THESIS

THE OSTEOGENIC CHARACTERIZATION AND CRYOPRESERVATION OF EQUINE BONE MARROW DERIVED MESENCHYMAL STEM CELLS WITH SCAAV-EQUINE-BMP-2

Submitted by

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ABSTRACT

THE OSTEOGENIC CHARACTERIZATION AND CRYOPRESERVATION OF EQUINE BONE MARROW DERIVED MESENCHYMAL STEM CELLS WITH SCAAV-EQUINE-BMP-2

Optimizing the environment of complex bone healing and improving treatment of catastrophic bone fractures and segmental bone defects remains an unmet clinical need both human and equine veterinary medical orthopedics. Animal models of fracture repair often involve small rodents, as historically significant large animal models, like the dog, continue to gain favor as companion animals. This trend continues despite the well-documented limitations in comparing fracture repair in humans, as few similarities exist. Study design, number of studies, and availability of funding also continues to limit large animal studies. Osteoinduction is often therapeutically targeted to incite new bone growth. Osteoinduction with recombinant BMP-2 (rhBMP-2) results in robust bone formation; although, long-term quality is scrutinized due to poor bone mineral quality. Gene therapy continues to gain popularity among researchers to augment bone healing, and with the approval of the first cell-based gene therapy treatment in South Korea, the clinical reputation of gene therapy is under scrutiny. Progenitor cell therapies and the content variation of patient-side treatments (e.g. PRP and BMAC) are being studied in humans, while the immunologic properties of autologous and allogeneic treatments are being studied in the horse.

The objective of this Masters Thesis was to determine whether scAAV-equine-BMP-2 transduced cells would induce osteogenesis in equine bone marrow derived mesenchymal stem cells (BMDMSCs) *in vitro*, and if these cells could be cryopreserved in an effort to osteogenically prime them as an "off-the-shelf" gene therapeutic approach for fracture repair. Our study found that transgene expression is altered by cell expansion, as would be expected by a transduction resulting in episomal transgene expression, and that osteoinductive levels could still be achieved five days after recovery. Further, protein expression continues up to fourteen days after initial transduction. This is the first evidence that cryopreservation of genetically modified BMDMSCs would not alter the osteoinductive potential or clinical use of allogeneic donor cells in cases of equine fracture repair. Future directions should include *in vivo* pilot data aimed at elucidating whether or not these cells expedite clinical healing. Other genes and gene combinations (e.g. PTH 1-34 and BMP-2) could be introduced to the expression vector backbone and compared to the vector utilized in this study.

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CHAPTER 1:

Introduction and literature review[1]

Bone has a remarkable capacity for self-renewal and remodeling [1], and has evolved to serve many mechanical, endocrine, and homeostatic functions [2]. Although normal bone remodels in response to adverse conditions such as changing biomechanical forces, microdamage, and fracture, about 5-10% of fractures do not heal conventionally even with clinical interventions resulting in non-union [3]. Thus, there is an unmet clinical need for novel approaches to promote rapid repair of complicated long bone fractures and large bone defects. The degree of soft tissue injury and type of fixation utilized, host factors such as age, diabetes, NSAID use, and osteoporosis limit osteogenesis *in vivo;* often these limiting factors result in clinical sequelae such as increased infection rate, risk of nonunion, and inability to maintain quality of life [4; 5].

Increasing osteogenesis has been explored through targeted overexpression of growth factor and exogenous hormone delivery—therapeutics mainly aimed at osteoinduction, a substance that results in the commitment of progenitor cells down an osteoblastic lineage. One way osteogenic induction is achieved *in vivo* is through delivery of growth factors that result in accelerated osteoblast generation from native progenitor cells, and therefore, accelerated bone formation. Bone formation and bone healing can be achieved through various pathways; therefore, a cursory signaling summary of the growth factors to be discussed, BMP-2 and PTH, is provided.

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Bone morphogenic proteins, part of the transforming growth factor-β superfamily, induce bone formation through binding complexes of serine threonine kinase receptors to initiate cell signaling [6]. The most studied osteogenic BMPs, 2, 4, and 7 bind the same complex of receptors [6]. Subsequent SMAD 1/5/8 phosphorylation allows nuclear translocation and binding to specific DNA elements to activate transcription of osteoblast-specific genes [7]. Osteogenesis may also occur through activation of TAK-1 and TAB1, which are crucial upstream regulators of MKK its activation of osteogenic gene transcription via p38/MAPK [8]. Both canonical (R-smad) and non-canonical (MKK) osteogenic BMP signaling results in the transcription of RunX2, Dlx5, and Osx [9]. Bone anabolism via PTH occurs through canonical WNT signaling. WNT-PTH crosstalk results in β-catenin stabilization, nuclear translocation, and subsequent transcription of genes to improve bone formation while decreasing bone resorption. Non-canonical WNT bone anabolism is often achieved with planar cell polarity crosstalk and is implicated in PTH 1-34 response to strain and during skeletal morphogenesis [10]. Further discussion of the signaling pathways involved in osteogenesis for bone healing can be reviewed with these references [3; 8; 11].

This review describes approaches used to promote osteogenesis in pathologic and osteoporotic fractures and segmental bone defects using BMP-2 and PTH. Use of appropriate pre-clinical animal models, recombinant protein therapy, gene therapy, and the use of progenitor cells are discussed. Scaffolding materials for bone have recently been comprehensively reviewed and will not be discussed in this manuscript [12; 13].

Animal Models:

Research in animal models is a critical component for translation to human clinical trials. No perfect model exists that exactly replicates fracture healing in humans; however, animal models may be utilized to answer specific clinical questions. Table 1.1-1.2 provides a descriptive summary of common animal model advantages and disadvantages, and Figure 1.1 provides pictorial representations of common pre-clinical models and the method most often utilized to study osteogenesis in segmental bone defects.

Mouse models in fracture repair are often utilized because the ability to purchase and or design specific genotypes and phenotypes affords researchers the ability to study cells with specific characteristics [14]. This is often done through genetically manipulated knock out models and inbred strains [15]. Nonetheless, murine bone lacks haversian systems, and it is unknown how this may affect pathophysiologic pathways of bone injury and healing when compared to humans [16] (Table 1.1). Similarly, rat bone is also devoid of haversian systems [16]. The rat is a popular model for delayed and non-union fracture repair models, as well as growth factor use in fracture repair. Similar to the mouse, inbred strains of rats may be purchased from commercial vendors, and housing, anesthesia, and pain management are inexpensive compared to large animal models. While it is often assumed inbred rat strains are genetically homogenous, there is some genetic heterogeneity within inbred populations with large variation in the number of single nucleotide repeats (SNPs) [17]. How SNP variation within genetically inbred populations affects baseline variation is unknown. Other limitations of the rat include size and decreased elasticity compared to human bones [18; 19]. It is unknown if the lack of haversian systems alters local reaction and nutrient or waste shuttling during pathology (Table 1.1). The rabbit offers similar advantages as the mouse and rat, such as ease of housing, anesthesia, and pain monitoring [20]; although they too

are dissimilar in size and body weight when compared to humans. Compared with mice and rats, rabbits are a more outbred species. This necessitates larger numbers in a given study to reach statistical power and significance because of individual variation. However, rabbit bone contains haversian systems and more closely replicates large animal models of bone structure. Therefore, success in rabbits may predict success in a larger animal model (Table 1.2).

When comparing bone composition between human, canine, swine, bovine, ovine, poultry, and rodent bone, canines' most closely resemble human bone composition when ash weight, extractable proteins, and IGF-1 content are considered [21]. Some studies have found that trabecular bone turnover is higher in canines than in humans, and that there is an age related decrease in the remodeling capabilities [21]. At the microstructure level, the secondary osteon structure and presence of plexiform bone adjacent to periosteum, especially during callus formation, allows canine cortical bone to withstand greater compressive forces than human bone [22]. Finally, the increased standing of canines as companion animals increases ethical concerns for their continued use in orthopedic research (Table 1.2).

Sheep are more similar in body weight to humans compared to the models discussed thus far, and the dimensions of their bones allow them to be suitable for surgical implants and biomaterial studies. However, their long bone trabecular density is 1.5-2 times greater than humans, conferring more inherent mechanical strength; and, because they are quadrupeds, weight distribution is dissimilar to humans [22]. Despite these limitations, sheep have some advantages. For example, when sheep age, their bone physiology resembles that of humans, with increases in osteoporotic or osteopenic bone loss [22; 23]. Differences in bone healing as it relates to age in

sheep, and stark difference in nutrition status should be taken into careful consideration when researchers are considering the sheep as a large animal model for bone repair (Table 1.2).

The horse is an FDA recommended model for osteoarthritis and comparative joint research [24], and availability to measure *in vivo* bone strain as well as the similar haversian remodeling suggest the horse is a good pre-clinical model for fracture repair despite cost-associated drawbacks [25; 26]. However, horses exhibit rapid periosteal expansion with plexiform bone that is unlike fracture healing in humans [27] (Table 1.2) and the use of minimally-weight bearing metacarpal bones should be considered (Figure 1.1).

References for Table 1.1: [21; 22; 28; 29]

References for Table 1.2: [16; 20-22; 28-30]

Recombinant Proteins:

Recombinant protein therapy is the use of purified therapeutic protein applied to bone defects to produce union (e.g. rhBMPs) or administered systemically to increase osteo-anabolism (e.g. PTH1-34). Production of recombinant proteins is done through a variety of bacterial (*Escherichia coli*), eukaryotic (yeast) or mammalian expression systems (Chinese Hamster Ovary cells (CHO), Human Embryonic Kidney cells (HEK), and AD293 cells (a derivative of HEK cells). In 2002 and 2004, respectively, the recombinant proteins to be discussed, PTH (1-34) and rhBMP-2, were approved for use in osteoporosis treatment and open tibial fractures in humans [31; 32] after extensive preclinical animal studies that demonstrated clinical efficacy (Table 1.3 and 1.4). The proteins remain of clinical, ethical, and socioeconomic interest [33; 34].

BMP-2: In 1965, Marshall Urist first discovered that proteins within bone could induce osteoid formation when he placed demineralized bone matrix in muscle tissue [35]. The proteins that were able to induce osseous metaplasia were given the family name "Bone Morphogenic Proteins." Since the advent of gene sequencing, BMPs have been further characterized by nucleotide similarity and are thus grouped accordingly (e.g. BMP-2/4 and BMP-5/6/7/8) [36]. The most potent osteoinductive agent available to clinicians' today is BMP-2. Table 1.3 and 1.4 summarizes seminal studies that supported FDA approval of rhBMP-2 for open tibial fractures. RhBMP-2 is most commonly combined with bovine collagen [37] and provides an exogenous supraphysiologic dose of osteoinductive growth factor to overcome the challenging clinical environment it is often placed in (e.g. open tibial fractures).

Despite the use of supraphysiologic doses, results are variable [38]. Contributing factors include the short half-life, potential for improper folding or post-translational modification that can reduce biologic activity [39], and the presence of natural BMP-2 antagonists, such as Noggin [40]. Likewise, there are strong species-associated dose requirements for osteogenesis—the recommended dose to induce osteogenesis in humans (1.5 mg/mL) is at least 3.75 times greater than the required dose in rodents (0.02-0.4 mg/mL) [41]. Furthermore, rhBMP-2 therapy is complicated as it is often cost-prohibitive, is not covered by insurance [42], is associated with a high degree of inflammation and ectopic bone formation [43], and widespread off-label use is documented and often results in unwanted side effects [44; 45].

RhBMP-2 use often results in bony union, but continuous corticies and non-remodeled trabeculae are often thin [46]; as the ultimate goal of fracture healing is to have mechanically

functional bone, rhBMP-2 generated bone quality is in question. Osteolysis and subsistence are reported in spinal fusion [47-49]—although, spinal use of rhBMP-2 is beyond the scope of this review, it is prudent to note similar findings found in long-bone fracture repair—principally cystic bone formation. There are accounts of greater trabecular bone spacing [50] and evidence of BMP-2 signaling induced osteoclastogenesis and inflammatory cytokine expression induction [51-53]—traits not overcome, and potentially worsened, by supraphysiologic rhBMP-2 doses. There are reports of BMP-2 induced adipogenesis [54] and clinical accounts of adipose tissue scattered throughout BMP-2 regenerated bone [55]. Although the concentration of BMP-2 required to overcome its native antagonists is unknown, the current research trends to low-dose BMP-2 with moderate success [56; 57]. Furthermore, phase II and III clinical trials were completed with objectives to decrease the dose of rhBMP-2 from 1.5mg/mL to 1.0mg/mL in patients undergoing internal fixation surgeries to repair closed diaphyseal tibial fractures. To its favor, retrospective analyses of on-label spinal fusion and open tibial fracture surgeries show a decreased rate of secondary interventions when rhBMP-2 is used instead of autograft alone. Additionally, operation time and hospital stay were reduced with rhBMP-2 use [38; 58].

There is no current consensus on rhBMP-2 treatment, utilization, or effectiveness among clinicians or researchers. Further study is needed to elucidate if combination therapy may allow a lower dose of rhBMP-2, if rhBMP-2 could be more effective if administered via another mechanism (e.g. gene therapy), and if bone quality and subsistence limit the long-term effectiveness of treatment.

PTH 1-34: The production of recombinant PTH 1-34, teriparatide, the amino terminal of the full-length PTH peptide (84 amino acids), is utilized to encourage osteo-anabolism when administered systemically and intermittently for the treatment of osteoporosis.

Off-label, PTH (1-34) has been studied in clinical trails to assess fracture repair [59]; however, therapy is limited by dosing ambiguities and mixed results. In one human clinical trial treating distal radius fractures, a higher dose (40μg) of PTH 1-34 was no better than the vehicle control, while the lower dose (20μg) shortened time to cortical continuity [59]. In patients with pelvic fractures, a dose of 100μg of PTH (1-84) and concurrent vitamin D and calcium supplementation accelerated fracture healing and functional outcome [60]. In rat tibial fractures, lower doses (60μg) of PTH 1-34 produced less external callus volume and ultimate load when compared to higher doses (200μg) [61]. These ambiguities and mixed results have led to the termination of several of the clinical trials in long bone fracture repair; although, PTH (1-34) may still be clinically indicated in other clinical scenarios. For instance, PTH (1-34) increases bone formation around implants, helping them assimilate into grafts [62-65] and increases flexural thickness and overall cortical thickness [66] predominantly through the proliferation of bone lining cells [67] and inhibition of osteoblast apoptosis [68]. It may also regulate bone formation around bone implants through strain specific osteoblastic induction [10].

An important limitation of PTH (1-34) is that treatment is approved for only 24 months of use due to an increased incidence of osteosarcoma and a dose-related increase in osteoblastoma and osteoma in female and male Fischer rats (https://www.accessdata.fda.gov/drugsatfda_docs/label/2008/021318s015lbl.pdf). While this

limitation may not be relevant in long bone fracture since patients should be well-within healthy bone remodeling, patients at risk of osteoporotic fractures may require long-term therapy. References for Table 1.3 and 1.4: [30-32; 61; 69-87]

Gene Therapy:

The transformation of cells, as observed by differing phenotypes, dates back to at least 1928, and was first observed and characterized in bacteria by Frederick Griffith [88]. Today, the term gene therapy encompasses a variety of techniques that utilize viruses, plasmids, and gene activated matrices to deliver therapeutic cDNA into host cells. This review will focus on gene therapy utilizing viruses only. Targets of gene therapy may be somatic or germ cells, and the distinction is important as the FDA currently allows gene therapy on somatic cells only. In challenging bone-healing environments it would be beneficial to deliver therapeutic osteo-anabolic genes over several weeks as opposed to only a few days.

The purpose of viral gene therapy is to either (1) replace defective native gene sequence, or to (2) provide an extra gene copy and drive over expression. Although different vectors and their associated therapies are designed with specific therapeutic targets in mind, transduction should result in transgene expression at therapeutic quantities [89] and be highly specific to the cellular target [90]. Viral gene therapy utilizes the efficiency of viruses to gain entrance into cells and to have quick production of protein from the genetically modified cell. When compared to transfection by plasmid or another cDNA containing element, the efficiency of viral vectors to transduce target cells is superior [91]; further, vectors with different serotypes have been shown to selectively transduce a number of cell types, including mesenchymal stem cells, chondrocytes,

and synoviocytes [92]. Gene therapy to induce bone formation has been delivered *in vivo* directly to a defect as a suspension [93; 94], *in vivo* lyophilized to an allograft implant scaffold [95], or through an *ex vivo* approach, where cell-type transduction is controlled *in vitro*, and then applied to a defect some time later in a dual surgery process (traditional) [89; 96], or tissue may be selectively isolated, transduced, and re-implanted in a single surgery (expedited) [91; 97; 98]. Figure 1.2 shows how a traditional, allogeneic *ex vivo* approach might be utilized to study fracture repair *in situ.*

Therapeutic genes used in gene therapy were initially successful in their use as recombinant proteins, and were then explored with gene therapy because it was hypothesized they may better effect clinical success with an alternate delivery [99]. At the site of large bone defects, recombinant proteins require specific temporal and spatial delivery mechanisms to decrease diffusion of the protein from the site of interest. While genetically modified cells will produce protein that also diffuses, the continuous and persistent production that is achieved for the lifetime of the genetically modified cell eliminates the need to deliver one-time supraphysiologic doses as occurs with recombinant protein therapy. Further, transduction and gene production by host cell machinery is more likely to undergo genuine post-translational modification, and may have greater biological activity compared to their recombinant counterparts [39; 100].

Despite removal of virulence factors from therapeutic vectors, there is concern viruses may revert to pathogenicity if they transduce a cell that has previously been, or becomes co-infected with another virus and that allows pathogenic replication within the patient. Some vectors, especially commonly used adenoviruses are pro-inflammatory even after removal of virulence factors, a trait attributed to the production of non-therapeutic, non-pathogenic viral genes [101; 102].

The following viruses have been the most commonly used in segmental bone defects as delivery vectors. Table 1.5 provides seminal gene therapy references utilizing BMP-2 and PTH (1-34) as therapeutics for segmental bone defects.

Adeno vectors: The non-enveloped adenovirus is a double stranded DNA (dsDNA) virus. Several of the early transcript genes of adenovirus are required for adeno-associated vectors to replicate, although the two are unrelated. Adenoviruses are relatively ubiquitous and do not cause any known disease in humans, making them incredibly useful during the early experiments of gene therapy. However, adenoviruses do elicit a large immune response, leading to immune destruction of transduced target cells [103; 104]. Newer vector constructs are often utilized.

Adeno-associated vectors: The adeno-associated virus (AAV) is a small, single-stranded DNA parvovirus that elicits minimal immunogenic reaction. The many serotypes available add to its allure as a therapeutic vector since targeted tissue tropism is conferred [90]. Serotype 2 is the most commonly utilized serotype in musculoskeletal tissues and is used with serum free media for maximal transduction. Several genes including BMP-2 [105], BMP-4 [106], and BMP-7 [107] are used in AAV vectors to induce osteogenesis, although BMP-2 is by far the most widely used due to its ability to induce *de novo* osteogenesis *in vitro* [108] and *in vivo* [109]. However, it has been observed that AAV vectors might not produce enough protein to heal large segmental defects. As previously mentioned, AAV vectors utilize genes from Adenovirus (termed "helper genes") to ensure viral replication; although, high titers of recombinant adeno-associated viral vectors have been produced in the absence of adenovirus helper genes [110].

Self-complementary adeno-associated vectors: Self-complementary adeno-associated virus (scAAV) is an AAV vector that has been engineered to contain coding and non-coding strands of DNA. Therefore, scAAV does not require DNA polymerase to produce a complementary DNA strand before mRNA is produced. This ultimately results in more efficient protein expression [111]. scAAV vectors have been used *in vivo* to produce the interleukin-1 receptor antagonist (IL-1ra) protein transgene in normal joints [112]; and it has been shown that repeat dosing can be achieved without immunogenic reaction when the serotype of the repeat dose is modified [112]. In bone healing, scAAV vectors have been used to deliver various DNA molecules that showed improved bone integration histologically. Yazici et al used scAAV2.5-BMP-2 coated allografts to increase incorporation when compared to autograft [95]. Further, Yazici showed that scAAV-BMP-2 treated femurs had increased torsional rigidity when compared to the control femur in post mortem analysis [95]. In contrast to integrated vectors, scAAV vectors remain episomal within the nucleus and the transgene is not replicated with subsequent cell divisions. Although this may seem like a disadvantage, scAAV transduced cells increased IL-1ra transgene expression in the equine model 183 days after *in vivo* injection [112]. Therefore long-term expression results from scAAV gene therapy even though it does not integrate into the cells genome. Figure 1.2 shows how scAAV may be used clinically to augment bone healing.

Lentiviral vectors: Lentiviral vectors are RNA viruses that transduce dividing and non-dividing cells, and each virion inserts two transgene copies into host chromosomes. Host chromosome integration leads to prolonged transgene expression, however insertion is currently not controlled and may lead to insertional mutagenesis. Insertional mutagenesis occurs when the viral transgene

integrates near potential proto-oncogenes, altering the nuclear regulation of transcripts and ultimately resulting in unwanted neoplasia. This random insertion into the genome affects the safety profile of this vector [91] and could limit its efficacy; however non-integrating lentiviral vectors have been produced [113; 114] and studies are being performed using antiviral pro-drugs that are metabolized to toxic compounds within transduced cells only [115]. Such advancements in vector technology show an effort to provide a clinical approach to eliminating therapeutic cells after the transgene is no longer needed.

Retroviral vectors: Retroviral vectors are RNA viruses that preferentially transduce and integrate into the genome of actively dividing cells. While bone is in a constant state of turnover, it is not dividing at a rate that allows it to be a suitable target of retroviral vectors delivered *in vivo*. However, similar to lentiviral vectors, transgene expression may also be mutagenic [116; 117].

References for Table 1.5: [89; 93-98; 118-123]

Progenitor/Stem Cells

The term "stem cell" exists in scientific literature dating back to 1868. Stem cells, or perhaps more correctly, tissue progenitor cells, utilized in modern research are derived from a variety of tissues (e.g. are digested away from lipo-aspirate, muscle, blood vessels, and other organs [124; 125]) and are selected for by adherence to culture plates. To properly denote plastic adherent tissue-based cells isolated from any source as true mesenchymal stem cells, characterization would include expression of CD105, CD73, and CD90 [126]. Additionally, cells would lack expression of the following markers: CD45, CD34, CD14/11b, CD79-alpha/CD19, and Human Leukocyte Antigen-DR [126], must self-renew, re-populate, and undergo tri-lineage differentiation *in vitro* [127].

Ease of isolation, replication potential (telomere length, pluripotent markers), planned therapeutic use, and other clinical indications often dictate the site of progenitor cell harvest, though there is evidence to suggest differences in cell differentiation between progenitor cells from different sources [128-130]. While each source can undergo tri-lineage differentiation into adipocytes, chondroblasts, and osteoblasts, there is evidence of lineage biases of progenitor cells isolated from bone marrow when osteoinduction is the goal [131] and it is likely epigenetic gene regulation confers some sort of tissue-source memory, as some groups have successfully changed the osteogenic differentiation capacity of adipose derived progenitor cells to rival bone marrow derived progenitors through use of histone deacetylase inhibitors [132].

Patients presenting with several risk factors for non-union formation or those presenting for a second surgery to repair a failed fracture consolidation are considered to have clinical indications for progenitor cell therapies [133]; however, patients cannot receive culture-expanded BMDMSCs unless they are part of a clinical trial. In lieu—clinicians are utilizing patient-side progenitor cell therapies, such as bone marrow aspirate concentrate and stromal vascular fraction—increased concentrations of cytokines and a small population of stem cells characterize both treatments [134]. While these patient-side treatments are minimally manipulated and autogenic, they have the potential to be heavily influenced by patient co-morbidities.

It is currently unknown how many progenitor cells are needed to affect segmental bone healing, from what source progenitor cells should come from, at what concentration, and in what vehicle should cells be delivered in. It is known those progenitors cells alone and without a carrier are not sufficient to alter segmental defects, and alone are not a suitable intervention. Studies have

found correlations between number of osteoprogenitor cells, concentration of fibroblast colony forming units, and final fracture consolidation [135-137] although there remains no consensus on how many cells are needed to fill a defect. In general, it is considered that mineralized callus formation is correlated to the number of progenitor cells within the bone marrow aspirate, especially when utilized in the absence of concentration [138]. Therefore, it is reasonable to postulate that culture-expanded cells may be especially helpful in instances of healing segmental bone defects when higher concentrations of cellular therapies have affected clinical success [139; 140].

Bone marrow aspirate concentrate (BMAC) is one minimally manipulated therapy, and considered an alternative to autologous bone graft (ABG). ABG has many well-documented co-morbidities and an upper limit of available graft material [141]. BMAC contains several cell type precursors, including platelet alpha-granules that contain numerous growth factors, and a small population of mesenchymal stem cells [142]. Recently, one group found that nucleated cell counts of BMAC samples were not predictive of colony forming units, suggesting the healing property of BMAC is correlated to growth factors contained within platelet granules such VEGF and IL-1ra [134]. In an equine model it was demonstrated that the addition of culture-expanded BMDMSCs and autologous PRP resulted in bone formation in chondral defects [143]. The role of nucleated cells, expanded BMDMSCs, and platelets (and platelet-based patient-side therapies) remains to be elucidated but may have the potential to encourage bone healing. It is judicious to note that none of these therapies have been tested in a challenging bone healing environment.

Conclusion

Promotion of osteogenesis to heal segmental bone defects and osteoporosis in humans and animals is a complex issue. While many studies have been performed, there are a myriad of unanswered questions. Successful clinical therapies may predominantly move towards cell-based growth factor delivery systems that address the need for sustained osteoinduction, especially in large defects to augment risk factors for non-unions. The barrier to this may be regulations that are associated with FDA rules governing autologous and allogeneic cell implantation in addition to genetically modified cells. The frequencies of nonunions (5-10% of fractures annually) remain an unmet challenge with serious socioeconomic impact, and the aging population necessitates that a more critical emphasis must be put on osteoporotic fractures and disease progression. Cellular-based therapeutic approaches require further intensive investigation, as there is no clear solution. Future testing in preclinical models and additional clinical trials of bone repair will elucidate safety and efficacy of alterations in dosing and route of administration of the recombinant proteins BMP-2 and PTH 1-34, gene therapy, and progenitor cell therapies.

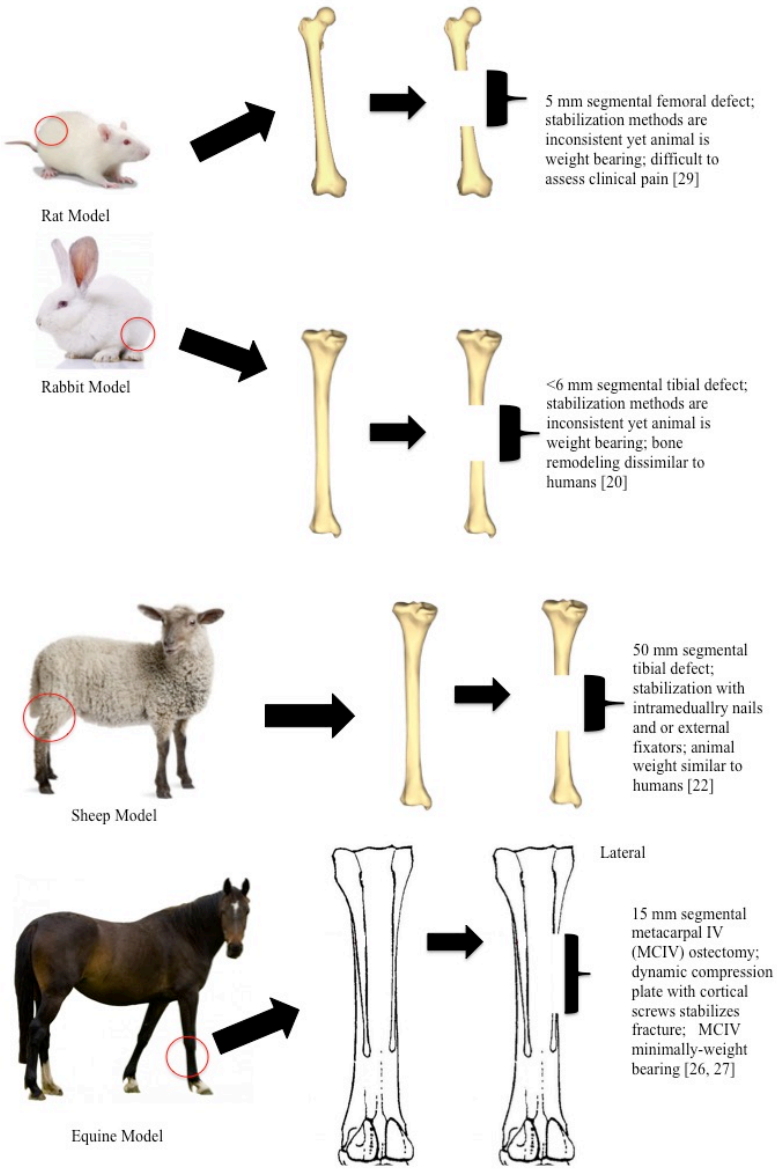

Figure 1.1 describes common animal models, the corresponding segmental defect size, and the type of fracture stabilization utilized for segmental bone research.

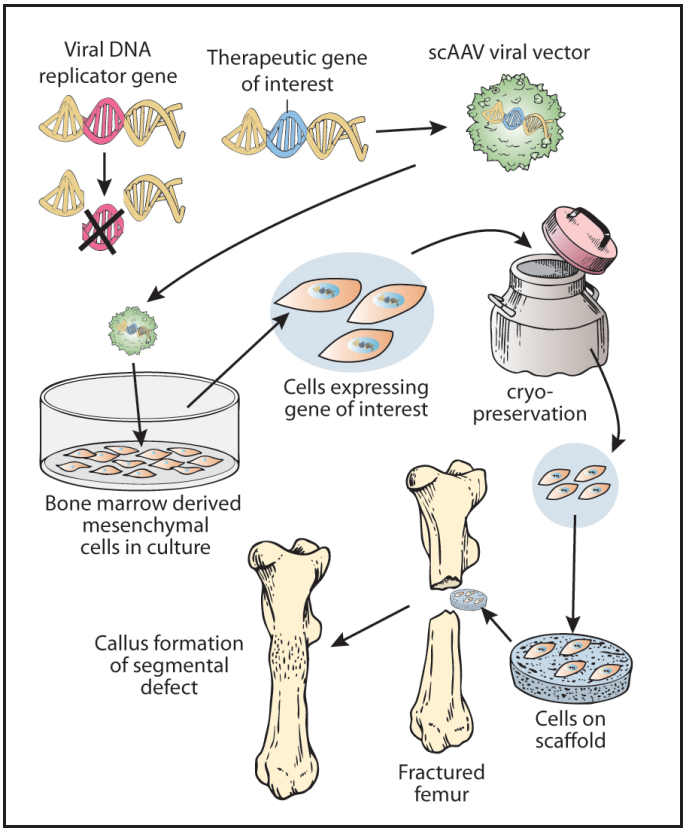

Figure 1.2 describes how an *ex vivo* technique may be utilized with cryopreservation to provide a gene therapy approach to segmental bone defects in fracture repair.

Table 1.1: Small Animal Model Advantages, Disadvantages, and Translational Relevance

Animal Model	Advantages	Disadvantages	Cost ($-$$$$)	Most common bone used	Potential Implications of Differences (when humans are considered)	References for Further Reading
Mouse	Low cost, genetic engineering of knock-out models allows for highly specific pathway and disease study; homogeneity confers statistical power; biomarker availability; Genetic engineering of knockout or epitope-tagged strains are helpful in quantifying evidence of changes in promoter specificity, response to transcription activators and inhibitors, and in heterozygous systems, cis or trans regulatory elements may also be explored.	Lack Haversian systems; skeleton is modeling driven due to permanently open growth plates at the epiphysis of long bones; low on phylogenetic scale.	$	Femur	Persistent epiphysis is a good model for juvenile pathology; light weight, difficult to monitor pain response to implants or fixators	22; 28; 29
Rat	Genetically homogenous strains are available, use of xenogenic cells and xenogenic gene sequences in gene therapy studies; homogeneity confers statistical power; biomarker availability. The femur offers a larger proportion of soft tissue coverage than the tibia, and can replicate the soft tissue trauma seen as a risk factor for non-union development in humans.	Lack Haversian systems; skeleton is modeling driven due to permanently open growth plates at the epiphysis of long bones; low on phylogenetic scale. Inconsistent use of internal fixation or use of only k-wire/intramedullary pins fails to provide axial or rotational stability.	$	Femur	Persistent epiphysis is a good model for juvenile pathology; light weight; immune response altered when using non-immuno-competent breeds	21; 22; 28; 29

Table 1.2: Medium and Large Animal Model Advantages, Disadvantages, and Translational Relevance

Animal Model	Advantages	Disadvantages	Cost ($-$$$$)	Most common bone used	Potential Implications of Differences (when humans are considered)	References for Further Reading
Rabbit	Bone density is similar to humans; bone contains Haversian systems	Size and shape differs greatly from humans; very fast remodeling compared to humans; differences in composition of the microstructure; vascular longitudinal tissue structure. External fixators are used inconsistently, the tibia is more commonly used than the femur, and bones that carry less weight are frequently used in growth factor studies (such as the ulna)	$$	Femur, Tibia	Faster remodeling may confound the expected speed of healing in the human	16; 20; 28; 29
Canine	Most similar bone density, extractable protein content (such as IGF-1) and ash weight when compared to humans.	Regarded as companion animal; trabecular bone may withstand greater compressive forces than human bone due to the increased plexiform bone structure adjacent to the periosteum	$$$	Femur	Mixed microstructure, specifically in the vicinity of the periosteum confers greater mechanical strength.	16; 21; 22; 28; 29
Sheep	Most similar body weight when compared to humans; size of bone and weight of animal replicates conditions for human implants and prostheses	High content of plexiform bone conferring greater ability to withstand compression in early life; Haversian remodeling is favored with age; prior to haversian remodeling, sheep bone is comprised mostly of a combination of woven and lamellar bone (primary structure). Ruminant digestive tract affects nutrient cycling and delivery compared to monogastrics; seasonally polyestrous cycle alters bone metabolism.	$$$	Tibia	Mixed microstructure, specifically in the vicinity of the periosteum confers greater mechanical strength; Haversian remodeling increases with age in sheep—average age and sex of sheep must be considered when extrapolating to differing populations of human patients. Human bone structure is mostly secondary osteons formed by the replacement of existing bone. It has been suggested the mechanical differences exist since primary bone structure is formed through cartilage mineralization.	22; 23
Horse	Most similar mechanical loading of the musculoskeletal system when compared to humans.	Immediately weight bearing; high cost of anesthesia, housing, and routine care. Studies conducted in small cohorts yield lower statistical power, but confer high scientific evidence; seasonally polyestrous cycle alters bone metabolism	$$$$	Metacarpal IV (MCIV)	Success in the horse likely confers success in the human; if MCIV is used, one must be careful to immediately extrapolate evidence to a persistently weight bearing bone	30

Table 1.3: Preclinical Studies Utilizing RhBMP-2 or PTH (1-34) Are Grouped According to the Animal Model Used and Described

Pre-Clinical Animal	Recombinant BMP-2	Recombinant PTH (1-34)	Selected References for Further Reading:
Mouse	*In vitro*: culture with 100 ng/mL rhBMP-2 induced osteocalcin gene and protein expression after 4 days of treatment, and increased intracellular [cAMP] in response to 1-34 PTH (400ng/mL) after 8 days of treatment. [73] *In vivo*: the addition of 5 or 20 µg rhBMP-2 to porous poly-D,L-lactide-co-glycolide implanted intra-muscularly in immunocompromised nude mice resulted in production of marrow-like tissue, and a greater area of new bone when compared to the carrier matrix alone. [69]	*In vivo*: luciferase tagged BMDMSCs were generated into ossicles and implanted in immunocompromised mice. 40µg/kg/day PTH (1-34) was given SQ for 3 weeks. Three out of four PTH (1-34) treated groups showed significant increases in total bone area within implanted ossicles. [71]	BMP-2: [69; 73] PTH (1-34): [70-72]
Rat	*In vitro*: rhBMP-2 increased cellular response to PTH (1-34) in an osteoblastic cell line (C20) and in a cell line (C26) capable of undergoing myogenic, adipogenic, and osteogenic differentiation. rhBMP-2 also increased the cellular response to PTH (1-34) in both cell lines, a characteristic of osteoblastic cells. [76] *In vivo*: 5mm segmental femoral defects showed radiographic, histologic, and mechanical evidence of union through endochondral bone formation in a dose dependent manner with 11µg rhBMP-2 in guanidine hydrochloride extracted demineralized bone matrix. [31]	*In vivo*: In a closed fracture model, 200µg/kg/day for 20 or 40 days increased ultimate load and external callus volume. 60µg/kg/day for 40 days increased ultimate load and external callus volume. [61] Treatment with 30µg/kg/day for 21 days resulted in increased torsional strength, stiffness, bone mineral content and density, and callus volume. [74] Doses as low as 10µg/dg/day for 28 days increased bone mineral content and density, ultimate load to failure of fracture callus. [32]	BMP-2: [31; 75; 76] PTH (1-34): [32; 61; 74]
Rabbit	*In vivo*: empty defects, autologous bone graft, and rhBMP-2 (0µg, 17µg, 35µg, and 70µg) loaded onto poly (DL-lactic acid) implant were loaded onto 20mm radial defects in 96 New Zealand White rabbits. After 8 weeks, autogenous bone graft, 35µg and 70µg rhBMP-2 groups made an equivalent amount of bone and had restored normal architecture. [81]	*In vivo*: PTH (1-34) increases cortical bone mass and mechanical strength in female rabbits after 140 days without adversely affecting serum Ca/P ratios. 40µg/kg/day increased ultimate force, stiffness, and work; while 10µg/kg/day had a lower elastic modulus not different from the control. [78] When treated for only 70 days with 10µg/kg/day, cortical bone porosity was not increased and cortical bone strength was increased. [79]	BMP-2: [77; 81] PTH (1-34): [78; 79]

Table 1.4: Preclinical Studies Utilizing RhBMP-2 or PTH (1-34) Are Grouped According to the Animal Model Used and Described

Canine	*In vivo*: bilateral 25mm radial osteotomies were filled with autologous bone graft or a collagen sponge with 0μg, 150μg, 600μg, or 2,400μg rhBMP-2. Dose dependent osteoinduction was observed, with higher doses resulting in heterotopic bone formation and cyst-like voids. By 12 and 24 weeks, biomechanical parameters were equal to autologous bone graft. Minimum effective dose of rhBMP-2 in collagen was determined to be between 0 and 150μg. [80]	*In* vivo: 5μg/kg/day increased titanium alloy implant fixation in the proximal tibia. Increased shear stiffness and energy absorption were observed after 4 weeks of daily SQ PTH (1-34) treatment. [83] A combination of the bisphosphonate, Zoledronic Acid and PTH (1-84) at 2.3 μg/μL and 0.1μg/μL, respectively, increased polar moments of inertia in a canine osteosarcoma cell line, xenografted into athymic rats. [82]	BMP-2: [80] PTH (1-34): [82; 83]
Caprine	*In vivo*: bilateral closed fractures were created in 16 goats, and 1 cm of periosteum was excited proximal and distal to the fracture. Absorbable collagen sponge with 0.86mg rhBMP-2 or buffer were applied to the anteriomedial aspect of the fracture, or wrapped circumferentially around it without any stability. Increased callus volume, and moderate increases in strength and stiffness were noted by torsional toughness and higher radiographic scores in the wrapped rhBMP-2 treated tibiae. [87]		BMP-2: [87] PTH (1-34): none authors are aware of
Equine	*In vivo*: defects made in the II and IV metatarsals were left empty, filled with autologous bone graft, or calcium phosphate cement/matrix with 2mg or 0.5 mg rhBMP-2, respectively. The combination of calcium phosphate and rhBMP-2 had greater maximum torque to failure in torsion scores. Histology suggested increased bone volume and more mature bone in rhBMP-2 treated sites. [85]	1mg of PTH (1-34) was covalently attached to 1.5mL volume fibrin hydrogel and implanted into a 5.5mm subchondral bone cyst that communicated with the joint in the proximal interphalangeal joint. Mild lameness was evident 9 weeks post operatively. 8 months post operatively the cyst was filled with radiopaque material, and the animal ambulated normally. [84]	BMP-2: [30; 85; 86] PTH (1-34): [84]

Table 1.5: Pre-Clinical Animal Studies Are Described and Grouped According to Method of Transgene Administration

Gene Therapy Modality	Pre-Clinical Studies	Therapeutic Gene	References for Further Reading
In vivo: suspension	In rats, femoral defects were treated with Ad-BMP-2 at the time of defect creation or 24 hours after. Mean bending and mean stiffness were increased, and bone mineral content was similar to contralateral control femora. In rabbit ulnae, segmental defects were treated 7 days after defect creation with Ad-BMP-6. At 6 and 8 weeks, treated ulnae had increased bone area, mineral content, and were stronger in torsional stiffness than control ulnae. In the equine, Ad-BMP-2/7 treated metacarpal IV did not heal better than untreated controls.	BMP-2, BMP-2/7, BMP-6	93; 94; 119; 120
In vivo: GAM	In mice, scAAV-BMP-2 lyophilized to allograft healed segmental femoral defects, cortical shells formed were equivalent to un-fractured femurs and had equivalent torsional rigidity. PTH (1-34) on a collagen sponge increased periosteal bone in equine cortical defects after 13 weeks, but did not heal subchondral defects exposed to the articular surface.	BMP-2, PTH (1-34)	95; 118
Ex vivo: traditional	Utilizing the traditional *ex vivo* technique, rat femoral defects treated with LV-BMP-2 had significantly higher peak torque and torque to failure biomechanics than femora treated with BMDMSCs alone. Further, a variety of cell types including muscle and human adipose can be transduced and utilized in mice and rats to produce bony union after 8 weeks.	BMP-2, BMP-4	89; 96; 122; 123
Ex vivo: expedited	Muscle, fat, and buffy coat hematologic components were transduced the same day as defect creation and implanted into critical sized bone defects. Radiographic union was displayed as early as 10 days and was earlier when compared to *ex vivo* traditional methods. Within 8 weeks, bone volume and torsion testing suggest mechanical stability comparable to control femora.	BMP-2, LMP-1	97; 98; 121

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CHAPTER 2:

Thesis Experiments[1]

Conventional clinical management of segmental bone defects in humans continues to result in 5-10% of fractures forming non-unions. This persistent gap in clinical success necessitates innovative approaches to bone repair. Active areas of research have recently been reviewed[1] and often involve the use of novel biologic therapies. In parallel, fracture repair in the horse and the plight of recovery is a major challenge because of chronic pain, the development of support limb laminitis, and association with ischemia and infection, which can result in euthanasia of the patient. Achieving successful repair of the affected limb is associated with severity of the fracture, the animal's temperament, and compliance as well as the mechanical limits of implants[2]. Further, equine fracture repair cases exhibit an increased incidence of infection ranging from 28 to 52.6% of cases due to the paucity of soft tissue coverage and often-traumatic fracture etiology[2]. Thus, there is an unmet clinical and ethical obligation of veterinarians to study novel approaches to promote rapid repair of complicated long bone fractures and large bone defects. Further, because of the challenges associated with fracture repair in the horse, and parallels between poor soft tissue coverage and blood supply to the distal limb of horses and the limbs of humans, a translational incentive exists such that success in the equine model may help to heal the 5-10% of human fractures that do not heal despite clinical intervention[1].

[1] This chapter is published in part: Ball AN, Phillips JN, McIlwraith CW, Kawcak CE, Samulski RJ, Goodrich LR. Genetic modification and cryopreservation of equine bone marrow derived progenitor cells with scAAV-BMP-2 induces osteogenesis *in vitro*. *Journal of Orthopaedic Research, Special Edition: Stem Cells* [Accepted December 12, 2018].

Mesenchymal stem cells derived from bone marrow aspirates (BMDMSCs) are often combined with growth factors to induce bone formation and accelerate healing[3]. There are notable successful studies in both human[4] and equine literature[5]. The most prominently studied growth factor in bone healing is bone morphogenic protein-2 (BMP-2), a member of the Transforming growth factor-β superfamily. When BMDMSCs and BMP-2 are combined, progenitor cells are encouraged to differentiate into osteoblasts (osteoinduction), and, exogenous progenitor cells (if provided) are a source of osteogenic cells (osteogenesis)[3]. Recombinant BMP-2 (rhBMP-2) is utilized for its osteoinductive nature in human fractures and is provided in a bovine collagen matrix to encourage native BMDMSC migration (osteoconduction) and osteoinduction[6]. However, rhBMP-2 is met with varying clinical success[7] and has well documented limitations such as ectopic bone formation, increased inflammation and edema, and a relatively short half-life[7]. In equine fracture repair cases, use of rhBMP-2 is often cost prohibitive[8].

Gene therapy is considered an alternative to recombinant protein therapy. BMDMSCs can be genetically modified to produce therapeutic proteins in quantities that affect clinical outcomes in bone[9]. Further, phase II and III clinical trials are ongoing and are testing the efficacy of gene therapy in challenging musculoskeletal environments such as osteoarthritis and rheumatoid arthritis[10]. Often the rate-limiting factor of gene therapy is vector transduction efficiency and complementary DNA second strand synthesis when vectors, such as rAAV, provide only the template cDNA[11]. Self-complementary adeno associated virus (scAAV) bypasses this step through providing both the template and coding DNA in a single transduction, allowing therapeutic protein production to begin within hours of transduction[12]. Further, scAAV has been

shown to selectively transduce target BMDMSCs [13] and produce high levels of therapeutic proteins even in BMDMSCs with low proliferation rates [9].

Our ability to overcome the limitations associated with vector serotype, transduction efficiency, and second-strand synthesis, precedes the clinical availability of a cell-based genetically modified therapeutic for fracture repair in the horse remains unmet for a number of reasons: (1) catastrophic fractures are often operated on within 24 hours of presentation and this is not enough time to harvest, expand, and transduce an autologous bone marrow sample as a source of BMDMSCs; (2) most equine patients have not undergone autologous stem cell harvesting and then had their cells culture expanded and cryopreserved; (3) and there are anecdotal reports of varying degrees of success, inflammation, and immune response to both autologous and allogeneic culture-expanded stem cell use [14-16]. Therefore, a potential alternative to improve the clinical success of equine fracture repair might be an allogeneic, "off-the-shelf", genetically modified BMDMSC biologic that has been assessed for its ability to augment bone repair.

Similar to the clinical manifestations in horses are a high rate of mal-union and non-union fractures, often estimated at 5-10% of annual fractures in humans [1]. Growth factor delivery to augment osteogenesis in this population of patients has recently been reviewed [1]. If our study and future *in vivo* studies in the horse provide "proof-of-principal", the cryopreservation of genetically modified BMDMSCs may be a future area of application in people. This is supported by the recent approval of the first gene therapy treatment for osteoarthritis, approved in Korea, and undergoing phase III clinical trials in the United States [10]. While the approved protocol is a gene delivered with a lentiviral vector, the cells are treated such that they do not undergo

replication following implantation—an effort to prevent any virus-associated insertional mutagenesis and subsequent neoplasia. The success of the commercialization of gene therapy in Korea and the clinical trial in the United States will be paramount to the field of gene therapy.

The objectives of the current study were: (1) to evaluate scAAV-BMP-2 osteogenic induction in equine BMDMSCs *in vitro*, and (2) to investigate if selective cryopreservation of scAAV-BMP-2 cells would not reduce the BMP-2 delivery capacity of the genetically modified cells following recovery *in vitro*. We hypothesized that 1) transduction with 48,000 viral particles per cell of scAAV-equine-BMP-2 would result in *in vitro* osteogenesis in equine BMDMSCs and 2) that the cryopreservation of the genetically modified cells would not impact the clinical delivery of the BMP-2 transgene.

Materials and Methods

Cell Culture and Harvesting:

Mesenchymal progenitor cells from 5 skeletally mature (between the ages of 2 and 5) horses were isolated aseptically from the sternum as previously described [17]. Cells that had undergone between 12 and 16 population doublings were used for each objective. Cells were plated in a 48 well plate at 50% confluency (37,500 cells/cm^2) and equilibrated overnight. Cells reached 80% confluency (60,000 cells/cm^2), were washed twice with PBS, and then transduced in serum free Dulbecco's Modified Eagle Media (DMEM) containing 48,000 viral particles per cell (vpc) scAAV-equine-BMP-2 or 8,000 vpc scAAV-GFP for 3 hours. Cells in non-vector control groups (rhBMP-2, osteogenic, and negative controls) were incubated in incomplete media during this time. Once the transduction was complete, serum-free media was aspirated, and complete

DMEM was added. Media was changed on day 2 and 5. Media supplementation was as follows (Table1):

BMP-2 Protein Expression:

BMP-2 protein expression was evaluated on days 7 (pre and post cryopreservation) and 14 (only pre-cryopreservation) using R&D Systems Human BMP-2 DuoSet ELISA (DY355). The tested media supernatant was collected from 2 wells of the 48-well plate after 48 hours of incubation. All samples were tested in triplicate.

Morphology:

Morphology was graded on days 7 (pre and post cryopreservation) and 14 (only pre-cryopreservation) using the descriptive table provided. Scoring was adapted from Zachos et al [18]. The grading scores were statistically treated as numerical variables according to Table 2.2. Morphology scoring was performed by two independent reviewers.

Staining:

Cells were stained for alkaline phosphatase and extracellular matrix calcium deposition with alizarin red on day 7 pre and post cryopreservation. Staining quantification was not performed in this study and did not undergo statistical analysis.

Alkaline Phosphatase Expression:

Alkaline phosphatase (ALP) lysate expression was evaluated on days 7 (pre and post cryopreservation) using Sensolyte® pNPP Alkaline Phosphatase Assay Kit (Catalog#AS-72146). Since alkaline phosphatase expression was only performed on the two horses undergoing objective 2 testing (cryopreservation), statistical analysis was not performed due to low sample size (n=2). All samples were tested in triplicate.

Statistics:

The experiment was designed as a split plot, and was analyzed as a mixed model. In objective 1, the horse (n=5) was utilized as the blocking variable (fixed), day (n=2) as the whole plot factor (fixed), and treatment (n=5) as the sub-plot factor (random). In objective 2, the horse (n=2) was utilized as the blocking variable (fixed), cryopreservation ("yes" or "no") as the whole plot factor (fixed), and treatment (n=5) as the sub-plot factor (random). The cells of the 2 horses utilized in objective 2 were also used in objective 1, and those data points (BMP-2 protein expression, alkaline phosphatase lysate expression, and morphology scoring) from objective 1 were compared to scores obtained during objective 2. BMP-2 protein expression values were log transformed based on variance, and the morphology scoring variables were analyzed without transformation. Alkaline Phosphatase lysate data did not undergo statistical analysis. The statistical program R was utilized to analyze the data with a significance value of $p < 0.05$.

Objective 2 Progenitor Cell Selection based on Horse:

Data from Objective 1 was analyzed and the progenitor cells from two horses (n=2) producing the greatest amount of BMP-2 protein expression and were chosen to undergo the cryopreservation studies. This was done to emulate a clinical allogeneic treatment where a screening process for osteogenic donor cells might be performed. All assays were performed as described in Objective 1. Cells were transduced as described in objective 1 and then cryopreserved 48 hours later. Cells were removed from their 48-well plate with Accumax, treatment groups were pooled, and frozen at -80°C for 24 hours, and then transferred to liquid nitrogen for at least 48 hours. Cells were cryopreserved in 95% BSA and 5% DMSO at a concentration of $5x10^6$cells/mL. To recover, small volumes (20-150 uL) of expansion media were added until 1mL total volume was reached. Cell viability (Table 2.3) was assessed using a

Trypan Blue assay and cells were counted using a hemocytometer. Cells were then placed in a T25 flask overnight in expansion media, and plated in 48 well plates at 80% confluency and allowed to equilibrate for 12 more hours. Cells were then incubated according to their respective treatment groups. Addition of the appropriate media constituted day 2.

Results

Objective 1

Protein Expression: ScAAV-equine-BMP-2 cells produced significantly more protein than any other group ($p<0.0001$) (Figure 1.1). Cells treated with rhBMP-2 treated cells (n=5) produced similar amounts of BMP-2 protein as scAAV-equine-BMP-2 genetically modified cells, as would be expected because they were incubated in constant concentrations (100 ng/mL d0-2, 50 ng/mL d2-7).

Morphology and Staining: ScAAV-equine-BMP-2, rhBMP-2, and osteogenic control cells became cuboidal and nodular arrangements of cells were observed (Figure 2.4). Morphology scoring (adapted from Zachos et al [18]) was significantly different between scAAV-equine-BMP-2, rhBMP-2, osteogenic controls and scAAV-GFP and negative controls (Figure 2.3). Alizarin red staining was done qualitatively and differences were apparent (Figure 1.2).

Objective 2

Protein expression: Seven days after transduction, and 5 days following recovery from cryopreservation, media supernatant was collected and analyzed for BMP-2 protein content (Figure 2.4). ScAAV-equine-BMP-2 cells produced significantly more protein than any other group ($p<0.0001$). Following cryopreservation, scAAV-equine-BMP-2 cells produced significantly less BMP-2 protein when compared to transduced cells that were not cryopreserved

(Table 2.4) likely due to the population doubling and loss of transgene expression in daughter cells; therefore, an expected population doubling and loss of episomal transgene expression in daughter cells could account for this decrease. Cryopreserved rhBMP-2 treated cells produced similar amounts of BMP-2 protein when compared to non-cryopreserved cells, as would be expected because they were incubated in constant concentrations regardless of population expansion; therefore, mitotic daughter cells were exposed to the same concentration of rhBMP-2.

Morphology and Staining: Seven days after transduction, scAAV-equine-BMP-2, rhBMP-2, and osteogenic control cells remained cuboidal and formed nodular congregations. This remained consistent following cryopreservation (Figure 2.5). Morphology scoring [18] remained consistent following cryopreservation. (Figure 2.6).

Alkaline Phosphatase Lysate:

Seven days after transduction, scAAV-equine-BMP-2, rhBMP-2 and osteogenic control cells produced more alkaline phosphatase on average than scAAV-GFP and negative control cells (Figure 2.7).

Discussion

Equine BMDMSCs are well known to undergo induced differentiation into adipocytes, chondroblasts, and osteoblasts cells *in vitro*[17]. The objective of the present study was to determine the osteogenic capacity of scAAV-equine-BMP-2 and to compare osteoblastic characteristics between other clinical treatment modalities (e.g. rhBMP-2) and *in vitro* standards (osteogenic media).

Viral gene therapy, the transfer of cDNA to a host cell, traditionally uses one of 4 well-studied techniques to augment bone healing. (1) *Ex vivo* traditional techniques harvest, expand, and transduce autologous or allogeneic cells in culture prior to placing them in a defect[19; 20]. Controversies of this technique include debates about the immune-modulating properties of allogeneic vs. autologous cells in the horse [21; 22], and the time consuming nature of autologous expansion for timely fracture repair. To bypass the need for an allogeneic donor and to bypass the time consuming nature of cell expansion, a modified (2) *ex vivo* expedited technique was developed in which cells are harvested and transduced using a single "same-day" technique [23]. However, *ex vivo* expedited techniques provide fewer cells, and in cases where bone marrow aspirate concentrate (BMAC) would be used, a very small population of BMDMSCs exists [24]. Further, nucleated cell counts of BMAC have not been predictive of colony forming unit cells [25], and so the true efficacy of BMAC may lie in the growth factors provided. Therefore, in challenging bone-healing environments where delivery of cells may modulate biomechanical properties of the bone earlier in fracture healing, an expanded population would be advantageous. (3) *In vivo* suspension techniques transfer a virus capable of transducing the native cell population, either directly [26], percutaneously at the completion of surgery, or within 2 weeks of surgery [27; 28]. (4) Finally, some groups have utilized viral vectors lyophilized to allografts to selectively transduce cells that infiltrate the fracture callus and improve the biomechanical properties of allografts and their potential to undergo remodeling[9].

All concerns considered, important clinical needs of equine clinical fracture cases are (1) that horses are operated on within 24 hours of presentation; (2) horses would benefit from earlier biomechanical stability of the fracture environment aimed to decrease secondary

complications[29], and (3) a biologic, gene therapeutic approach may provide strong osteoinductive and osteogenic elements to equine fracture repair. While the controversy of autologous and allogeneic cell sources remains [21; 22; 30], clinical equine practice in the case of fracture repair is most amenable to allogeneic cell use. Further recent studies by Colbath et al as well as others suggest a high safety profile of allogeneic MSCs [22; 31; 32]. Sources of mesenchymal progenitors (e.g. adipose vs. bone marrow) also show signs of epigenetic memory that can be altered to improve osteogenesis in adipose derived mesenchymal progenitors with the use of histone deacetylase inhibitors[33]. This evidence, although indirect, is supportive that certain cell lines from specific donors may be epigenetically superior and responsive to osteogenic, chondrogenic, or tenogenic signals. Moreover, equine clinicians appreciate variance between patient's reactions to stem cell treatments (e.g. presence of inflammatory flare, successful regeneration of target tissues); further, in human medicine with recombinant protein use, it is often that osteogenic responses are not as uniform *in situ*[7; 34; 35].Therefore, we hypothesized that allogeneic cells could be pre-screened for osteogenic capability and BMP-2 delivery, and that these cells could be genetically modified and subsequently cryopreserved as an "off-the-shelf" biologic available for a wide variety of clients.

We first observed that scAAV-equine-BMP-2 transduced cells produced significantly more BMP-2 protein than any other treatment group (Figure 1.1). This is in agreement with several other groups utilizing a traditional *ex vivo* transduction technique [36; 37]. Importantly, the one-time scAAV-equine-BMP-2 transduction produced significantly more protein than what is clinically available today without repeat administration of rhBMP-2. Further, collagen-sponge retention *in vivo* ranges from 10-75%, and is dependent on multiple factors such as pH, carrier type, and

carrier weight [38]. While this study did not incorporate the use of a carrier, protein elution from genetically modified cells loaded onto a carrier would be expected to remain the same, and protein delivery would then be dependent on cell delivery, and not dictated by hydrolysis and elution characteristics of various scaffolds. It is true that long-term implants are available, allowing for slow-release of rhBMP-2 and other growth factors *in vivo* for several weeks [39]. However, steady state levels remain difficult to achieve, immune modulation to growth factor efficacy on slow-release scaffolds has not been quantified, and is likely patient dependent. In our study, the every other day media changes provided a constant concentration of available growth factors (e.g. rhBMP-2, ascorbic acid, βGP, and dexamethasone) that were likely higher than what would be available *in vivo*, yet transduced cells still produced more BMP-2 protein. Further, continued high levels of transgene expression at day 14 *in vitro* is promising when considering BMP-2 delivery *in vivo*, and other studies utilizing similar scAAV vectors, have provided therapeutic transgene expression levels up to 183 days after cell transduction in normal equine joints [40], while other studies have reported transgene expression ranging from 2 weeks in fibroblasts [41], and up to 2.35 years in the trabecular meshwork of the anterior segment of cynomolgus monkeys [40-42].

A common osteoblastic marker and important enzyme for callus mineralization is alkaline phosphatase. Staining for alkaline phosphatase in BMP-2 treated groups (scAAV-equine-BMP-2 and rhBMP-2 groups) show a clear predilection for this osteoblastic marker (Figure 1.2). Alkaline phosphatase exists in a membrane bound form on osteoblasts, and is secreted into mineralized matrices. [43] As a functional marker of osteoblastogenesis, membrane-bound bone-specific alkaline phosphatase was quantified (Figure 2.7). When using equine mesenchymal

cells, dexamethasone has been a key component of inducing osteoblastogenesis in our lab and others [17; 44], while human mesenchymal cells [45] and common culture cell lines have produced ALP in response to BMP-2 treatment [46]. Although statistical analysis was not performed on ALP lysate samples due to small sample size, scAAV-equine-BMP-2, rhBMP-2, and osteogenic control cells performed similarly. Cryopreserved cells that were thawed and retested followed similar patterns to BMP-2 protein expression, with cryopreserved cells producing less overall protein. Taking into consideration overall BMP-2 protein expression in cryopreserved cells (Figure 2.4), morphology scoring (Figure 2.5 and 4b), and ALP lysate production (Figure 2.7), cryopreserved genetically modified mesenchymal stem cells retain functional characteristics of osteoblasts. Further *in vivo* studies will need to be conducted to elucidate if these patterns confer clinical significance in the horse.

While there are techniques employed to expedite autologous cells and gene therapy [23; 47], there have been no attempts the authors are aware of to screen and cryopreserve allogeneic genetically modified BMDMSC treatments. The authors of this study provide the first evidentiary support that the cryopreservation of scAAV-equine-BMP-2 cells does not affect their osteoinductive potential. Figure 2.4 illustrates that despite cryopreservation, transgene protein expression is preserved. While there was some decrease in transgene expression, the authors hypothesize this is due to cell-expansion during recovery. All scAAV transgenes remain episomal within genetically modified cells, and are not passed to daughter cells during mitosis. This is in contrast to the utilization of lentiviral vectors, where investigators are exploring ways use systemic anti-viral treatments to program apoptosis in genetically modified cells after transgene use is no longer needed [48]. Total eradication of therapeutic cells (as evaluated by luciferase marker genes)

has yet to be achieved in a pre-clinical animal model. Genetically modified cells in phase II and phase III clinical trials within the US are exposed to radiation preventing them from replicating after genetic modification to avoid concerns of insertional mutagenesis [49] and are only being performed for rheumatoid or post-traumatic osteoarthritis management.

In this experiment, cells were recovered overnight in flasks and then equilibrated in a 48-well plate before experimental days were counted again (approximately 24 hours). Based on average BMDMSC doubling time (1-2 days)[50], the cell population would have expanded during this time. Further evidence of this was shown in our scAAV-GFP transduced cells, in which a decreased number of fluorescent cells were observed following recovery (data not shown). Due to the heavy staining (Figure 2.5), lack of morphological differences (Figure 2.6), and retention of osteoinductive levels of protein expression (Figure 2.4), it is reasonable to hypothesize that the residual BMP-2 production from the original transduced population could still be providing the necessary cues to induce osteoblastogenesis in this new population, and would likely do so in the bony microenvironment *in vivo*.

Conclusion:

This is the first published study that demonstrates the utility of screening allogeneic cells for donor responsiveness to gene therapy aimed at inducing osteogenesis *in vitro*. We have provided evidence that an *ex vivo* transduction technique, employed with a screening process, produces detectable levels of BMP-2. This is the first evidence of an "off-the-shelf" gene therapeutic approach for fracture repair in orthopedics, translational to human and veterinary medicine. Further work remains to be done to establish the therapeutic efficacy of BMP-2 genetically

modified allogeneic cells in clinical cases of fracture repair, and use of an equine pre-clinical model, translational to humans, should be performed to establish therapeutic efficacy *in vivo*.

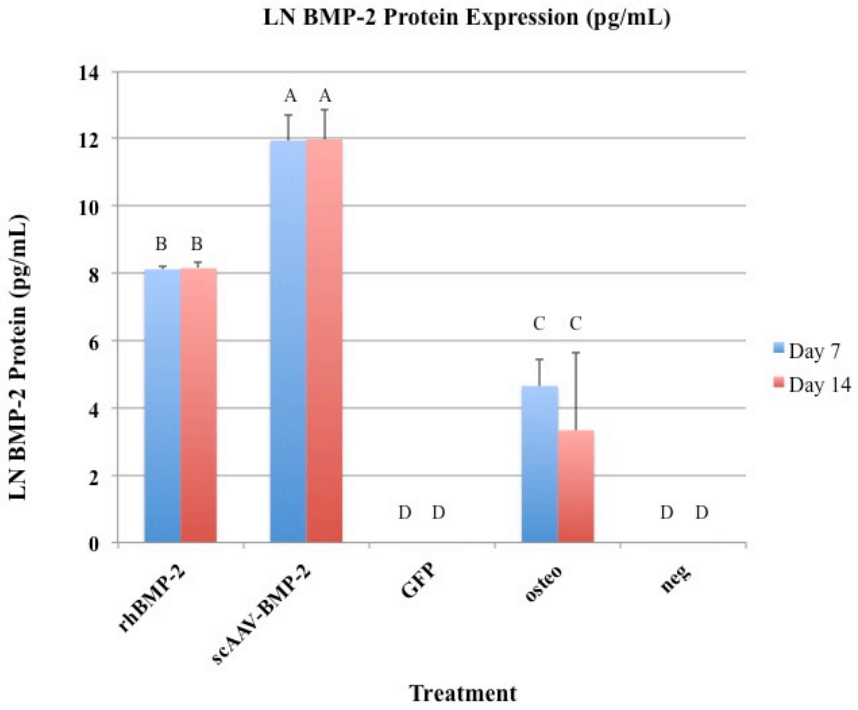
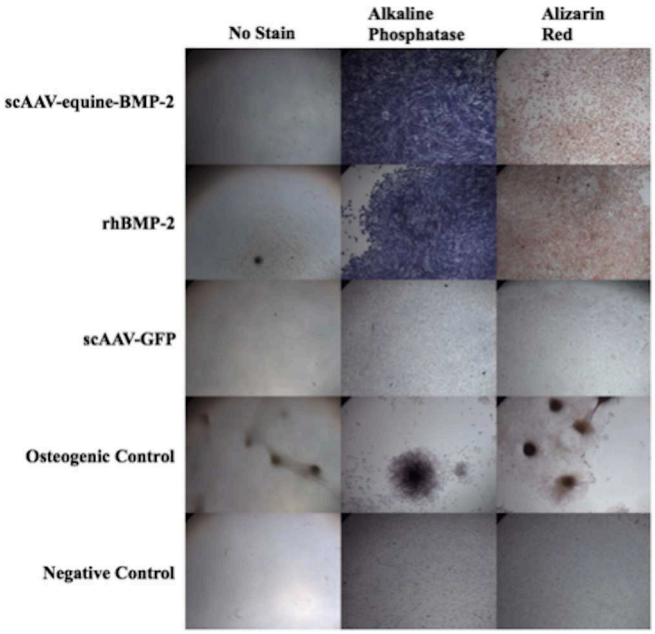


Figure 2.1: BMP-2 protein expression (pg/mL): Error bars denote standard deviation from the mean. Letter changes denote significant differences between groups ($p < 0.0001$). ScAAV-equine-BMP-2 transduced cells produced significantly more BMP-2 than any other group. Transgene expression persisted without significant changes 14 days.



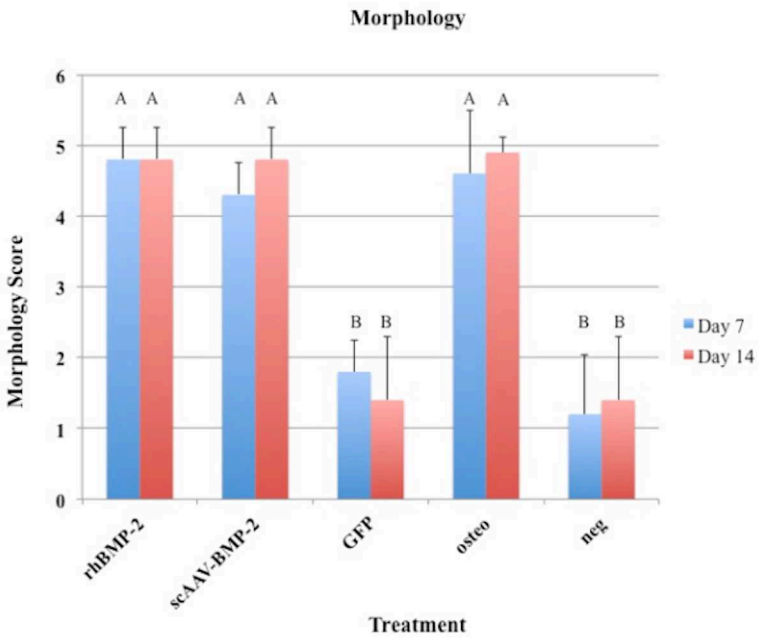


Figure 2.2 and 2.3: 2.2: MSCs in culture treated with scAAV-equine-BMP-2, rhBMP-2, and osteogenic media exemplify osteogenic morphologic changes at day 7 following transduction. 2.3: Morphology Scores: Error bars denote standard deviation from the mean. Letter changes denote significant differences between groups. ScAAV-equine-BMP-2 transduced cells, rhBMP-2 treated cells, and osteogenic control cells appeared significantly more osteogenic than GFP transduced cells and negative controls ($p<0.0001$). The morphological changes were evident by day 7.

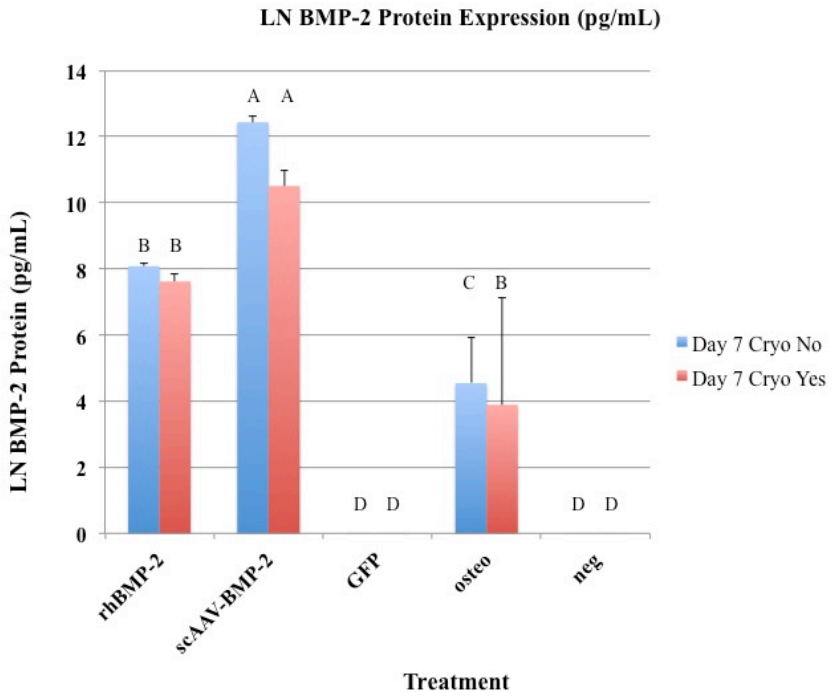
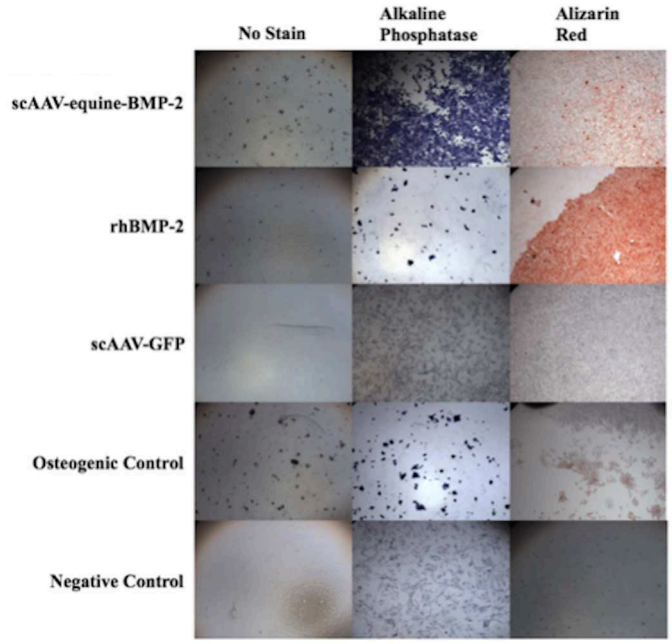


Figure 2.4: BMP-2 protein expression (pg/mL): Error bars denote standard deviation from the mean. Letter changes denote significant differences between groups ($p < 0.0001$). ScAAV-equine-BMP-2 transduced cells produced significantly more BMP-2 than any other group. Transgene expression persisted after cryopreservation.



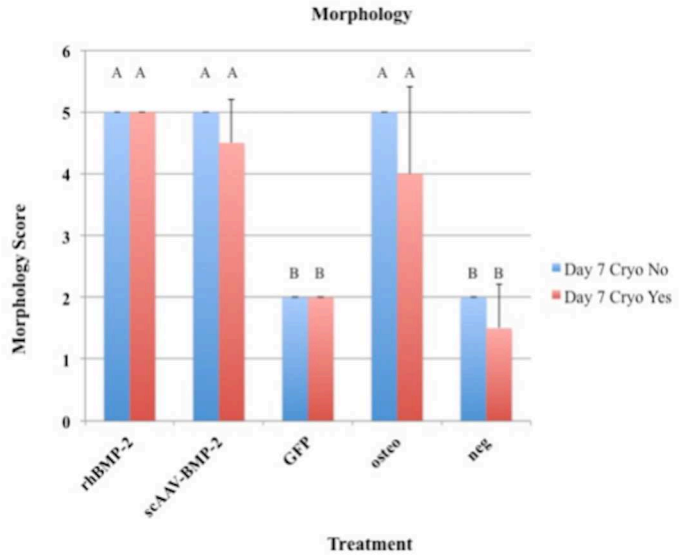


Figure 2.5 and 2.6: 2.5: MSCs in culture treated with scAAV-equine-BMP-2, rhBMP-2, and osteogenic media exemplify osteogenic morphologic changes at day 7 following transduction, cryopreservation, and recovery. 2.6: Morphology Scores: Error bars denote standard deviation from the mean. Letter changes denote significant differences between groups ($p < 0.0001$). This article is protected by copyright. All rights reserved Cryopreservation did not affect how scAAV-equine-BMP-2 transduced cells, rhBMP-2 treated cells, and osteogenic control cells appeared morphologically when compared to scAAV-GFP and negative controls ($p<0.0001$). The morphological changes were evident by day 7 following transduction, cryopreservation, and recovery.

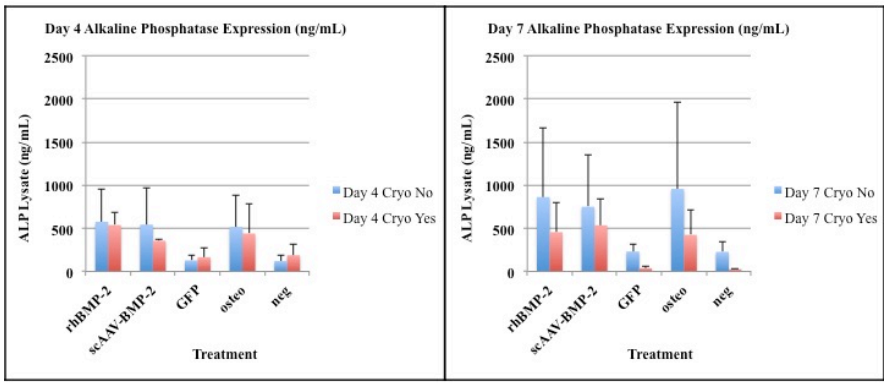


Figure 2.7: Side by side comparison of day 4 and day 7 alkaline phosphatase expression (ng/mL). Expression patterns between cryopreserved and non-cryopreserved cells follow a similar trend when compared to BMP-2 protein expression (Figure 2.4).

Table 2.1

Group	Media Supplementation
rhBMP-2 (100ng/mL for 2 days, 50ng/mL following)	Complete DMEM with 170μM ascorbic acid, 2mM β-glycerol phosphate (βGP)
scAAV-equine-BMP-2	Complete DMEM with 170μM ascorbic acid, 2mM β-glycerol phosphate (βGP)
scAAV-GFP	Complete DMEM with 170μM ascorbic acid, 2mM β-glycerol phosphate (βGP)
Osteogenic Control	Complete DMEM with 170μM ascorbic acid, 2mM β-glycerol phosphate (βGP), $1x10^{-9}$ M dexamethasone
Negative Control	Complete DMEM with 170μM ascorbic acid, 2mM β-glycerol phosphate (βGP)
Expansion Media	Complete αMEM with 4ng/mL recombinant FGF-2

Table 2.2

Score	Description
0	Dead, completely detached cells
1	Rounded, detaching cells
2	Progenitor cell with normal, fibroblastic appearance
3	Cells congregating and/or cuboidal; ≤25% of culture is osteogenic
4	Cells congregating and/or cuboidal; 26-75% of culture is osteogenic
5	Cells congregating and/or cuboidal; ≥75% culture is osteogenic

Table 2.3

Treatment Group	Viability
rhBMP-2	96%
scAAV-equine-BMP-2	97%
scAAV-GFP	99%
Osteogenic Control	99%
Negative Control	99%

Table 2.4

Treatment	Day 7 Obj. 1 (non-cryopreserved) (pg/mL BMP-2)	Day 7 Obj. 2 (cryopreserved) (pg/mL BMP-2)
rhBMP-2	3,230.57	2,038.55
scAAV-eBMP-2	251,495.85	36,620.825
scAAV-GFP	Below limit of detection	Below limit of detection
Osteogenic Control	94.1	48.837
Negative Control	Below limit of detection	Below limit of detection

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SUMMARY AND CONCLUSIONS

In summary, this compilation of experiments critically evaluated the *in vitro* capability of scAAV-equine-BMP-2 to induce osteoblastogenesis in transduced equine BMDMSCs. Further, the effect of cryopreservation on genetically modified BMDMSCs was characterized for the first time in our lab; thus, the objectives of this work were met.

The study revealed that cryopreservation and cell expansion does not affect the morphology or stain uptake for alkaline phosphatase, nor for calcium deposition within the extracellular matrix. Protein expression, as evaluated by a BMP-2 ELISA, is affected by cryopreservation. This decrease in protein expression is likely due to cellular expansion, as scAAV vectors remain episomal following transduction, and is less likely a direct effect of cryopreservation. In the small sample size tested, the decrease in protein expression was not statistically significant.

The next logical step in characterization of the vector is to perform a pilot study comparing transduced BMDMSCs that have been cryopreserved with transduced cells that have not been cryopreserved, and BMDMSCs alone. Other appropriate controls (e.g. empty defects, scAAV-GFP transduced cells, rhBMP-2) should be included. This could be performed in CSU's MCIV segmental bone defect model. Finally, with the horse being an established translational research animal for humans, and the need for innovative approaches to non-union fractures and segmental bone defects in the human medical field, the exploration of cryopreserved scAAV-BMP-2 cells might be considered might be characterized in human BMDMSCs.