Published in final edited form as:

J Tissue Eng Regen Med. 2012 November ; 6(10): e43–e50. doi:10.1002/term.1594.

Adeno-Associated Virus–Coated Allografts: A Novel Approach for Cranioplasty

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Abstract

Bone autografts are considered the gold standard for cranioplasty although they lead to comorbidity. Bone allografts are more easily obtained but have low osteogenic potential and fail to integrate into healthy bone. Previously, we showed that by coating long-bone allografts with freeze-dried recombinant adeno-associated virus (rAAV) vector encoding for an osteogenic gene, enhanced osteogenesis and bone integration were achieved. In this study our aim was to evaluate the bone repair potential of calvarial autografts and allografts coated with either single-stranded rAAV2 vector (SS-rAAV-BMP2) or self-complementary pseudotyped vector (SC-rAAV-BMP2) encoding for bone morphogenetic protein (BMP)–2 in a murine cranioplasty model. The grafts were implanted into critical defects in the calvariae of osteocalcin/luciferase (Oc/Luc) transgenic mice, which allowed longitudinal monitoring of osteogenic activity using bioluminescence imaging (BLI). Our results showed that the bioluminescent signal of the SC-rAAV-BMP2–coated allografts was 40% greater than that of the SS-rAAV-BMP2–coated allografts ($p < 0.05$) and that the bioluminescent signal of the SS-rAAV-BMP2–coated allografts was not significantly different from the signals of the autografts or uncoated allografts. Micro-computed tomography (μ CT) confirmed the significant increase in osteogenesis in the SC-rAAV-BMP2 group compared with the SS-rAAV-BMP2 group ($p < 0.05$), indicating a significant difference in bone formation when compared with the other grafts tested. In addition, histological analysis revealed extensive remodeling of the autografts. Collectively, these results demonstrate the feasibility of craniofacial regeneration using SC-rAAV-BMP2–coated allografts, which may be an attractive therapeutic solution for repair of severe craniofacial bone defects.

Keywords

gene therapy/therapeutics; craniomaxillofacial surgery; tissue engineering; imaging; bone remodeling/regeneration; bone graft(s)

1. Introduction

Bone tissue has regenerative capabilities that enable the self-repair of fractures and tissue loss. The vast majority of fractures can heal either on their own or with the aid of standard orthopedic procedures (Einhorn, 2005). In extreme situations, however, in which bone loss due to trauma or tumor surgery results in a critical-size bone defect—one spanning more than 2 cm—complete regeneration will not occur (Hollinger and Kleinschmidt, 1990). Nontraditional approaches, such as gene therapy for bone healing (Lieberman, *et al.*, 2002), can be considered potential treatments that will resolve this clinical problem (Awad, *et al.*, 2007).

Cranioplasty is required for the repair of calvarial bone defects. It is considered one of the oldest surgical procedures recorded, dating back to the times of the Celts (Sanan and Haines, 1997). The goal of this procedure is to reconstruct the cranial vault in order to protect brain tissue and recover an aesthetic appearance. Several implant materials have been used in cranioplasty over the years, ranging from autografts and allografts to alloplasts and xenografts (Prolo and Oklund, 1991). Although there is no doubt that fresh autografts are considered the best option for cranioplasty (Artico, *et al.*, 2003, Thesleff, *et al.*, 2011), their availability is limited and their use is associated with donor site morbidity (Summers and Eisenstein, 1989, Younger and Chapman, 1989). Alloplastic materials such as polymethylmethacrylate, hydroxyapatite, and titanium mesh are widely used because of the limitations of autografts that we just mentioned, especially in custom-made cranioplasties (Thesleff, *et al.*, 2011). However, the use of calcium phosphate and hydroxyapatite recently was reported to result in complications in at least 9% of cases (Afifi, *et al.*, 2010, Wong, *et al.*, 2011), and when polymethylmethacrylate and titanium mesh implants were compared to autografts in human patients, autogenous calvarial bone grafts were found to have better mechanical, biologic, and immunologic properties (Sahoo, *et al.*, 2010). Bone allografts are readily available from tissue banks and could be processed to provide large structural grafts. Yet, both experimental and clinical studies have shown that processed bone allografts fail to remodel and incorporate with host bone (Burchardt, *et al.*, 1978, Chalmers, 1959, Goldberg and Stevenson, 1993). Despite this, processed allografts remain a common choice for severe bone defect repair because of the limitations related to autografts. Unfortunately, the limited bone formation and remodeling achieved using structural allografts are directly associated with a 25% to 35% 3-year failure rate and nonunion (Berrey, *et al.*, 1990, Younger and Chapman, 1989). For those allografts that survive, the failure rate at 10 years has been documented to be as high as 60% (Wheeler and Enneking, 2005).

While experimental models have demonstrated that these limitations can be overcome by seeding the allograft with mesenchymal stem cells (MSCs) transduced with bone morphogenetic protein (BMP) (Xie, *et al.*, 2007), technical hurdles in obtaining, culturing, and placing these cells within the allograft have led investigators to pursue acellular gene therapy approaches.

The first method of acellular gene therapy for bone healing involved the use of a gene-activated matrix, in which naked plasmid DNA was physically entrapped in a polymer matrix sponge (Bonadio, *et al.*, 1999). Alas, this approach suffered from very low transfection efficiency. This difficulty was overcome by the use of recombinant adeno-associated virus (rAAV), which can be freeze-dried onto any implantable surface without losing its infectivity (Ito, *et al.*, 2005). When using self-complementary (SC) rAAV, up to a six-fold increase in transfection efficiency is achieved compared with single-stranded (SS) rAAVs (Jayandharan, *et al.*, 2008). By coating allografts with an rAAV vector that expresses angiogenic, osteogenic, and osteoclastogenic genes, one can induce vascular ingrowth, new bone and marrow formation, and osteoclastic remodeling of necrotic cortical allografts (Ito,

et al., 2005, Koefoed, *et al.*, 2005). Moreover, an rAAV serotype that has tropism for MSCs (SC-rAAV2.5-BMP2) was recently used successfully for cell transfection as well as for femur graft coating (Yazici, *et al.*, 2011).

It is clear, therefore, that for many clinical conditions a more complex and robust tissue-engineering approach to enhancing bone formation is required. “Revitalizing” rAAV-coated allografts (i.e. improving the allografts’ osteogenic capacity and integration, thereby achieving the clinical outcomes usually associated with autografts) represents a potential solution in the treatment of large segmental defects in long bones. Yet, long bones heal by endochondral ossification, as opposed to craniofacial bones, which heal through intramembranous bone formation. In addition, long bones are rich with bone marrow cells that can be activated by a potent osteogenic transgene, while the presence of these cells is limited in the cranial bones. We therefore seek to investigate whether a rAAV-coated allograft-based approach could be effective in craniofacial bone repair. Here we used rAAV vector encoding for the *bone morphogenetic protein-2 (BMP2)* gene, a member of the BMP family that induces endochondral bone formation by stimulating the differentiation of mesenchymal progenitor cells (Hogan, 1996). To validate our hypothesis, we compared the use of allografts coated with rAAV-BMP2 vectors with the use of uncoated allografts or autografts in cranioplasty.

2. Materials and Methods

2.1 Murine cranioplasty model

The Institutional Animal Care and Use Committee of The Hebrew University approved all procedures used in this study. Preparation of calvarial bone defects, allografts, and autografts (each 5-mm in diameter) was performed as previously described (Pelled, *et al.*, 2010) in 23 osteocalcin/luciferase (Oc/Luc) transgenic female mice (FVB/N background, 10–16 weeks old). The mice were anesthetized by intraperitoneal injection of ketamine and xylazine (0.85% ketamine/0.15% xylazine at 1 μ l/g body weight). A 5-mm-diameter circular full-thickness defect (a nonhealing critical-size defect) was created at the lambda suture of the calvaria by using a standard 5-mm-diameter trephine with minimal penetration of the dura mater. When autografts were used the bone was excised and replaced. When allografts were used the bone was devitalized and then coated with viral vector as described elsewhere (Yazici, *et al.*, 2011). Four different types of bone grafts were implanted: 1) SC-rAAV-BMP2-coated allograft (8 animals); 2) SS-rAAV-BMP2-coated allograft (5 animals); 3) uncoated allograft (5 animals); and 4) autograft (5 animals). Bone grafts were prepared in the following manner. For the SC-rAAV-BMP2-coated allografts and the SS-rAAV-BMP2-coated allografts, the implants were coated with 10^9 particles/mm² viral vector and prepared as previously described (Pelled, *et al.*, 2010, Yazici, *et al.*, 2011). For the uncoated allografts, the implants were prepared from C57BL/6 mice in the same manner as the rAAV-BMP2-coated allografts. Autografts consisted of the same bone that was removed from the host defect site in the calvaria. In all cases the bone graft was placed in the calvarial defect and the scalp was sutured.

2.2. *In vivo* imaging of *Luc* expression

Bioluminescence analysis was performed in a manner previously described (Iris, *et al.*, 2003) on Days 2, 4, 8, 10, 14, 17, 21, 24, and 28 after graft implantation. Quantification of the bioluminescent signal focused on one region of interest (ROI) encompassing most of the calvaria, within which lay the defect site, as well as another ROI at the animal’s tail area, which was used as an internal control. We reduced any background signal from both of these ROIs and divided the value of the signal obtained from the defect-site ROI by the

value of the signal obtained from the control ROI. In this manner we obtained a final value of calculated luminescence.

2.3 Evaluation of bone formation *in vivo*

New bone formation at the defect site was evaluated using micro-computed tomography (μ CT) (Steinhardt, *et al.*, 2008). Calvariae were harvested on Day 28 and scanned using a Desktop Cone-Beam μ CT Scanner (μ CT 40; Scanco Medical AG, Bassersdorf, Switzerland). Microtomographic slices were acquired at 1000 projections and reconstructed at a spatial nominal resolution of 16 μ m. A constrained 3D Gaussian filter ($\sigma = 0.8$ and support = 1) was used to suppress some noise found in the volumes. Bone volume (BV) and bone mineral density (BMD) were calculated in a constant circular ROI, measuring 7.5 mm in diameter, which contained within it the initial defect site.

2.4 Histological analysis

Bones were fixed in 4% formalin for at least 24 hr, decalcified using a 0.5-M EDTA solution (pH 7.4), passed through an increasingly graded series of ethanol baths and embedded in paraffin. Sections (5 μ m) were cut using a motorized microtome (Leica Microsystems, Nussloch, Germany). Hematoxylin and eosin (H&E), Alcian blue, and tartrate-resistant acid phosphatase (TRAP) staining procedures were performed as previously reported (Aslan, *et al.*, 2006, Tiyyapattanaputi, *et al.*, 2004, Turgeman, *et al.*, 2001).

2.5 Statistical analysis

Bioluminescence data were compared by performing a repeated-measures analysis of variance (ANOVA) using a general linear model (GLM) procedure of the SAS statistical package (SAS Institute Inc., Cary, NC, USA), in which the within-subject factor is the time of sampling (specific day) and the between-subject factor is the treatment group. Next, the Ryan-Einot-Gabriel-Welsch multiple range test was performed for a pair-wise comparison between groups. Probability values from the ANOVA were adjusted using the Bonferroni method according to the number of comparisons. μ CT data are expressed as means $\pm$ standard errors and were tested for significance by performing the Student t-test, in which the minimal criterion for significance was determined to be a probability value of less than 0.05.

3. Results

3.1 Osteogenic effect of rAAV-BMP2 calvarial allograft transplantation

Oc/Luc transgenic mice, which harbor the *luciferase* (*Luc*) gene under the control of the osteogenic tissue-specific promoter osteocalcin (Iris, *et al.*, 2003), were used to evaluate the osteogenic effect of rAAV-BMP2 on implantation of a calvarial allograft. In this model, the osteogenic activity in transgenic mice is reflected by *Luc* expression, which is activated by the osteocalcin promoter and is quantified using the BLI system. The *Luc* signal from all experimental groups was collected from a specific ROI containing the calvarial defect site, at several time points up to 28 days after graft implantation, in order to create an expression trend line (Figure 1A, B). Two types of rAAV vectors encoding human *BMP2* were used and compared in this experiment: SC-rAAV-BMP2 and SS-rAAV-BMP2. Implantation of both vector-coated allografts had a significant effect ($F=1.75$; p [intervention group $\times$ day] = 0.0272) on osteogenesis during the study period. Multiple post-hoc comparisons found the observations of the SS-rAAV-BMP2 group to significantly differ ($P=0.0345$) from those of the SC-rAAV-BMP2 group. The control groups, Autografts and Uncoated Allografts showed expression trend line, which was similar to the SS-rAAV-BMP2 regarding intensity and dynamics, but was not statistically different. The trend line for the SC-rAAV-BMP2

group showed a peak of expression on Day 8, which decreased in the following days. The trend line for the SS-rAAV-BMP2 group peaked later, on Day 10, and also decreased afterwards. ROIs in the SC-rAAV-BMP2 group displayed a 40% increase in the level of *Luc* expression on Day 8 compared to ROIs in the SS-rAAV-BMP2 group (Figure 1). Multiple *post hoc* comparisons found that the SS-rAAV-BMP2 group significantly differed ($p = 0.0345$) from the SC-rAAV-BMP2 group. In the Autograft and Uncoated Allograft control groups, the expression trend line was similar to that in the SS-rAAV-BMP2 group with regard to intensity and dynamics, and there was no statistical difference between that group and controls (Figure 1).

3.2 Quantification of new bone formation induced by rAAV-BMP2-coated allografts in Oc/Luc mice

The osteogenic effect following transplantation of the bone grafts resulted in new bone formation in defect site, which was validated by μ CT. The μ CT analysis, which focused on the specific ROI containing the graft, showed significantly higher bone volume values in the SC-rAAV-BMP2 group than in all other groups, indicating greater bone formation and implant integration. In the SC-rAAV-BMP2 group bone volume reached a mean value of $13.6 \pm 0.49 \text{ mm}^3$, whereas bone volume was only $10.49 \pm 0.53 \text{ mm}^3$ in the SS-rAAV-BMP2 group and similar values in the autograft and uncoated allograft groups (Figure 2). We also calculated the bone mineral density of the newly formed tissue, but there was no significant difference between groups (Figure 3). A visual analysis of three-dimensional (3D) images depicting the healing site (Figure 4) reveals a similar picture to that generated from the bone volume data.

3.3 Qualitative histological analysis of allografts implanted in Oc/Luc mice

Calvaria samples were harvested 28 days after graft implantation, and following dissection and μ CT scanning these samples were subjected to histological analysis. Standard H&E as well as TRAP and Alcian blue staining procedures were performed. Figure 5 represents a schematic view of an axial section, in which the red region represents the host calvaria, the light blue region represents the graft, and the green region is newly formed tissue. In all groups areas of newly formed bone tissue were found (Figure 5). In the control groups this newly formed tissue (represented by the green area) was mostly located in the graft-host junction (Figure 5C and D), whereas in the rAAV-coated allograft groups (Figure 5A and B) newly formed bone was also found surrounding the graft. Cells typically involved in bone formation, such as osteocytes and osteoblasts, were observed mostly in the rAAV-coated allograft groups, as can be seen in H&E-stained tissues (Figure 6A, a, and B, b).

Revitalization markers, such as bone marrow, blood vessels, and osteoclasts, were also detected, again mostly in the rAAV-BMP2-coated allografts, as demonstrated by TRAP and Alcian blue staining (Figure 7A–H). In those two groups, newly formed tissue between the graft and host and under the graft displayed the combined characteristics of connective tissue and ossification (Figure 7I–L).

4. Discussion

To date, autografts present the gold standard treatment in the clinical setting for bone reconstruction in the craniofacial area (Jaffe, *et al.*, 1991). Yet, due to limitations associated with autograft harvest, alloplasts are commonly used for bone defect repair (Thesleff, *et al.*, 2011). Structural allografts, which could be an alternative, suffer from limited life spans and a high failure rate. However, using a method previously described (Ito, *et al.*, 2005, Koefoed, *et al.*, 2005, Tiyyapatanaputi, *et al.*, 2004, Yazici, *et al.*, 2011), allografts can be revitalized by coating the cortical surface with a freeze-dried rAAV that encodes for bone remodeling genes. In the current study our aim was to define, for the first time, the potential

of rAAV-BMP2-coated allografts to induce bone repair in a murine model of cranioplasty. Since cranial bones differ significantly from long bones and are poor in bone marrow, it was not possible to predict the efficiency of AAV-coated allografts for fracture repair in calvarial bone defects.

Based on our previous work using an rAAV-Luc vector (Pelled, *et al.*, 2010), following graft implantation we expected to see a local and transient rAAV transduction confined to the boundary of the postoperative hematoma that would reach a peak at 1 week and termination on Day 35. This expression pattern is optimal for bone formation because the first week following the creation of a bone defect is an “inflammatory” phase dedicated to catabolism of damaged tissue (Einhorn, 2005). The expected peak transgene expression at 1 week (Pelled, *et al.*, 2010) is viewed to be ideal to jumpstart the “reparative” phase of fracture healing, which is known to commence at this time (Einhorn, 2005). Finally, the cessation of gene expression by Day 35 alleviates concerns about unregulated long-term gene expression and allows the final “remodeling” phase of fracture healing to occur naturally. Importantly, biodistribution assays previously performed ensure that the vector does not migrate from the surgical site (Pelled, *et al.*, 2010).

In this study, we evaluated the potential of rAAV-BMP2-coated allografts as a novel treatment for cranioplasty using a real-time *in vivo* imaging approach based on the use of transgenic mice harboring the *Luc* marker gene under the regulation of the human osteocalcin promoter (Iris, *et al.*, 2003). Using this model we found maximal expression of osteocalcin in all experimental groups within the first 10 days. In particular, osteocalcin expression in the SS-rAAV-BMP2 group peaked on Day 10, whereas in the SC-rAAV-BMP2 group it peaked on Day 8 as it did in the control groups. Overall, the SC-rAAV-BMP2-coated grafts induced higher levels of osteocalcin expression than the SS-rAAV-BMP2-coated grafts. Our results support previous findings (Yazici, *et al.*, 2011) affirming that the double-stranded phase of the rAAV vector (i.e. the transition from single-strand DNA to double-strand DNA, which takes place after the entrance of viral DNA into the host cell nucleus and before its integration into the host DNA and its transcription) is the rate-limiting step of AAV transfection (Dai and Rabie, 2007). This could also be the reason why, in our study, osteocalcin expression was similar in the control groups to the signal measured in the SS-rAAV-BMP2 group. Nevertheless, the higher (albeit not significantly higher) osteocalcin expression found in the SC-rAAV-BMP2 group, compared with the non-coated Allograft and Autograft groups, demonstrated an advantage of coated allografts for bone repair in this model.

This advantage was supported when a μ CT analysis was used to determine the extent of new bone formation in each group. The results clearly showed that more bone volume formed in calvarial defects 28 days after graft implantation in the SC-rAAV-BMP2 group than in all other groups. A similar advantage of SC-rAAV-BMP2-coated allografts over SS-rAAV-BMP2-coated allografts was also demonstrated in a femoral defect model (Yazici, *et al.*, 2011). Not surprisingly, we did not see a difference between the SS-rAAV-BMP2 group and either control group.

Qualitative histological analysis revealed a variety of bone cells as well as revitalization factors in the coated allograft sections. This observation is supported by previous studies showing the ability to revitalize the allograft (which is in fact a dead bone that serves only as a scaffold) without any immune response (Ito, *et al.*, 2005, Koefoed, *et al.*, 2005, Tiyyapatanaputi, *et al.*, 2004, Yazici, *et al.*, 2011). BMP2-coated allografts displayed an extensive area of newly formed bone tissue located in the graft-host junction as well as below and above the graft compared with the control groups. Newly formed tissue around the coated allografts was rich in revitalization markers and bone cells, indicating mature

bone compared to the less mature tissue that appeared in the Uncoated Allograft group. Future in-depth investigations into the mechanism of calvarial bone repair using AAV-coated allografts should include a quantitative analysis of the distinct cells and repair tissue types within the repair zone.

In this study we did not encounter any histological evidence of an immune response against the rAAV vectors that we used. Since rAAV vectors can elicit an immune response or an immune tolerance, depending on various factors as recently reviewed (Mays and Wilson, 2011), it is clear that an in-depth analysis of the immune response to rAAV-coated allografts should be performed in the future.

In conclusion, the data collected in this study support the feasibility of using rAAV-coated implants for cranioplasty and potentially for other bone augmentation and regeneration procedures in the craniofacial complex. Since those implants were also found to be efficient in a long-bone model (Yazici, *et al.*, 2011), it is feasible that additional preclinical studies could pave the way for application of this therapeutic strategy in the clinical setting.

Acknowledgments

This work was supported by the following research grants from the National Institutes of Health: R21DE017096, R01DE019902, AR46545, AR54884, and AR54041. We thank Mr. Eran N. Choman and Dr. Dori Pelled for help with the statistical analyses.

References

- Afifi AM, Gordon CR, Pryor LS, Sweeney W, Papay FA, Zins JE. Calcium phosphate cements in skull reconstruction: a meta-analysis. *Plast Reconstr Surg.* 2010; 126:1300–1309. [PubMed: 20885250]
- Artico M, Ferrante L, Pastore FS, Ramundo EO, Cantarelli D, Scopelliti D, Iannetti G. Bone autografting of the calvaria and craniofacial skeleton: historical background, surgical results in a series of 15 patients, and review of the literature. *Surg Neurol.* 2003; 60:71–79. [PubMed: 12865021]
- Aslan H, Zilberman Y, Kandel L, Liebergall M, Oskouian RJ, Gazit D, Gazit Z. Osteogenic differentiation of noncultured immunisolated bone marrow-derived CD105+ cells. *Stem Cells.* 2006; 24:1728–1737. [PubMed: 16601078]
- Awad HA, Zhang X, Reynolds DG, Guldberg RE, O'Keefe RJ, Schwarz EM. Recent advances in gene delivery for structural bone allografts. *Tissue Eng.* 2007; 13:1973–1985. [PubMed: 17518728]
- Berrey BH Jr, Lord CF, Gebhardt MC, Mankin HJ. Fractures of allografts. Frequency, treatment, and end-results. *J Bone Joint Surg Am.* 1990; 72:825–833. [PubMed: 2365716]
- Bonadio J, Smiley E, Patil P, Goldstein S. Localized, direct plasmid gene delivery in vivo: prolonged therapy results in reproducible tissue regeneration. *Nat Med.* 1999; 5:753–759. [PubMed: 10395319]
- Burchardt H, Jones H, Glowczewskie F, Rudner C, Enneking WF. Freeze-dried allogeneic segmental cortical-bone grafts in dogs. *J Bone Joint Surg Am.* 1978; 60:1082–1090. [PubMed: 363723]
- Chalmers J. Transplantation immunity in bone homografting. *J Bone Joint Surg Br.* 1959; 41-B:160–179. [PubMed: 13620723]
- Dai J, Rabie AB. The use of recombinant adeno-associated virus for skeletal gene therapy. *Orthod Craniofac Res.* 2007; 10:1–14. [PubMed: 17284242]
- Einhorn TA. The science of fracture healing. *J Orthop Trauma.* 2005; 19:S4–6. [PubMed: 16479221]
- Goldberg VM, Stevenson S. The biology of bone grafts. *Semin Arthroplasty.* 1993; 4:58–63. [PubMed: 10148544]
- Hogan BL. Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes Dev.* 1996; 10:1580–1594. [PubMed: 8682290]
- Hollinger JO, Kleinschmidt JC. The critical size defect as an experimental model to test bone repair materials. *J Craniofac Surg.* 1990; 1:60–68. [PubMed: 1965154]

- Iris B, Zilberman Y, Zeira E, Galun E, Honigman A, Turgeman G, Clemens T, Gazit Z, Gazit D. Molecular imaging of the skeleton: quantitative real-time bioluminescence monitoring gene expression in bone repair and development. *J Bone Miner Res.* 2003; 18:570–578. [PubMed: 12619943]
- Ito H, Koefoed M, Tiyyapatanaputi P, Gromov K, Goater JJ, Carmouche J, Zhang X, Rubery PT, Rabinowitz J, Samulski RJ, Nakamura T, Soballe K, O'Keefe RJ, Boyce BF, Schwarz EM. Remodeling of cortical bone allografts mediated by adherent rAAV-RANKL and VEGF gene therapy. *Nat Med.* 2005; 11:291–297. [PubMed: 15711561]
- Jaffe KA, Morris SG, Sorrell RG, Gebhardt MC, Mankin HJ. Massive bone allografts for traumatic skeletal defects. *South Med J.* 1991; 84:975–982. [PubMed: 1882275]
- Jayandharan GR, Zhong L, Li B, Kachniarz B, Srivastava A. Strategies for improving the transduction efficiency of single-stranded adeno-associated virus vectors in vitro and in vivo. *Gene Ther.* 2008; 15:1287–1293. [PubMed: 18496572]
- Koefoed M, Ito H, Gromov K, Reynolds DG, Awad HA, Rubery PT, Ulrich-Vinther M, Soballe K, Guldberg RE, Lin AS, O'Keefe RJ, Zhang X, Schwarz EM. Biological effects of rAAV-caAlk2 coating on structural allograft healing. *Mol Ther.* 2005; 12:212–218. [PubMed: 16043092]
- Lieberman JR, Ghivizzani SC, Evans CH. Gene transfer approaches to the healing of bone and cartilage. *Mol Ther.* 2002; 6:141–147. [PubMed: 12161179]
- Mays LE, Wilson JM. The complex and evolving story of T cell activation to AAV vector-encoded transgene products. *Mol Ther.* 2011; 19:16–27. [PubMed: 21119617]
- Pelled G, Ben-Arav A, Hock C, Reynolds DG, Yazici C, Zilberman Y, Gazit Z, Awad H, Gazit D, Schwarz EM. Direct gene therapy for bone regeneration: gene delivery, animal models, and outcome measures. *Tissue Eng Part B Rev.* 2010; 16:13–20. [PubMed: 20143927]
- Prolo DJ, Oklund SA. The use of bone grafts and alloplastic materials in cranioplasty. *Clin Orthop Relat Res.* 1991; 268:270–278. [PubMed: 2060219]
- Sahoo N, Roy ID, Desai AP, Gupta V. Comparative evaluation of autogenous calvarial bone graft and alloplastic materials for secondary reconstruction of cranial defects. *J Craniofac Surg.* 2010; 21:79–82. [PubMed: 20061970]
- Sanan A, Haines SJ. Repairing holes in the head: a history of cranioplasty. *Neurosurgery.* 1997; 40:588–603. [PubMed: 9055300]
- Steinhardt Y, Aslan H, Regev E, Zilberman Y, Kallai I, Gazit D, Gazit Z. Maxillofacial-derived stem cells regenerate critical mandibular bone defect. *Tissue Eng Part A.* 2008; 14:1763–1773. [PubMed: 18636943]
- Summers BN, Eisenstein SM. Donor site pain from the ilium. A complication of lumbar spine fusion. *J Bone Joint Surg Br.* 1989; 71:677–680. [PubMed: 2768321]
- Thesleff T, Lehtimäki K, Niskakangas T, Mannerström B, Miettinen S, Suuronen R, Ohman J. Cranioplasty with adipose-derived stem cells and biomaterial: a novel method for cranial reconstruction. *Neurosurgery.* 2011; 68:1535–1540. [PubMed: 21336223]
- Tiyyapatanaputi P, Rubery PT, Carmouche J, Schwarz EM, O'Keefe RJ, Zhang X. A novel murine segmental femoral graft model. *J Orthop Res.* 2004; 22:1254–1260. [PubMed: 15475206]
- Turgeman G, Pittman DD, Müller R, Kurkalli BG, Zhou S, Pelled G, Peyser A, Zilberman Y, Moutsatsos IK, Gazit D. Engineered human mesenchymal stem cells: a novel platform for skeletal cell mediated gene therapy. *J Gene Med.* 2001; 3:240–251. [PubMed: 11437329]
- Wheeler DL, Enneking WF. Allograft bone decreases in strength in vivo over time. *Clin Orthop Relat Res.* 2005; 435:36–42. [PubMed: 15930919]
- Wong RK, Gandolfi BM, St-Hilaire H, Wise MW, Moses M. Complications of hydroxyapatite bone cement in secondary pediatric craniofacial reconstruction. *J Craniofac Surg.* 2011; 22:247–251. [PubMed: 21233736]
- Xie C, Reynolds D, Awad H, Rubery PT, Pelled G, Gazit D, Guldberg RE, Schwarz EM, O'Keefe RJ, Zhang X. Structural bone allograft combined with genetically engineered mesenchymal stem cells as a novel platform for bone tissue engineering. *Tissue Eng.* 2007; 13:435–445. [PubMed: 17518596]
- Yazici C, Takahata M, Reynolds DG, Xie C, Samulski RJ, Samulski J, Beecham EJ, Gertzman AA, Spilker M, Zhang X, O'Keefe RJ, Awad HA, Schwarz EM. Self-complementary AAV2.5-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. [PubMed: 21206485]

Younger EM, Chapman MW. Morbidity at bone graft donor sites. *J Orthop Trauma.* 1989; 3:192–195. [PubMed: 2809818]

Figure 1. Osteogenic effect of rAAV-BMP2-coated allograft transplantation in the site of a calvarial defect

Osteogenic activity in transgenic mice is reflected by *Luc* expression, which is activated by the osteocalcin promoter and quantified using the BLI system. The bioluminescent signal was collected from a specific ROI containing the calvarial defect up to 28 days after graft implantation. A repeated-measures ANOVA shows a significant effect ($F=1.75$; p [intervention group $\times$ day] = 0.0272) of treatment on bone formation during the study period. The rAAV-coated allograft groups, SS-rAAV-BMP2 and SC-rAAV-BMP2, differed significantly from each other throughout the experiment ($p = 0.0345$) (A). Representative images obtained on Day 8 (B). White circles represent the ROI used for the analysis.

Figure 2. μ CT analysis of newly formed tissue: bone volume

Bone volume (BV) quantification was calculated within a specific ROI containing the calvarial defect. In the SC-rAAV-BMP2 group, the ROI displays significantly more new bone formation than that found in ROIs in all other groups. * $p < 0.001$, ** $p < 0.005$, *** $p < 0.01$.

Figure 3. μ CT analysis of newly formed tissue: bone mineral density

Quantification of bone mineral density was calculated within the same ROI. No significant differences between groups were noted. HA = hydroxyapatite.

Figure 4. μ CT analysis of newly formed tissue: images

3D images of specimens representing the median from each group demonstrate the status of defect healing on Day 28 postimplantation. White circles represent the ROIs used for the quantitative analyses.

Figure 5. Schematic view of newly formed tissue in the calvarial defect model

Calvaria samples were subjected to **qualitative** histological analysis 28 days after graft implantation. Light blue = graft, green = new bone, red = host bone, and dark blue = bone marrow. Large areas of newly formed bone tissue were found in the SS-rAAV-BMP2 (A) and SC-rAAV-BMP2 (B) groups as opposed to the control groups. There is a small area of newly formed tissue in the Autograft group (C), and this area is mostly located at the graft-host junction or underneath the graft. There is a very small amount of newly formed tissue in the Uncoated Allograft group (D). Note that these are representative samples from the defect analyzed in Figure 2.

Figure 6. Qualitative histological analysis of new bone formation in a calvarial defect model
 Calvarial bone sections were stained using H&E. A, a: SS-rAAV-BMP2 group. B, b: SC-rAAV-BMP2 group. C, c: Autograft group. D, d: Uncoated Allograft group. G = graft bone, H = host bone, Arrows: black indicates osteoblasts and white indicates osteocytes. Original magnifications $\times 10$ (panels A, B, C, and D) and $\times 40$ (boxes enlarged and shown in panels a, b, c, and d).

Figure 7. Allograft revitalization qualitative analysis

Calvarial tissue sections were subjected to H&E, Alcian blue, and TRAP staining (left, middle, and right columns, respectively). The revitalization markers that were mostly observed are bone marrow compartments, blood vessels, and osteoclasts (black arrowheads, black arrows, and white arrows, respectively). These markers were mostly found in rAAV-BMP2-coated grafts (A, E, and I: SS-rAAV-BMP2 grafts; B, F, and J: SC-rAAV-BMP2 grafts) and were less prevalent in uncoated grafts (C, G, and K: Autografts; D, H, and L: Uncoated Allografts).