INTRODUCTION: Exogenously control and monitoring of transgene expression in vivo is of great importance for gene therapy and for the study of gene function in vivo. In such a system, the transgene expression can be controlled time wise at a defined level of expression, thus controlling regeneration process. Segmental bone defects and nonunion fractures are relatively common in the skeleton and represent a great challenge for modern orthopedics. Osteogenic growth factors, thus controlling regeneration process. Segmental bone defects and nonunion fractures are relatively common in the skeleton and represent a great challenge for modern orthopedics. Osteogenic growth factors, limit the success of this technology. Gene therapy approaches based on AAV vectors and genetically engineered mesenchymal stem cells (MSCs) can efficiently induce the healing of segmental and nonunion bone defects, by combining gene regulated, efficient and long-term expression properties. We hypothesize that tetracycline-controlled expression systems (Tet-On and Tet-Off) encoding the hBMP-2, can induce regulated bone regeneration in calvarial segmental defects.

METHODS: The Institutional Animal Care and Use Committee approved all procedures consistent with the guide for the care and use of laboratory animals. We used two Tetracycline-controlled gene expression systems: 1) A Tet-On system was delivered by the human parvovirus AAV-2 (Adeno Associated Virus type-2). Two vectors were constructed: a tetracycline induced, transactivator vector and a hBMP-2 vector. Each vector was encapsidated separately, yielding two viruses. In this Tet-On system, mRNA transcription was activated in the presence of the inducer, leading to hBMP-2 protein expression, and was repressed in its absence. 2) A Tet-Off system was based on genetically engineered MSCs harbouring the inducible human BMP-2 expression vector (ptTATop-BMP2), which has a bi-directional promoter (TATA sequence) and uses elements of the tetracycline regulatory system as described by Gossen and Baron (Gossen et al., 1994; Baron et al., 1995). Both systems were regulated by administration of doxycycline (Dox). For the calvarial segmental defect assay, we used 18 male CD1 nude mice. The mice were anesthetized, and a 5-mm-diameter full-thickness circular calvarial defect, which is a non-healing critical-sized defect, was created in the lambda suture of the calvaria. The mice were divided into 3 groups, each group was further subdivided to Dox treated and non-treated groups. Dox was orally administered through the drinking water, at a saturating concentration (Moutsatsos et al., 2001) in order to induce or repress the expression of hBMP-2, depending on the Tet-system used ("On"/"Off"). Group 1 was implanted with collagen sponge (Duragen, Integra Neuro Sciences, NJ) loaded with 1×10^5 cells harboring the BMP-2 Tet-Off system, Groups 2 and 3 received collagen sponge only. The scalp was sutured with a 4-0 nylon suture, and the animals were allowed food and activity. At four and ten days post-operation, mice in Group 2 were injected with 5×10^5 PFUs of rAAV-hBMP-2 and rAAV-rtA (1:1 ratio) at the defect site. Four weeks following transplantation of the cells (Group 1) or first injection with the rAAV (Group 2), the mice were sacrificed and the calvarial specimens were dissected free from the soft tissue for digital imaging and histological analysis. In order to measure bone regeneration in a quantitative and noninvasive manner, we utilized the cooled charged coupled device (CCCD) camera. This is a well-established non-invasive, quantitative imaging system. Briefly, the model consists of transgenic mice (hOC-Luc) harboring the luciferase (Luc) marker gene under regulation of the human osteocalcin (hOC) promoter. In these mice the luciferase is expressed and monitored by the CCD Camera in a tissue specific manner, specifically in sites of osteogenic differentiation and bone regeneration. 18 hOC-Luc mice were quantitatively monitored non-invasively at 7, 14, 21, and 28 days by the CCD camera as was previously described (Bar et al., 2002). The results were confirmed by luciferase bioluminescent assay and real time quantitative RT-PCR of Luc gene.

RESULTS: In mice treated with rAAV-BMP-2 injection (Group 2), calvarial specimens exhibited a radio-opaque focus in the regeneration site at day 10, post injection. Histological analysis using histochemical staining (Masson’s Trichrome) confirmed osteogenic tissue formation, while control group showed no sign of osteogenic regeneration in the segmental bone defect site. At 28 days, mice of both rAAV-BMP-2 and genetically engineered MSCS treated experimental groups (Group 1 and Group 2) were sacrificed and analysed. Mice in Group 1 that were treated with the genetically engineered mesenchymal stem cell-based regulated gene expression system, showed massive bone formation and complete bone regeneration (Fig.1), while Group 2, the rAAV-BMP-2 treated mice, showed only partial regeneration (Fig.2). The control subgroups and the nontreated mice (Group 3) showed no sign of osteogenic regeneration in the segmental bone defect site. Results were analysed by quantitative digital faxitron imaging and histological analysis. Fluorescent Histomorphometry of Bone Tissue was assessed using Calcein Green Labeling. As a baseline for the quantitative and noninvasive experiment, utilizing the CCD Camera and the hOC-Luc transgenic mouse model, we were able to show luciferase expression in the segmental defect site (Fig.3). Indicating the activation of osteocalcin promoter as part of the biological regeneration process, that takes place at the nonunion fracture site. This preliminary result establishes our model of real time non-invasive and quantitative monitoring of bone regeneration related gene expression. We are currently analysing the results obtained from the calvarial segmental defect in hOC-Luc mice, quantified using the CCD camera.

DISCUSSION: Exogenously regulated expression systems can be potentially used to control bone regeneration and repair in non-healing bone defects (Moutsatsos et al., 2001). The controlled expression of the hBMP-2 enabled us to regulate bone formation in vivo, using AAV and genetically engineered MSCs systems (Tet-On and Tet-Off respectively). The difference in the results obtained when using AAV (Group 2) vectors and genetically engineered MSCS (Group 1) may be attributed mainly to the fact that genetically engineered MSCs have a double effect, both a paracrine and autocrine, while the rAAV-2 system delays on transducing cells recruited to the regeneration site. The effect of hBMP-2 in inducing bone formation was monitored in a real time, non-invasive and quantitative system that enables us to better understand the biological process during bone regeneration and repair. Our data demonstrate a regulated and monitored system for inducing bone regeneration in calvarial segmental defects and nonunion fractures. This system may be potentially developed and utilized for controlling bone regeneration and for developing gene therapy platforms for skeletal repair. We conclude that controlled and biosafe gene therapy platform for bone regeneration and repair can be achieved using Tet-regulated hBMP-2 gene.