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14. ABSTRACT
The central hypothesis of this application is that posterolateral spine fusion can be successfully achieved with a novel and simple minimally invasive percutaneous injection. We propose that this can be done by injection of AdBMP2 transduced human fibroblasts possessing an icasp9M that have been micro-encapsulated with osteoclast degradable hydrogel into the desired fusion site. During this first year, we have nitiated the identification of the phenotype of the monocyte-like cells that appear to be capable of bone degradation. We have demonstrated in MSCs, which have a stably integrated icasp9M, can be rapidly induced to undergo apoptosis, after delivery of a chemical inducer of dimerization (CID) after encapsulation in PEG hydrogel. The next step is to start in vivo testing of this system. Additionally we have developed the methodology to non-invasively locate the hydrogel microsphere placement, optically, with respect to the newly forming bone. This has lead to a publication. We have also initiated studies to fuse the spine using our cell based gene therapy system encapsulated in the PEG hydrogel microspheres, in both mouse and rat. We will continue to complete these studies in the upcoming year.

15. SUBJECT TERMS- BMP2, Spine fusion, PEG Hydrogel, Gene Therapy, Adenovirus.

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INTRODUCTION: The treatment of many spinal problems involves stabilization of the spine by applying bone grafts to the posterior elements of the spine. The objective of these procedures is to induce bone to bridge between adjacent vertebral bodies and "fuse" the vertebrae into a larger bone mass. Posterolateral fusion of the spine is the most commonly performed of all the types of spine fusion and is useful for the treatment of scoliosis, instability and painful degenerative conditions of the spine. We recently demonstrated that adenovirus transduced cells expressing BMP2, when injected into the paraspinous musculature, could rapidly form new bone at the targeted location and efficiently fuse vertebral bone at a desired site, within 2 weeks. Encapsulation of these cells in poly(ethylene glycol)-diacrylate (PEGDA) hydrogels allowed for longer survival of the cells *in vivo*, did not result in inflammation, which otherwise completely ablate new bone formation/fusion, and maintained the cells at the target location. Thus our preliminary data demonstrates the ability to induce new bone formation at the desired fusion location without need for any surgical intervention. Here we propose to engineer additional safety features into the material by using an inducible caspase 9 (icasp9_M), which when activated will induce apoptosis within the transduced cells. Further, the hydrogel will also possess an osteoclast selective protease site which allows for removal of the biomaterial during bone remodeling.

BODY:

Task 1: To characterize the ability of the molecular therapy system to rapidly induce fusion of the new bone with the vertebra, through induction of osteoclast progenitors (OCP) – monocyte progenitors. The proposed studies in this aim are an extension from our previous findings that monocyte-like cells that are not multinucleated osteoclasts were observed at the fusion site between the new heterotopic bone, and skeletal bone (figure 1). These cells appeared to be contributing to the remodeling of the fusion structure, and may be critical to enhancing fusion between the two bones. We propose that if we could characterize and isolate

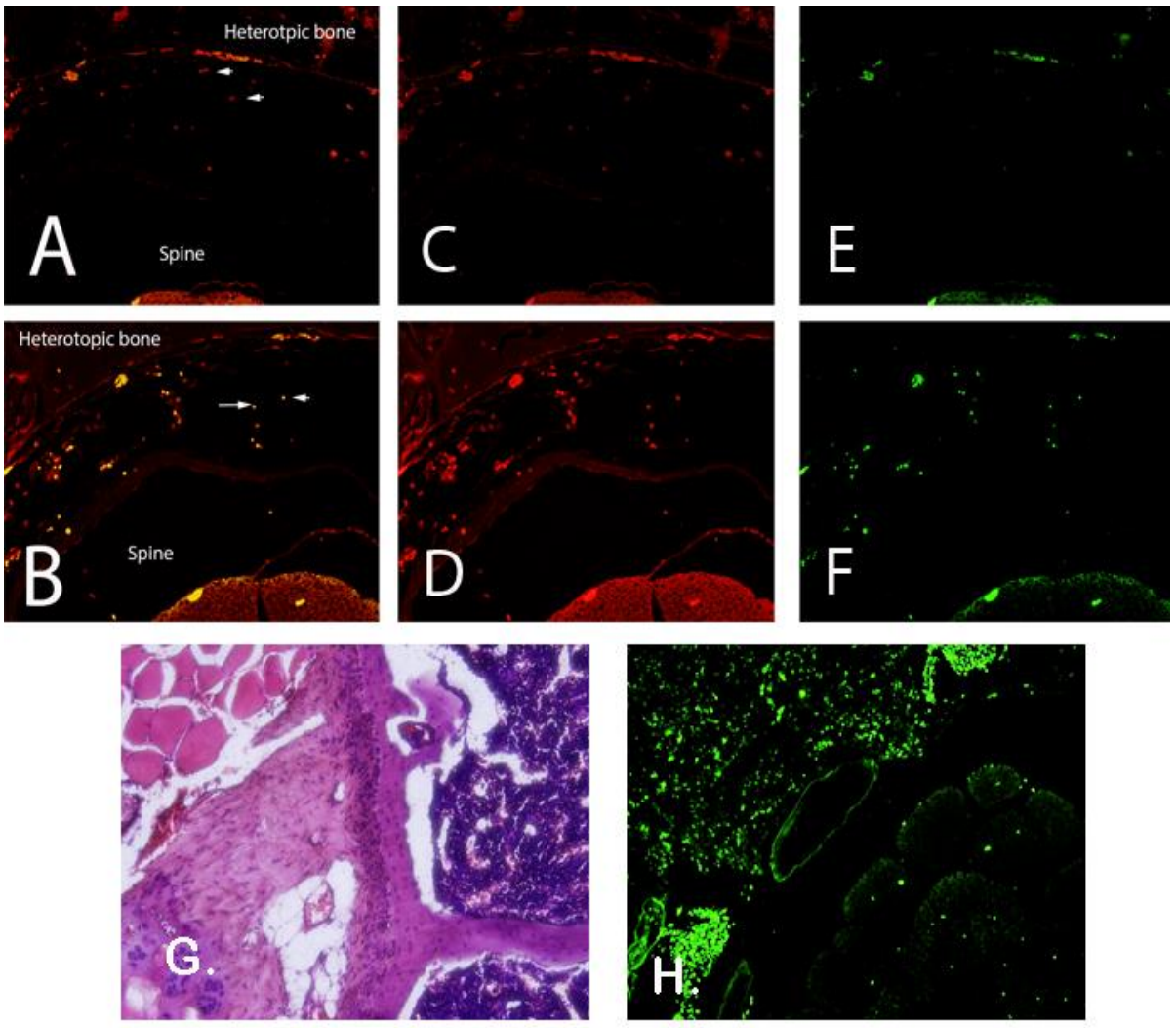

Figure 1: Representative photomicrographs of tentative vertebral fusion with the heterotopic bone. The slides were immunostained for **(A and C)** CD11b (red), **(A and E)** fractalkine (green) and merged in **(A)**. In panels **(B and D)** fractalkine (red), **(B and E)** LyC6 (green), and **(G)** hematoxylin and eosin for viewing. Panel H depicts a nerve with positive staining for LyC6 in the perineurium. These are representative photomicrograph (10X) of a sample taken 4 weeks after the initial injection of AdBMP2 transduced cells.

these cells, they may be beneficial in enhancing spine fusion, in other model systems as well as our own. One of the first steps is to determine their phenotype and determine if they are actually specialized M2 macrophages expanded to aid in matrix remodeling and fusion.

Therefore we have secured animal approval for the studies, and performed spine fusion in mice to generate tissue sections for initial phenotypic characterization of these spines. In these experiments, mice (n= 4) were injected with AdBMP2 or Adempty transduced cells and then spines isolated at weekly intervals (1-6 weeks) and serial sections generated, that represent the fusion process in the animals. We then initiated immunohistochemical staining with key antibodies that will be used for fluorescence activated cell sorting (FACs). These antibodies (CD11b ^{lo}, fractalkine^{+}, Ly6C^{+}), are surface antigens that can readily allow us to sort populations of cells for testing in bone resorption assays. After initial optimization of the antibodies, we observed co-localization of fractalkine^{+} and Ly6C^{+} in the cells, but as expected they were not expressing CD11b (figure 1). These cells were found lining the junction between the heterotopic bone and the new bone (figure 1, panel B). What was further intriguing was that cells associated with the perineurium of the nerve were also LyC6^{+} but not fractalkine^{+}. It is unclear whether these cells may function as an earlier progenitor, or whether they are just a separate population. We will continue to characterize this phenptype.

The next step is to isolate and further characterize the phenotype of these cells specifically to demonstrate their potential M2 nature. Also we will collect the various positive and negative populations and confirm their bone resorption ability using a standard assay kit (Bone Resorption Assay Kit; CosmoBio Co, Ltd) that uses a fluoresceinated calcium phosphate-coated plate. Additionally, we will isolate and test these cells *in vitro* to determine whether they respond to Lipopolysaccharide (LPS) and the proinflammatory cytokine interferon-γ (IFNγ) to promote a classically activated M1 macrophage expressing IL-12 or conversely if exposure to IL-4 or IL-13 will promote an "alternatively activated" M2 phenotype that expresses IL-10. We predict that those studies will be completed within the next year.

Task 2: To increase the safety and controllability of the procedure through selective ablation of the cellular component of the microspheres followed by osteoclast specific resorption on the polymer, and bone healing.

We first wanted to introduce and inducible caspase 9 (icasp9$_M$) into the delivery cells so that the BMP2 production could be regulated by systemic delivery of a chemical inducer of dimerization (CID). This in turn would cause the icasp9$_M$ to become activated and initiate an apoptotic pathway leading to cell death. Therefore we generated human mesenchymal stem cells that possess a stably integrated icasp9$_M$ as well as a GFP reporter. We also obtained an MSC cell line that does not possess the icasp9$_M$ for use as an additional control. In these first experiments we determined the timing of cell death (figure 2) and determined whether this would attenuate the BMP2 expression (figure 3). We observed immediate killing of the cells greater than 95% after exposure to the CID, however, the vehicle did not lead to any significant cell death. We next looked at BMP2 expression after deliver of a single dose of CID on day 1 (figure 3). BMP2 activity was assessed through an assay in which the bone marrow cell line W20-17 will undergo osteogenic differentiation, and up regulate alkaline phosphatase activity, in the presence of active BMP2. The results suggest that the single dose of CID was able to ablate the expression of BMP2 in the cells, but the cells were capable of being efficiently transduced to produce active BMP2.

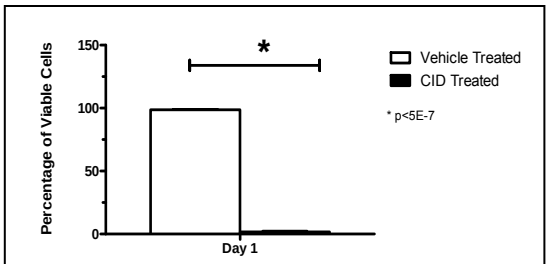

Figure 2: Cell death was immediate following delivery of the 0.1 nM chemical inducer of dimerization (CID)

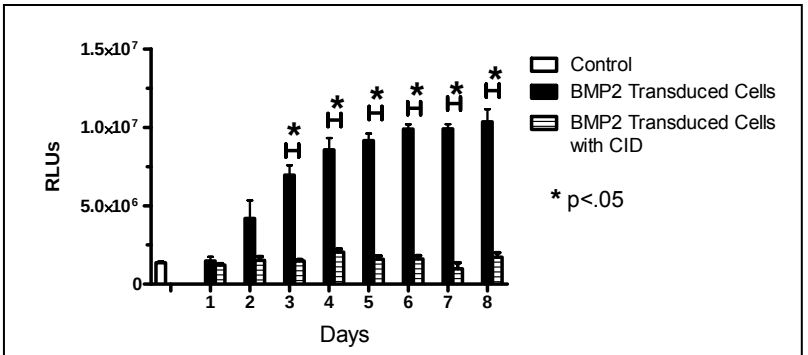

Figure 3: BMP2 activity in the presence and absence of CID. W20-17 cells were exposed for 3 days with media isolated from MSCs + icasp9$_M$ transduced with AdBMP2 (5000vp/cell) and in the presence of absence of CID. The CID was delivered on day 1, and remained in the cultures, throughout the assay.

We next attempted to encapsulate the cells and determine if they were capable of producing BMP2 to confirm that the CID would freely diffuse into the hydrogel, and activate the icasp9$_M$ similarly to the unencapsulated cells. As seen in figure 4, although the preliminary data appears to be variable, the encapsulated cells appear to produce similar amounts of the BMP2 as the unencapsulated cells. We next looked at the ability of the encapsulated cells to undergo apoptosis in the presence of CID (figure 5). These preliminary experiments suggested that within 24 hours approximately 60-75% of the cells were observed expressing the dsRED in the MSCs + icasp9$_M$, but was reduced to approximately 40% of the cells expressing the dsRED after delivery of the 0.1nM CID. Alternatively, approximately 80% were observed expressing the dsRED in the control MSCs lacking the inducible caspase 9 regardless of the presence of CID. Although preliminary these studies suggest that the CID is able to induce apoptosis in the encapsulated cells. Further studies are ongoing to follow up and optimize the kinetics and delivery of CID to the encapsulated cells.

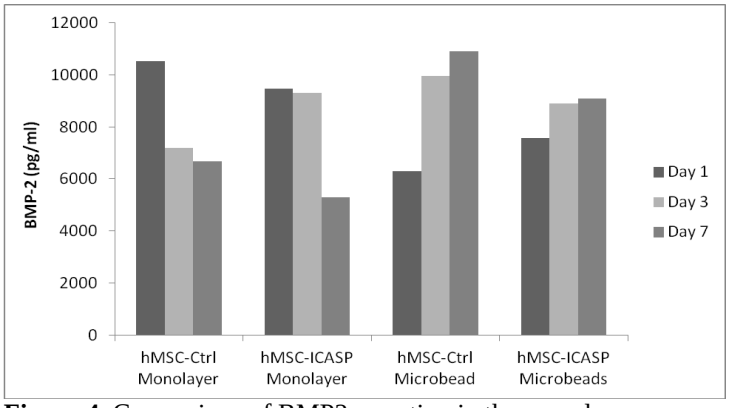

Figure 4. Comparison of BMP2 secretion in the monolayer versus the encapsulated cells.

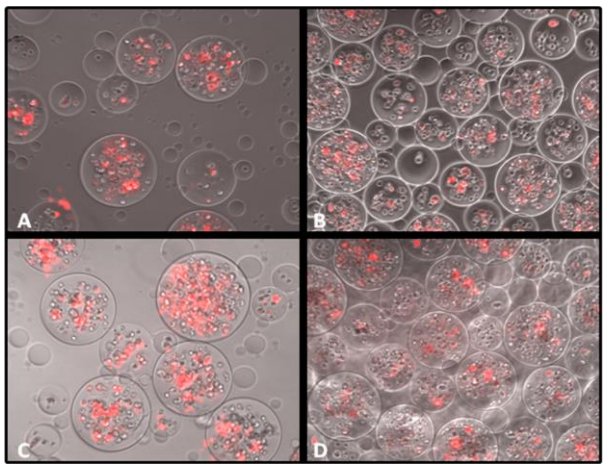

Figure 5: Comparison of dsRED expression in MSCs or MSCs + icasp9$_M$ in the presence of CID. Cells were transduced with AddsRED (5000vp/cell) and cell death was scored as the absence of red color. **(A)** hMSC-Ctrl w/o CID; **(B)** ICASP w/o CID; **(C)** hMSC-Ctrl w/ CID; **(D)** hMSC-ICASP w/ CID.

We also performed a very preliminary study to look at whether delivery of the CID could then suppress bone formation *in vivo*. In these studies, the MSCs or MSCs + icasp9$_M$ were transduced with AdBMP2 (5000vp/cell) and then injected into the rear hind-limb of NOD/Scid mice. Bone formation was detected using x-ray approximately 15 days after the initial injection (figure 6). Although there was significant variability within groups (Table 1), the trends suggest that deliver of the CID was able to suppress the bone formation when delivered earlier in the reaction (figure 6, panels A and B). However, when delivered at 11 days after initial induction of HO, the majority of the samples had significant HO (figure 6, panel C). We are currently repeating a many of these experiments to gain optimize the CID dose, and delivery time, with enough replicates to afford statistical analysis and publication. We predict that those experiments will be completed within the next year. Additionally we will initiate similar experiments in the GPSG or degradable hydrogel microspheres.

Control MSCs	**Samples with HO**
No CID	100%
CID delivered on day 1	50%
CID delivered on day 4	75%
CID delivered on day 11	50%
icasp9$_M$ MSCs	
No CID	100%
CID delivered on day 1	25%
CID delivered on day 4	25%
CID delivered on day 11	75%

Table 1: Preliminary analysis of the HO in the presence or absence of CID in MSCs and MSCs + icasp9$_M$

Task 3: To assess and compare bone quality of the skeletal and new bone during and after completion of the fusion.

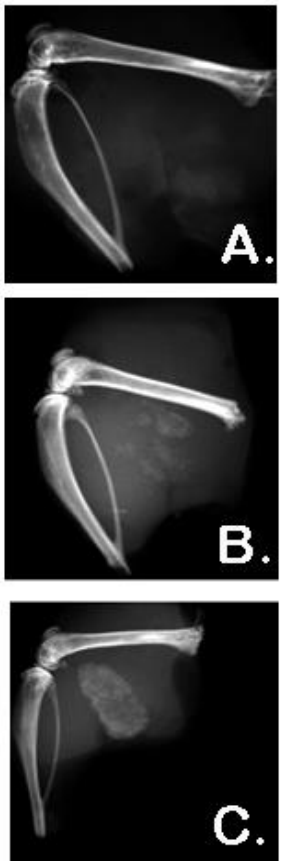

Figure 6: Radiological evaluation of bone formation 15 days after animals received AdBMP2 transduced MSCs + icasp9$_M$ followed by systemic delivery of CID **(A)** day 1, or

To accomplish this, we have developed a non-invasive optical imaging methodology to determine the optimal dose of microspheres with respect to their placement, cell viability and resultant bone formation. Therefore we altered our approach by incorporating an Alexafluor dye into the PEG hydrogel that could be detected optically. To further confirm viability of the cells, we transduced the cells with AdIFP and AdBMP2, so that we could follow the cells. Initial experiments were performed to then detect the presence of the two reporters with respect to the newly forming bone. Since this involved both optical imaging and co-alignment with microCT, we had to develop novel methodology to integrate the systems. These experiments lead to a publication of this methodology (see appendix). We propose in the next year to implement this technology using a newer reporter virus that does not interfere with the BMP2 expression, and will provide more robust bone formation. Further we will also add microPET imaging for early bone formation through detection of MMP9, which we have previously shown highlights the region where the bone matrix will be placed. From these initial findings we observed the critical nature of placement of the materials, so in this upcoming year we will focus on confirming these studies, quantifying cell viability *in vivo,* and determining optimal conditions for stabilized fusion in our rat models.

KEY RESEARCH ACCOMPLISHMENTS: We have:

- Approval of animal experiments
- Have initiated characterization of monocyte-like cells
- Have obtained stable MSC cell lines possessing the inducible caspase 9 (icasp9$_M$)
- Have demonstrated the ability of these cells to secrete BMP2 after transduction with adenovirus
- Have shown that these cells are viable after encapsulation
- Have shown that these cells respond to the CID (or activator of apoptosis) by undergoing cell death, and through the lack of BMP2 within the culture supernatant
- Have started to characterize the optimal dose of the CID to induce similar rapid cell death and suppression of the BMP2
- Have started to characterize the kinetics of in vivo delivery of CID, and resultant suppression of bone formation.
- Have developed methodology for optical detection of integrated near infrared reporters in the hydrogel microspheres
- Have developed methodology for co-localizing the optical data with microCT data of resulting bone formation.
- Published this work

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

- Lu Y, Darne C, Tan I, Zhu B, Hall M, Lazard Z, Davis AR, Simpson S, Sevick-Muraca EM and Olmsted-Davis EA. Far-red fluorescence gene reporter tomography for determination of placement and viability of cell-based gene therapies. Optics Express (in Press).

CONCLUSION:

We have initiated experiments to isolate and characterize the potential M2 monocyte cells that we propose are involved in rapid remodeling of the skeletal bone matrix for integration to the newly formed bone

fusion mass. In these experiments we have identified the initial cells and found that they possess several markers of the M2 lineage, which will be useful for fluorescence activated cell sorting. Once we have isolated these cells we will test their ability to resorb bone through a standard assay, and will also confirm that they behave similarly to M2 monocytes in their ability to respond to IL4 rather than proinflammatory cytokines such as LPS. These experiments are ongoing and we have not run into any problems that would prevent us from reaching our proposed goals. We have begun to establish optimal parameters for encapsulation of the MSCs + icasp9M in the PEG hydrogel microspheres that will lead to optimal bone formation. This included a number of preliminary in vitro experiments. We predict this work will be completed in the next year and we will initiate studies with the degradable form of the hydrogel. We will also continue to introduce the hydrogel microspheres through an injection into our model of spine fusion. However, due to the size and amount of microspheres, the mouse model is not practical. We have obtained fusion, but with the non-degradable form of microspheres, the fusion mass is large and often times less robust due to the large tissue area that the microspheres encompass. We have thus initiated experiments in rat models that can accommodate the large number of microspheres. In these studies, we have observed significant changes in HO as compared to the mouse. Mice readily form bone in the absence of BMP2 however, in the rat models, they do not form HO, but only orthotopic bone. We have been investigating the steps of bone formation even orthotopically in the rat models, and found that we can induce bone formation through delivery of additional cells either isolated specifically from peripheral nerves or periosteum. We are currently testing whether these cells can be bypassed by deliver of additional agents that lead to nerve remodeling (MMP9) (1)(2), which have been shown to enhance the expansion of the peripheral nerve/periosteal cells *in vivo*, and thus would potentially allow us to avoid having to deliver an additional cell type.

REFERENCES: List all references pertinent to the report using a standard journal format (i.e. format used in *Science, Military Medicine,* etc.).

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