
F
GFP positive cells (%)
Ethanol 80% (%)

Figure 4.4. Effect of increasing Ad5F35eGFP concentrations without or with GeneJammer on proportions of GFP expressing cells (A), RFI (B) and percentage of non viable cells (C). [a,b,c,d,] Bars with different letters denote significant differences ($P<0.05$; ANOVA) within GeneJammer treated group (n=3). * [and] ** denote differences between control and GeneJammer treated groups within each viral concentration (n=3; $p<0.05$; ANOVA). [a,b] Bars with different letters denote significant differences ($P<0.05$; Kruskal-Wallis Test followed by Dunn's Multiple Comparisons Test in B and ANOVA in C). Results are expressed as mean ± SEM from 3 independent replicates.

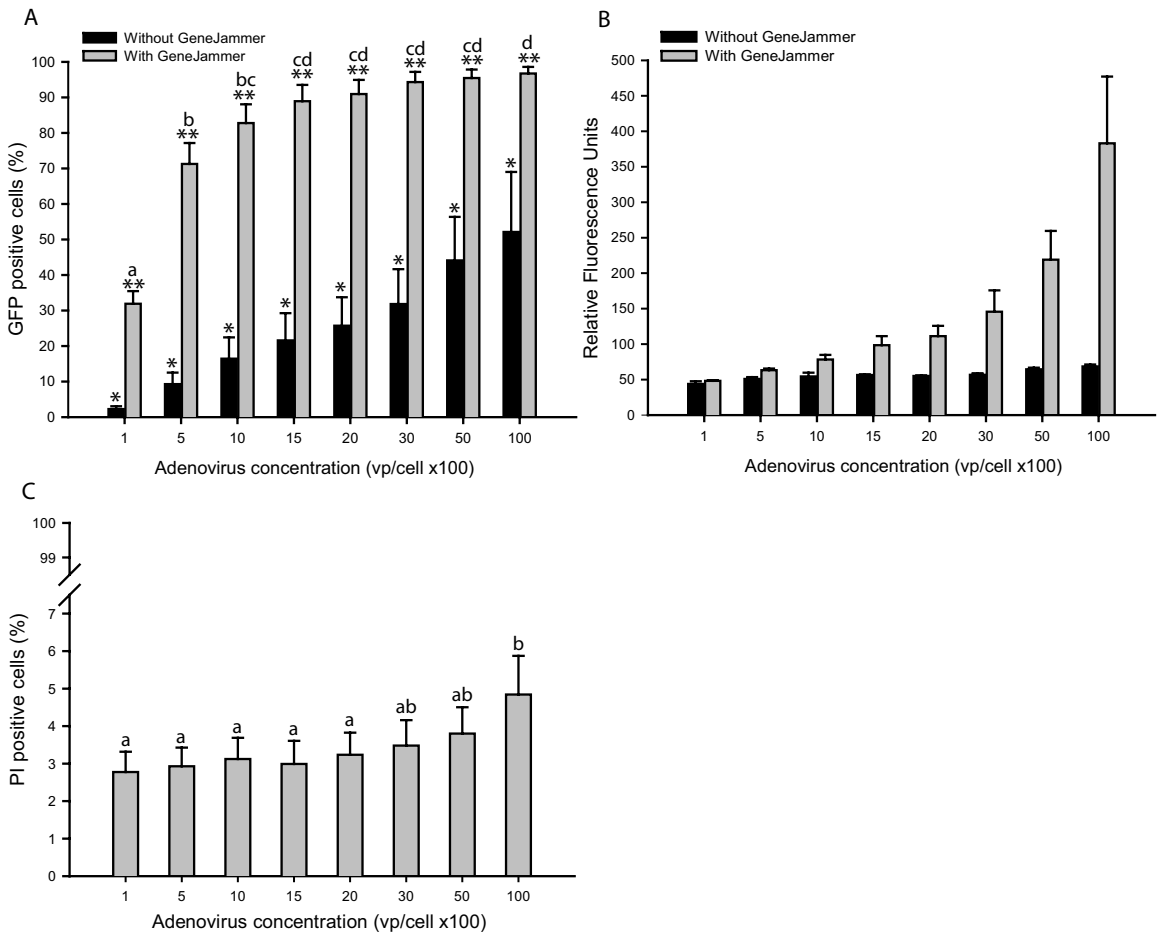

A
Without GeneJammer
With GeneJammer
GFP positive cells (%)
Adenovirus concentration (vp/cell x100)
B
Relative Fluorescence Units
C
PI positive cells (%)

Figure 4.5. Effect of FBS on transduction of pMSCs with Ad5F35eGFP vector without or with GeneJammer on proportions of GFP expressing cells, RFI (A) and percentage of non viable cells (B). [a,b,c,] Bars with different letters denote significant differences ($P<0.05$; ANOVA) within GeneJammer treated group (n=3). [A,B,C] Bars with different capital letters denote significant differences ($P<0.05$; ANOVA) within control group (n=3) across different FBS concentrations. * [and] ** denote differences between control and GeneJammer treated groups within each FBS concentration (n=3; $p<0.05$; ANOVA). Values are mean ± SEM from 3 independent replicates.

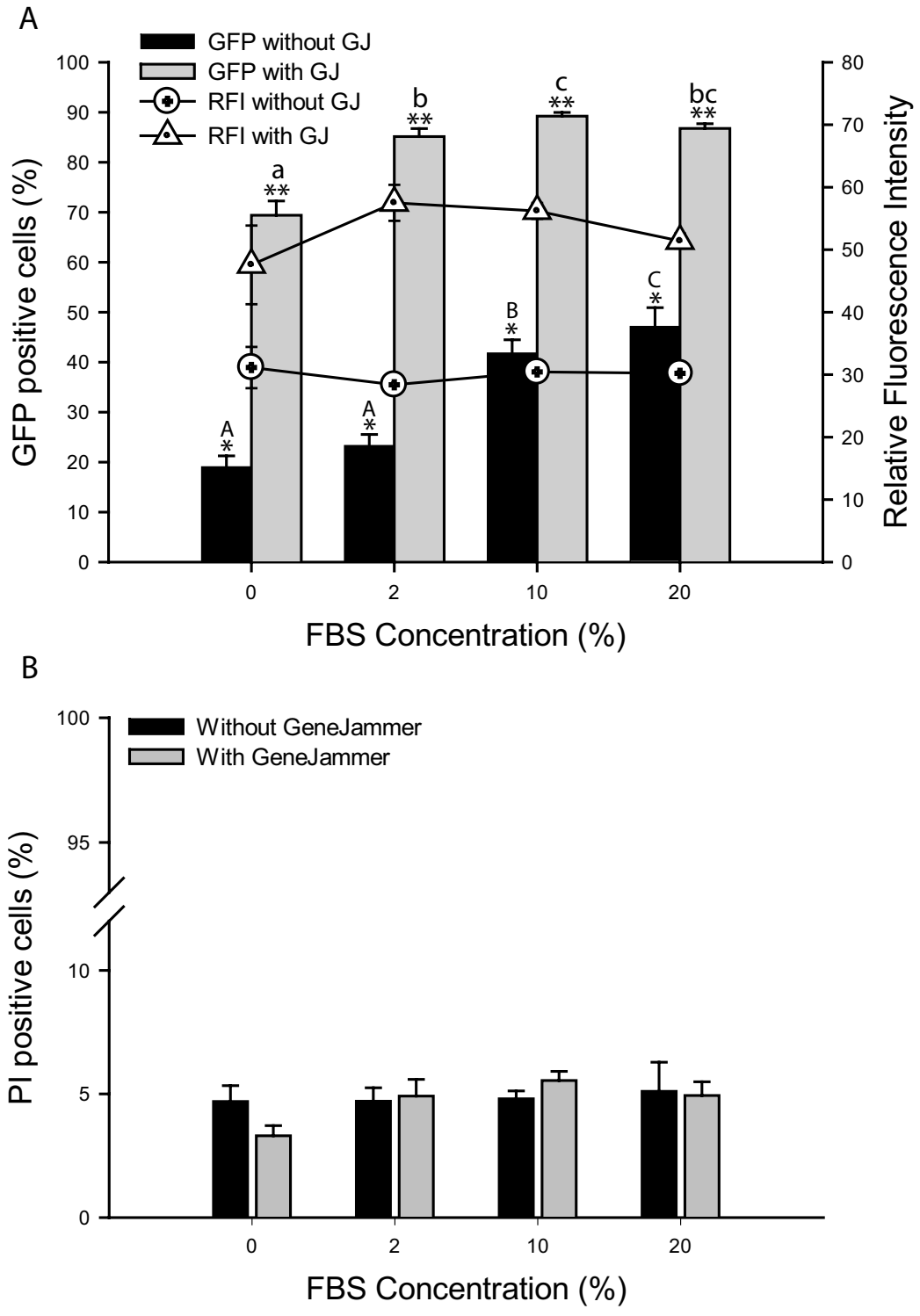

A
GFP without GJ
GFP with GJ
RFI without GJ
RFI with GJ
GFP positive cells (%)
Relative Fluorescence Intensity
FBS Concentration (%)
a
b
c
bc
A
B
C
B
Without GeneJammer
With GeneJammer
PI positive cells (%)
FBS Concentration (%)

Figure 4.6. Transduction of pMSCs with Ad5eGFP without or with GeneJammer. Effect of transduction of pMSC cultures with Ad5eGFP in medium containing 1.6% GeneJammer on percentage of GFP positive cells, RFI and proportion of non-viable cells (A). Photomicrographs of pMSCs 24 h after transduction with Ad5F35-EGFP without (B) or with 1.6% GeneJammer (C). Representative dot plots of control (D) or GeneJammer transduced pMSCs (C) showing distribution of cell populations based on GFP intensity (X axis) and PI staining (Y axis). Very low proportions of cells are non-viable (top left quadrants). A larger percentage of cells are viable and GFP positive in GeneJammer-treated group (90.3%, bottom right quadrant in E) compared with that in the control (31.1%, bottom right quadrant in B). [a,b and *,**] denote a significant difference at $P<0.05$ (Student's *t*-test; n=3). NS: non-significant difference.

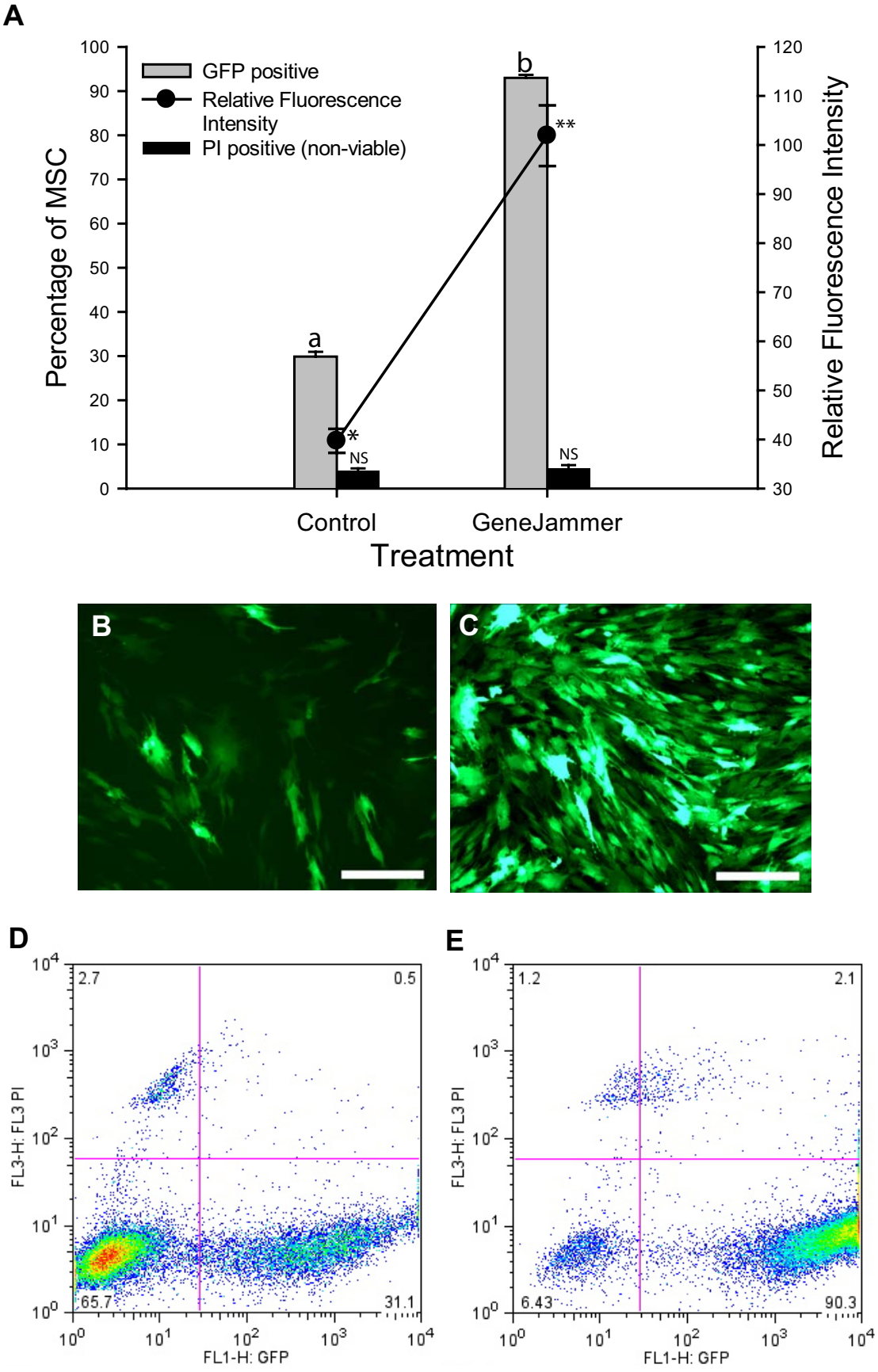

A
GFP positive
Relative Fluorescence Intensity
PI positive (non-viable)
Percentage of MSC
Relative Fluorescence Intensity
a
b
*
**
NS
NS
Control
GeneJammer
Treatment
B
C
D
E
FL3-H: FL3 PI
FL1-H: GFP
2.7
0.5
65.7
31.1
1.2
2.1
6.43
90.3

Figure 4.7. Effect of Lipofectamine on Ad5F35eGFP transduction efficiency of pMSCs (percentage of GFP positive cells and relative fluorescence intensity), percentage of non-viable cells (A) and mean SSC-height values (B) determined by flow cytometry. Representative histograms of the frequency of events at different SSC-height values in the control (red curve), GeneJammer-trated (blue curve) and Lipofectamine 4 uL are shown in (C). [a,b,c,d,e] Bars with different letters denote significant difference ($P<0.05$; ANOVA followed by HSD). Values are mean ± SEM from 4 independent replicates.

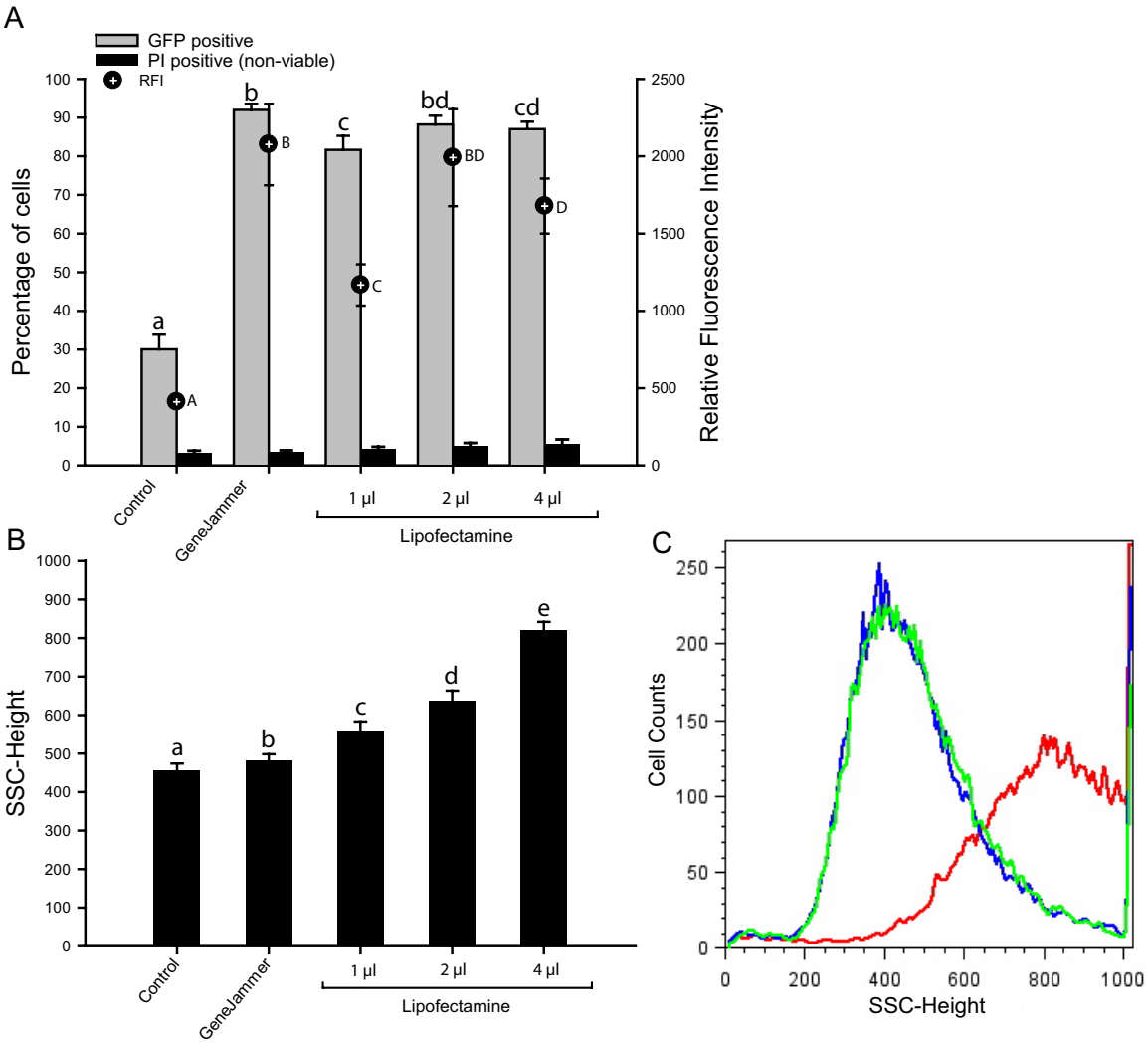
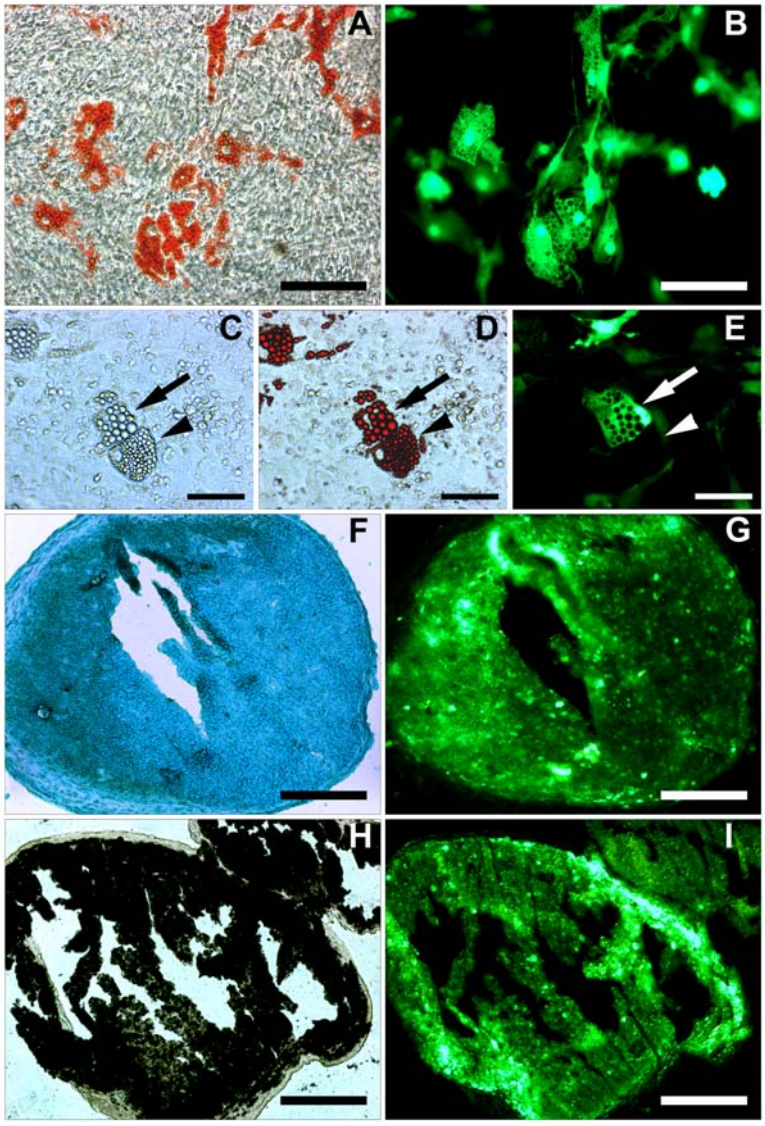

A
GFP positive
PI positive (non-viable)
RFI
Percentage of cells
Relative Fluorescence Intensity
a
b
c
bd
cd
A
B
C
BD
D
Control
GeneJammer
1 µl
2 µl
4 µl
Lipofectamine
B
SSC-Height
a
b
c
d
e
Control
GeneJammer
1 µl
2 µl
4 µl
Lipofectamine
C
Cell Counts
SSC-Height

Figure 4.8. In vitro differentiation of pMSCs transduced with Ad5F35eGFP in medium containing GeneJammer 1.6%. pMSCs were transfected, induced to differentiate into lipogenic, chondrogenic or osteogenic lineages and differentiation assessed by histochemical stains. In lipogenic cultures GFP fluorescent cells (B and E) with characteristic refringent lipid droplets were evident by phase contrast microscopy (C). These cells stained positive for the lipid stain ORO (A and D). However, not all cells with lipid accumulation were GFP positive. Panels (C), (D) and (E) show the same microscopic field photographed in phase contrast (unstained), bright field (stained with ORO) and UV light (GFP fluorescence) respectively. Arrows point to the same differentiated cell (note the lipid droplets in (C) and ORO stained droplets in (D)) expressing GFP (E). Arrowheads point to a lipogenic differentiated pMSC (C and D) that resulted non-fluorescent (E). Cryosections from cell masses cultured in chondrogenic medium stained positive with alcian blue (F) and fluoresced under UV light (G). Similarly, cryosections from cell masses cultured under osteogenic conditions turned black upon staining with von Kossa and (H) and fluoresced under UV light (I). Bar = 100 μm (A and B), 50 μm (C-E) and 200 μm (F-I).


A
B
C
D
E
F
G
H
I

CHAPTER 5

A NOVEL COMPOUND ENABLES HIGH-LEVEL ADENOVIRUS TRANSDUCTION IN THE ABSENCE OF AN ADENOVIRUS-SPECIFIC RECEPTOR[1]

[1] Pablo Bosch, Christine M. Fouletier-Dilling, Alan R. Davis, Jessica A. Shafer, Steven L. Stice, Zbigniew Gugala, Francis H. Gannon, and Elizabeth A. Olmest-Davis, Submitted to *Human Gene Therapy*, 06/18/2005

OVERVIEW SUMMARY

Adenovirus (Ad) vectors are very useful in gene transfer ranking second behind DNA transfection as a DNA delivery method. However, host range and tissue tropism restrict the use of specific Ad vectors sometimes necessitating the lengthy construction of new vectors with more appropriate cell specificity. This is particularly important in bone formation using Ad type 5 carrying the gene for bone morphogenetic protein 2 (Ad5BMP2) where inefficient transduction can result in the total lack of bone formation. We describe here a novel methodology using GeneJammer® that increases the transduction efficiency of adenovirus vectors in general in a receptor independent fashion. We show that the use of this compound enables bone to be formed readily *in vivo* in immunocompetent animals.

ABSTRACT

Viral vectors are extensively used to deliver foreign DNA to cells for applications ranging from basic research to potential clinical therapies. A limiting step in this process is virus uptake and internalization into the target cells which is mediated by membrane receptors. Although it is possible to modify viral capsid proteins to target the viruses, such procedures are complex and often unsuccessful. Here we present a rapid, inexpensive system for improving transduction of cells including those that lack receptors for adenovirus fiber proteins. Addition of GeneJammer® during the adenovirus transduction led to both a significant increase in the total number of transduced cells as well as the level of transgene expression per cell. Studies using cell lines deficient in adenovirus receptors demonstrated that addition of GeneJammer® provided a novel cellular-entry mechanism for the virus. These findings were tested in a cell-based gene therapy system for the induction of bone, which is contingent on high level expression of the transgene. Inclusion of GeneJammer® in Ad5BMP2 transduction of human mesenchymal stem cells and a murine osteoblastic cell line demonstrated a correlating increase in bone formation. The results suggest a novel and versatile method for achieving high level transduction using adenovirus.

INTRODUCTION

Osteoinductive cytokines comprise the bulk of known osteoinductive agents as a class of relatively small secreted molecules that are capable of promoting the formation of bone. Some of the most heavily studied of these factors are the bone morphogenetic proteins (BMPs), which are part of the TGF-β superfamily and were originally isolated based on their ability to induce ectopic bone. Recently Turgeman *et al.* (2002) suggested that BMP2 may also be capable of enhancing adult mesenchymal stem cells to form bone, providing a possible role in treatment of osteoporosis. The human recombinant proteins BMP2 and BMP7 (rhBMP2 and rhBMP7) have been recently approved for restricted clinical use, however large doses of these recombinant proteins are required to induce adequate bone repair, suggesting that the mode of BMP delivery still requires further optimization. Furthermore, BMP induced osteogenesis even under ideal conditions does not approach the efficiency of bone formation seen during normal fracture repair (Franceschi *et al.*, 2004). Also, delivery of these proteins requires a carrier such as collagen sponge carriers (Bonadio *et al.*, 1999) which can also elicit responses that often influence the bone formation process.

Alternatively, gene therapy approaches have been developed to circumvent the problems associated with delivering the protein itself (Musgrave *et al.*, 2000; Olmsted-Davis *et al.*, 2002). Studies using both non-viral and viral delivery systems have successfully induced bone formation in several animal models. Park *et al*., (2003) used liposomes to transfect mesenchymal stem cells to heal mandibular defects in rats by an *ex vivo* strategy which expressed markers of new bone matrix such as osteopontin and osteocalcin within 2 weeks following gene transfer. Musgrave *et al.*, (2000) have shown

primary bone marrow mesenchymal stem cells (hBM-MSCs) transduced with Ad5-BMP2 produced no radiographic ossification. We demonstrated (Olmsted *et al.*, 2001) that Ad5 elicits only minimal amounts of BMP2 synthesis upon transduction of hBM-MSCs due to the lack of adenovirus specific receptors. Further we previously demonstrated a critical link between the amount of BMP2 expressed in the tissue and the production of bone (Gugala *et al.*, 2003). Therefore, the poor efficiency of delivery and transient expression of the gene are significant challenges that need to be overcome for these strategies to be truly effective.

Consequently, several approaches have been undertaken in order to circumvent these problems. A limiting step in adenoviral uptake is indeed the entry into the target cells which is mediated by the coxsackie -adenovirus receptor (CAR) and cellular $\alpha_V\beta$ integrins. In many cell types, these receptors are either absent or expressed at low levels leading to poor transduction efficiency by adenovirus. For instance, CAR expression is highly variable and most stem cells and fibroblastic cells are poorly infected by adenoviral vectors due to low expression of CAR (Hidaka *et al.*, 1999; Sakurai *et al.*, 2003).

Recently investigators have attempted to circumvent this problem by introducing genetic fiber modifications in Ad which provides an alternate cellular entry route. Chimeric Ad5 vectors possessing fiber proteins derived from subgroup B Ad serotypes such as Ad35 have become increasingly popular as gene transfer vectors because they can efficiently deliver genes to cell types that are refractory to Ad5 infection (Mizuguchi and Hayakawa, 2002; Olmsted-Davis *et al.*, 2002; Gao *et al.*, 2003). These groups B adenovirus have been shown to utilize CD46 as a primary attachment receptor (Gaggar *et*

al., 2003; Segerman *et al.*, 2003). However, receptors for these chimeric viruses appeared to be absent on most murine cells (Mallam *et al.*, 2004), which reduce their versatility and high titers of the purified fiber modified viruses are difficult to produce.

Polycations and cationic lipids, which form complexes with adenoviral particles, have also been used to facilitate *in vitro* transduction of a range of cell types (Lanuti *et al.*, 1999; Toyoda *et al.*, 2001). Similarly, adenoviral infection of primitive human hematopoietic cells can be strongly enhanced by several cationic lipids (Harrison *et al.*, 1995; Byk *et al.*, 1998; Marit *et al.*, 2000). Others have been using bilamellar cationic liposomes to protect adenovectors from preexisting humoral immune responses (Yotnda *et al.*, 2002).

The aim of this study was to investigate the role of a commercial polyamine-based compound (GeneJammer®, Stratagene, La Jolla, CA) on enhancement of adenoviral transduction of several cell types *in vitro*. We show a significant increase in both the total number of transduced cells and level of transgene expression per cell, when the transduction is carried out in the presence of GeneJammer®. Studies using cell lines deficient in adenovirus receptors demonstrated that addition of GeneJammer® provided a novel cellular-entry mechanism for the virus. We further tested this methodology in our cell based gene therapy system for induction of bone formation. The compound increased cell transduction and achieved a high level of functional BMP2 protein expression, in both murine and human cell types. Further, this increase in BMP2 production correlated with increased bone formation *in vivo*. This finding greatly advances our goal of developing a cell-based gene therapy system for the induction of bone formation. GeneJammer® transfection reagent markedly increases the efficiency of

adenovirus-mediated gene transfer in cells deficient in adenovirus receptors through an alternative pathway for virus entry into the cell. Hence this methodology provides a rapid inexpensive technique for transduction of adenovirus-refractive cells.

MATERIALS AND METHODS

Cell culture

Cell lines: Chinese hamster ovary cells (CHO), the human lung carcinoma cell line (A549), and murine osteoblast cell line (MC3T3-E1), were obtained from American Type Culture Collection. Human lung carcinoma cells (A549) were propagated in Dulbecco's Modified Eagles Medium (DMEM) (Biowhittaker, Walkersville, MD), supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Life Technologies Inc., Gaithersburg, MD); CHO cells and MC3T3-E1 were propagated in RPMI and αMEM respectively supplemented as described above.

The W20-17 mouse stromal cell line (a gift from Genetics Institute, Cambridge, MA) was propagated as described by Thies *et al*., (1992). Briefly, the cells were grown in DMEM supplemented as described above and cultured at a subconfluent density in order to maintain the phenotype. All cell types were grown at 37°C and 5% CO_2 in humidified air.

Human bone marrow mesenchymal stem cells: Discarded human bone marrow products were obtained from healthy donors in compliance with all state and federal regulations following Institutional Review Board approval. Mononuclear cells were isolated by gradient density centrifugation on Ficoll-Paque™ PLUS (Amersham

Pharmacia Biotech, Piscataway, NJ) and washed twice with Dulbecco's Phosphate Buffered Saline (PBS) (Life Technologies Inc., Gaithersburg, MD) prior to culturing. Cells were plated at a density of 5 x 10^6 cells/cm^2 (Jaiswal *et al.*, 1997) in DMEM supplemented with 10% FBS and antibiotics-antimycotics as described above. Early adherent fibroblastic cells appeared within two days of culture, and after one week dead cells and debris were removed by washing with PBS and cells were passaged prior to confluence. Several vials of these cells were frozen in Origen® dimethyl sulfoxide freeze medium (Igen International Inc., Gaithersburg, MD).

Human Mononuclear Peripheral blood cells (hPBMCs): Whole peripheral blood was obtained from healthy donors in compliance with all state and federal regulations following Institutional Review Board approval. Approximately 10-30 ml of blood were collected into heparinized tubes, and then layered onto 20 ml of Ficoll-Paque^{TM} PLUS density gradient (Amersham Biosciences, Piscataway, NJ). Cells were centrifuged at 770 x g for 30 minutes at room temperature and the hPBMCs band was carefully removed washed with PBS and resuspended at a concentration of 3 × 10^6 cells/ml.

Transduction of cells with adenovirus in the presence or absence of GeneJammer®

Adenoviruses: Replication defective E1-E3 deleted first generation human type 5 adenovirus (Ad5) and/or modified forms in which the normal fiber protein has been substituted for the human adenovirus type 35 fiber (Ad5F35) were constructed to contain cDNAs for either BMP2 or GFP in the E1 region of the virus (Olmsted *et al.*, 2001). The virus particles to plaque forming units ratio are: 55, 76, 8, 132, 200 for Ad5BMP2, Ad5F35BMP2, Ad5eGFP, Ad5F35eGFP and Ad5-empty respectively, and all viruses were shown to be negative for replication competent adenovirus.

Cell transduction: Adherent cells (1 x 10^6) were transduced with adenovirus at three different virus concentrations (2,500, 5,000 and 10,000 vp/cell) with or without 1.2% GeneJammer®. This concentration of the polyamine compound was optimized based upon percentage of GFP transduction (Data not shown). Briefly, 15 µl of GeneJammer® or PBS was added to 500 µl of DMEM medium without supplements and incubated for 10 minutes at room temperature. The virus was then added at indicated concentrations and the mixture further incubated for 10 minutes at room temperature. This virus-GeneJammer® mixture was added to approximately 1 x 10^6 cells along with 750 µl of DMEM supplemented with 10% FBS and antibiotics/antimycotics. The cells were incubated at 37°C for 4 hours and then the mixture was diluted with 3 ml of fresh medium containing FBS. hPBMCs (1 x 10^7) were transduced with 5000 vp/cell of adenovirus in the presence or absence of 1.2% GeneJammer® as described above.

Flow cytometry

Transduced cells were trypsinized, washed and resuspended in PBS, 48 hours after the initial transduction. Dead cells and debris were excluded from analysis based on propidium iodide (PI) fluorescence. Flow cytometric analysis was performed with a FACSCalibur cytometer (Becton Dickinson, San Jose, CA). Percentage of GFP positive cells was quantified using a CellQuest™ software (Becton Dickinson, San Jose CA), on a minimum of 10^5 cells per sample (n=3). Relative fluorescence intensity (RFI) of the GFP positive cell population was also determined for each sample. All data were taken in triplicate and reported as mean and standard deviation. Significance was determined between samples transduced with and without GeneJammer® (n=3) using a Student's t

test with 95% confidence interval ($p<0.05$) (GraphPad InStat software, GraphPad Software, Inc.,San Diego, CA).

A549, hBM-MSCs and CHO cells were analyzed for CAR, αV integrin and CD46 expression. Briefly the cells at a concentration of 1 x 10^6/ml were incubated with the primary antibody: CAR (ab9891, ABCAM, Inc., Cambridge, MA), αV integrin (MAB1953Z, Chemicon International, Inc., Temecula, CA) or CD46 (CBL488, Chemicon International, Inc., Temecula, CA) for 15 minutes on ice. The secondary antibody, FITC-conjugated rat anti-mouse (Cat. No 553354, BD Biosciences, Pharmigen, San Jose, CA) was diluted 1/200 and cells incubated with the secondary antibody for 15 minutes on ice. Cell pellets were washed in PBS and the samples analyzed by flow cytometry.

Quantification of BMP2

BMP2 protein was measured in the culture supernatant taken from cells 72 hours after transduction with Ad5BMP2 (at varying doses) in the presence or absence of GeneJammer®. Briefly 10^6 cells were transduced as described above and culture supernatant collected and assayed using the Quantikine® BMP2 Immunoassay from R&D systems (DBP200, R&D Systems Inc., Minneapolis, MN). A monoclonal antibody specific for BMP2 has been coated onto the microplate wells. Standards and samples (in triplicate) are pipetted into the wells and any BMP2 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for BMP2 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and a color develops in proportion to the amount of BMP2 bound in the initial step. The color

development is stopped and the intensity of the color is measured by reading the microplate at 450 nm. A standard curve was drawn by plotting the optical density for the standards versus the concentration of the standards and BMP2 concentration in samples was extrapolated from this standard curve.

Alkaline phosphatase assay

W20-17 cells were assayed for alkaline phosphatase activity three days after addition of either the supernatant of cells infected with Ad5BMP2, Ad5-empty or medium control by a chemiluminescent procedure (Olmsted *et al.*, 2001). Cellular alkaline phosphatase was extracted by three freeze-thaw cycles in 100 μl/cm^2 of 25 mM Tris-HCl, pH 8.0 and 0.5% Triton X-100 and the activity was then measured by addition of CSPD® Ready-to-use with Sapphire II enhancer (Tropix, Inc., Bedford, MA) to the samples. The light output from each sample was integrated for 10 seconds after a 2 second delay by the luminometer (TD-20/20, Turner Designs, Sunnyvale, CA). Alkaline phosphatase levels were recorded in relative luminescence units (RLU) and normalized to protein content with the BCA assay using bovine serum albumin to derive a standard curve. Data are presented as percent induction above unstimulated basal control cells. Statistical analysis was performed as described previously. Briefly all data were taken in triplicate and reported as mean and standard deviation. A Student's t test with 95% confidence interval ($p<0.05$) was done between the untreated control and each experimental condition.

Heterotropic bone assay

hBM-MSCs were transduced with Ad5BMP2 (2500 vp/cell) or an Ad5-empty (2500 vp/cell) which is a control adenovirus type 5 vector that lacks a transgene in the E1

deleted region. Briefly, cells were removed by trypsin, resuspended at a concentration of 5 x 10^6 cells/100 µl PBS, and then injected into the hind limb quadriceps muscle of NOD/SCID mice (2 animals per group). All animals were euthanized two weeks after injection and the hind limbs were harvested, the skin removed, and the limbs placed in formalin. Bone formation was analyzed by X-ray analysis using a Faxitron Specimen Radiography System Model MX-20 (83 seconds at 31 kV; Faxitron, Wheeling, IL). All animal studies were performed in accordance with standards of the Baylor College of Medicine, Department of Comparative Medicine after review and approval of the protocol by the Animal Use and Care Committee.

RESULTS

Enhanced viral transduction in the presence of GeneJammer®

Adenovirus transduction of coxsackie-adenovirus receptor (CAR)-negative cell lines is extremely inefficient requiring large amounts of virus, and resulting in low level expression of the desired transgene. To enhance virus uptake into the cells, we tested the ability of the commercially available polyamine complex, GeneJammer® to enhance virus transduction. Accordingly, CAR-negative hBM-MSCs (10^6 cells) were transduced with Ad5eGFP virus at three different doses (2,500 vp/cell, 5,000 vp/cell, and 10,000 vp/cell) in the presence or absence of GeneJammer® (Fig. 5.1A). An adenovirus type 5 lacking any transgene (Ad-empty) was used as a negative control. Transduction in presence of GeneJammer® increased the number of the hBM-MSCs transduced cells for all concentrations of virus (Fig. 5.1A). The lowest viral vector concentration of 2,500 vp/cell resulted in the most dramatic increase in the percentage of GFP expressing cells (15% GFP+ cells in the control group and 95% of GFP+ cells in GeneJammer-treated

group). Further we observed an increase in the number of transduced cells with a corresponding increase in virus concentration in the absence of GeneJammer®, however, in the presence GeneJammer®, the maximum number of transduced cells (95-100%) was found at all viral concentrations.

Since multiple virus particles can enter the same cell, we also analyzed the samples transduced in the presence of GeneJammer®, in which we obtained 95-100% cell transduction, to determine if the intensity of GFP expression increased with virus concentration. As shown in Figure 5.1B, the intensity of GFP expression from the cells transduced with GeneJammer®, increased with virus dose. Since 100% of the cells were transduced at all virus doses in this population, the increase in GFP intensity presumably represents an increase in the number of virus particles each cell is taking up. The data suggests that GeneJammer® not only enhances the number of cells taking up the virus (Fig. 5.1A) but also the total amount of virus entering any given cell (Fig. 5.1B).

The compound GeneJammer® allows adenovirus to enter cells lacking the receptor for fiber

Two potential models exist as to the mechanism by which the polyamine enhances virus uptake. First, GeneJammer® may aid in virus binding to its receptor, therefore potentially acting as a co-receptor for the virus internalization. Alternatively, this compound may bind to the virus and promote a novel entry route into the cell. To determine which of these is most likely, we compared the transduction efficiency of various cell types known to have differential expression levels of CAR and αV integrin (Ad5) or CD46 (Ad5F35), in the presence or absence of GeneJammer®. The three cells lines chosen have the following receptor characteristics: A549 cells express high levels of

CAR, αV integrin, and CD46; hBM-MSCs lack CAR but express αV integrin, and express moderate levels of CD46, and CHO cells express little to no adenovirus receptors (Table 1). As expected, Ad5eGFP (2,500 vp/cell) transduced 100% of the receptor positive A549 cells, while less than 15% of the receptor negative CHO cells were transduced (Fig. 5.2A and C). The hBM-MSCs which are αV integrin positive-CAR negative yielded approximately 10% of the cells expressing GFP at this same viral dose (Fig. 5.2B). Parallel transductions done in the presence of GeneJammer® showed significant enhancement in transduction in both the cells lacking CAR but expressing αV integrin, as well as the receptor negative cells (Fig. 5.2B and C). The results (Fig. 5.2C) show approximately 95% of the transduced CHO cells were expressing GFP when the adenovirus was delivered in the presence of the polyamine, suggesting that the molecular mechanism is not adenovirus receptor associated but rather, a novel pathway for virus entry into the cell. We did not see any significant changes between the groups in the A549 cells (Fig. 5.2A) due to the fact that the cells in the absence of GeneJammer® have been maximally transduced. However, the data suggest that these two systems for virus entry do not appear to inhibit one another (Fig. 5.2A).

Next we chose to determine if the virus entry into the cells via the polyamine was specific to the adenovirus type 5 capsid, so similar experiments were conducted using the altered fiber virus Ad5F35eGFP. The results were similar to those obtained with the Ad5 vector, suggesting similar mechanism that is not dependant on adenovirus type 5 fiber for entry into the cell (Fig. 5.2).

BMP2 expression and bone formation in the presence of GeneJammer®

We previously reported the inability of Ad5BMP2 to transduce hBM-MSCs which resulted in low level expression of BMP2 and lack of bone formation *in vivo* (Olmsted-Davis *et al.*, 2002). Additionally, since eGFP has been engineered to be extremely stable, whereas BMP2 is significantly less stable at both the RNA and protein level, the enhancement by the polyamine may be overestimated when eGFP is used as a transgene. To determine this, we measured the amount of BMP2 secreted into the culture supernatant after transduction of hBM-MSCs with Ad5BMP2, in the presence or absence of GeneJammer®. Expression and secretion of BMP2 in hBM-MSCs was enhanced after combined GeneJammer® Ad5BMP2 transduction. Culture supernatants were collected 72 hours after initial transduction and BMP2 quantified by ELISA. The results (Fig. 5.3A) show increased BMP2 protein in culture supernatant taken from the hBM-MSCs transduced in the presence of the polyamine compared with those from hBM-MSC cultures where GeneJammer® was omitted during transduction. BMP2 was not detected in the culture supernatant from either cells alone or those transduced with the control virus Ad-empty, demonstrating that BMP2 expression in all cases specifically corresponded with viral transduction (Fig. 5.3A).

BMP2 protein in culture supernatant was further assayed for functional activity using the W20-17 cell based assay (Thies *et al.*, 1992; Blum *et al.*, 2001; Olmsted *et al.*, 2001). In this assay, the murine bone marrow cell line W20-17 was exposed to the culture supernatant from hBM-MSCs that were transduced in the presence or absence of GeneJammer®. This cell line responds to exogenously added BMP2 by increasing the expression of alkaline phosphatase. Culture supernatants from hBM-MSCs as well as

those transduced with the Ad-empty control virus were also included in the analysis to verify that the W20-17 response was BMP2 specific. We observed a 12-fold increase in the relative chemiluminescence in the supernatant of W20-17 cells exposed to media from hBM-MSCs transduced with Ad5BMP2 (2,500 vp/cell) in the presence of GeneJammer® as compared to those samples transduced in the absence of the compound (Fig. 5.3B). At the virus concentrations of 5,000 and 10,000 vp/cell, we observed a 3.5 and 0.8 fold increase respectively in the cells transduced in the presence of the polyamine (Fig. 5.3B). Alkaline phosphatase activity in the samples exposed to the culture supernatant from the cells transduced in the presence of GeneJammer® were maximally activated, whereas exposure to culture supernatant from cells transduced in the absence of the polyamine appeared to increase with viral dose (Fig. 5.3B).

We have previously shown that hBM-MSCs transduced with Ad5BMP2 induces little to no detectable bone formation while those cells transduced with Ad5F35BMP2 elicited significant bone in the same two week time period suggesting that transduction efficiency of the cells was critical to bone formation *in vivo* (Olmsted-Davis *et al.*, 2002). To further confirm that GeneJammer® can significantly increase Ad5BMP2 transduction efficiency and BMP2 production in the hBM-MSCs without altering cell viability *in vivo*, we chose to compare these two cell populations in our heterotopic bone assay (Olmsted-Davis *et al.*, 2002). Briefly, hBM-MSCs ($5x10^6$) transduced with either Ad5BMP2 or the control vector Ad5-empty in the presence or absence of GeneJammer® were injected into the hind quadriceps muscle of NOD/SCID mice. After two weeks, the injected tissues were isolated and bone formation determined by radiological analysis. Mice that received cells transduced with Ad5BMP2 in the presence of GeneJammer® had

detectable mineralized bone, whereas those that received cells transduced in the absence of the polyamine had none (Fig. 5.3C). In no cases did we detect bone in mice receiving cells transduced with the Ad-empty control vector (data not shown). The data suggest that bone formation correlated with enhanced transduction efficiency and expression of BMP2.

Next we determined the effect of the GeneJammer® on adenovirus transduction of the murine cell line MC3T3-E1. These cells are a C57BL/6 derived cell line that can be propagated *in vitro*, and used *in vivo* in C57BL/6 mice without eliciting a graft versus host response in the animals. However, they do not possess CAR or CD46 (data not shown), and therefore are not readily transduced with Ad5 or Ad5F35 vectors. To overcome this barrier the cells were transduced with Ad5BMP2 at varying doses (2,500, 5,000 and 10,000 vp/cell) in the presence or absence of GeneJammer®. Culture supernatant was removed approximately 72 hours after transduction and assayed for functional BMP2 activity using the W20-17 cells. Supernatant from cells transduced with Ad5BMP2 at a 5000 vp/cell in the presence of the compound, yielded a significant elevation approximately 1.5 fold in alkaline phosphatase activity as compared to similar amounts of culture supernatant taken from the parallel samples of cells transduced in the absence of GeneJammer® (Fig. 5.4A). Further, the level of alkaline phosphatase induction was similar to that obtained from culture supernatant removed from hBM-MSCs after transduction with Ad5F35BMP2 (Fig. 5.4A).

We have observed that we can make copious amounts of bone in immunodeficient NOD/SCID mice (Olmsted-Davis *et al*, 2002; Gugala *et al*, 2003) after transduction of Ad5F35BMP2 into a number of human cells. However, in our current investigations we

wished to inject transduced cells into immunocompetent mice. To aid in this process we transduced and injected murine (MC3T3-E1) rather than human cells. Approximately 5 x 10^6 MC3T3-E1 cells were transduced with either Ad5BMP2 (5,000 vp/cell) or Ad5-empty (5,000 vp/cell) in the presence or absence of GeneJammer® and injected into the hind quadriceps muscle of C57BL/6 mice (n=4). Figure 5.4B shows the results of radiological analysis of the rear hind limbs of only two of the mice, since all mice gave identifcal results, approximately two weeks after injection of the transduced cells. Limbs of mice that received the cells transduced with Ad5BMP2 in the presence of GeneJammer® exhibited detectable mineralization while the limbs injected with cells transduced in the absence of the polyamine showed little to no detectable bone. These findings demonstrate the ability of BMP2 transduced cells to elicit bone formation even in mice with a normal immune system. Further, the results suggest once again that the level of Ad5BMP2 transduction of the cells is crucial to elicit bone formation.

Transduction of human hPBMCs in the presence of GeneJammer®

A potentially attractive gene therapy system to treat musculoskeletal defects would be to efficiently transduce a patient's own blood cells to express high levels of BMP2. In previous studies we (Yotnda *et al.*, 2001) and others (Schroers *et al.*, 2004), have found that human blood is very difficult to transduce with standard adenovirus type 5 vectors. Although the chimeric vector Ad5F35 greatly improves the transduction efficiency, we chose to determine if we could enhance the human hPBMCs transduction with the addition of GeneJammer®. Human PBMCs were grown in 6-well dishes and transduced with an Ad5F35eGFP virus at a concentration of 5,000 vp/cell in the presence or absence of GeneJammer®. It was observed a 14% increase in transduction efficiency

of hPBMC by Ad5F35eGFP in the presence of GeneJammer® (Fig. 5.5A). We then measured the amount of BMP2 secreted into the culture supernatant after transduction of hPBMCs with Ad5F35BMP2, in the presence or absence of GeneJammer®. The results show a smaller but statistically significant increase (0.5 fold) in BMP2 protein in culture supernatant taken from the hPBMCs transduced in the presence of this compound (Fig. 5.5B). BMP2 was not detected in the culture supernatant from either cells alone demonstrating that the BMP2 expression in all cases specifically corresponded with viral transduction (Fig. 5.5B). The level of BMP2 activity was also measured in this culture supernatant using the W20-17 cell assay. Cells were transduced with Ad5F35BMP2 at a concentration of 5000 vp/cell in the presence or absence of GeneJammer®. We observed a similar small but significant (0.4 fold) increase in expression of alkaline phosphatase activity, which is induced in W20-17 cells in response to BMP2, demonstrating an increase in BMP2 production by the hPBMCs transduced in the presence of GeneJammer® (Fig. 5.5C).

DISCUSSION

Much emphasis in tissue engineering of bone has been focused on developing gene therapy approaches to induce bone formation. However, many critical obstacles still stand in the pathway of developing such a system for clinical use, such as efficient transduction of either delivery cells or target tissues, sequestration of the cell or vector system to the specific site, and avoidance of immune surveillance and clearing prior to obtaining the desired bone induction.

We and others have taken a viral-based gene therapy approach to deliver BMP2 ectopically for induction of new bone formation (Olmsted-Davis *et al.*, 2002; Gugala *et*

al., 2003; Tsuda *et al.*, 2003; Zhu *et al.*, 2004). Since BMP2 is a secreted protein, the transduced cells function as a delivery vehicle (Gugala *et al.*, 2003), but they provide additional safety, since no free virus would be delivered to patients, which reduces both immune clearance and acute reaction to the virus itself. Ideally these cells would be readily obtainable from patients, such as human peripheral blood which would avoid additional immune response to foreign cells. However, we have previously demonstrated that bone formation was linked to the level of BMP2 secretion, with high levels of BMP2 inducing more bone formation than low. Hence transduction methodology must be versatile enough to provide high level transduction of a variety of species required for preclinical development, as well as the cell types found in human blood.

Here we present a rapid, easy, inexpensive versatile method for achieving high level adenovirus transduction of cells from various species, including hPBMCs, by inclusion of the DNA transfection reagent, GeneJammer®. Comparison of eGFP expression after transduction with Ad5eGFP or Ad5F35eGFP showed significant increase in both the number of cells and the intensity of transgene expression in the presence of GeneJammer® independent of fiber type. Interestingly, in experiments in which 100% of the cells were transduced at all viral doses in the presence of GeneJammer®, we observed an increase in GFP intensity corresponding with increasing amounts of adenovirus. As shown Figure 5.1B, at 2,500 vp/cell, 100% of the cells are transduced. However, the mean fluorescence intensity of eGFP expression is approximately 10^3 eGFP fluorescence units, whereas at 5,000 vp/cell the mean is greater than 10^4 eGFP fluorescence units. Further, at the highest virus concentration (10,000 vp/cell) approximately 60% of the cells expressed equal or greater than 10^4 eGFP

fluorescence units as compared to the lower dose (5,000 vp/cell) in which only 30% of the cells express this level of eGFP intensity. These results suggest that the mechanism by which GeneJammer® assists adenovirus entry into cells, is not limiting at the doses studied and that it can also increase the total number of adenovirus particles entering any individual cell resulting in higher levels of expression per cell.

Since adenovirus fiber protein is involved in receptor binding and internalization, two likely models exist as to how the compound enhances cellular transduction. First, GeneJammer® may bind the virus and allow cellular entry through either an alternative receptor or a non-receptor mediated pathway. Alternatively the compound may enhance adenovirus binding to its normal receptor, functioning as a co-receptor.

To determine which of the two tentative mechanisms was involved, we selected three different cell lines which had varying levels of adenovirus receptors and transduced them in the presence or absence of GeneJammer®. As expected Ad5eGFP was able to infect 100% of the CAR positive cell line A549, but were less efficient at transducing the hBM-MSCs, which possesses only the α_v receptor; with 10% of the cells positive for eGFP expression. Further, in the CHO cells that are negative for both adenovirus-specific receptors, we observed no cells expressing eGFP. However, when GeneJammer® was included in the transduction reaction, approximately 100% of the cells were found to be expressing the transgene in all three cell types. A significant enhancement in expression was achieved in the cell lines that lacked one of both adenovirus type 5-specific receptors, while in the receptor positive cell lines, the transduction efficiency remained the same, presumably due to the fact that it was maximal under both circumstance. Interestingly, the results suggest that GeneJammer®

is not enhancing viral entry through its normal receptor/co-receptor mediated route but rather utilizing a novel mechanism, since 100% of the cells were transduced in the cell line lacking adenovirus type 5 specific receptors. This finding is consistent to other studies where uptake of adenovirus via hexon protein binding to dipalmityl phosphatidyl choline resulted in cellular internalization via an alternative mechanism that does not utilize receptor binding (Balakireva *et al.*, 2003). However, the authors suggested that this is a mechanism utilized by adenovirus for entry in the lung, since alveolar epithelial type II cells synthesize the major component of pulmonary surfactant, disaturated phosphatidylcholine upon adenovirus infection. Further they suggest that although the cells do possess adenovirus-specific receptors, it is the production of this compound which leads to the high transduction efficiency found with these cell types including A549 cells (Balakireva *et al.*, 2003). Further studies are required to determine if GeneJammer® functions through similar mechanisms.

GeneJammer® significantly enhanced expression of eGFP when Ad5F35eGFP was used in the transductions. Again this suggests that the mechanism by which GeneJammer® enhances virus entry into the cell is not specific to fiber type 5. As expected A549 cells which possess the adenovirus type 35 specific receptor CD46, 100% of cells transduced in the presence or absence of the compound. Interestingly, we observed approximately the same transduction efficiency in both hBM-MSCs and CHO cells with Ad5F35eGFP even though they differ significantly in the level of CD46. One possible explanation is that the anti-human CD46 antibody used in these experiments may not detect hamster CD46. The lower level of induction by GeneJammer® is in part due to the higher transduction efficiency of the Ad5F35eGFP vector in its absence with

approximately 55-60% of the cells transduced. Ad5eGFP transduction of CHO cells, however, is low in the absence of GeneJammer®, and it has been shown previously (Bergelson *et al.*, 1997; Davison *et al.*, 1999) that CHO cells lack CAR, because CHO cells can be transduced by Ad5 vectors when these cells are transfected with a vector that elicits the expression of CAR.

We next measured the level of BMP2 production in cells transduced with Ad5BMP2 to determine if GeneJammer® could enhance the expression of this protein. There was a significant enhancement in BMP2 production, and activity *in vitro* and *in vivo* (Fig. 5.3A, B and C). Interestingly, in the assay for functional BMP2, media collected from hBM-MSCs transduced with Ad5F35BMP2 stimulated alkaline phosphatase to a level of approximately 5,000 chemiluminescence units, while the same cells transduced with Ad5BMP2 in the presence of GeneJammer® elevated alkaline phosphatase to a level of 9,000 chemiluminescence units demonstrating that this universal system could actually lead to increased BMP2 activity than parallel systems using the chimeric Ad5F35BMP2 (Olmsted-Davis *et al.*, 2002). As expected this high level expression of BMP2 after transduction of the hBM-MSCs with Ad5BMP2 in the presence of GeneJammer® led to significant *in vivo* bone formation after implantation, whereas the cells transduced in parallel in the absence of GeneJammer®, did not induce detectable mineralization or osteoid by histology. Again this verifies our previous finding (Gugala *et al.*, 2003) that the level of BMP2 expression dictates the formation of bone and is linked to the transduction efficiency of the cells. Hence developing a system which would routinely provide high level transduction of cells with adenovirus independent of vector type is an essential component to developing a gene therapy

system to induce bone formation. Further, the data also demonstrates that there are no apparent deleterious effects on these processes or the BMP2 protein itself due to the potential presence of residual compound in the media or to the animals after injection of these transduced cells suggesting that this is safe for conducting preclinical trials.

Next we determined the effects of this compound on Ad5BMP2 transduction efficiency of a murine cell line, MC3T3-E1 by measuring BMP2 activity in the culture supernatant using the W20-17 assay. Previous attempts to achieve high level transduction of this cell line have failed due in part to the lack of adenovirus-specific receptors for either Ad5 or Ad5F35 vectors (Mallam *et al.*, 2004) . However, use of this or other syngeneic murine cells is critical in pre-clinical studies using Ad5BMP2 transduced cells, since it is derived from the C57BL/6 strain and does not elicit a graft versus host reaction in recipient C57BL/6 mice (data not shown). The results demonstrate that GeneJammer® provides a similar enhancement of expression in these murine cells as that observed in the hBM-MSCs. Interestingly, at 2,500 vp/cell neither transduction methodology led to detectable levels of BMP2 activity in the culture supernatant, presumably due to potential low level transduction of the MC3T3-E1 cells. However, at the higher doses of Ad5BMP2 we did observe significant increases in alkaline phosphatase activity correlating with BMP2 activity in the culture supernatant presumably due to increased transduction of the MC3T3-E1 cells in the presence of GeneJammer®. Culture supernatant taken from MC3T3-E1 cells transduced with Ad5BMP2 (5,000 vp/cell or 10,000 vp/cell) in the presence of GeneJammer® led to at least the same or higher levels of alkaline phosphatase and/or BMP2 activity than those achieved using culture supernatants collected from the Ad5F35BMP2 (2500 vp/cell)

transduced hBM-MSCs (Fig. 5.3B) which consistently produces the level of BMP2 necessary to induce bone formation *in vivo*. These results suggest that the cells are transduced to express BMP2 at a level capable of eliciting bone formation in the immuno-incompetent mice. When the MC3T3-E1 cells transduced with Ad5BMP2 (5,000 vp/cell) in the presence of the compound were implanted *in vivo* into C57BL/6 mice, we observed significant bone formation, suggesting that this level of BMP2 is also sufficient to induce bone formation in immune competent mice (Fig. 5.4B). However, when samples transduced in parallel but in the absence of GeneJammer® were transplanted we did not observe any bone formation again presumably due to the low level of BMP2 expression from these cells. Further these results suggest that high level BMP2 production is essential regardless of the immune profile of the mice. Interestingly this is contradictory to a previous report which suggests that low level virus transduction of cells, with potent osteo-inductive agents may prevent significant immune response allowing longer transgene expression and greater bone induction (Kim *et al.*, 2003).

Finally we tested the ability of GeneJammer® to improve the transduction efficiency of hPBMCs. The results indicated that 32% of the cells expressed GFP after transduction with Ad5F35eGFP in the presence of GeneJammer® as compared with 15% of the total population when the compound was omitted. We did not observe detectable levels of eGFP expression in hPBMCs after transduction with Ad5eGFP in either case (data not shown). This supports previous findings by our laboratory (Yotnda *et al.*, 2001) and others (Schroers *et al.*, 2004) that demonstrated approximately 50% of the lymphoid populations could be transduced using Ad5F35 vectors as compared to 5% when Ad5 vectors were used. Interestingly, since lymphoid cells make up approximately 28-60% of

the hPBMCs, the 15% transduction we obtained using the Ad5F35 vectors may represent transduction of the lymphoid component. However, further studies are needed to determine whether GeneJammer® enhances transduction of the lymphoid component of hPBMCs or whether it transduces another population of cells perhaps lacking the CD46 receptor.

Since we are interested in using the hPBMCs to deliver BMP2 to specific sites for the production of bone, the exact nature of the cells transduced in peripheral blood are not essential to our efforts. Therefore we determined whether hPBMCs transduced with Ad5F35BMP2 in the presence of absence of BMP2 or GeneJammer® could make detectable levels of BMP2. Our results showed approximately 0.4 fold more BMP2 in culture supernatant collected from hPBMCs transduced in the presence of GeneJammer®, as compared to those in the absence. This result was reproducible and statistically significant. Further, we observed a similar enhancement in alkaline phosphatase activity in the W20-17 cells after exposure to culture supernatant collected from hPBMCs transduced in the presence of GeneJammer® as compared to similar samples transduced in the absence of this compound, supporting the idea that the BMP2 detected in the culture supernatant is functionally active.

We conclude that the use of GeneJammer® as described in this study can significantly enhance adenovirus entry into cells even in those lacking adenovirus-specific receptors. Thus this technique provides investigators the ability to transduce a wide range of cells through a rapid, inexpensive method, offering substantial versatility which was previously lacking. Such advances are critical in the translation of cell-based gene therapy systems from small animal models into preclinical and clinical testing.

REFERENCES

BALAKIREVA, L., SCHOEHN, G., THOUVENIN, E., and CHROBOCZEK, J. (2003). Binding of adenovirus capsid to dipalmitoyl phosphatidylcholine provides a novel pathway for virus entry. J Virol **77**, 4858-4866.

BERGELSON, J.M., CUNNINGHAM, J.A., DROGUETT, G., KURT-JONES, E.A., KRITHIVAS, A., HONG, J.S., HORWITZ, M.S., CROWELL, R.L., and FINBERG, R.W. (1997). Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science **275**, 1320-1323.

BLUM, J.S., LI, R.H., MIKOS, A.G., and BARRY, M.A. (2001). An optimized method for the chemiluminescent detection of alkaline phosphatase levels during osteodifferentiation by bone morphogenetic protein 2. J Cell Biochem **80**, 532-537.

BONADIO, J., SMILEY, E., PATIL, P., and GOLDSTEIN, S. (1999). Localized, direct plasmid gene delivery in vivo: prolonged therapy results in reproducible tissue regeneration. Nat Med **5**, 753-759.

BYK, T., HADDADA, H., VAINCHENKER, W., and LOUACHE, F. (1998). Lipofectamine and related cationic lipids strongly improve adenoviral infection efficiency of primitive human hematopoietic cells. Hum Gene Ther **9**, 2493-2502.

DAVISON, E., KIRBY, I., ELLIOTT, T., and SANTIS, G. (1999). The human HLA-A*0201 allele, expressed in hamster cells, is not a high-affinity receptor for adenovirus type 5 fiber. J Virol **73**, 4513-4517.

FRANCESCHI, R.T., YANG, S., RUTHERFORD, R.B., KREBSBACH, P.H., ZHAO, M., and WANG, D. (2004). Gene therapy approaches for bone regeneration. Cells Tissues Organs **176**, 95-108.

GAGGAR, A., SHAYAKHMETOV, D.M., and LIEBER, A. (2003). CD46 is a cellular receptor for group B adenoviruses. Nat Med **9**, 1408-1412.

GAO, W., ROBBINS, P.D., and GAMBOTTO, A. (2003). Human adenovirus type 35: nucleotide sequence and vector development. Gene Ther **10**, 1941-1949.

GUGALA, Z., OLMSTED-DAVIS, E.A., GANNON, F.H., LINDSEY, R.W., and DAVIS, A.R. (2003). Osteoinduction by ex vivo adenovirus-mediated BMP2 delivery is independent of cell type. Gene Ther **10**, 1289-1296.

HARRISON, G.S., WANG, Y., TOMCZAK, J., HOGAN, C., SHPALL, E.J., CURIEL, T.J., and FELGNER, P.L. (1995). Optimization of gene transfer using cationic lipids in cell lines and primary human CD4+ and CD34+ hematopoietic cells. Biotechniques **19**, 816-823.

HIDAKA, C., MILANO, E., LEOPOLD, P.L., BERGELSON, J.M., HACKETT, N.R., FINBERG, R.W., WICKHAM, T.J., KOVESDI, I., ROELVINK, P., and CRYSTAL, R.G. (1999). CAR-dependent and CAR-independent pathways of adenovirus vector-mediated gene transfer and expression in human fibroblasts. J Clin Invest **103**, 579-587.

JAISWAL, N., HAYNESWORTH, S.E., CAPLAN, A.I., and BRUDER, S.P. (1997). Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. J Cell Biochem **64**, 295-312.

KIM, H.S., VIGGESWARAPU, M., BODEN, S.D., LIU, Y., HAIR, G.A., LOUIS-UGBO, J., MURAKAMI, H., MINAMIDE, A., SUH, D.Y., and TITUS, L. (2003). Overcoming the immune response to permit ex vivo gene therapy for spine fusion with human type 5 adenoviral delivery of the LIM mineralization protein-1 cDNA. Spine **28**, 219-226.

LANUTI, M., KOURI, C.E., FORCE, S., CHANG, M., AMIN, K., XU, K., BLAIR, I., KAISER, L., and ALBELDA, S. (1999). Use of protamine to augment adenovirus-mediated cancer gene therapy. Gene Ther **6**, 1600-1610.

MALLAM, J.N., HURWITZ, M.Y., MAHONEY, T., CHEVEZ-BARRIOS, P., and HURWITZ, R.L. (2004). Efficient gene transfer into retinal cells using adenoviral vectors: dependence on receptor expression. Invest Ophthalmol Vis Sci **45**, 1680-1687.

MARIT, G., CAO, Y., FROUSSARD, P., RIPOCHE, J., DUPOUY, M., ELANDALOUSSI, A., LACOMBE, F., MAHON, F.X., KELLER, H., PLA, M.,

REIFFERS, J., and THEZE, J. (2000). Increased liposome-mediated gene transfer into haematopoietic cells grown in adhesion to stromal or fibroblast cell line monolayers. Eur J Haematol **64**, 22-31.

MIZUGUCHI, H., and HAYAKAWA, T. (2002). Adenovirus vectors containing chimeric type 5 and type 35 fiber proteins exhibit altered and expanded tropism and increase the size limit of foreign genes. Gene **285**, 69-77.

MUSGRAVE, D.S., BOSCH, P., LEE, J.Y., PELINKOVIC, D., GHIVIZZANI, S.C., WHALEN, J., NIYIBIZI, C., and HUARD, J. (2000). Ex vivo gene therapy to produce bone using different cell types. Clin Orthop Relat Res, 290-305.

OLMSTED, E.A., BLUM, J.S., RILL, D., YOTNDA, P., GUGALA, Z., LINDSEY, R.W., and DAVIS, A.R. (2001). Adenovirus-mediated BMP2 expression in human bone marrow stromal cells. J Cell Biochem **82**, 11-21.

OLMSTED-DAVIS, E.A., GUGALA, Z., GANNON, F.H., YOTNDA, P., MCALHANY, R.E., LINDSEY, R.W., and DAVIS, A.R. (2002). Use of a chimeric adenovirus vector enhances BMP2 production and bone formation. Hum Gene Ther **13**, 1337-1347.

PARK, J., RIES, J., GELSE, K., KLOSS, F., VON DER MARK, K., WILTFANG, J., NEUKAM, F.W., and SCHNEIDER, H. (2003). Bone regeneration in critical size defects by cell-mediated BMP-2 gene transfer: a comparison of adenoviral vectors and liposomes. Gene Ther **10**, 1089-1098.

SAKURAI, F., MIZUGUCHI, H., and HAYAKAWA, T. (2003). Efficient gene transfer into human CD34+ cells by an adenovirus type 35 vector. Gene Ther **10**, 1041-1048.

SCHROERS, R., HILDEBRANDT, Y., HASENKAMP, J., GLASS, B., LIEBER, A., WULF, G., and PIESCHE, M. (2004). Gene transfer into human T lymphocytes and natural killer cells by Ad5/F35 chimeric adenoviral vectors. Exp Hematol **32**, 536-546.

SEGERMAN, A., ATKINSON, J.P., MARTTILA, M., DENNERQUIST, V., WADELL, G., and ARNBERG, N. (2003). Adenovirus type 11 uses CD46 as a cellular receptor. J Virol **77**, 9183-9191.

THIES, R.S., BAUDUY, M., ASHTON, B.A., KURTZBERG, L., WOZNEY, J.M., and ROSEN, V. (1992). Recombinant human bone morphogenetic protein-2 induces osteoblastic differentiation in W-20-17 stromal cells. Endocrinology **130**, 1318-1324.

TOYODA, K., NAKANE, H., and HEISTAD, D.D. (2001). Cationic polymer and lipids augment adenovirus-mediated gene transfer to cerebral arteries in vivo. J Cereb Blood Flow Metab **21**, 1125-1131.

TSUDA, H., WADA, T., ITO, Y., UCHIDA, H., DEHARI, H., NAKAMURA, K., SASAKI, K., KOBUNE, M., YAMASHITA, T., and HAMADA, H. (2003). Efficient BMP2 gene transfer and bone formation of mesenchymal stem cells by a fiber-mutant adenoviral vector. Mol Ther **7**, 354-365.

TURGEMAN, G., ZILBERMAN, Y., ZHOU, S., KELLY, P., MOUTSATSOS, I.K., KHARODE, Y.P., BORELLA, L.E., BEX, F.J., KOMM, B.S., BODINE, P.V., and GAZIT, D. (2002). Systemically administered rhBMP-2 promotes MSC activity and reverses bone and cartilage loss in osteopenic mice. J Cell Biochem **86**, 461-474.

YOTNDA, P., CHEN, D.H., CHIU, W., PIEDRA, P.A., DAVIS, A., TEMPLETON, N.S., and BRENNER, M.K. (2002). Bilamellar cationic liposomes protect adenovectors from preexisting humoral immune responses. Mol Ther **5**, 233-241.

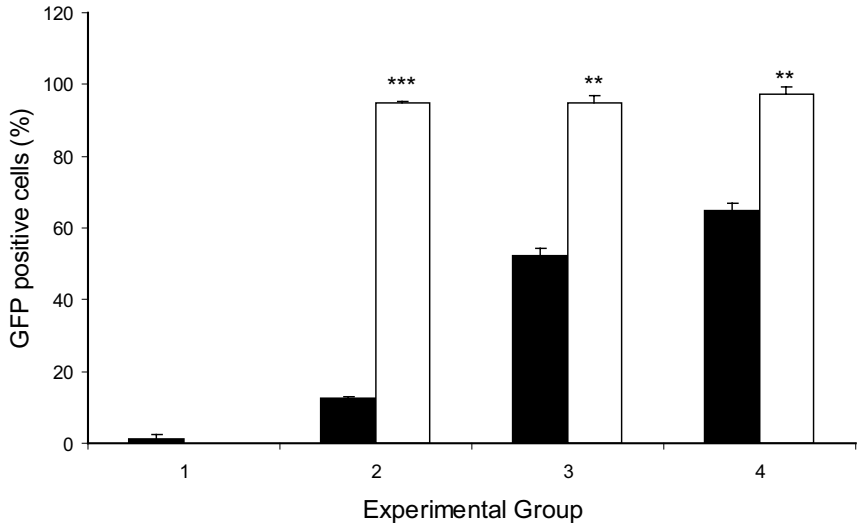
YOTNDA, P., ONISHI, H., HESLOP, H.E., SHAYAKHMETOV, D., LIEBER, A., BRENNER, M., and DAVIS, A. (2001). Efficient infection of primitive hematopoietic stem cells by modified adenovirus. Gene Ther **8**, 930-937.

ZHU, W., RAWLINS, B.A., BOACHIE-ADJEI, O., MYERS, E.R., ARIMIZU, J., CHOI, E., LIEBERMAN, J.R., CRYSTAL, R.G., and HIDAKA, C. (2004). Combined bone morphogenetic protein-2 and -7 gene transfer enhances

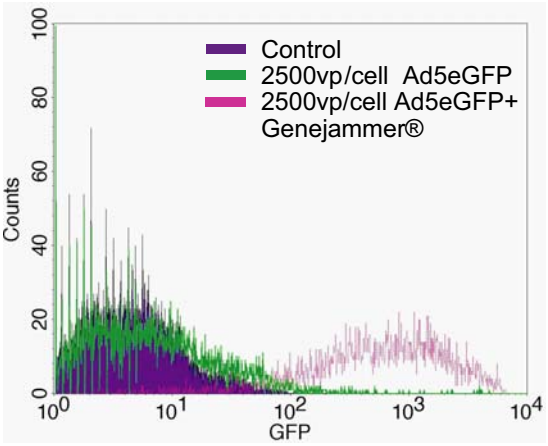
osteoblastic differentiation and spine fusion in a rodent model. J Bone Miner Res **19**, 2021-2032.

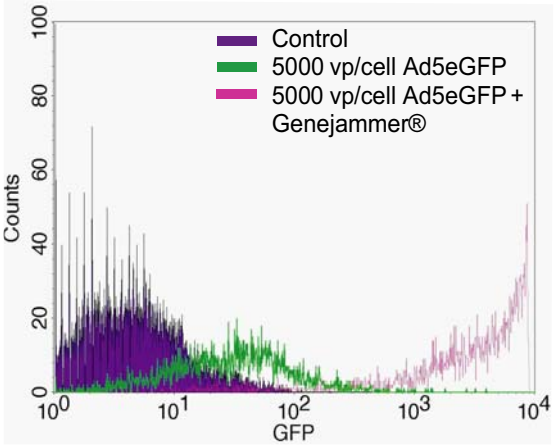
FIG. 5.1. (A) Flow cytometric quantification of GFP expression after transduction of hBM-MSCs with Ad5-empty (bar 1), Ad5eGFP 2,500vp/cell (bar 2), Ad5eGFP 5,000vp/cell (bar 3), Ad5eGFP 10,000vp/cell (bar 4). In the absence ■ or presence ▭ of GeneJammer®. The percentage of GFP positive cells is depicted as the average eGFP fluorescence where n=3. Errors bars represent means ± standard deviation for n=3. *** represent $p<0.001$ and ** represent $p<0.01$; Student *t* test. **(B)** GFP fluorescence intensity shifts in the flow cytometry profiles of eGFP expression in the hBM-MSCs transduced with either 2,500 vp/cell, 5,000 vp/cell, or 10,000 vp/cell Ad5GFP in the presence of GeneJammer® shown in **(A)**. In all samples 100% of the cells were found to express eGFP.

A


GFP positive cells (%)
120
100
80
60
40
20
0
***
**
**
1
2
3
4
Experimental Group

B


Control
2500vp/cell Ad5eGFP
2500vp/cell Ad5eGFP+
Genejammer®
Counts
GFP


Control
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5000 vp/cell Ad5eGFP +
Genejammer®
Counts
GFP

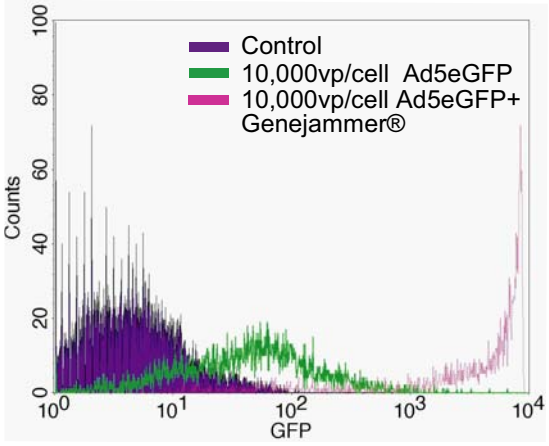

Control
10,000vp/cell Ad5eGFP
10,000vp/cell Ad5eGFP+
Genejammer®
Counts
GFP

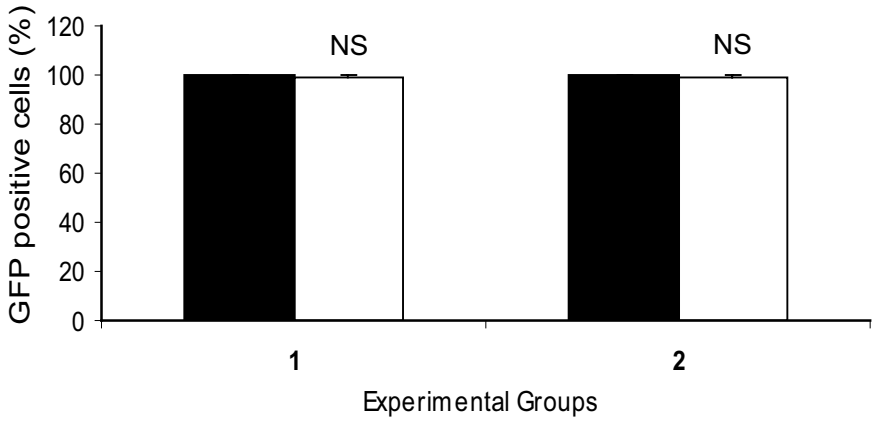
TABLE 5.1: Adenovirus receptors expression on selected cell lines

Cell line	Cell Surface Receptor		
	CAR	Integrin αV	CD46
A549	93.28% ± 1.28	99.26% ± 0.53	99.27% ± 0.79
hBM-MSCs	*0.34% ± 0.50	*87% ± 4.70	31.78% ± 3.54
CHO	1.77% ± 0.27	2.04% ± 0.16	0.19% ± 0.05

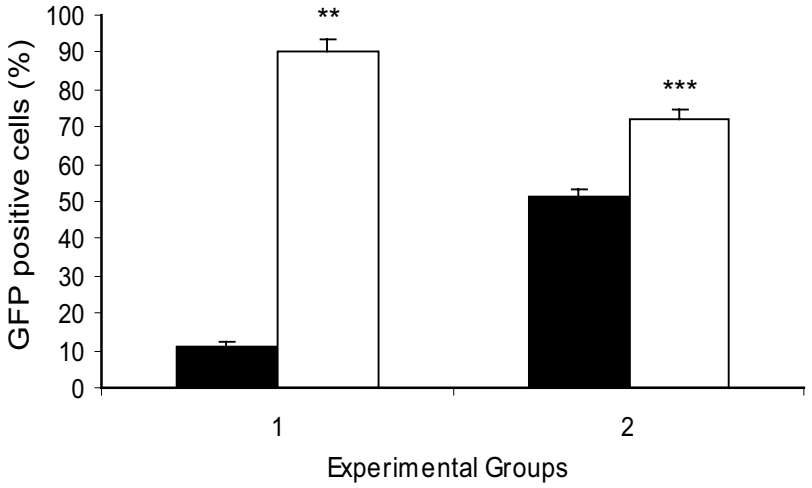
Numbers represent mean percentage of cells expressing each receptor ± SD (n=3)

FIG. 5.2. Flow cytometry analysis of GFP expression of A549 cells **(A)**, hBM-MSCs (B) and CHO cells **(C)** transduced with Ad5eGFP 2,500 vp/cell (bar 1), Ad5F35eGFP 2,500 vp/cell (bar 2). In the absence ■ or presence □ of GeneJammer®. The percentage of GFP positive cells was depicted as the average GFP fluorescence where n=3. Errors bars represent means ± standard deviation for n=3. *** represent $p<0.001$ and ** represent $p<0.01$; Student *t* test.

A


GFP positive cells (%)
120
100
80
60
40
20
0
NS
NS
1
2
Experimental Groups

B


GFP positive cells (%)
100
90
80
70
60
50
40
30
20
10
0
**
***
1
2
Experimental Groups

C

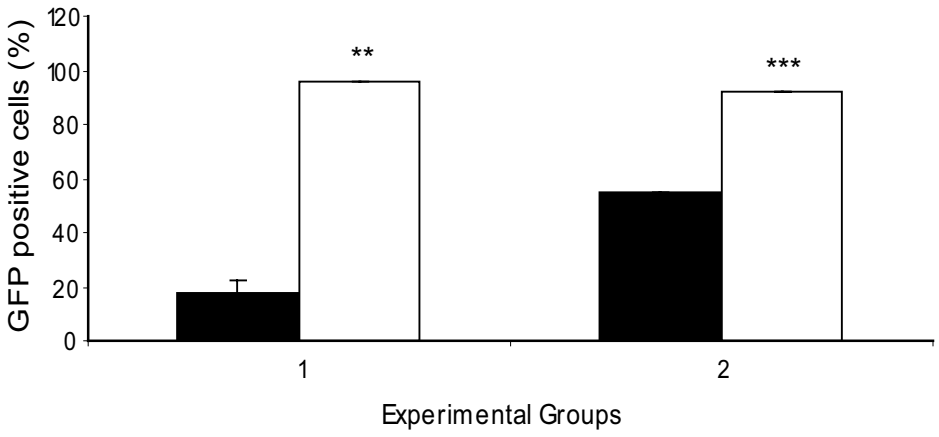
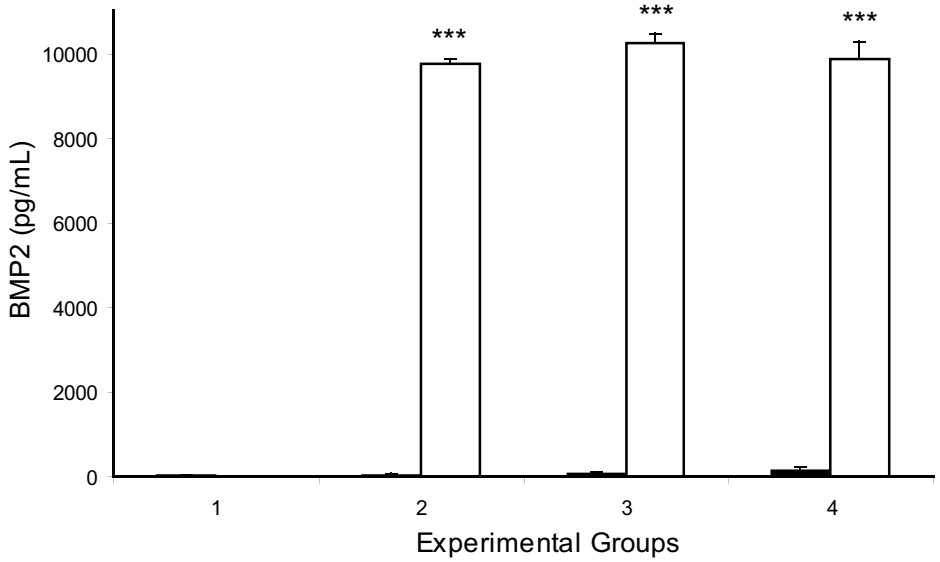
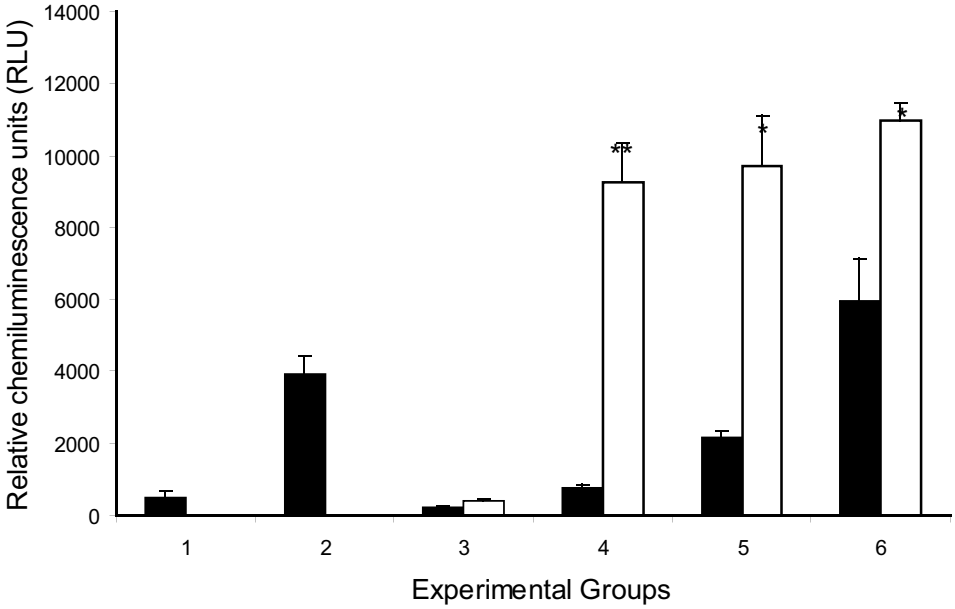

GFP positive cells (%)
120
100
80
60
40
20
0
**
***
1
2
Experimental Groups

FIG. 5.3. Quantification of BMP2 protein **(A)**, or activity *in vitro* **(B)** or *in vivo* **(C)**. **(A)** BMP2 protein concentration was determined in the culture supernatants using the Quantikine BMP2 assay (R&D systems). The concentration of BMP2 was extrapolated from a standard curve using known concentrations of recombinant BMP2. BMP2 concentrations in the supernatant are reported as pg/ml, n=3. **(B)** BMP2 activity was measured in culture supernatant taken from hBM-MSCs alone (bar 1), hBM-MSCs transduced with a control Ad5F35BMP2 (2500vp/cell) (bar 2), or those cells transduced with Ad5-empty (5,000 vp/cell) bar 3, Ad5BMP2 (2,500 vp/cell), bar 4, Ad5BMP2 (5,000 vp/cell) bar 5, Ad5BMP2 (10,000 vp/cell) bar 6 by determining the increase in alkaline phosphatase activity in W20-17 cells 72 hours after the exposure. In the absence ■ or presence □ of GeneJammer®. Alkaline phosphatase activity is depicted as the average relative chemiluminescence units (RLU) where n=3. Errors bars represents means ± standard deviation for n=3. *** represent $p<0.001$ and ** represent $p<0.01$; Student *t* test. **(C)** Radiologic analysis of heterotropic bone formation in NOD-SCID mice after intramuscular injection of hBM-MSCs transduced with Ad5BMP2 (5,000 vp/cell) + GeneJammer® **(C1)** or Ad5BMP2 alone **(C2)**.

A


***
***
***
10000
8000
6000
4000
2000
0
BMP2 (pg/mL)
1
2
3
4
Experimental Groups

B

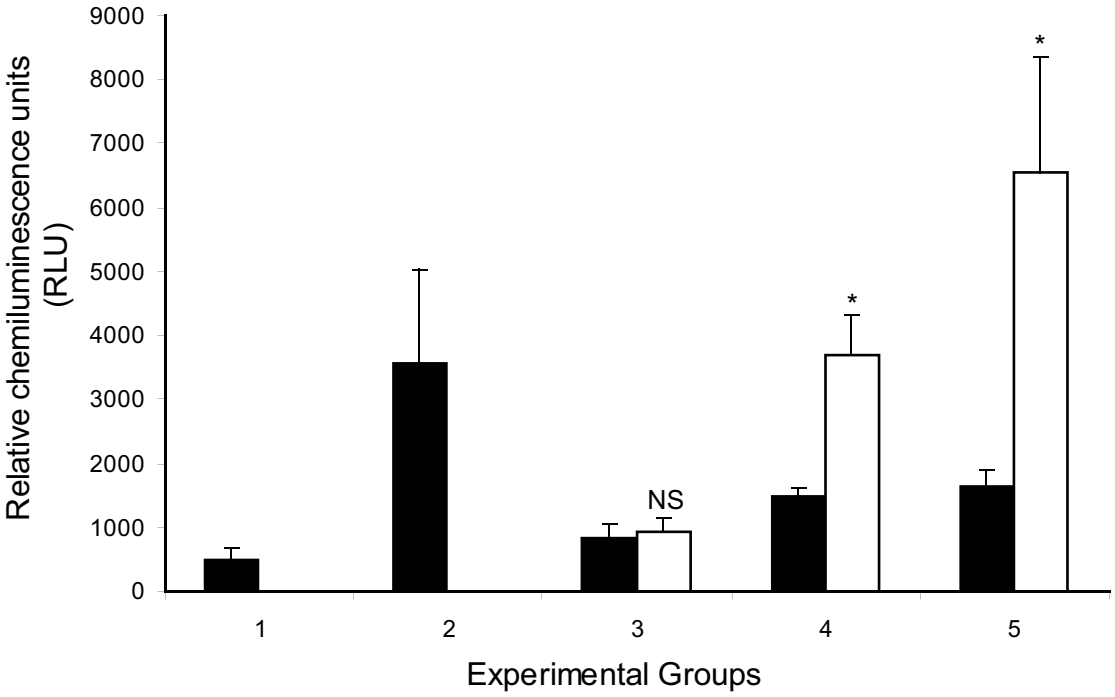

14000
12000
10000
8000
6000
4000
2000
0
Relative chemiluminescence units (RLU)
1
2
3
4
5
6
Experimental Groups

C


1
2

FIG. 5.4. Analysis of BMP2 activity both i*n vitro* and *in vivo.* Analysis of BMP2 activity in **(A)** culture supernatant taken from MC3T3-E1 cells (bar 1) or those transduced with Ad5F35BMP2 (2,500 vp/cell) bar 2, Ad5BMP2 (2,500 vp/cell) bar 3, Ad5BMP2 (5,000 vp/cell) bar 4, Ad5BMP2 (10,000 vp/cell) bar 5. Alkaline phosphatase activity is depicted as the average relative chemiluminescence (RLU) where n=3. Errors bars represents means ± standard deviation for n=3. * represent $p<0.05$; Student *t* test.. In the absence ■ or presence □ of GeneJammer®. *In vivo* analysis of BMP2 activity **(B)** was determined radiologically in C57BL/6 mice that received MC3T3-E1 cells transduced with Ad5BMP2 (5,000 vp/cell) in the presence **(B1)** or absence **(B2)** of GeneJammer®.

A


Relative chemiluminescence units (RLU)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
NS
*
*
1
2
3
4
5
Experimental Groups

B

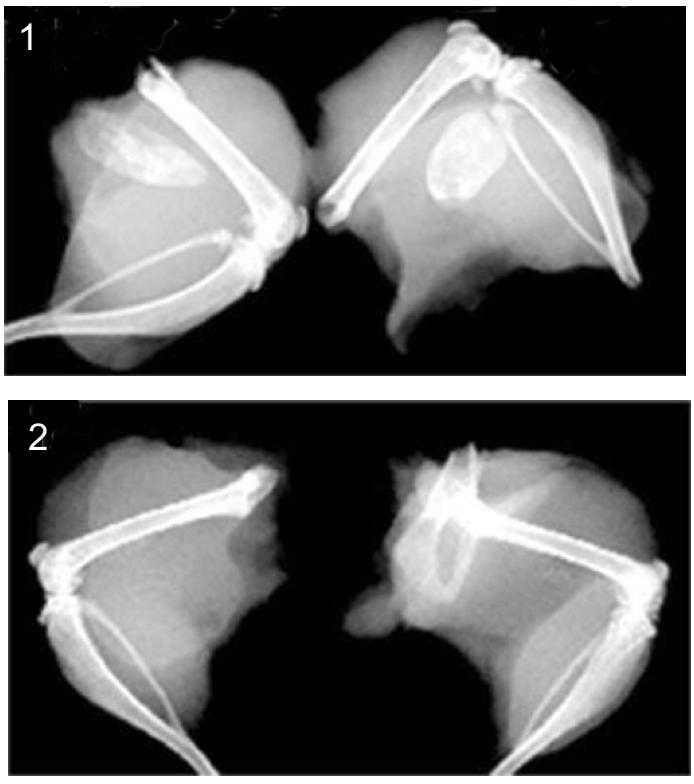
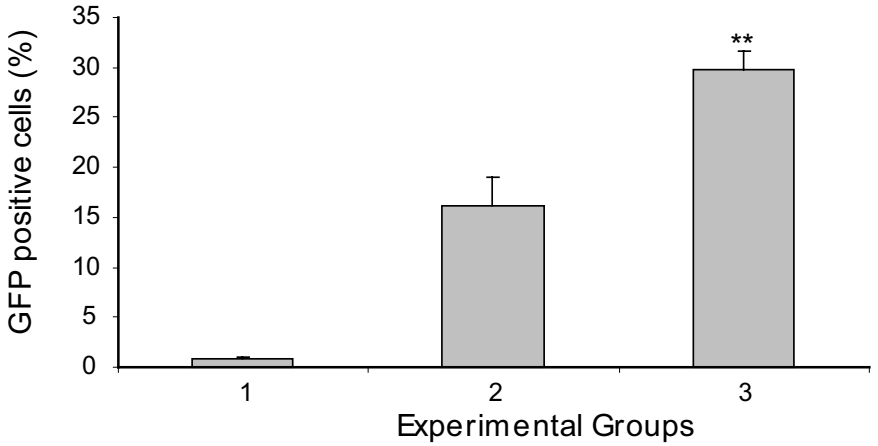
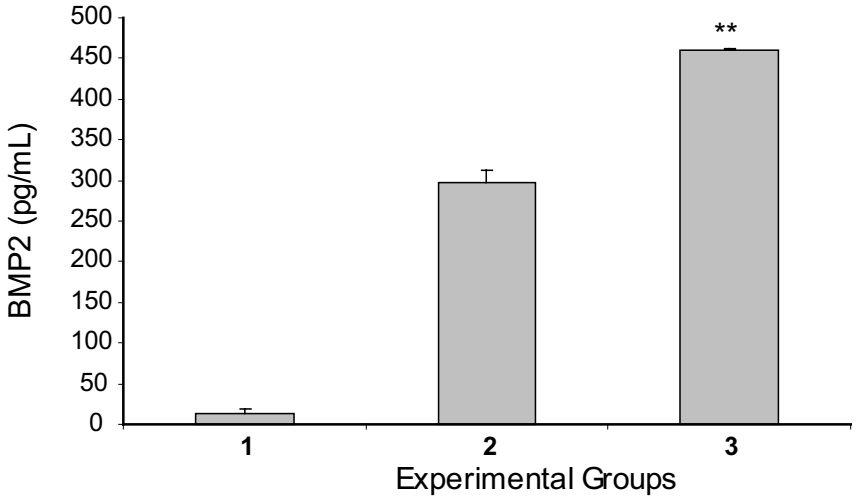

1
2

FIG. 5.5. Transduction efficiency of hPBMCs in the presence or absence of GeneJammer®. **(A)** Flow cytometric analysis of GFP expression in cells or quantification of BMP2 **(B)** protein or **(C)** activity in culture supernatant from hPBMCs transduced with Ad5F35HM4 (5,000 vp/cell) which lacks any transgene (bar 1), Ad5F35eGFP (5,000 vp/cell) (bar 2), or Ad5F35eGFP 5000 vp/cell + GeneJammer® (bar 3). **(C)** BMP2 activity was determined by the induction of alkaline phosphatase in the W20-17 cells. Bar 4 represents the control, media from W20-17 alone. Alkaline phosphatase activity is depicted as the average relative chemiluminescence units (RLU). Errors bars represents means ± standard deviation for n=3.

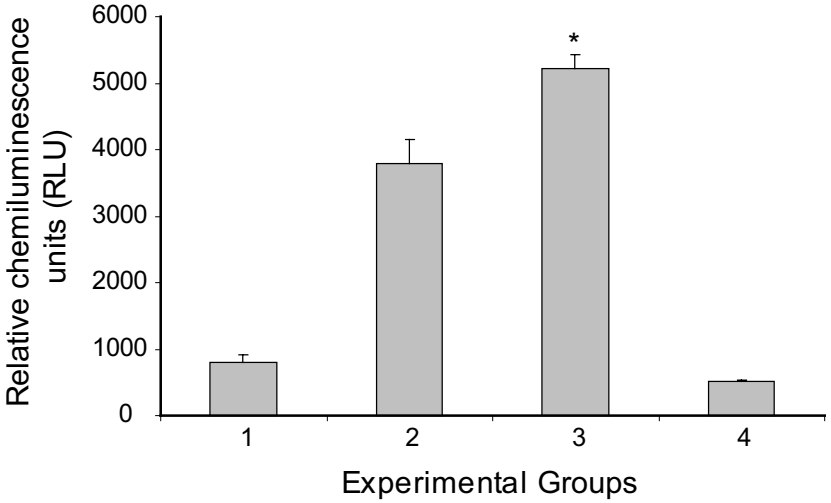
A


GFP positive cells (%)
35
30
25
20
15
10
5
0
**
1
2
3
Experimental Groups

B


BMP2 (pg/mL)
500
450
400
350
300
250
200
150
100
50
0
**
1
2
3
Experimental Groups

C


Relative chemiluminescence units (RLU)
6000
5000
4000
3000
2000
1000
0
*
1
2
3
4
Experimental Groups

CHAPTER 6

CONCLUSIONS

Adult bone marrow contains mesenchymal stem cells (MSC), also referred as marrow stromal cells, which constitute an integral component of the bone marrow microenvironment that support hematopoiesis. In addition, these pluripotent stem cells can form in vitro and in vivo connective tissues including bone, cartilage and fat. Because the relative ease for establishing in vitro cultures of MSCs and their ability to extensively proliferate under in vitro conditions, MSCs hold great promise as a readily available cell source for development of cell-based strategies for the treatment of human diseases. A particularly attractive therapeutic approach is the use of cultured genetically modified MSCs as vehicles for delivery of therapeutic agents. In this regard, development of appropriate large animal models to address biosafety concerns associated with these novel protocols will be instrumental for future human clinical use. Therefore, the overall goal of the studies that comprise this dissertation was to establish a pig model where to study transient and stable genetic modifications of cultured MSCs. Finally, gene delivery methods developed for pig MSCs (pMSC) were tested in human MSCs and rodent cell lines.

The objectives of the first study were: a) to establish porcine MSC lines and identify appropriate in vitro culture conditions; b) to study non-viral and viral-based

methods for gene delivery in porcine MSC and c) to determine pMSC reprogramming potential using somatic cell nuclear transfer (SCNT). Ten adult pMSC lines were established from pig bone marrow using a minimally invasive aspiration technique. The MSC identity of fibroblast-like cells isolated from pig bone marrow was confirmed by immunophenotyping and multipotential differentiation capacity. Bone marrow-derived fibroblastic cells highly expressed a marker for cells of mesenchymal origin (CD90) but they did not express the hematopoietic marker CD11b. When MSCs were exposed to appropriate inductive conditions in vitro, they differentiated into fat, cartilage and bone tissues. These adult stem cells exhibited vast proliferative potential in vitro (~ 30 cell doublings until senescence) and underwent transient and stable genetic modification with non-viral and viral vectors. Of particular interest is the highly efficient transduction of MSCs with a non-integrating human adenovirus (Ad5F35eGFP) and adeno-associated vectors. Nuclear transfer results suggest that pMSC can undergo nuclear reprogramming to support blastocyst development after being transferred to enucleated MII oocytes. All these characteristics along with favorable clonal cell propagation properties make pMSCs an attractive source of cells for large animal preclinical testing. These findings will lead to future autologous cell/gene therapy studies comparing easily cultured genetically modified adult pMSC to isogeneic embryonic cells derived via SCNT, thus addressing cell rejection issues in non murine models for disease and tissue repair.

Since adenovirus very rarely integrates into the host genome, replication-defective recombinant adenoviral vectors are considered efficient expression vectors particularly for those applications, such as bone regeneration, in which transient transgene expression is the desired outcome. MSCs are normally poorly transduced by adenovirus vectors due

to the lack or low expression of specific adenoviral receptors. Therefore, the objectives of the second study were: a) to optimize adenoviral transduction efficiency of bone marrow pMSC using a commercial polyamine-based transfection reagent (GeneJammer®, Stratagene, CA, USA); and b) to determine if transduced cells retain the ability to differentiate in vitro. It is described in this study a highly effective adenoviral transduction system for cultured pMSC which are normally poorly infected by human adenovectors. Complexing human adenovectors with GeneJamme, a commercial polyamine-based tranfection reagent, led to high transgene expression levels in pMSCs while minimizing adverse cell reactions. Furthermore, transduced MSCs retained multipotential differentiation capacity in vitro. In light of the multiple applications of MSC in areas of gene therapy and targeted delivery of therapeutics, results from this study will facilitate addressing multiple biological questions and safety concerns in a large animal model before this protocols move into human clinical trials.

The excellent gene delivery efficiency achieved in pMSCs transduced with adenovirus vectors complexed with GeneJammer prompted us to perform experiments to determine if similar methods would enhance adenovirus infection of human and rodent cell lines. Additionally, the effect of GeneJammer on transduction efficiency was tested in a cell-based gene therapy system for the induction of bone, which is contingent on high level expression of the transgene. As it was demonstrated in the second study (Chapter 4), addition of GeneJammer® during adenoviral transduction of human MSCs and rodent cell lines led to both a significant increase in the total number of transduced cells as well as the level of transgene expression per cell. Studies using cell lines deficient in adenovirus receptors demonstrated that addition of GeneJammer® enhance viral cellular

entry by an adenovirus receptor-independent mechanism. Human and murine cell lines infected with adenovectors carrying the gene for bone morphogenetic protein 2 (BMP2), an osteoinductive cytokine, in presence of GeneJammer achieved higher levels of functional BMP2 expression both in vitro and in vivo. High level of BMP2 expression after transduction of human MSCs in presence of GeneJammer led to the production of detectable amount of mineralized tissue in the heterotopic bone assay whereas cells transduced without GeneJammer did not induce mineralization.

In summary, the use of GeneJammer® as described in this study can significantly enhance adenovirus entry into cells even in those lacking adenovirus-specific receptors. Thus this technique provides investigators the ability to transduce a wide range of cells through a rapid, inexpensive method, offering substantial versatility which was previously lacking. Such advances are critical in the translation of cell-based gene therapy systems from small animal models into preclinical and clinical testing.

Future Studies

In addition to the great potential of MSC in the area of human therapeutics, these adult stem cells are being considered as potential cell source for somatic cell nuclear transfer research. The same features that make MSC an excellent source for cell-mediated therapy are also attractive attributes for a nuclear donor cells for somatic cell nuclear transfer transgenesis, i.e., easily isolated and cultured, clonal propagation and amenable to genetic manipulation.

In the first study presented in Chapter 3 of this dissertation we have established that porcine pMSC can be isolated from bone marrow aspirates using conventional cell culture techniques, proliferate extensively in vitro and undergo transient and stable

genetic modification with non-viral and viral vectors. It is predicted that transient expression of endogenous or exogenous genes in donor cells transduced by adenovirus vectors will represent an important tool to manipulate donor cell physiology in vitro. These novel adenovirus-based approaches could open new possibilities for controlling cell processes such as cycle progression, DNA methylation or apoptosis in NT donor cells, which eventually will lead to improvements in the efficiency of cloning and a better understanding of reprogramming processes. One important question that has not been addressed in our study is whether adenoviral-transduced cells can undergo nuclear reprogramming when transferred to appropriate cytoplasts. Comparison of in vitro and in vivo development of embryos reconstructed with non-transduced or transduced donor cells will provide insights into nuclear reprogramming of adenovirus infected cells.

The use of integrating viral vectors like adeno-associated virus (AAV) is emerging as a highly effective alternative method for stable gene modification of cultured cells. These cells can be later used as nuclear donors to produce cloned animals bearing the genetic transformation. We present strong evidence that pMSCs and fibroblasts transduced with an AAV vector carrying the GFP transgene can lead to high percentage of cells expressing the transgene for extended time. Whether the AAV vector integrated at specific sites of the cell host genome and the number of copies of the virus per genome was not addressed in our study and warrant further investigation. Production of nuclear transfer transgenic animals from of AAV-gene modified cells will represent an important step for future use of AAV vectors in animal transgenesis. Our data also suggest that pMSCs can be, at least partially reprogrammed after transfer to enucleated MII oocytes to support embryo development to the blastocyst stage. Further experiments will be needed

to confirm this preliminary finding and compare the in vitro and in vivo developmental ability of embryos reconstructed with fibroblasts or pMSC.

A therapeutic area in which MSCs promise to play a preponderant role is in ex vivo gene therapy. In these strategies, MSCs obtained from the patient own bone marrow are propagated ex vivo, genetically modified and reintroduced into the patient for treatment of various diseases. Development of efficient gene delivery systems for MSCs remains a significant challenge to overcome for future clinical use. In the second and third studies presented in Chapters 4 and 5 of this dissertation we describe a highly efficient method for adenoviral gene delivery into cultured MSCs from pigs, human and rodents. Addition of a polyamine-base compound (GeneJammer) during adenovirus transduction of MSCs led to enhanced infection efficiency of these cells which are otherwise inefficiently infected by adenovirus. First we develop methods to achieve high transduction rates in porcine MSCs and then we adapted these methods to human MSCs. We have chosen the pig as the study species because its importance as large animal model for gene therapy. In adenoviral transduction experiments with pig MSCs presented in Chapter 4, GFP was used as the reporter transgene to determine transfection efficiency and transgene expression in cells exposed to different experimental conditions. Since GFP has been engineered to be extremely stable it is predicted that expression of transgenes codifying for functional proteins, such as BMP2 which are less stable at both the RNA and protein level will lead to different patterns of gene expression. Therefore, transduction experiments of porcine MSC with adenovirus carrying a trangene for a biologically active protein with potential therapeutic use (e.g., BMP2) will provide valuable information about levels and time course of gene expression. In addition, the pig

model will be useful for in vivo studies of cell distribution after local or systemic MSC transplantation, patterns of transgene expression and cell doses needed to achieve a therapeutic effect. Finally, the positive effect of GeneJammer on transduction of human MSCs with adenovectors carrying the BMP2 transgene as demonstrated in the heterotopic bone formation assay warrant further confirmation in animal models of bone regeneration. It is hoped that results from these proposed studies will help to advance mesenchymal stem cell-based therapy strategies closer to routine human clinical use.