Three-dimensional multipotent progenitor cell aggregates for expansion, osteogenic differentiation and ‘in vivo’ tracing with AAV vector serotype 6

J.R. Ferreira, M.L. Hirsch, L. Zhang, Y. Park, R.J. Samulski, W-S. Hu and C-C. Ko

INTRODUCTION

Stem cell-based clinical applications require both a large quantity of cells and an effective expansion technology with a reproducible differentiation protocol for bone formation and regeneration of large skeletal defects. Multipotent adult progenitor cells (MAPCs) are bone marrow-derived stem cells characterized by high levels of CD31 and Oct4. This latter marker is crucial for their extensive self-renewal ability as revealed in embryonic stem cell cultures. Besides its self-renewal advantage, MAPCs also present more homogeneity when compared with mesenchymal stem cells (MSCs), and the clinical grade human MAPCs offer therapeutic relevance as a immunomodulatory treatment tool for certain diseases. However, little is known about the clinical potential of MAPCs to form bone tissue, and also on the phenotypic and genotypic expression pattern during osteogenic differentiation. Furthermore, combined applications of MAPCs and adeno-associated viral (AAV) vectors in bone tissue engineering have been deferred until AAV cytotoxicity and transduction efficiency are thoroughly investigated.

Recently, the rat MAPC (rMAPC) line has been shown to have a high expansion rate when cultured as static three-dimensional (3D) cell aggregates. These cell aggregates retained their pluripotency state for at least 16 days. More importantly, researchers have demonstrated that 3D static aggregate stem cell cultures are robust bioprocesses for MAPC expansion that offer safety advantages over suspension cultures. Unlike suspended aggregates, static embryonic stem cell cultures do not form invasive tumors after ‘in vivo’ transplantation. Moreover, adult stem cells (like MSCs) are known to differentiate in the presence of bone signaling cues, such as dexamethasone and bone morphogenetic protein-2 (BMP-2) through wnt/β-catenin pathways. In our preliminary studies with 2D cultures, dexamethasone positively modulated the osteogenic differentiation...
of rMAPCs, but not exogenous recombinant human BMP-2, a potent osteogenic growth factor. Thus, it would be important to test the reproducibility of dexamethasone osteogenic effects in 3D aggregate systems, as the 3D system better mimics the 'in vivo' environment with a higher expansion capability. Interestingly, 3D cultures of human embryonic stem cells have also been confirmed as a viable alternative to monolayer 2D systems, and possess a significant 'in vivo' reproducibility after long-term culture. Therefore, this study focused on investigating rMAPC 3D aggregate static long-term cultures for cell expansion and osteogenic differentiation induced by dexamethasone.

In addition to applications of MAPCs for characterization of bone formation in large skeletal defect models, the therapeutic potential of transgenic MAPCs is also of particular interest. For example, multipotent or differentiated MAPCs could be altered to express or secrete endogenous factors involved in local bone formation, such as BMPs as in other bone fracture healing models. In addition, MAPCs could be labeled (that is, with the green fluorescent protein) for 'in vivo' tracing of cell replication and migration 'in vivo'. Currently, viral gene delivery vectors have demonstrated success for the efficient transduction of a wide variety of cell types 'in vivo' and 'ex vivo'. In particular, recombinant adeno-associated virus (rAAV) offer a broad tropism and have demonstrated success in 'in vivo' applications. While several studies have demonstrated MSC transduction by single-strand rAAV serotypes 2, 3 and 6 'in vitro', to our knowledge none have evaluated the transduction of self-complementary (sc) rAAV vectors in MAPCs for 'in vitro' and/or 'in vivo' applications in large calvaria bone defects within a non-load-bearing environment. These critical-size large defects (CSD) do not heal by themselves, and therefore, they may need a combination of MAPC-based bone tissue engineering approaches and AAV vector technologies for bone formation to occur.

Thus, our hypotheses test whether a 3D cell culture system can provide a large number of rMAPCs for (1) osteogenic differentiation with dexamethasone supplementation and (2) rAAV-mediated gene transfer for 'in vivo' bone formation in calvaria large defects. The transduction efficiency of rAAV serotypes 2, 3 and 6 in MAPCs (Supplementary Figure S1). This latter marker is highly expressed in undifferentiated MAPCs, and therefore is used as a hallmark with Oct4 to confirm the undifferentiated state of MAPCs. This was also confirmed at the transcript level, where expression of Oct4 and CD31 remained high at day 4, when compared with baseline levels before expansion started (Supplementary Figure S1).

AAV cytotoxicity and transduction efficiency on rMAPC aggregates To investigate gene delivery to rMAPCs in the 3D aggregate culture, a scCMV-enhanced green fluorescent protein (GFP) expression cassette was packaged in capsids previously reported to be capable of MSC transduction. After self-complementary AAV (scAAV)-GFP vector transduction at a multiplicity of infection (MOI; viral genomes per cell) of 100, no differences in cell viability were observed between undifferentiated and differentiated rMAPC aggregates for serotypes AAV3 and AAV6 (Figure 2a). For AAV2, viability of differentiated rMAPC aggregates was similar to untransduced cells (Figure 2a). However, there was a significant decrease in cell viability when comparing AAV2-treated undifferentiated and differentiated rMAPC cultures (P < 0.01) (Figure 2a).

In general, the transduction efficiency of different scAAV serotypes, as measured by a GFP + phenotype, was greater in differentiated aggregate cultures compared with undifferentiated cells (Figure 2b). Among the tested serotypes, AAV6 serotype presented the highest percentage of GFP + cells after 3 days when compared with AAV2 (P = 0.0073) and AAV3 (P = 0.0068). Expression of GFP increased up to day 7 in AAV6-transduced aggregates (Figure 1a). After day 4, the number of viable cells decreased substantially, which is possibly related to the low volume of the growth medium (Figure 1a) (P < 0.001). Also, the doubling time was consistently 37 h through all 4 days of the 3D aggregate culture, and then decreased substantially thereafter. The population of viable cells outstand the dead/damaged cells seen by the large viable-associated red fluorescence 2D plane of the aggregate (Figure 1b). Also, rendering a 3D volume view of the aggregate revealed a large population of viable cells clustered together to form a rounded aggregate as seen in the 2D view (Figures 1b and c).
cultures (Figure 3a), and afterwards, cells continued to express GFP through day 14 (Figures 3b-e).

Bone-like matrix derived from dexamethasone-treated rMAPC aggregates

As effective gene delivery was demonstrated in 3D cultures, we next sought to investigate the effects of dexamethasone on the bone matrix generation, and mineralization. Cell/matrix cryosections were analyzed after differentiation of rMAPC aggregates was induced by osteogenic media with and without dexamethasone (OMD and OM, respectively) and by basal media (BM) without dexamethasone and osteogenic supplements. Unlike in OM and BM treatment, dexamethasone-treated aggregate cultures were highly immunoreactive to collagen type I antibody and a collagenous fiber network could be observed (Figure 4a). The orange/redness of collagen fibrils could also be appreciated under polarized light after Picro-Sirius Red staining, consistent with a high level of maturation of the collagen (Supplementary Figure S2c).

Electron microscopy images of the rMAPC aggregate matrices at day 38 confirmed the abundant matrix mineralization in dexamethasone cultures (Figure 4b), indicated by large areas of long spindle-like crystals (white arrows). On demineralized sections at an earlier time point (day 27), few matrix demineralized areas were observed, as well as a densely compacted matrix that possessed regions with typical collagen D-periodicity (data not shown). On the other hand, matrix-formed crystals were scarce and short in length in cultures grown in the absence of dexamethasone (Figure 4d). In rMAPC aggregate matrices grown in BM, no crystals were observed (Figure 4f).

All these matrix features in OMD cultures resembled natural bone tissue. On the contrary, aggregate cultures without dexamethasone (both OM and BM cultures) showed poorly mineralized matrices with scarce collagen type I (Figures 4c and e). In addition, all matrices in dexamethasone medium stained poorly for lipid deposits (red deposits stained by Oil Red O in Supplementary Figure S2a) and for proteoglycans (orange/red areas stained by safranin O in Supplementary Figure S2b).

Dexamethasone increases expression of mesoderm and bone markers in rMAPC aggregates

After undergoing a dexamethasone-enriched osteogenic differentiation protocol for 38 days in 3D cultures, Oct4 mRNA levels in rMAPCs decreased significantly to an average ΔCT of approximately 2 compared with baseline levels (fold change = -0.7; Figure 4g) (P = 0.019). This observation was consistent with other literature and was seen at a lesser extent in cultures treated without dexamethasone (OM: average ΔCT = 3; fold change = 0.9), suggesting that dexamethasone is inducing rMAPC differentiation. The α-fetoprotein (Afp) transcript, which is usually upregulated on differentiated cells, was expressed at high levels in OM, but...
without significance and to a lesser degree in dexamethasone-enriched media (OMD) with significance ($P < 0.01$) (Supplementary Figure S3). At the terminus of the differentiation process, the endothelial-specific marker $CD31$ was significantly downregulated in both media without dexamethasone ($P = 0.003928$) and with dexamethasone ($P = 0.035557$), ruling out a possible endothelial commitment (Figure 4h). Also, Kdr, a mediator of VEGF-induced endothelial proliferation and differentiation, was found downregulated in dexamethasone-supplemented cultures (Figure 4h). More importantly, mesoderm-specific transcripts such as $BMP-2$ ($P = 0.039$) and $BMP-4$ ($P = 0.015$) were found highly upregulated by dexamethasone, and to lesser extent in OM without dexamethasone (Figure 4h). Essential mid- to late-stage osteogenesis markers signaling molecules were assessed, particularly $Col1a2$ (collagen type I $\alpha2$ chain), osteopontin ($OSP$) and osteocalcin ($OCN$) (Figure 4i). The late bone-specific gene, $OCN$ (Figure 4i), was found highly upregulated with dexamethasone when compared with medium without dexamethasone ($P = 0.014$). Collagen type 1 ($Col1a2$) and $OSP$ early bone markers were overexpressed relative to baseline (Figure 4i); however, transcript expression was without significance for $Col1a2$, perhaps due to the ongoing late stage of bone development (38 days) on dexamethasone culture conditions (OMD). In summary, overexpression of mesodermal and late bone-specific genes in OMD confirmed their osteogenic potential under dexamethasone supplementation in this 3D culture system.

Nuclear colocalization of $\beta$-catenin increased in rMAPC aggregates treated with dexamethasone.

After confirming the osteogenic potential of rMAPC aggregates, we assessed the activation of the wnt/$\beta$-catenin pathway, crucial
on skeletal development. Expression of nuclear β-catenin was evaluated during the osteogenic differentiation in medium with and without dexamethasone. To confirm the translocation of the β-catenin to the nucleus and inhibition of β-catenin degradation, the expression of the activated form of glycogen synthase kinase 3 (GSK3) (p-GSK3 Tyr216/279) that sequesters and negatively regulates β-catenin activation was also assessed. The β-catenin is only activated when a wnt signal is present and p-GSK3 kinase activity is inhibited. Upon activation, β-catenin is mainly found free and colocalized with the nucleus, and this was demonstrated only in dexamethasone-differentiated rMAPC cells (Figure 5a).

Conversely, in OM, β-catenin was found outside the nucleus and closely associated with the plasma membrane, which suggests that this molecule is inactive, and therefore, no wnt signaling occurred (Figure 5b). These observations were supported by the lack of expression of the β-catenin-negative regulator p-GSK3 in dexamethasone-treated cultures (Figure 5c) and by its abundant expression in culture media without dexamethasone (OM) (Figure 5d).

AAV6 transduction for ‘in vivo’ rMAPC tracing and bone formation

After efficient osteogenic differentiation conditions were set for the aggregates, we then used the AAV6 serotype to both trace the differentiated aggregates ‘in vivo’ and study its effects on bone formation. The ability of scAAV6 to label differentiated rMAPCs for ‘in vivo’ tracing was investigated on a macro-porous hydroxyapatite-based 3D scaffold. In these experiments, osteogenically differentiated rMAPC or OD-rMAPC aggregates (dexamethasone treated at day 38 of differentiation) were transduced with scAAV6-CMV-enhanced green fluorescent protein at an MOI of 100. On the following day, these transduced aggregates were loaded into macro-porous hydroxyapatite-based scaffolds, which were then implanted in a rat calvaria CSD bone formation model. At 4 and 10 days after ‘in vivo’ implantation of porous scaffolds with AAV6-transduced rMAPC, tracing of transduced cells revealed GFP + cells on the porous areas around the scaffold materials (Figures 6a–e). The number of GFP + cells appeared to be higher at 10 days than at 4 days of post-implantation (Figure 6f).

Importantly, untransduced and AAV6-transduced rMAPCs generated an osteogenic-like tissue expressing late specific bone markers (such as OSP and OCN) within the macro-pores of the 3D scaffold after 10 days (Figures 7a, b and d, e). Furthermore, transplanted AAV6-transduced rMAPCs formed a bone-like collagenous matrix with osteocyte-like cells entrapped in lacunae within the macro-pores (Figures 7g and h). Also, newly formed bone was visualized at the native bone-scaffold interface 10 days after implantation of transduced cells (Figure 7i). These observations indicate that AAV6 transduction allows for ‘in vivo’ bone formation and mediates stable gene transfer to differentiated rMAPC aggregates, which remains strong post-implantation while not affecting the osteogenetic differentiation of rMAPCs. Lastly, Oct4 expression was not detected in GFP + cells ‘in vivo’ after 10 days, and therefore the presence of cell growth from day 4 to 10 was not related to the presence of immortalized rMAPCs (Figure 8).

DISCUSSION

Stem cell-based approaches are valuable tools not only for the formation of new tissues like bone, but also when used as models of bone disease and to understand bone development.14-18 Rat and human MAPCs have been used as cell-based therapies for newly formed tissues in rodent studies,17,18 and MAPC-based clinical trials have been initiated to take advantage of MAPC immunomodulatory effects (ClinicalTrials.gov NCT00677859, NCT00677222). This study successfully combined for the first time OD-rMAPC- and AAV-vector-mediated gene delivery for the final purpose of bone formation in a calvaria CSD model.

Rat calvaria ‘in vivo’ CSD models require a large number of cells, which often rely on growth in cell scale-up bioprocesses.19,20 Despite several innovations in these robust bioprocesses, progress has been slow owing to cellular heterogeneity, including karyotypic abnormalities associated with prolonged ‘ex vivo’
culture times, which make stem cells unfit for clinical use.\textsuperscript{21–23} However, in this study we used a cell line (rMAPCs) with high self-renewal ability and homogeneity, and thus we were able to expand rMAPCs on static 3D aggregate culture systems for a short time period (4 days). Recently, rMAPCs have shown to possess a high expansion rate with a doubling time of about 23 h in 3D suspension cultures.\textsuperscript{6} In our study, the doubling time was consistently 37 h through all 4 days of the 3D aggregate culture. The lower doubling time reported by Subramanian et al.\textsuperscript{3} is probably related to the different cell counting protocol used.

Determining the viable cell population was a more appropriate tracer of osteogenic-differentiated rMAPC cells after AAV6-GFP transduction in a calvaria bone CSD formation model. Macro-porous 3D hydroxyapatite-based scaffolds carried untransduced cells (a) or AAV6-GFP-transduced cells (b–e), and GFP expression was visualized by immunohistochemistry with anti-GFP antibody after 4 days (b) and 10 days (c) \textit{in vivo} and with isotype IgG antibody after 4 (d) and 10 days (e). Sections were stained with DAB chromogen and counterstained with hematoxylin. Quantification of rMAPC-GFP + cells \textit{in vivo} (f) by light microscopy counting, mean from five technical replicates. Hap, hydroxyapatite-based biomaterial. Nuclei counterstained with hematoxylin. Magnification: \times 100.

In preliminary monolayer culture studies, dexamethasone stimulated rMAPCs to form a bone-like matrix \textit{in vitro} and increased ALP activity at early stages. In this study, dexamethasone-enriched medium induced the translocation of the β-catenin molecule to the nucleus during rMAPC differentiation (Figure 5a), suggesting the activation of the wnt/β-catenin pathway. Elucidating the role of dexamethasone in the activation of the β-catenin pathway is important, as this pathway was found to be essential in skeletal development, and stem cell fate.\textsuperscript{26–28} Here in 3D aggregate cultures, we found that dexamethasone also increased the nuclear β-catenin at the terminus of rMAPC osteogenic differentiation, like in MSCs.\textsuperscript{30} The free translocation of β-catenin to the nucleus was confirmed by the absence of GSK3\textsuperscript{30} (Figure 5c). Besides the presence of wnt, the β-catenin signaling requires the inhibition of GSK3 kinase activity, as the active form of GSK3 targets β-catenin for proteasomal degradation.\textsuperscript{29} The GSK3–β-catenin degradation complex is activated when a specific tyrosine is phosphorylated at GSK3.\textsuperscript{31,32} Conversely, in medium without dexamethasone (OM), the β-catenin was not found in the nucleus in the presence of an abundant p-GSK3 kinase, which targets β-catenin for degradation and restricts its nuclear translocation. The concomitant activation of the β-catenin pathway and inhibition of the GSK3 kinase activity has been reported and extensively reviewed in the literature.\textsuperscript{29,33–35} The interaction of the BMP and wnt pathways seems to be particularly complex in bone development.\textsuperscript{30,35,36} The existence of different levels of crosstalk between BMP and wnt has been recently confirmed during the differentiation of the \textit{Drosophila} mesoderm and of other stem cell lineages.\textsuperscript{30,35,37} Nevertheless, these complex interactions are far away from being fully understood and further research is mandatory for later bone regeneration applications.

AAV vectors are proving to be increasingly effective because of their superior safety profile and \textit{in vivo} sustained transgene expression,\textsuperscript{38,39} including their ability to transduce non-dividing primary cells\textsuperscript{40} and certain adult stem cell lineages.\textsuperscript{11,12,41}
The results present herein suggest that safe, efficient and long-term OD-rMAPC aggregate transduction is feasible through 14 days ‘in vitro’ and 10 days ‘in vivo’ for rat calvaria defects (end points of the study) through the use of scAAV6 vectors. Importantly, gene transfer with scAAV6 did not affect the osteogenic differentiation of rMAPCs or the proliferation of these osteoblast-like cells (Figure 7). Undifferentiated aggregates displayed reduced transduction efficiency with the three analyzed scAAV serotypes, and an increased cytotoxic trend, particularly with scAAV2 (Figure 2). To our knowledge, this is the first report comparing the scAAV transduction efficiency in undifferentiated versus differentiated MAPCs using a 3D culture system. As mentioned previously, the therapeutical potential of human MAPC cells is currently being tested in clinical trials. This fact plus the stable transduction efficiency seen here with scAAV6 makes the MAPC 3D aggregate system an ideal cell-based therapy for AAV6-mediated gene delivery. Compared with the previous literature, levels of AAV6 transduction efficiency on rMAPCs were significantly higher (15–27% up to day 7) at lower MOIs (100) than with human MSCs at MOIs of $10^7$ (<0.5%). Yet, further studies are needed focusing on AAV transduction efficiency of human MAPCs because efficient gene transfer decreases significantly from non-human primates to humans when using marrow-derived adult stem cells (MSCs). More interestingly, our 3D hydroxyapatite macro-porous scaffolds loaded with untransduced and transduced OD-rMAPC aggregates generated a bone-like tissue 10 days after implantation in calvaria CSD models that do not heal by themselves (Figure 7). These findings indicate that this scAAV6 technology could be used with rMAPCs for future bone regeneration approaches with porous 3D hydroxyapatite-based scaffolds. Nevertheless, optimal conditions for the complete regeneration of this calvaria defect model by MAPCs have yet to be established, as well as for load-bearing defect models (e.g., femur).

In conclusion, the results demonstrate that rMAPCs in this 3D culture system can be a feasible model to generate effectively...
rMAPC Growth and Osteogenic Medium

Briefly, rMAPC growth medium consisted of a 60/40 (v/v) mixture of low glucose Dulbecco’s modified Eagle’s medium (Gibco, Carlsbad, CA, USA) and MCDB-201 (Sigma, St Louis, MO, USA) supplemented with 0.026 μg ml⁻¹ ascorbic acid 3-phosphate (Sigma), linoleic acid-bovine serum albumin (Sigma) (103 μg ml⁻¹ bovine serum albumin and 81.3 μg ml⁻¹ linoleic acid), insulin-transferrin-selenium (Sigma) (final concentration 10 μg ml⁻¹ insulin, 5.5 μg ml⁻¹ transferrin, 0.005 μg ml⁻¹ sodium selenite), 0.02 μg ml⁻¹ dexamethasone (Sigma), 4.3 μg ml⁻¹ β-mercaptoethanol and 2% (v/v) qualified fetal bovine serum (HyClone, Logan, IL, USA). Three growth factors were also added to the media: human platelet-derived growth factor (PDGF-BB; R&D Systems, Minneapolis, MN, USA) (10 ng ml⁻¹), mouse epidermal growth factor (Sigma) (10 ng ml⁻¹) and mouse leukemia inhibitory factor (10.3 U ml⁻¹) (ESGRO, Chemicon, Billerica, MA, USA). All media were also supplemented with 100 IU ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin (Lonza, Walkersville, MD, USA).

BM composition consisted of 60/40 (v/v) mixture of low glucose Dulbecco’s modified Eagle’s medium and MCDB-201 supplemented with linoleic acid-bovine serum albumin (103 μg ml⁻¹ bovine serum albumin and 81.3 μg ml⁻¹ linoleic acid), insulin-transferrin-selenium (final concentration 10 μg ml⁻¹ insulin, 5.5 μg ml⁻¹ transferrin and 0.005 μg ml⁻¹ sodium selenite), 4.3 μg ml⁻¹ β-mercaptoethanol and 10% fetal bovine serum. BM did not have dexamethasone supplementation. Osteogenic medium (OM) was composed of BM with the addition of 10 mM of β-glycerophosphate (Sigma) and ascorbic acid at 0.2 mM (Sigma) with or without dexamethasone supplementation at 10⁻² M (OMD and OM, respectively).43

3D cell culture systems

rMAPCs were cultured either as 3D aggregates or as monolayer (2D) cell culture systems in an incubator at 37 °C, 5% CO₂ and O₂ while in growth media, and when differentiation media (OMD, OM, BM) was supplemented, cell cultures were changed to normal O₂ levels.

For the 3D aggregate system, 1 - 2 × 10⁴ MAPC cells were seeded in suspension with MAPC growth medium in 96-well rounded bottom ultra-low attachment plates (Costar, Corning Inc. Life Sciences, Lowell, MA, USA), using a modification of a forced aggregation method reported previously.3,44 Cells in suspension were centrifuged at 1400 r.p.m. for 4 min to allow cells to settle to the bottom of the well and form aggregates over time. Aggregates were allowed to grow for 5 days, and cell viability and diameter of aggregates was monitored at days 1 - 5 (see protocol below). Then at day 4, ten 3D aggregates were transferred to each well on 24-well ultra-low attachment plates (Costar), and the medium was completely changed to either BM, OM or OMD. OM and BM had no dexamethasone supplementation. The medium change took place every 2 - 3 days. All of these three culture conditions (BM, OM and OMD) were evaluated for osteogenic differentiation. Differentiation was terminated at both 27 and 38 days.

For the 2D monolayer cultures, 8 × 10⁴ rMAPCs were seeded with rMAPC growth medium in 24-well flat plates with regular attachment properties (Costar). Cells were allowed to grow for 4 - 5 days until they reach the 80 - 90% of confluency for mesodermal differentiation. Then, growth medium was completely replaced by either OMD, OM or BM conditions, to study the osteogenic potential of the rMAPC aggregates using these three culture conditions. Media change took place every 2 - 3 days. All these culture conditions (OM and OMD) were evaluated for osteogenic differentiation. Differentiation was terminated at both 21 and 42 days.

Cell viability and rMAPC 3D aggregate diameter

During the 4 days of rMAPC aggregate growth, cell viability and proliferation in the aggregates was assessed by a CellTiter 96 Aqueous One Solution assay kit (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Briefly, this kit contains an MTS compound that is bioreduced by cells into a colored formazan product. The quantity of formazan product was measured by the amount of 490 nm of absorbance that is directly proportional to the number of living cells in culture. To perform the assay, 20 μl of CellTiter 96 solution was added to the media with gentle shaking. Plates were then
placed in an incubator for about 2 h at 37 °C. 5% CO₂ and 5% O₂. Background absorbance from negative controls (without cells) was typically 0.206-0.216 for all wells; this average background was subtracted from all absorbance values of experimental groups for normalization. Eight biological replicates were run at day 0, 2, 4 and 5. The cell number was determined by performing cell titration.

A Live/Dead Cell Viability Assay C12-resazurin/SYTOX Green kit (L34951; Molecular Probes, Eugene, OR, USA) was used to visualize the cell viability for all wells; this average background was subtracted from all absorbance absorbance from negative controls (without cells) was typically 0.206-0.216 for all wells; this average background was subtracted from all absorbance values of experimental groups for normalization. Eight biological replicates were run at day 0, 2, 4 and 5. The cell number was determined by performing cell titration.

Histological analysis of 3D aggregate matrices

On days 27 and 38, cell/matrix layers from 3D aggregate and 2D cultures were washed with calcium-free PBS 1 × and fixed. Cryosections with approximately 5 μm thickness were made from the rMAPC aggregate matrices. For collagen maturation, matrix cryosection slides were stained for 1 h in 0.1% solution of Sirius red dissolved in aqueous saturated picric acid (Electron Microscopy Sciences, Hatfield, PA, USA) for collagen identification. Slides were dehydrated in gradient ethanol, followed by xylene treatment and resin mounted. Stained cell/matrix layers were visualized and photographed with a Nikon Eclipse Ti-U with an Olympus DP70 camera (Japan) on polarized light.

For cartilage tissue analysis, cryosections were stained using a safranin O staining protocol. Briefly, cryosection slides were stained with Weigert’s iron hematoxylin working solution for 10 min, washed in water, stained with Fast Green solution for 5 min, rinsed quickly with 1× acetic acid solution for 10-15 s and stained in 0.1% safranin O solution for 5 min, followed by dehydration and mounting.

Lipid deposition in cell matrices was evaluated using Oil Red O staining protocol after fixation of cryosection slides. Briefly, cell/matrix layers were rinsed with 60% isopropanol, stained with freshly prepared Oil Red O working solution 15 min (Electron Microscopy Sciences), rinsed with 60% isopropanol and stained with Harris hematoxylin for nuclei counterstain. All cell/matrix layers were visualized under light microscopy (Nikon Eclipse Ti-U with an Olympus DP70 camera). Matrices stained with Procion-Sirius Red solution were observed under polarized light to view collagen organization and maturation. All experiments were run in triplicate.

Immunohistochemistry

rMAPC aggregates/matrix cryosections were fixed with 4% paraformaldehyde, rinsed, endogenous peroxidase activity was inhibited by incubating for 30 min with 0.3% H₂O₂, in 100% methanol, rehydrated and transferred to PBS–Triton X solution.

For collagen type I, avidin/biotin activity was blocked in the cryosections with Avidin–Biotin kit (Dako, Cermonteria, CA, USA), rinsed thrice with PBS plus Triton X and unsppecific antibody binding sites were blocked for 30 min with 0.4% fish skin gelatin in PBS. Then, cryosections were incubated overnight at 4 °C with rabbit primary antibody against rat collagen type I (NB600-408; Novus Biologicals, Littleton, CO, USA), rinsed thrice and incubated with secondary biotinylated goat anti-rabbit immunoglobulin G (IgG) antibody (NB730-B; Novus Biologicals) for 30 min at room temperature. The cell/matrix layer was incubated in ABC complex (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer’s protocol, rinsed thrice and DAB Chromogen solution (Dako) was added to the matrix layer for 5-20 min until brown color developed. Positive controls with collagen type I antibodies produced by osteoprogenitor cultures (MC3T3-E1) and negative controls containing a gelatin substrate were also stained. Cell/matrix layers were visualized under light microscopy.

For β-catenin and active form of GSK3α/β immobilization, cryosections were incubated overnight at 4 °C with goat primary antibody against rat β-catenin (C-18) (sc-1496; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or p-GSK3α/β ( Tyr279/Tyr216) (sc-11758; Santa Cruz Biotechnology), rinsed thrice and incubated with secondary donkey anti-goat DyLight649-conjugated IgG antibody (705-496-147; Jackson ImmunoResearch, West Grove, PA, USA) at 30 min at room temperature. Then, cryosections were counterstained with DAPI nuclei kit (Millipore, Billerica, MA, USA) for 5 min. Sections with primary rat osteoblast cultures were used as positive controls to view β-catenin translocation from the plasma membrane to the nucleus. β-catenin, GSK3α/β and nuclei were observed under fluorescence microscopy (Nikon Eclipse Ti-U with Nikon Digital sight DS-Q1Mc camera) with TRITC and DAPI filters, respectively; photos were taken for each filter and then merged together.

Transmission electron microscopy

On days 27 and 38 of differentiation, 3D aggregate cells/matrices were washed twice with PBS 1 × and fixed in 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). For decalcification, a 0.1 M EDTA solution was used for 2 days buffered with 0.15 M sodium phosphate, at pH 7.4, 4 °C. Then, samples were post-fixed in potassium ferrocyanide reduced osmium (1% osmium tetroxide/1.25% potassium ferrocyanide/0.15 M sodium phosphate, pH 7.4) for 1 h. For undecalcified samples, after the ethanol dehydration series, propylene oxide was used as an intermediate solvent, followed by incubation in a solution of propylene oxide and Polybed 812 resin for 2 h, samples stayed overnight in Polybed 812 resin, and then polymerization was achieved in the tube for 24 h at 60 °C. Ultrathin sections with 70 nm thickness were cut, stained with uranyl acetate and lead citrate, and observed using a LEO EM-910 transmission electron microscope operating at 80 kV (Carl Zeiss SMT, Peabody, MA, USA), and images were taken at ×2000-50,000 using a Gatan Orion Sc1000 CCD camera with Digital Micrograph 3.11.0 (Gatan Inc., Pleasanton, CA, USA) at the Microscopic Services Laboratory (MSL) at the University of North Carolina. Matrices/cell layer sections from differentiated osteoblast-like murine-derived clonal MC3T3-E1 cell lines were used as a positive control for comparison purposes with OD-MAPC1.
In vitro AAV transduction

Recombinant scAAV-CMV-enhanced green fluorescent protein vector serotypes 2, 3B and 6 were produced using our previously described triple transfection protocol in human embryonic kidney cells. Following transfection, the nuclear lysate was fractionated based on density using cesium chloride centrifugation. Fraction samples were then digested with DNAseI, subsequently followed by proteinase K digestion, and the remaining viral genomes were denatured in a sodium hydroxide solution as described. Alkaline gel electrophoresis and southern blotting were used to identify fractions with sc genomes, which were then pooled and dialyzed in PBS. The viral titer was then determined by quantitative PCR using the following GFP primer set: forward primer, 5'-AGCACAGAGCAC TTCTTCAAGTCC-3' and the reverse primer, 5'-TGTAGTGTACCCAGCTTG TGCC-3'. The final titer was then verified by southern blotting.

Cultures with 3D undifferentiated aggregates (after 4 days of aggregate expansion in GM) and 3D dexamethasone-differentiated 3D aggregates (after 15 days in OM plus dexamethasone) in 24 ultra-low attachment wells were incubated for 3, 7 and 14 days with serotypes AAV2-GFP, AAV3B-GFP and AAV6-GFP at MOIs (viral genomes per cell) of 100. After trypsinization, cell viability was assessed by 72 h of transduction using the CellTiter 96 Aqueous One Solution assay kit as recommended (Promega). Also, quantification of rAAV transduction efficiency was performed by evaluating GFP+ cells using flow cytometry with dead cell exclusion by FITC filter using the previously mentioned Nikon apparatus.

‘In vivo’ tracing and bone formation with AAV6-GFP-transduced OD-rMAPC aggregates

3D macro-porous hydroxyapatite-based scaffolds (diameter 8 mm and thickness 2 mm) developed previously in our lab were used to carry dexamethasone-treated OD-rMAPC aggregates after rAAV6 transduction at an MOI of 100 into an ‘in vivo’ bone large defect model in calvaria. The defect consisted in a 8-mm-diameter calvaria critical-size defect, which has been described. The 11- to 13-week-old Sprague-Dawley rats (Charles River, Wilmington, MA, USA) served as recipients of the rMAPC aggregate-loaded scaffolds. Rats were anesthetized and an 8-mm-diameter defect was created on the calvaria bone using a dental drill. Rats were divided into scaffold groups with untransduced cell aggregates and groups with AAV6-GFP transduced aggregates.

At 4 and 10 days after surgery, rats were euthanized and the specimens were retrieved for decalcification and immunohistochemical examinations with antibodies against rat GFP, Oct4, OSP, and rabbit IgG (negative isotype control). DAB chromogen solution (Dako) was added until brown color developed. Counterstaining with nuclear hematoxylin or Alcian blue was also performed. The number of GFP+ cells was counted in five random fields in each defect using a microscopy grid of 400 x 400 μm². The total number of cells was calculated towards a total defect area of 336 mm². Decalcified specimens were also evaluated microscopically for the formation of bone-like matrix and collagen at the hydroxyapatite-based scaffold macro-pores and at the native bone–scaffold interface using hematoxylin–eosin and Masson’s trichrome histological staining. The experimental protocol for all animal care and use in this study was reviewed and approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Statistics

Student’s t-tests were performed in all assays, except for gene expression data, where one-way analysis of variance was used for multiple statistical comparisons using the JMP9 software (SAS Institute, Cary, NC, USA). Statistical significance was defined at P < 0.05.

CONFlict of interest

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Sandra Horton at North Carolina State University for processing ‘in vivo’ tissue specimens for immunohistochemistry and Victoria Madden at UNC Microscopy Services Laboratory for processing in vitro cell cultures for electron microscopy. Also, we would like to acknowledge Dr Gannock Ukonkasemsin for her contribution towards the statistical analysis of this project. João Ferreira was supported by the doctoral fellowship of FCT-Portuguese Foundation for Science and Technology (SFRH/BD/36841/2007). This work was supported in part by grants from the: NIH/NIDCR (K08DE018695), NC Biotech Center, American Association for Orthodontist Foundation awarded to C-CK; Northwest Genome Engineering Consortium pilot awarded to MLH; and Wellstone (SUS4AR056933) and NIH (SR01A072176) awarded to RJS.

REFERENCES

1. Ulla-Montoya F, Kidder BL, Pauwelyn KA, Chase LG, Luttun A, Crabbé A et al. Comparative transcriptome analysis of embryonic and adult stem cells with extended and limited differentiation capacity. Genome Biol 2007; 8: R163.
2. Roobrouck VD, Clavel C, Jacobs SA, Ulla-Montoya F, Crippa S, Sohni A et al. Differentiation potential of human postnatal mesenchymal stem cells, mesangioblasts, and multipotent adult progenitor cells reflected in their transcriptome and partially influenced by the culture conditions. Stem Cells 2011; 29: 871–882.
3. Subramanian K, Park Y, Verfaille CM, Hu WS. Scalable expansion of multipotent adult progenitor cells as three-dimensional cell aggregates. Biotechnol Bioeng 2011; 108: 364–375.
4. Taiani JT, Krawetz RJ, Zur Nieden NI, Elizabeth Wu Y, Kallos MS, Matyas JR et al. Reduced differentiation efficiency of murine embryonic stem cells in stirred suspension bioreactors. Stem Cells Dev 2010; 19: 989–998.
5. Hong L, Sultana H, Paulius K, Zhang G. Steroid regulation of proliferation and osteogenic differentiation of bone marrow stromal cells: a gender difference. J Steroid Biochem Mol Biol 2009; 114: 180–185.
6. Zur Nieden NI, Kempka G, Rancourt DE, Ahr HJ. Induction of chondro-, osteo- and adipogenesis in embryonic stem cells by bone morphogenetic protein-2: effect of cofactors on differentiating lineages. BMC Dev Biol 2005; 5: 1–15.
7. Arpornmaeklong P, Brown SE, Wang Z, Krebsbach PH. Phenotypic characterization, osteoblastic differentiation, and bone regeneration capacity of human embryonic stem cell-derived mesenchymal stem cells. Stem Cells Dev 2009; 18: 955–968.
8. Yazici C, Takahata M, Reynolds DG, Xie C, Samulski RJ, Samulski J et al. Self-complementary AAV2-BMP2-coated femoral allografts mediated superior bone healing versus live autografts in mice with equivalent biomechanics to unfractured femur. Mol Ther 2011; 19: 1416–1425.
9. McCarty DM, Fu H, Monahan PE, Toulson CE, Naik P, Samulski RJ. Adeno-associated virus terminal repeat (TR) mutants generate self-complementary vectors to overcome the rate-limiting step to transduction ‘in vivo’. Gene Therapy 2003; 10: 2112–2118.
10. Gao R, Yan X, Zheng C, Goldsmith CM, Aflonce S, Hai B et al. AAV-mediated transfer of the human aquaporin-1 cDNA restores fluid secretion from irradiated miniature pig parotid glands. Gene Therapy 2011; 18: 38–42.
11. Chng K, Larsen SR, Zhou S, Wright JF, Martinelli-Wilks R, Risiko JE. Specific adeno-associated virus serotypes facilitate efficient gene transfer into human and non-human primate mesenchymal stem cells. J Gene Med 2007; 9: 22–32.
12. Stender S, Murphy M, O’Brien T, Stengard C, Ulrich-Vinther M, Soballe K et al. Adeno-associated viral vector transduction of human mesenchymal stem cells. Eur Cell Mater 2007; 13: 93–99; discussion 99.
13. Montes GS, Junqueira LC. The use of the Picrosirius-polarization method for the study of the biopathology of collagen. Mem Inst Oswaldo Cruz 1991; 86(Suppl 3): 1–11.
14. Triffitt JT. Osteogenic stem cells and orthopedic engineering: summary and update. J Biomed Mater Res 2002; 63: 384–389.
15. Mauney JR, Volloch V, Kaplan DL. Role of adult mesenchymal stem cells in bone tissue engineering applications: current status and future prospects. Tissue Eng 2005; 11: 787–802.
16. Kollerer B, Friedl G, Jandrositz A, Sanchez-Cabo F, Prokesch A, Paar C et al. Gene expression profiling of human mesenchymal stem cells derived from bone marrow during expansion and osteoblast differentiation. BMC Genomics 2007; 8: 70.
17. Ji KH, Xiong J, Fan LX, Hu KM, Liu HQ. Rat marrow-derived multipotent adult progenitor cells differentiate into skin epidermal cells ‘in vivo’. J Dermatol 2009; 36: 403–409.
OD-MAPC aggregates and AAV6 in bone bioengineering

JR Ferreira et al.