IMMUNOGENIC AND IMMUNOMODULATORY PROPERTIES OF INDUCED PLURIPOTENT STEM CELLS AND BONE MARROW-DERIVED MESENCHYMAL STEM CELLS

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IMMUNOGENIC AND IMMUNOMODULATORY PROPERTIES OF INDUCED PLURIPOTENT STEM CELLS AND BONE MARROW-DERIVED MESENCHYMAL STEM CELLS

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Advancement of stem cell therapy is dependent upon the practicality, safety, and efficacy of the cells being evaluated for clinical application. Over the past decade, the need for banked stem cells which are readily available for use at the time of a patient's diagnosis has become apparent. The overall goal of this dissertation research was to compare induced pluripotent stem cells (iPSCs) to bone marrow-derived mesenchymal stem cells (MSCs), first in terms of their ability to be generated from genetically diverse individuals, and then in terms of their immunogenic and immunomodulatory properties for potential allogeneic use.

It has previously been demonstrated in mice that genetic background affects the proliferation and differentiation rates of MSCs. The purpose of our first study was to determine if genetic background affects the efficiency of generating iPSCs from mice. Results of this study confirmed that genetic background does affect both the efficiency of generating iPSCs during the early stages of reprogramming as well as the pluripotent stability of the iPSCs during later stages of reprogramming. The results also confirmed the need to understand the immunogenic and immunomodulatory properties of these cells for potential allogeneic application given that it may not be feasible to generate iPSCs from all individuals or to wait for the time that it takes to generate iPSCs and then screen them for safety and efficacy.

The purpose of our second study, therefore, was to evaluate the in vitro immunogenic and immunomodulatory properties of murine iPSCs compared to MSCs using modified mixed leukocyte reactions. Our comparisons revealed that iPSCs generated through both lentiviral and *piggyBac* reprogramming methods had similar immunogenic properties as MSCs, and more potent immunomodulatory effects than MSCs. This information is critical when considering the use of iPSCs in the place of MSCs for both regenerative medicine and transplant medicine. Further studies must be performed, however, in order to determine if iPSCs retain their immunogenic and immunomodulatory properties upon differentiation into specific cell or tissue types.

With this knowledge, we then shifted the focus of our third study to the horse, which is a valuable model for the human immune response. The purposes of this study were to immunophenotype MSCs from horses of known MHC haplotype and to compare the immunogenicity of MSCs with differing immunophenotypes, particularly in regards to MHC class II expression, through modified mixed leukocyte reactions. Results of this study demonstrated for the first time the extreme heterogeneity that exists in MHC class II expression by equine MSCs and that MHC class II positive equine MSCs are capable of inciting an immune response in vitro. This knowledge is critical for the treatment of our equine patients as well as for studies using the horse as an animal model for human diseases. Future experiments to determine if we can modulate this MHC class II expression in culture will be of great interest prior to performing in vivo studies to examine the immune response to allogeneic equine MSCs, and ultimately to compare allogeneic equine MSCs to iPSCs both in terms of their immunogenic and immunomodulatory properties as well as their regenerative ability.

BIOGRAPHICAL SKETCH

Lauren Virginia Schnabel was born in Port Jefferson, New York on March 16, 1979 to Richard and Jane Schnabel. She graduated *Cum Laude* from Choate Rosemary Hall in 1996 and *Cum Laude* from Duke University in 2000, where she received her Bachelor's degree in Biology. Lauren was accepted into the Cornell University College of Veterinary Medicine in 2000 and received her D.V.M. degree in 2004. As a veterinarian, Dr. Schnabel began her specialization in large animal surgery through completion of an internship at Rood and Riddle Equine Hospital from 2004-2005. Following her internship, she returned to Cornell University as a postdoctoral fellow funded by a National Institutes of Health Comparative Medicine Training Grant in the laboratory of Dr. Lisa Ann Fortier where she performed research on regenerative therapies. She then went on to complete a Large Animal Surgery Residency at Cornell University College of Veterinary Medicine from 2006-2009 and achieved Diplomate status in the American College of Veterinary Surgeons in 2010. Following her residency, Dr. Schnabel began pursuing a Ph.D. degree in the laboratory of Dr. Fortier in the Graduate Field of Comparative Biomedical Sciences, Department of Clinical Sciences, funded for the first three years by a National Institutes of Health Comparative Medicine Training Grant (2009-2011) and by a Cornell University Graduate Research Assistantship (2011-2012). In 2012, she was awarded a five-year National Institutes of Health Mentored Clinical Scientist Research Career Development Award (K08). During her Ph.D. training, Dr. Schnabel was also awarded the Cornell University Biological and Biomedical Sciences Graduate Research and Teaching Fellowship, which she completed from 2011-2012 in order to further her professional development and prepare herself for an academic position consisting of research, clinical work, and teaching.

This dissertation is dedicated to my family and friends, whose love and support have given me the strength and courage to pursue my dreams.

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LIST OF ABBREVIATIONS

ADSVF	Adipose derived stromal vascular fraction
AP	Alkaline phosphatase
ANCOVA	Analysis of covariance
APC	Antigen presenting cell
APC (FACS)	Allophycocyanin
AT-MSC	Adipose-derived mesenchymal stem cell
bFGF	Basic fibroblastic growth factor (also known as FGF-2)
BM-MSC	Bone marrow-derived mesenchymal stem cell
BMC	Bone marrow concentrate
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFSE	5(6)-carboxyfluorescein diacetate N-succinimidyl ester
CIITA	Class II transactivator
c-Myc	Cellular-Myelocytomatosis
CMV	Cytomegalovirus
DMEM	Dulbecco's modified eagle's medium
ELA	Equine leukocyte antigen
ESC	Embryonic stem cell
FACS	Fluorescent activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate

FL1	Fluorescence channel 1
FL4	Fluorescence channel 4
GFP	Green fluorescent protein
GMFI	Geometric mean fluorescence intensity
^{hi}	High mean fluorescence intensity
IDO	Indoleamine 2,3-dioxygenase
IFN-ɣ	Interferon gamma
IGF-I	Insulin-like growth factor I
IgG	Immunoglobulin G
IL-10	Interleukin-10
iPSC	Induced pluripotent stem cell
Klf4	Kruppel-like factor 4
LFA-1	Lymphocyte function-associated antigen-1
LIF	Leukemia inhibitory factor
^{lo}	Low mean fluorescence intensity
MACS	Magnetic activated cell sorting
MEF	Mouse embryonic fibroblast
MHC	Major histocompatibility complex
MHC-M	Major histocompatibility-matched
MHC-MM	Major histocompatibility-misatched
MLR	Mixed leukocyte reaction
MSC	Mesenchymal stem/stromal cell
NK	Natural killer cell

Oct4	Octamer-binding protein 4
P	Passage
P (statistics)	P value
PBL	Peripheral blood leukocyte
PBS	Phosphate buffered saline
PE	Phycoerythrin
PE-Cy5	Phycoerythrin-cyanin-5
PerCP-Cy5.5	Peridinin-chlorophyll-protein complex-cyanin-5.5
PGE2	Prostaglandin E2
PRP	Platelet rich plamsa
RBC	Red blood cell
RPMI	Roswell Park Memorial Institute medium
S.D.	Standard deviation
SSEA1	Stage-specific embryonic antigen 1
Sox2	Sex determining region Y-box 2
TetO	Tetracycline operator
TGF-ß	Transforming growth factor beta
TTF	Tail tip fibroblast

LIST OF SYMBOLS

α	Alpha
β	Beta
©	Copyright
ɣ	Gamma
>	Greater than
≥	Greater than or equal to
<	Less than
≤	Less than or equal to
L	Liter
µg	Microgram
µL	Microliter
µm	Micron
mg	Milligram
mL	Milliliter
n	Number
ng	Nanogram
%	Percentage
±	Plus-minus
®	Registered
™	Trademark
x	Times

CHAPTER 1

GENERAL INTRODUCTION

Overall Goal of Dissertation Research

Advancement of stem cell therapy is dependent upon the practicality, safety, and efficacy of the cells being evaluated for clinical application. The overall goal of this dissertation research was to compare induced pluripotent stem cells (iPSCs) to bone marrow-derived mesenchymal stem cells (MSCs) first in terms of ability to be generated from genetically diverse individuals, and then in terms of immunogenic and immunomodulatory properties. The remainder of this chapter provides relevant background information, the rationale for this overall goal, and the specific objectives and hypotheses for the studies presented in chapters 2 through 4.

The laboratory mouse was ideal to address the questions of the first two studies presented (chapters 2 and 3) due to the variety of readily available inbred strains and the well-established reprogramming methods used to generate mouse iPSCs. With this knowledge, we then shifted our focus to the horse for the third study (chapter 4) in order to further immunophenotype equine MSCs for future use in immunologic and regenerative studies comparing iPSCs to MSCs. This knowledge on equine MSCs, along with very recent publications on the generation of equine iPSCs [1-3], makes the horse an ideal candidate for such studies.

The horse is superior to the mouse as a model for many reasons. The horse allows for non-invasive access to large quantities of samples such as blood, bone marrow, serum, and skin needed to generate and test iPSCs from intentionally major histocompatibility complex (MHC)-matched or mismatched animals. The amount of tissue available allows for thorough studies of biological, histological, mechanical, and functional outcome data. The horse is also a valuable model for the study of the human immune response because the horse and human genome have

high homology, and the horse and human have similar primary immunodeficiencies indicating that horses are a good choice to model the human immune response [4-8]. Most importantly, horses and humans form outbred species, unlike many laboratory animals.

Finally, proof of efficacy and safety in horses is commonly required by regulatory agencies prior to the approved use of regenerative therapies in humans because horses more closely approximate the situation in and response to treatment of human patients when compared to laboratory species [9]. The end of the chapter includes a review of the current status of clinical stem cell use in the horse. This review is important to understand what evidence presently exists to justify the use of stem cells in horses and how we as equine surgeons can advance the field of regenerative medicine as a whole by performing carefully controlled experimental and clinical trials that are then directly translatable to humans.

History of Bone Marrow-Derived Mesenchymal Stem Cell (MSC) Research

Adult bone marrow-derived mesenchymal stem cells (MSCs) were first discovered in experiments performed by Friedenstein and colleagues in the 1960s and 1970s [10-12]. In these experiments, they demonstrated that fibroblastoid cells could be isolated from the bone marrow and that these cells were able to adhere to plastic and form colonies [10]. Although Friedenstein also demonstrated the osteogenic potential of MSCs, it wasn't until the late 1990s that Pittenger and colleagues demonstrated the in vitro ability of MSCs to differentiate into cells of osteogenic, chondrogenic, and adipogenic lineages [13]. This seminal work, which also included characterization of cell surface markers on MSCs using flow cytometry, renewed worldwide interest in MSCs [11-13]. Despite a large body of research since that time, many unanswered questions remain concerning both the safety of MSCs for allogeneic applications and the

regenerative ability of MSCs for the treatment of naturally occurring diseases. Clinical trials in both humans and animals using MSCs have shown only modest results, leading many researchers to now believe that their positive effects on tissue repair are due to the soluble factors they secrete which may promote endogenous cell survival by inducing angiogenesis, decreasing the inflammatory and immune responses, and reducing apoptosis [14-19].

Immunogenic and Immunomodulatory Properties of Bone Marrow-Derived Mesenchymal Stem Cells

It has been demonstrated that MSCs have low immunogenicity when used autologously and that they possess significant immunomodulatory properties [20-25]. Many mechanisms for the immunosuppressive effects of MSCs have been described including inhibition of T cell proliferation, alteration of dendritic cell maturation, induction of regulatory lymphocytes, and apoptosis of CD8 positive T cells [21, 22, 26-28]. Although it was initially believed and demonstrated that allogeneic MSCs can be immune privileged due to these immunosuppressive properties [29-31], immune rejection of allogeneic MSCs has been reported [32-37].

The finding that MSCs are capable of alterations in their MHC class I and II expression profiles is likely to blame for these conflicting results. While adult MSCs generally display the phenotype of high MHC class I expression and low or negative MHC class II expression, MSCs from mice and humans with high MHC II expression levels have also been described [32, 33, 38]. Additionally, both MHC class I and class II expression levels on MSCs can be upregulated by proinflammatory cytokines such as IFN-ɣ [39, 40]. It is therefore necessary to carefully immunophenotype MSCs to be used in immunologic studies as those MSCs expressing high

levels of MHC class II are more likely to incite an immune response compared to those that are MHC class II negative.

In order to determine the in vitro immunogenicity and immunomodulatory capabilities of MSCs, researchers have relied on modified one-way mixed leukocyte reactions (MLRs) as described and used in this dissertation research. In MLRs, one population of cells is referred to as the responder cell population while another is referred to as the stimulator cell population. Responder cells are leukocytes that will divide in response to a stimulus and are typically harvested from either peripheral blood or lymphoid organs such as the spleen. Stimulator cells are the cells responsible for responder leukocyte proliferation and can either be leukocytes (classic MLR) or another cell type such as stem cells in this case. Stimulator leukocytes used in classic MLRs to determine histocompatibility or used in modified MLRs as controls must be irradiated or treated with Mitomycin C to prevent cell division. This cell division would otherwise be indistinguishable from that of the responder cells when using ^{3}H-thymidine labeling [41, 42]. Even with newer labeling methods such as 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE) used in this dissertation research, in which responder leukocytes alone are labeled prior to culture, stimulator leukocyte response and division is still undesirable due to media exhaustion [42]. In modified MLRs, stem cell division can be inhibited if using ^{3}H-thymidine for labeling but otherwise the cells can be seeded at low density at the establishment of cultures and left to divide provided that the amount of media is adequate. In order to examine the immunomodulatory effects of stem cells, classic MLRs are carried out in tissue culture wells with responder leukocytes of one haplotype and stimulator leukocytes of a different haplotype either alone (as a control) or in the presence of stem cells seeded on the bottom of the wells.

Antigen presentation in MLRs can occur via direct or indirect antigen presentation pathways [43-45]. Two direct antigen presentation pathways have been described. The first relies on the presentation of stimulator antigens by stimulator antigen presenting cells (APCs) to responder T cells. The second is caused by direct recognition of allogeneic stimulator MHC molecules by responder T cells. The indirect antigen presentation pathway relies on the presentation of shed stimulator MHC molecules (generally following cell death) by responder APCs [43-45]. For all of these pathways, the expression of MHC class I and II molecules by stem cells is critical to the MLR response elicited.

History of Induced Pluripotent Stem Cell (iPSC) Research

Induced pluripotent stem cells (iPSCs) were first generated from adult mouse fibroblasts by Takahashi and Yamanaka in 2006 using retroviral transfection of Oct3/4, Sox2, Klf4, and c-Myc factors under embryonic stem cell (ESC) culture conditions [46]. The iPSCs were shown to be ESC-like based on their capacity to form embryoid bodies in vitro and to form teratomas in SCID mice [46]. Shortly thereafter, it was also demonstrated that mouse iPSCs were able to give rise to germ line chimeras [47]. Reports of the generation of iPSCs from adult human fibroblasts quickly followed in the literature [48-50] and iPSCs became regarded as the most promising candidate for the clinical application of regenerative therapies [51].

The immediate and intense focus on iPSCs was due to the fact that they are pluripotent, unlike MSCs, and can be autologous or patient-specific, unlike ESCs. At a time when MSC clinical studies began showing either very modest regenerative potential or no beneficial effects at all, iPSCs were a new source of hope for the field. In addition, because iPSCs can be generated from somatic cells, they avoid the ethical concerns surrounding the isolation and use

of human ESCs [51], making them a more attractive option to the general public. It did not take long, however, for iPSCs to come under attack as concerns were raised over their safety in terms of genetic instability and tumorigenic potential [51, 52].

Retroviruses and lentiviruses are highly efficient at generating iPSCs but lead to genomic integration of the transgenes, some of which are known oncogenes such as Klf4 and c-Myc [51, 53]. For that reason, many groups have since successfully investigated the use of non-integrating reprogramming methods to generate iPSCs including the use of plasmids, proteins, adenoviruses, Cre-recombinase excisable lentiviruses, and transposases such as the *piggyBac* transposase [51, 53-59]. Similarly, many groups are investigating reprogramming methods that exclude the factors Klf4 and c-Myc [2, 51, 53, 60]. Nevertheless, it has become apparent that iPSC lines generated from any of these reprogramming methods have the potential for karyotypic and genomic aberrations, and therefore must be thoroughly screened for safety prior to clinical use [52, 57, 61-64].

Despite the tremendous potential placed on iPSCs for patient-specific use, the practicality of such autologous iPSC use has recently been called into question [57, 65]. Under optimal conditions, iPSCs take several months to culture expand and evaluate for pluripotency [48, 55, 57, 66]. The addition of screening for genetic instability adds even more time to this already extended process, making autologous iPSC use impractical for many of these diseases that would potentially benefit from stem cell therapy. This led us to two important questions concerning iPSCs that would have a significant impact on their future clinical application: (1) Does genetic background affect iPSC generation; and (2) What are the immunogenic and immunomodulatory properties of iPSCs? Our hypotheses for iPSCs were based on previous findings for ESCs, which are detailed below.

Effect of Genetic Background on Stem Cell Derivation

It has been previously demonstrated in mice that genetic background affects the ability to derive ESCs [67-69]. Some strains such as 129/Sv are noted for the ease in which ESC lines can be established, while others such as BALB/c are notoriously difficult to establish ESC lines from, requiring alternate culture conditions and a particularly high concentration of Leukemia Inhibitory Factor (LIF) to maintain pluripotency [67, 69]. Because of this precedence, our hypothesis was that genetic background would also affect the ability to generate iPSCs. Results of this study, presented in chapter 2, confirmed this hypothesis. The results also confirmed the need to understand the immunogenic and immunomodulatory properties of these cells for potential allogeneic application given that it may not be feasible to generate iPSCs from all individuals.

Both genetic background and age of the patient have also been shown to affect proliferation and differentiation rates of MSCs [70-72], suggesting that autologous MSC use also may not be feasible for some patients. Given these findings and the fact that the future of stem cell therapy is headed toward the use of banked stem cells that have been tested for both efficacy and safety [65, 73], we sought to compare the immunogenic and immunomodulatory properties of iPSCs and MSCs for potential use in regenerative and transplantation medicine.

Immunogenic and Immunomodulatory Properties of Embryonic Stem Cells (ESCs)

At the time of this dissertation research, no studies had been published examining the immunogenic properties of iPSCs. It has since been demonstrated, however, that undifferentiated iPSCs, like ESCs, express low or absent levels of MHC class I and are negative for MHC class II

expression [74]. Interestingly, it has also been shown that MHC class II expression on iPSCs is not upregulated by differentiation or by stimulation with IFN-ɣ [74]. The extent to which MHC class I expression can change upon iPSC differentiation or stimulation with proinflammatory cytokines is unknown. Several studies have shown that MHC class I expression increases upon iPSC differentiation and with IFN-ɣ stimulation, but often only to a level still much less than that of somatic cells [74-76]. The consequence of such a change in MHC class I expression is complex as a high expression level of MHC class I could lead to T cell activation, while a continued lack of MHC class I expression could potentially lead to iPSCs being targeted by natural killer (NK) cells in vivo [77]. Conflicting results have been reported for ESCs on this subject, with some groups reporting ESCs as susceptible to NK cell lysis, and others reporting that ESCs are neither susceptible to NK cell lysis nor capable of eliciting T cell responses [77, 78].

Since the start of this dissertation research, conflicting results have also been reported on the immunogenicity of iPSCs. While the first report on the immunogenicity of iPSCs revealed that undifferentiated autologous mouse iPSCs were immune rejected in a teratoma model study [79], two other reports since then have shown that both undifferentiated and differentiated syngeneic mouse iPSCs are non-immunogenic in vitro and in vivo [75, 76]. To date, no studies have examined the immunomodulatory properties of iPSCs even though it is known that ESCs are capable of immunosuppression through multiple mechanisms including expression of arginase I, prevention of dendritic cell maturation, and upregulation of regulatory T cells [80-83]. When considering the use of iPSCs as an alternative for MSC therapy, this information is critical. The purpose of the study presented in chapter 3, therefore, was to evaluate the in vitro immunogenic and immunomodulatory properties of iPSCs compared to MSCs using modified

mixed leukocyte reactions. Our hypothesis, based on prior ESC knowledge, was that undifferentiated iPSCs would have similar immunogenic and immunomodulatory properties as MSCs. Results of this study confirmed this hypothesis and have led us to design future studies (outside the scope of this dissertation) using the horse as model to then compare the regenerative ability of iPSCs and MSCs in vivo.

Development of Equine Induced Pluripotent Stem Cells

An exciting development to occur during the course of this dissertation research was the generation of equine iPSCs, first by Nagy et al. [1] and then by Khodadadi et al. [2] and Breton et al [3]. Nagy et al. reprogrammed equine fetal fibroblasts to iPSCs using the doxycycline-inducible *piggyBac* transposon reprogramming system. Both Khodadadi et al. and Breton et al. reprogrammed adult fibroblasts to iPSCs using a constitutive retrovirus or Moloney Murine Leukemia Virus, respectively [2, 3]. With the aid of these publications, and our collaborators from the Nagy laboratory, we are currently working towards generating iPSC from horses of known MHC haplotype to be used for future experiments and compared to the MSCs that we have already isolated from these horses as described in chapter 4 and discussed below.

Current Status of Clinical Stem Cell Use in The Horse (Modified from: Schnabel LV, Fortier, LA, McIlwraith CW, Nobert KM. Therapeutic use of stem cells in horses: Which type, how, and when? *Vet J.* In Press.)

The use of stem cells in veterinary medicine continues to increase at a pace that is more rapid than available scientific and clinical evidence [84-87]. Despite the widespread use of stem cells for the treatment of equine musculoskeletal disorders, there are very few reports of long-

term clinical data. Many experimental and clinical studies are lacking the proper control groups and are complicated by multimodal therapeutic approaches. In addition, there are impending changes in regulatory laws by the FDA, which eventually may limit the use of some or all types of equine stem cells [88, 89]. When considering our equine patients and also the fact that the horse is a valuable model for the human response to stem cell treatment, we must take caution in how future equine studies are designed and executed and how stem cells are used on a daily basis so as not to bias any findings.

The two most commonly used stem cells in equine veterinary medicine are adult bone marrow-derived and adipose tissue-derived mesenchymal stem cells (MSCs) [85, 86, 90]. Cells from either of these sources can be used after a culture period or after a brief centrifugation step for point-of-care treatment. Bone marrow aspirate is typically harvested from the sternum (marrow spaces 3 to 5) or ilium using a Jamshidi needle [91, 92]. The bone marrow aspirate can be cultured for approximately 2-3 weeks to obtain bone marrow-derived mesenchymal stem cells (BM-MSCs) or immediately centrifuged patient-side to produce bone marrow concentrate (BMC). BMC concentrates both stem cells and platelets compared to raw bone marrow aspirate, but yields a much lower number of stem cells compared to culture-expanded BM-MSCs [93]. Both BM-MSC isolation/culture services and BMC centrifugation systems are available commercially and are frequently used by equine practitioners [90, 93, 94].

Adipose tissue is generally harvested from the tail head region and then collagenase digested and either cultured for several weeks to obtain adipose tissue-derived MSCs (AT-MSCs) or processed commercially to isolate adipose-derived stem vascular fraction (AD-SVF) cells within 4-24 hours [90]. Although equine AT-MSCs have been well described in the literature [95-100], AD-SVF cells have been favored over AT-MSCs in clinical use most likely

due to their highly publicized commercial availability and short turnaround time from adipose tissue harvest to clinical application [90]. It is important to note, however, that because of the lack of a culture step, only a fraction (20 to 40%) of the AD-SVF cells are stem cells [98]. As detailed below, there is evidence to support the use of both cultured and processed adult MSCs for the treatment of equine musculoskeletal disorders including tendonitis and osteoarthritis.

Umbilical cord tissue-derived and placentally-derived MSCs have been assessed for safety in the literature primarily because of the fact that they are generally used in an allogeneic fashion [101, 102], but have not yet been evaluated for efficacy in the treatment of any equine disorder. Despite this fact, umbilical cord-derived MSCs are being used clinically and so are mentioned here. Only those stem cells for which peer-reviewed publications containing pre-clinical and/or clinical data exist, however, will be discussed further.

***Tendonitis*:** The use of cultured bone marrow-derived MSCs (BM-MSCs) for the treatment of equine tendonitis is supported in the literature both by experimental and clinical studies [103-107]. Peer-reviewed publications with clinical data only currently exist for BM-MSCs and not for other types of stem cells. Smith *et al.* (2003) first described the culture process and use of BM-MSCs for the treatment of naturally occurring superficial digital flexor tendonitis in a single case report of a polo pony [108]. In the first case controlled study on the use of BM-MSCs for the treatment of naturally occurring superficial digital flexor tendonitis by Pacini *et al.* (2007), 11 BM-MSC treated horses were compared to 15 control horses treated by traditional methods with both groups using the same rehabilitation protocol [104]. In this study, 9/11 (82%) of the BM-MSC treated horses returned to racing in 9-12 months and were still racing without re-injury at 2 years post-treatment compared to the control group in which all of the 15 horses had experienced a re-injury event within 1 year (median re-injury time of 7 months) [104].

In a later large clinical case series by Godwin *et al.* (2012) in which BM-MSC treated horses were compared to historical controls from the literature [109, 110], a significant reduction in re-injury rate was found for National Hunt horses treated with BM-MSCs compared to National Hunt horses treated with other therapies as previously reported by Dyson (2004) (25.7% compared to 56%, respectively) and O'Meara *et al.* (2010) (25.7% compared to 53%, respectively). Interestingly, no differences in the percentage of National Hunt horses treated with BM-MSCs compared to historical controls as reported by O'Meara *et al.* was found in terms of return to racing and completing 3 and 5 races. Only 8 Thoroughbred flat racehorses treated with BM-MSCs were compared to 3 Thoroughbred flat racehorses treated with medical therapies by Dyson *et al.* (2004). The small sample sizes in each study make translation of these findings to Thoroughbred flat racehorses difficult.

For the treatment of discrete core lesions, stem cells should be injected directly into the lesion under ultrasound guidance using aseptic technique [103-105, 107]. Use of a 20 or 23 gauge needle is recommended for direct injection based on work from our laboratory in which the use of needles smaller than 23 gauge resulted in significantly decreased BM-MSC viability compared to larger gauge needles. This effect is presumed to be due to increased mechanical shearing of the cells with decreased needle size [111]. For diffuse tendon or suspensory ligament lesions, or for multiple lesions within the same tendon/ligament or limb, administration of stem cells via intravenous regional limb perfusion should be considered based on a recent study by Sole *et al.* [112]. Either intravenous catheters or butterfly needles size 23 gauge or larger are recommended for this technique for ease of slow administration and to avoid decreased cell viability as discussed above. As reported by Sole et al. (2012), intra-arterial regional limb-perfusion should be avoided at this time due to the potential for arterial thrombosis [112].

Studies to determine the optimal number of stem cells for the treatment of tendon/ligament lesions in the horse have yet to be performed. A range of BM-MSC numbers has been described in the literature for therapeutic application with the most common being $10x10^6$ BM-MSCs per tendon lesion [103-105, 107]. Horses with unfavorable outcomes in both the Pacini et al. (2007) and Godwin et al. (2012) studies were injected with fewer BM-MSCs compared to horses with favorable outcomes, however these results are limited in sample size. It is likely that a range exists within which stem cell numbers are most effective. Dose-dependent response studies are needed in the horse to determine this range.

While all of the peer-reviewed publications on the use of BM-MSCs for the treatment of tendonitis have relied on a single injection, it is common in clinical practice to perform multiple injections depending on healing of the injured tendon. Our clinical practice is to perform a recheck examination of the horse at 30 days post initial injection and to repeat BM-MSC treatment if there is a less than fifty percent improvement in both the degree of lameness and ultrasonographic evaluation findings. A rehabilitation protocol is also essential for success and must be tailored to each individual horse according to physical examination and lameness evaluation findings in conjunction with ultrasonographic findings. For that reason, it might be prudent to provide owners with instruction only until the time of the horse's next recheck examination with specific but gradual increase in exercise as opposed to box stall rest, as has been described in the literature [107].

Further studies need to be performed to determine if the use of BM-MSCs is more or less effective than the use of platelet-rich plasma (PRP) for the treatment of tendonitis or if their effects are additive if used simultaneously. It is common in clinical practice to administer PRP at the time of diagnosis and bone marrow aspiration, with a second injection of BM-MSCs and

PRP when the cultured BM-MSCs are ready. Stem cells and PRP should not be mixed together in the same syringe as the PRP may clot prohibiting injection. To circumvent this possibility, they can be injected sequentially using the same needle, but not mixed together prior to injection. Our laboratory and others are currently investigating the chemotactic properties of biologics such as PRP for the recruitment of endogenous MSCs from normal tissue surrounding the injury site [113].

***Joint disease*:** The efficacy of stem cells for the treatment of equine osteoarthritis (OA) and cartilage injuries has been evaluated in the form of experimental and clinical studies and with more favorable results for bone marrow-derived cells than adipose-derived cells [93, 114-116]. In the only study published on the use of intra-articularly administered stem cells for the treatment of OA in the horse, Frisbie *et al.* (2009) created early OA using a carpal osteochondral fragment model [115, 117]. Injured joints were treated once with cultured BM-MSCs, AD-SVF cells, or saline. At 70 days post-treatment, no differences were found in cartilage biochemistry or histology between the groups. Joints injected with BM-MSCs had significantly less synovial effusion and lower concentrations of the pro-inflammatory modulator prostaglandin E2 (PGE2) in comparison to joints injected with either AD-SVF cells or saline [115]. Treatment with AD-SVF cells incited an inflammatory response demonstrated by increased concentrations of tumor necrosis factor alpha (TNF-α) [115]. A large clinical trial is needed to determine the effects of intra-articular administration of BM-MSCs on the progression of naturally occurring OA in horses non-responsive to routine treatments [86, 118] . The results of such a trial would be useful for further determining if and how stem cells should be used for the treatment of equine OA. It is possible that MSCs will have more of an effect on horses with advanced OA than that which has been observed in experimental OA models. It is also possible that the MSCs may

need to be differentiated prior to administration to be maximally effective for the treatment of OA [86, 118].

The efficacy of both cultured BM-MSCs and BMC has been evaluated for the treatment of experimentally induced full-thickness cartilage defects created on the lateral trochlear ridge of the femur in horses [93, 116] In the Wilke *et al*. (2007) study, BM-MSCs in fibrin grafted into full thickness cartilage defects resulted in significantly improved arthroscopic scores and biopsy assessments at 30 days compared to control defects treated with fibrin alone. At 8 months, however, no differences in BM-MSC treated and control defects were found [116]. It is unclear whether the results of these long-term assessments could have been affected by the trauma of the 30-day biopsy or if the effects of the BM-MSCs were truly short-lived. Using a similar study design, but without a biopsy, Fortier *et al.* demonstrated the effectiveness of BMC for repair of full-thickness cartilage defects [93]. Both short-term (3 month) and long-term (8 month) assessments revealed a significant improvement in the macroscopic and histologic scoring for BMC grafted and microfracture treated defects compared to control microfracture alone treated defects. At 8 months, BMC grafted defects also had significantly improved magnetic resonance imaging measurements compared to control treated defects [93].

McIlwraith *et al.* (2011) also recently evaluated the efficacy of BM-MSCs administered intra-articularly for the treatment of microfracture treated full-thickness medial femoral condyle cartilage defects [119]. Horses either received BM-MSCs with hyaluronan or hyaluronan alone 1 month after creation of the defects. After a year-long period of exercise, horses were euthanized. Horses treated with BM-MSCs had a significant increase in repair tissue firmness and concentration of aggrecan compared to control. No other significant differences were found

between treatment and control groups on magnetic resonance imaging and gross, histologic, and biochemical examinations [119].

In a clinical follow-up of thirty-three horses that received intra-articular BM-MSCs following arthroscopic surgery for the treatment of stifle injuries including medial femoral condyle cysts and meniscal damage, Ferris *et al.* reported a low morbidity rate equivalent to that of surgery alone or as reported for treatment with common chondroprotective agents [114]. For this case series, the authors also reported improved results for horses with meniscal injuries being able to return to work compared to previous reports [114].

For the treatment of osteoarthritis (OA), BM-MSCs can be injected intra-articularly either alone or in conjunction with products routinely used to treat OA such as hyaluronan. Current recommendation is to inject stem cells ($20x10^6$) in HA [22mg of Hyvisc (hyaluronate sodium, $3x10^6$ Da, Anika Therapeutics, Woburn, MA)] in an OA-affected joint on an as needed basis as would be performed if using corticosteroids. The frequency of injection would therefore vary depending on the individual horse and response to treatment. Administration of a non-steroidal anti-inflammatory agent (NSAID) prior to injection of BM-MSCs is recommended to reduce the risk of joint flare [114]. While the use of NSAIDs is prudent, the reported occurrence of joint flare following intra-articular of BM-MSCs is low (9%) and equivalent or less than that reported for HA products alone [114]. If a joint flare where to occur, treatment with NSAIDs alone is recommended unless there is concurrent evidence of injection requiring joint lavage [114].

For intraoperative treatment of cartilage defects, BM-MSCs or BMC can be grafted into a lesion under arthroscopic guidance [93, 116]. This requires the use of gas arthroscopy at the time of grafting. In most cases, a dual syringe injection system is used in which the cellular component is in one syringe and bovine thrombin is in the other syringe; when injected

simultaneously, they mix and form a clot that is molded to the contour of the lesion. Alternatively, autologous or commercial fibrin can be used to retain the cells. There are other cartilage grafting procedures, such as those using allogeneic chondrocytes in fibrin with insulin-like growth factor-I (IGF-I) that appear at least equally as efficacious [120, 121], but no direct comparative studies between the cartilage repair grafting procedures have been performed. In addition to the grafting techniques described, there is experimental evidence to support the use of BM-MSCs injected intra-articularly for the treatment of cartilage defects [119]. This application is most commonly used when a diagnosis of cartilage injury is made at the time of arthroscopy and no graft material is anticipated as needed or prepared for intra-operative application.

As discussed for the treatment of tendonitis, it is unclear at this time if the simultaneous use of biologics such as PRP would be of added benefit. It is also unclear what the optimal number of MSCs for the treatment of OA and cartilage defects is as no dose response studies have been performed to date. While published studies most commonly use a range of $10x10^6$ to $20x10^6$ BM-MSCs per joint/cartilage defect, cell numbers can be larger, particularly for the grafting of large defects.

Following intra-articular injection of stem cells for the treatment of OA in the absence of surgery, horses are returned to exercise after 24 hours similar to more routine treatment with corticosteroids and/or chondroprotective agents. Following a surgical stem cell grafting procedure, a rehabilitation protocol is established and is largely dependent on the size, location, and nature of the cartilage defect. Young horses treated for osteochondritis dissecans (OCD) lesions are generally confined to a box stall for 2 weeks post-operatively, following which time gradually increasing amounts of hand walking are initiated. At 3 months post-operatively, a veterinary examination is performed and radiographs of the affected joint are taken and

evaluated for healing prior to small paddock turnout for an additional month and then full turnout for another 2 months. Veterinary examination and radiographs at 6 months postoperatively are then used to determine if the horse can start training. Adult horses treated for traumatic lesions without surgery, may start handwalking as early as 7 days post-injection and are evaluated at 1 month post-injection to further define an exercise protocol dependent on the particular horse, the type and magnitude of articular damage, and the horse's discipline.

***Laminitis*:** There is a great deal of hope in the equine community that stem cells may be effective in the treatment of laminitis and many practitioners are currently administering stem cells from different sources to laminitic horses. It is important to acknowledge that no studies have been performed to evaluate the safety or efficacy of stem cells for the treatment of this devastating condition. While a recent study by Carter *et al.* (2011) demonstrated that laminitic horses have a loss of p63-positive epidermal stem cells compared to control non-laminitic horses, it is unknown whether administration of MSCs could aid in the repopulation of epidermal stem cells. It is also unknown if the administration of epidermal stem cells themselves harvested from other regions of the horse such as the skin could potentially be effective for the treatment of laminitis [122].

Intravenous regional limb perfusion of stem cells is currently being performed clinically for the treatment of laminitis. Owners will seek stem cell therapy as a cure for their horse because they know the devastating consequences of laminitis; and while it might be helpful, the underlying cause of the disease should be aggressively pursued with diagnostics in conjunction with medical management and nutritional therapies. Ideally, laminitic horses are treated in the acute phase in an attempt to decrease the inflammatory response, but this approach would require the use of banked-self stem cells or application of allogeneic cells. For the treatment of

laminitis, most horses are treated with stem cells twice, once as early as possible during an acute episode and again at 14 days after the first injection. As previously mentioned, there is currently no published data to evaluate the effectiveness of stem cells for laminitis and therefore administration techniques, cell types, and cell numbers also have not been evaluated.

***Considerations and cautions for future use of stem cells in the horse*:** Early administration of stem cells is presumed to be advantageous rather than attempt treatment when fibrous scar tissue is formed. This paradigm can be accomplished with a point-of-care heterogeneous cell product such as BMC, but it often cannot be realized for culture-expanded MSCs unless allogeneic cells are used. Although there is evidence in the literature that MSCs have anti-inflammatory and immunomodulatory properties [15, 16, 20, 21, 123-125], it remains unclear if equine MSCs incite an immune response in vivo if used allogeneically, especially if administered repeatedly.

Preliminary studies by Carrade *et al.* examined the use of umbilical cord- and placentally-derived MSCs suggested that such cells do not incite a cellular immune response, even after repeated injections [101, 102]. It is important to recognize that MSCs from neonatal sources are likely more immune-privileged than those derived from adult sources, and that the horses used in these studies were not major histocompatibility class (MHC) haplotyped. This means that although the cells evaluated were not "self", they might not have been allogeneic in the true sense of being of a different MHC haplotype, which would affect their immunogenicity. In addition, it is critical to understand that not all MSCs from the same source are identical and that MHC class II expression can vary dramatically depending on the horse and on how many times the cells have been passaged. Our work, as presented in chapter 4 of this dissertation, using MHC haplotyped horses revealed that passage 2 BM-MSCs are highly heterogeneous in MHC class II expression (range 0 - 98% positive), and that increasing MHC II expression is directly

related to increasing responder T cell proliferation in vitro. Interestingly, BM-MSCs from some horses can be negative for MHC class II expression from passage 2 onwards, but the majority of horses are positive for MHC class II expression until passage 4 or even later passages. These findings are especially important when considering that most adult source MSCs such as BM-MSCs are used clinically at very low passage number to maintain stemness. Identification of a universal stem cell donor whose low passage BM-MSCs are MHC class II negative would be of clinical value. The fact that BM-MSCs from most horses are MHC class II positive for multiple passages and that the amount of MHC class II expression is directly correlated to immune response in vitro emphasizes the fact that extreme caution must be exercised in the application of allogeneic stem cells from both a safety and regulatory standpoint.

The therapeutic application of stem cells in equine veterinary medicine holds great promise, but there are also a lot of unanswered questions at this time. Research efforts must be directed towards determining the optimal number of stem cells for each specific clinical application as well as the route of administration, dosing formulation, and dosing schedule. Particular points of interest are the safety of allogeneic stem cells and the interactions of stem cells with biological products, including the ability of biologics to potentially attract stem cells to the site of injury. The idea of a universal BM-MSC donor that would provide the ability to use stem cells immediately during the acute phase of the injury or at the time of diagnosis is an exciting one for the field of regenerative medicine.

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CHAPTER 2

GENETIC BACKGROUND AFFECTS INDUCED PLURIPOTENT STEM CELL GENERATION

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Abstract

The influence of genetic background on the ability to generate induced pluripotent stem cells (iPSCs) has the potential to impact future applications, but has yet to be examined in detail. The purpose of this study was to determine if genetic background affects the efficiency of generating iPSCs during early reprogramming as well as the pluripotent stability of the iPSCs during later stages of reprogramming. Mouse embryonic fibroblasts (MEFs) were isolated from six strains of mice (NON/LtJ; C57BL/6J; DBA/2J; BALB/cJ; 129S1/SvlmJ; CAST/EiJ) that were selected based on genetic diversity and differences in ability to produce embryonic stem cell (ESC) lines. MEFs were reprogrammed via doxycycline-inducible lentiviral transduction of murine *Oct4, Klf4, Sox2,* and *c-Myc*. Differences in efficiency to generate iPSCs were assessed on primary transformation plates by comparing the total number of colonies, the percentage of colonies positive for alkaline phosphatase staining and the percentage of cells positive for SSEA1. iPSC colonies were expanded to establish doxycycline-independent cell lines whose pluripotency was then evaluated via ability to form teratomas in NOD.CB17-*Prkdc*^{scid}/J mice. Proliferation of non-transduced parent MEFs from each strain was also examined over ten days under conditions that simulated reprogramming. NON/LtJ and CAST/EiJ strains were more efficient than other strains in generating iPSCs for all parameters measured on primary transformation plates and parent MEFs from these strains were more proliferative than those from other strains. Doxycycline-independent iPSC lines were established using standard conditions for all strains except BALB/cJ, which required a higher concentration (5x) of Leukemia Inhibitory Factor (LIF). iPSCs from all strains were capable of producing teratomas in NOD.CB17-*Prkdc*^{scid}/J mice. The results of this study suggest that genetic background does affect iPSC generation and pluripotent stability. In addition, our results demonstrate that strain

differences in efficiency to generate iPSCs during the early stages of reprogramming are correlated with those observed in proliferation of parent MEFs. These findings have important implications both for future iPSC applications as well as for future investigation into determining the genes responsible for reprogramming efficiency and stability.

Introduction

The iPSC field continues to make rapid advances in terms of optimizing reprogramming methods to circumvent clinical safety issues and characterization of the genetic and epigenetic composition of established iPSC lines [1-4]. The influence of genetic background on the ability to generate iPSCs, as well as the stability and quality of derived iPSCs for downstream applications, also has the potential to impact the future applications. However, the role of genetic background has yet to be examined in significant detail. The effect of genetic background on pluripotency has precedence in mice; it is well documented that there are dramatic strain differences in ability to produce embryonic stem cell (ESC) lines [5-8].

Many of the mouse iPSC studies to date have used mouse embryonic fibroblasts (MEFs) from transgenic mice of an undefined or hybrid background [9], or have used MEFs or tail tip fibroblasts (TTFs) derived from animals originally produced from hybrid ESCs [9-16]. Few studies have used MEFs or TTFs from a pure inbred strain [17-19]. To our knowledge, only one study to date has directly compared the ability of two different inbred strains to generate iPSCs [17]. In this study, Hanna *et al*. found that MEFs from NOD/ShiLtJ mice, a strain previously considered nonpermissive for ESC derivation, were capable of generating iPSCs, but that these iPSCs were dependent on exogenous transgene expression unlike the iPSCs derived from control 129Sv/Jae MEFs [17]. The authors determined that the NOD/ShiLtJ iPSCs were dependent upon ectopic expression of either KLF4 or c-MYC using constitutive lentiviruses, and that the cells were able to overcome this factor dependence when cultured in media supplemented with any of the following proteins or small molecules: WNT3a, which promotes iPSC derivation in the absence of c-MYC [20]; CHIR99021, a GSK3b inhibitor; or Kenpaullone, a GSK3b and CDK1/cyclin B inhibitor which has been shown to replace KLF4 during iPSC reprogramming

[17,21]. As the authors concluded, these results suggest that genetic background can affect the pluripotent stability of iPSCs and that reprogramming and culture conditions may have to be modified for certain strains [17].

The purpose of this study was to determine if genetic background affects the efficiency of generating iPSCs during early reprogramming as well as the pluripotent stability of the iPSCs during later stages of reprogramming. We chose six different inbred strains of mice to examine based on their genetic diversity [22-24] and on their differences in ability to produce ESC lines [5-8]. These six strains included five classical laboratory strains (NON/LtJ, C57BL/6J, DBA/2J, BALB/cJ, and 129S1/SvlmJ) and one wild-derived inbred strain (CAST/EiJ) (**Figure 2.1**). Because 129-derived substrains such as 129S1/SvlmJ support facile ESC line derivation while both C57BL/6J and BALB/cJ mice do not [5-8], we reasoned that these strains would be useful for assessing potential differences in reprogramming efficiency. In addition, three of the strains (C57BL/6J, 129S1/SvlmJ, and CAST/EiJ) are progenitors of the Collaborative Cross that is proving effective for analyzing complex genetic phenotypes [25,26]. Knowledge on the potential differences between these strains in their ability to generate iPSCs and their pluripotent stability might therefore be amenable to genetic analysis.

In this study, we show that iPSC lines can be generated from all six of the strains examined using a lentiviral reprogramming system [27-29] and that these cell lines are capable of forming teratomas in NOD.CB17-*Prkdc*^{scid}/J mice. We demonstrate, however, that there are strain differences in efficiency of iPSC induction, growth, and maintenance requirements, and that these strain differences correlate with proliferative ability of the parental MEFs.

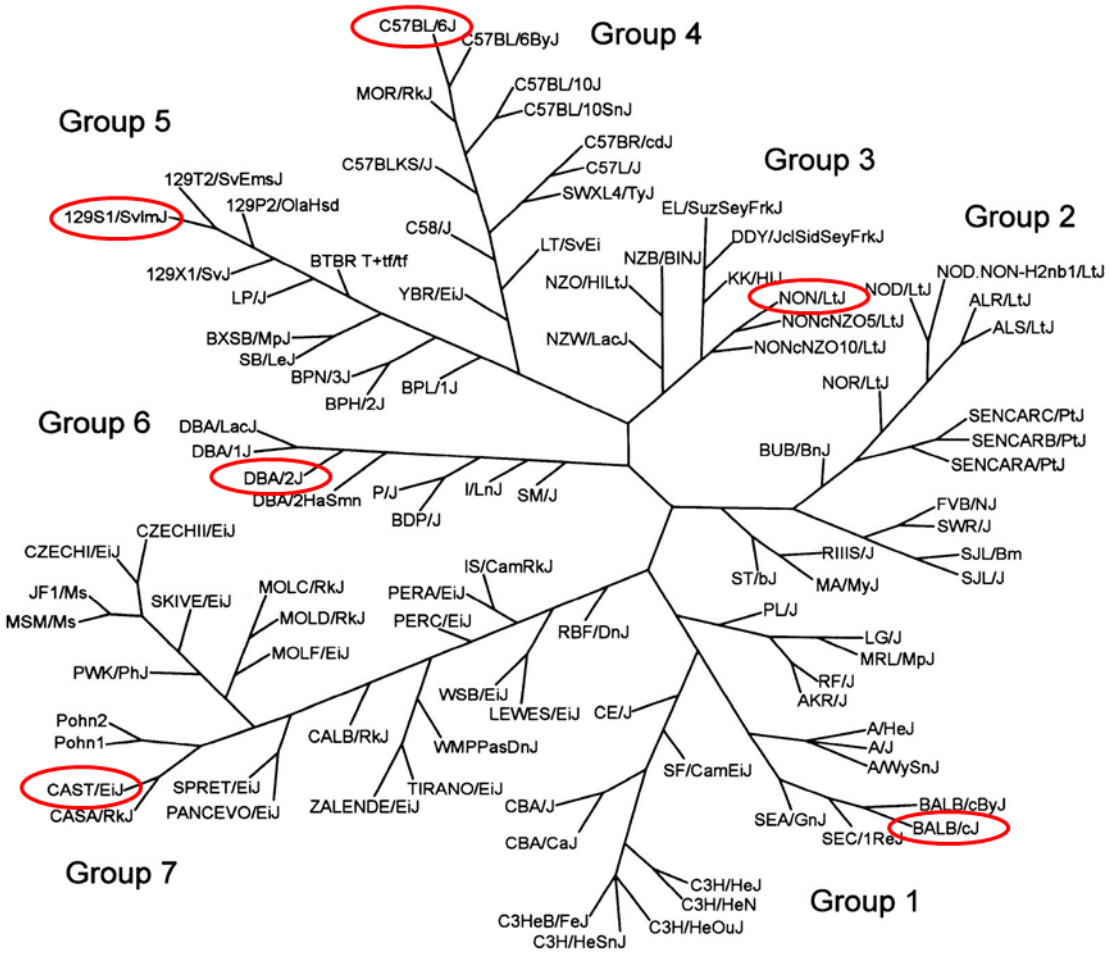


Figure 2.1. Mouse family tree. The seven mouse groups as described as by Petkov *et al.* [22] with the strains examined in this study circled in red. Group 1, Bagg albino derivatives; Group 2, Swiss mice; Group 3, Japanese and New Zealand inbred strains; Group 4, C57/58 strains; Group 5, Castle's mice; Group 6, C.C. Little's DBA and related strains; Group 7, wild-derived strains. (Modified from Petkov *et al.* and reprinted with permission [22]. The length and angle of the branches were optimized for printing and do not reflect the actual evolutionary distances between strains.)

Materials and Methods

Mice: Male and female mice from all six strains examined (NON/LtJ; C57BL/6J; DBA/2J; BALB/cJ; 129S1/SvlmJ; CAST/EiJ) were purchased from The Jackson Laboratory (Bar Harbor, ME). For each strain, breeder trios were established for timed matings such that embryonic day 13.5 embryos could be collected and processed to generate MEFs. NOD.CB17-*Prkdc*^{scid}/J mice, used for teratoma formation assays, were also purchased from The Jackson Laboratory (Bar Harbor, ME). The use of mice in this study was approved by the Institutional Animal Care and Use Committee of Cornell University.

MEF culture: Embryonic day 13.5 embryos were isolated from the uteri of pregnant mice, lavaged with phosphate buffered saline (PBS), and eviscerated. Each embryo was then gently homogenized in MEF media (high glucose Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum (FBS), penicillin (100 units/mL), and streptomycin (100μg/mL)) and the resultant cell suspension was transferred to a 100mm tissue culture plate and incubated at 5%CO_2, 90% humidity, and 37°C. The plates were washed with PBS and the media changed daily until the MEFs were confluent, at which time they were trypsinized, resuspended in freeze media (DMEM with 10% FBS and 10% DMSO), and cryopreserved until further use. All experiments were performed using MEFs derived from two different embryos for each strain.

Lentiviral constructs: Lentiviral vectors for doxycycline-inducible transgene expression were constructed as previously described [27-29] using a FUW-based plasmid with a tetracycline operator (TetO) and a constitutive CMV promoter. Briefly, the viral packaging plasmids psPAX2 and pMD2.G (Addgene 12260 and 12259, Cambridge, MA) as well as the plasmids encoding the reverse tetracycline transactivator (M2rtTA; Addgene 20342, Cambridge MA) and

the mouse factors *Oct4*, *Sox2*, *Klf4* and *c-Myc* (Addgene 20323, 20326, 20322 and 20324 respectively, Cambridge, MA) were purified from bacterial cultures. The vectors were then prepared by co-transfecting the viral packaging plasmids with plasmids encoding the reverse tetracycline transactivator and the reprogramming factors into 293T cells using the FuGENE®6 Transfection Reagent (Roche Applied Science, Indianapolis, IN). Viral supernatants were collected at 48 and 72 hours, concentrated using an Amicon Ultra-15 Centrifugal Filter Unit with an Ultracel-30 membrane (Millipore, Billerica, MA), filtered through a 0.45um filter, and stored in liquid nitrogen until used.

Reprogramming of MEFs and iPSC culture: Passage 2 (P2) MEFs from each strain were seeded onto gelatin-coated tissue culture plates at a density of $6.75x10^3$ cells/cm^2 in MEF media and allowed to adhere for 24 hours [27]. The culture media was then replaced with fresh MEF media supplemented with the viral supernatant described above. Following 24 hours of incubation with the viral supernatant, the culture media was changed to ESC media (KnockOut™ DMEM (Gibco, Grand Island, NY) supplemented with 15% KnockOut™ Serum Replacement (Gibco, Grand Island, NY), recombinant LIF, MEM non-essential amino acids solution (100µm), 2mM GlutaMAX™ (Gibco, Grand Island, NY), 0.1 mM 2-Mercaptoethanol, penicillin (100 units/mL), streptomycin (100µg/mL), and doxycycline (2µg/mL; Sigma, St. Louis, MO)). Cells destined for flow cytometric analysis and for expansion were kept on original 6-well plates while cells destined for AP staining and colony counting were trypsinized and passaged onto gelatin-coated 60mm tissue culture plates seeded with feeder cells (Cs irradiated C57BL/6J x 129S1/SvImJ1 MEFs) prior to the start of reprogramming with doxycycline. For all plates, ESC media was refreshed daily during reprogramming.

AP staining and colony counting: AP staining was performed directly on the 60mm plates using the Vector Red Alkaline Phosphatase Substrate Kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's directions. Both the number of AP stained colonies and the total number of colonies on the plates was quantified using bright field microscopy at 100x magnification. Colonies were identified based on the following morphological criteria: well defined-border; three-dimensionality; and tightly packed cells. A grid system was used on the plates to facilitate colony counting. Each plate was counted twice and the mean number of AP stained colonies and the mean total number of colonies was determined. The percentage of AP stained colonies was determined by dividing the mean number of AP stained colonies by the mean total number of colonies and then multiplying by one hundred.

Flow cytometric analysis: Cells from the 6-well primary transformation plates were trypsinized, washed with PBS, fixed in 4% paraformaldehyde, washed again, and resuspended in blocking buffer (TBS buffer, 0.1% Triton X-100, and 1% BSA) overnight at 4°C. The cell pellet was then washed, resuspended in unconjugated primary antibody for 1 hour at 4°C, washed, and resuspended in a secondary fluorescent-conjugated antibody for an additional 1 hour at 4°C. Cells were resuspended in blocking buffer and analyzed on a BD LSR II (Becton Dickinson Immunocytometry Systems, San Jose, CA) flow cytometer and FACSDiva software (Becton Dickinson Immunocytometry Systems, San Jose, CA). Data was collected on $1x10^4$ cells. Double staining with primary antibodies against SSEA1 (Millipore MAB4301, Billerica, MA) and LIN28 (Abcam Inc. ab46020, Cambridge, MA) with respective FITC (SouthernBiotech 1010-02, Birmingham, AL) and PerCP-Cy5.5 (Santa Cruz Biotechnologies sc-45101, Santa Cruz, CA) conjugated secondary antibodies was performed with resultant quadrant statistics including percentage of positive cells in each quadrant. Calibration of the flow cytometer and

setting of gates was performed using non-transduced P2 MEFs as negative controls and established 1-A4 (C57BL/6J x 129S1/SvlmJ) iPSCs and v6.4 (C57BL/6J x 129S4/SvJae) ESCs [28] as positive controls. The 1-A4 iPSC line was generated in our laboratory and validated via teratoma formation in NOD.CB17-*Prkdc*^{scid}/J mice and ability to generate germline chimeras through blastocyst injection.

iPSC line generation: iPSC colonies from 6-well primary transformation plates were picked with pipette tips into individual wells of 96-well tissue culture plates containing trypsin. The trypsin was neutralized with DMEM and 10% FBS, and the cells within each well were then transferred to individual wells of 96-well tissue culture plates seeded with feeder cells in ESC media and expanded. Doxycycline was removed from the media at the 6-well plate stage (around P7) in order to establish doxycycline-independent cell lines from each strain. The cells were then further expanded (P10-P15) in order to reach the cell numbers necessary for teratoma formation assays and for cryopreservation of stock from each strain.

Teratoma formation and histological analysis: iPSCs from one doxycycline-independent cell line from each strain were trypsinized, pelleted and suspended at $1x10^7$ cells/mL in MEF media. 150µl of the cell suspension ($1.5x10^6$ cells) was injected subcutaneously into the flank of a NOD.CB17-*Prkdc*^{scid}/J mouse. For each strain, a total of 6 injections were performed in 3 NOD.CB17-*Prkdc*^{scid}/J mice (both flanks of each mouse were injected). Four to 5 weeks post injection, tumors were surgically dissected, fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. All histologic sections were reviewed by a board certified veterinary pathologist (T.L.S.) for teratoma formation.

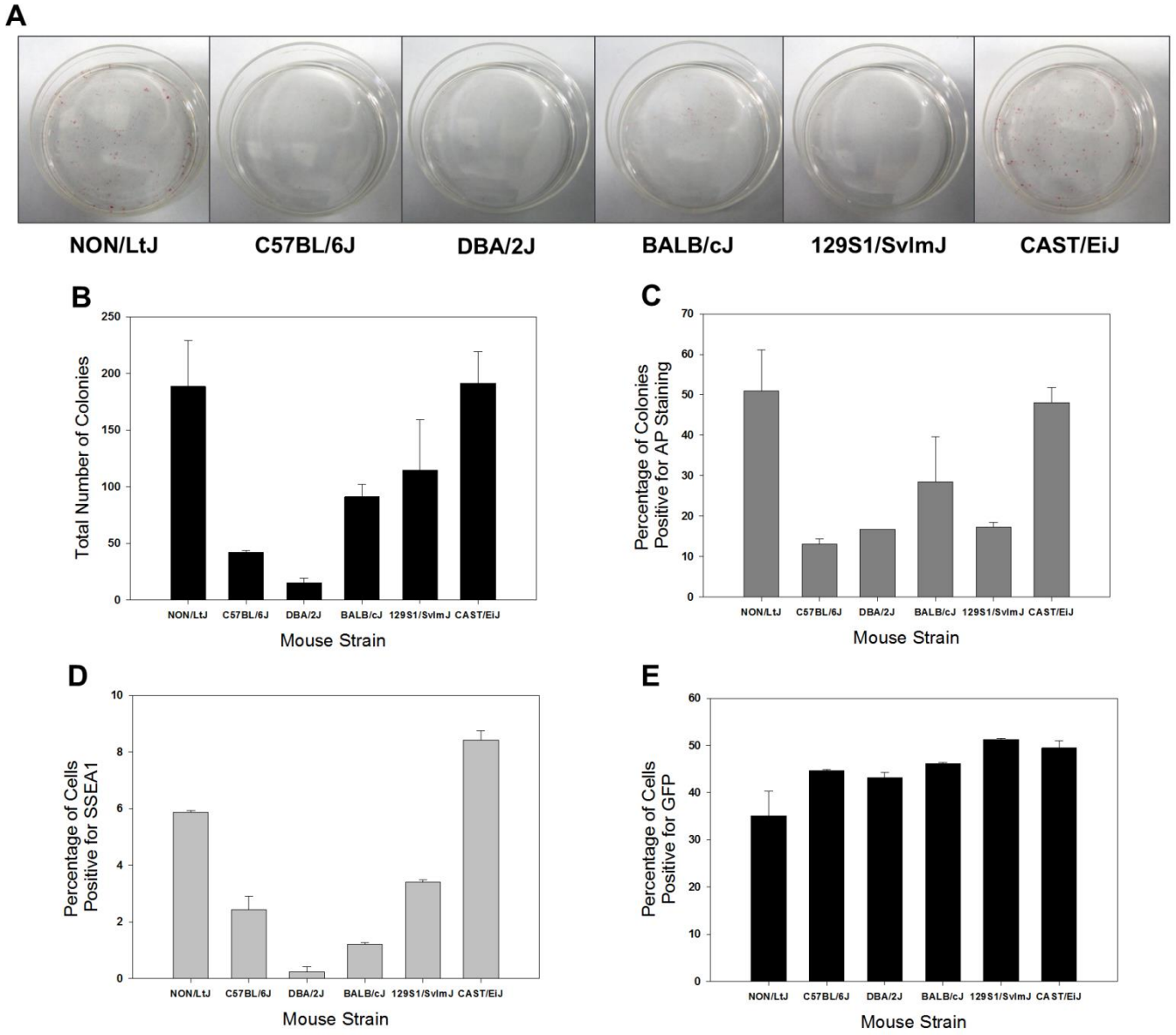
MEF proliferation assays*:* Proliferation of non-transduced parent P2 MEFs from each strain was examined every 2 days over a total of 10 days. $1.9x10^5$ MEFs were seeded on each 60mm tissue

culture plate to be cultured and later harvested at the indicated time points to perform cell counts. MEFs were maintained in standard MEF media for the first 24 hours and then the media was changed to ESC media supplemented with doxycycline to simulate reprogramming conditions for the remainder of the assay. Assays were performed using MEFs derived from 2 different embryos for each strain.

Results and Discussion

Strain differences in efficiency to generate iPSCs are manifested in the early stages of reprogramming: In order to assess potential strain background effects on iPSC generation during early reprogramming, the primary transformation and 60mm plates were evaluated for the total number of colonies, the percentage of colonies positive for AP staining, and the percentage of cells positive for SSEA1 and LIN28 expression. On both the 60mm plates in which the cells were used for AP staining and colony counting and the 6-well primary transformation plates in which the cells were used for flow cytometric analysis, gross differences in the generation of iPSC colonies were observed such that cells had to be stained and counted, or harvested for flow cytometry, after only 8 days of reprogramming in order to avoid overconfluency of cells from the most efficient strains (**Figure 2.2A**). This time point was much earlier than expected based on the doxycycline-inducible lentiviral reprogramming system literature in which colonies are generally passaged or picked off of primary transformation plates around 13-21 days for expansion and/or evaluation [27, 29-30], and stresses the differences that can be observed when using strains of diverse genetic backgrounds. Because the iPSCs were harvested at this very early time point of 8 days, the resultant LIN28 expression was negative in the iPSCs from all six strains and only SSEA1 expression was included in the final analysis. This finding is consistent

Figure 2.2. Strain differences during early iPSC reprogramming. Gross strain differences in efficiency to generate iPSC colonies were observed after 8 days of MEF reprogramming as visualized in these photographs with alkaline phosphatase (AP) staining (A). Strain differences were quantified by total number of colonies (B), percentage of colonies positive for alkaline phosphatase staining (C) and percentage of cells positive for SSEA-1 (D). NON/LtJ and CAST/EiJ strains were consistently more efficient than other strains in generating early iPSCs. These differences were not believed to be due to strain differences in MEF transducibility, as demonstrated by the percentage of cells positive for GFP on day 8 following transduction with a lentiviral GFP vector (E). Experiments were performed using MEFs derived from 2 different embryos for each strain (data presented as mean ± S.D.).


A
NON/LtJ
C57BL/6J
DBA/2J
BALB/cJ
129S1/SvlmJ
CAST/EiJ
B
Total Number of Colonies
250
200
150
100
50
0
NON/LtJ C57BL/6J DBA/2J BALB/cJ 129S1/SvlmJ CAST/EiJ
Mouse Strain
C
Percentage of Colonies Positive for AP Staining
70
60
50
40
30
20
10
0
NON/LtJ C57BL/6J DBA/2J BALB/cJ 129S1/SvlmJ CAST/EiJ
Mouse Strain
D
Percentage of Cells Positive for SSEA1
10
8
6
4
2
0
NON/LtJ C57BL/6J DBA/2J BALB/cJ 129S1/SvlmJ CAST/EiJ
Mouse Strain
E
Percentage of Cells Positive for GFP
60
50
40
30
20
10
0
NON/LtJ C57BL/6J DBA/2J BALB/cJ 129S1/SvlmJ CAST/EiJ
Mouse Strain

with the literature in which LIN28 is used as a marker for more established iPSCs and ESCs [31-33] as confirmed by our control iPSC (1-A4) and ESC (v6.4) lines.

During early reprogramming, MEFs from all of the strains formed cell colonies exhibiting typical iPSC morphology that were alkaline phosphatase (AP) positive within 8 days after the start of reprogramming. However, the total number of colonies and the percentage of AP-positive colonies varied dramatically between the strains. Notably, NON/LtJ and CAST/EiJ strains were more efficient than other strains (**Figures 2.2B and 2.2C**). Similarly, the percentage of cells positive for SSEA1 varied between the strains but paralleled the previous two parameters with NON/LtJ and CAST/EiJ having the highest percentage (**Figure 2.2D**). As expected, the percentage of cells positive for SSEA1 was low (between 0.11 and 8.64%) at this early time point of 8 days after the start of reprogramming for all strains. Using the same doxycycline-inducible lentiviral reprogramming system, Brambrink *et al.* previously demonstrated that SSEA1 expression appears between 3 and 9 days of reprogramming, whereas AP activity appears within 3 days of reprogramming [27]. Brambrink *et al.* also showed that after 9 days of reprogramming, about 7% of AP-positive cells were also SSEA1-positive [27]. This percentage of SSEA1 positive cells is consistent with our findings.

To ensure that the differences amongst strains in reprogramming efficiency were not due to differences in lentiviral infection, P2 MEFs were seeded on 6-well plates at the same density as they were for reprogramming, transduced with a lentiviral GFP vector (Addgene 14883, Cambridge, MA) and maintained under reprogramming conditions. After 8 days, the cells were trypsinized and the percentage of GFP positive cells was determined using flow cytometry. The percentage of GFP positive cells was very similar for all strains, ranging from 35.10±5.23% for NON/LtJ MEFs to 51.25±0.21% for 129S1/SvImJ1 MEFs, suggesting that the strain differences

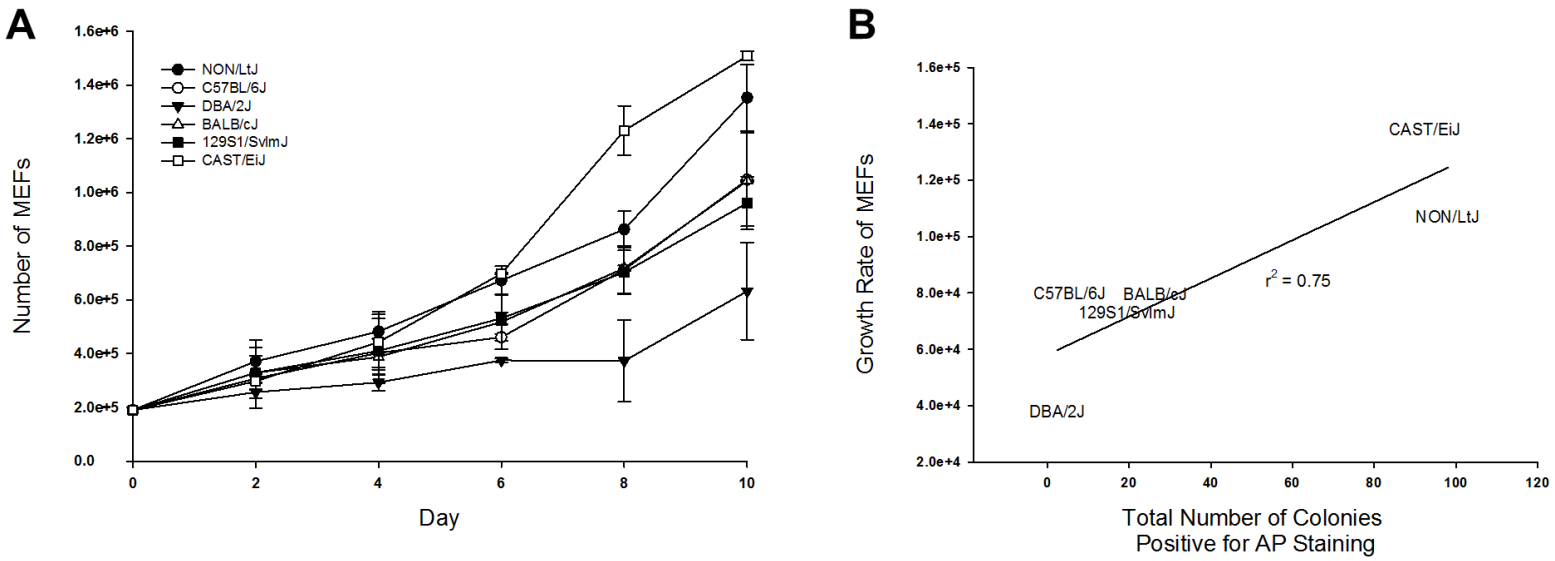
in efficiency to generate iPSCs were not due to strain differences in MEF transducibilty (Figure 2E).

Differences in proliferation of parent non-transduced MEFs correlate with differences in efficiency to generate iPSCs during early reprogramming: Proliferation of non-transduced parent P2 MEFs was examined every 2 days over a total of 10 days in order to determine if genetic differences in MEF proliferation could potentially be affecting the efficiency of iPSC generation (**Figure 2.3A**). Strain differences in MEF proliferation were observed over the 10 day period and a positive correlation was found between MEF growth rate and efficiency to generate iPSCs during early reprogramming. This is demonstrated in **Figure 2.3B** where MEF growth rate and total number of colonies positive for AP staining are compared with a resultant r^2 value of 0.75. In particular, NON/LtJ and CAST/EiJ MEFs were the most proliferative and most efficient in generating iPSCs while DBA/2J MEFs were the least.

Interestingly, fibroblasts capable of increased proliferation through *Trp53* deletion have increased iPSC generation efficiency [34-36]. It is possible that MEFs of the most efficient strains found in this study, NON/LtJ and CAST/EiJ, have a reduced rate of senescence compared to the other strains which is allowing for more effective reprogramming. The fact that the most proliferative MEFs were of the CAST/EiJ strain is also of interest as this wild-derived inbred strain is the most genetically distinct strain that we examined.

The finding of this study that cellular proliferation rate is correlated with iPSC generation efficiency is consistent with those of Ruiz *et al.* in which the induction of cellular proliferation (through downregulation of pRb) increased human iPSC reprogramming efficiency [36]. In that study, Ruiz et al. also elegantly demonstrated that cell cycle arrest (through induction of the arrest inducers p15, p16, or p21) inhibits reprogramming and actually drives iPSCs towards

Figure 2.3. Strain differences in MEF proliferation. Strain differences in MEF proliferation were observed over 10 days and were well correlated with the observed strain differences in efficiency to generate iPSCs during early reprogramming. Non-transduced parent P3 MEFs from each strain were seeded at $1.9x10^5$ cells per 60mm tissue culture plate on Day 0 and then counted every 2 days over a total of 10 days (A). MEFs were maintained in standard MEF media for the first 24 hours after which the media was changed to ESC media supplemented with doxycycline to simulate reprogramming conditions. MEFs derived from 2 different embryos were evaluated for each strain and (data presented as mean ± S.D.). The growth rate of the MEFs from each strain was determined from the slope of the linear regression curve fitted to the data set in (A) for each strain. The growth rate of each strain was then plotted against the total number of colonies positive for AP staining and a line of best fit determined, revealing a moderately strong positive correlation between iPSC generation efficiency and MEF proliferation as indicated by the r^2 value (B).


A
Number of MEFs
Day
NON/LtJ
C57BL/6J
DBA/2J
BALB/cJ
129S1/SvImJ
CAST/EiJ
1.6e+6
1.4e+6
1.2e+6
1.0e+6
8.0e+5
6.0e+5
4.0e+5
2.0e+5
0.0
0
2
4
6
8
10
B
Growth Rate of MEFs
Total Number of Colonies
Positive for AP Staining
CAST/EiJ
NON/LtJ
C57BL/6J
BALB/cJ
129S1/SvImJ
DBA/2J
r2 = 0.75
1.6e+5
1.4e+5
1.2e+5
1.0e+5
8.0e+4
6.0e+4
4.0e+4
2.0e+4
0
20
40
60
80
100
120

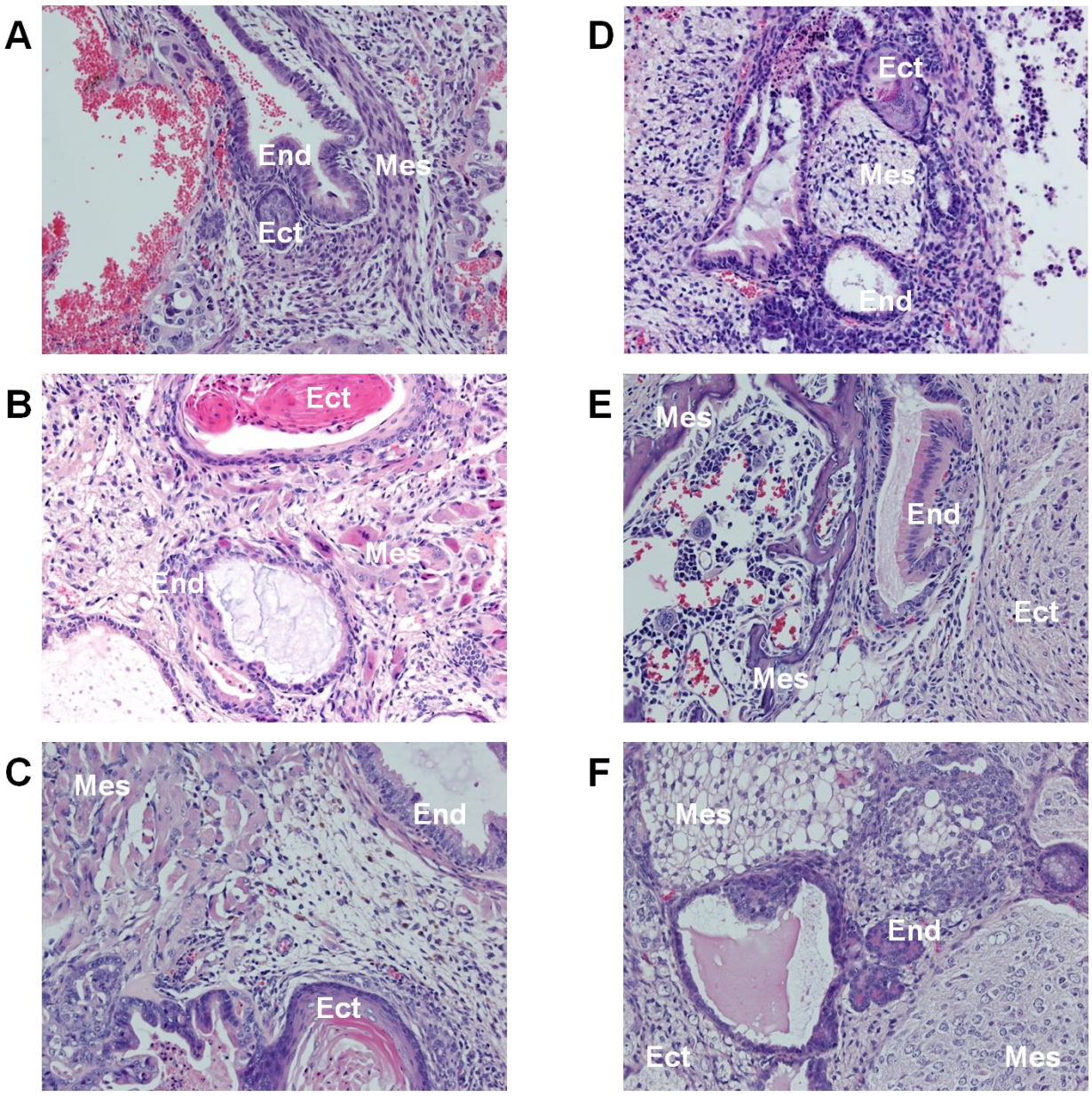
irreversible differentiation [37]. A potential follow-up study to this one in order to further elucidate the mechanisms behind the differences in genetic background effects on iPSC efficiency would be to alter the cellular proliferation of the MEFs for each strain, either through induction or arrest, and then examine the iPSC generation efficiency.

BALB/cJ iPSCs require a higher concentration of LIF than other strains for cell line expansion and doxycycline independence*:* In order to determine if genetic background affects the pluripotent stability of iPSCs during later stages of reprogramming, iPSC lines from all six strains were established and further expanded without doxycycline supplementation. Doxycycline-independent iPSC lines could be established using our standard conditions and ESC media for all strains except BALB/cJ, which were established only when supplemented with a higher concentration (5x) of LIF. This finding suggests that BALB/cJ iPSCs may have reduced pluripotent stability and is consistent with the BALB/cJ ESC literature in which BALB/cJ ESC lines were established only when using a 5x higher concentration of LIF than that needed for other strains [5-7]. The mechanism behind this requirement for increased LIF supplementation in BALB/cJ cells has yet to be identified.

Doxycycline-independent cell lines from all strains are capable of producing teratomas in SCID mice: Doxycycline-independent cell lines from all the strains were capable of producing teratomas in NOD.CB17-*Prkdc*^{scid}/J mice by 5 weeks post injection, thereby demonstrating pluripotency (**Figure 2.4**). For all strains, the cell lines were between P10 and P15 and were the initial cell lines chosen for the teratoma assay. None of the cell lines from any of the strains required a repeat set of injections or the assay to be repeated with a different cell line.

iPSC lines in this study were not evaluated for their ability to generate chimeras or for germline competence, making direct comparison to the ESC literature on the effect of genetic

Figure 2.4. Pluripotency of iPSCs derived from MEFs of each strain. Doxycycline-independent cell lines from all the strains were capable of producing teratomas in NOD SCID mice by 5 weeks post injection as shown in these histologic images, all of which are stained with hematoxylin and eosin and viewed at 200x magnification. (A) = NON/LtJ; (B) = C57BL/6J; (C) = DBA/2J; (D) = BALB/cJ; (E) = 129S1/SvlmJ; (F) = CAST/EiJ. Tissues from all three germ layers were identified on each section as indicated by the labels: Ect = Ectoderm; Mes = Mesoderm; End = Endoderm.


A
End
Mes
Ect
D
Ect
Mes
End
B
Ect
Mes
End
E
Mes
End
Ect
Mes
C
Mes
End
Ect
F
Mes
End
Ect
Mes

background difficult beyond the finding of reduced pluripotent stability already discussed for the BALB/cJ strain. While the 129S1/SvlmJ strain was moderately efficient in generating IPSCs compared to the other strains in this study and the 129S1/SvlmJ iPSC line was readily able to form teratomas in NOD.CB17-*Prkdc*^{scid}/J mice, a conclusion cannot be drawn from this data as to whether or not this strain is as useful for generating iPSCs as it has been shown to be for generating ESCs for transgenic applications [6,7]. In addition, the two most efficient strains in this study, NON/LtJ and CAST/EiJ, are strains which have not been examined for their ability to generate ESCs, making them intriguing candidates for future studies.

Conclusions

Our comparison of six different inbred mouse strains has revealed that genetic background does affect both the efficiency of generating iPSCs during the early stages of reprogramming as well as the pluripotent stability of the cells during later stages of reprogramming. These findings suggest that genetic background must be considered when interpreting results of iPSC studies in the literature and that iPSC derivation may need to be customized for different strains. In addition, our findings suggest that the proliferation rate of the fibroblasts is positively correlated with iPSC generation, suggesting a possible simple laboratory screening parameter to predict iPSC generation efficiency.

The two most efficient strains in this study, NON/LtJ and CAST/EiJ, may prove useful in the future for deriving iPSCs for transgenic purposes, as iPSCs from these strains appear to be robust. One limitation to this study, however, was that we did not evaluate the iPSCs lines for their ability to generate chimeras and for germline competence. This information is essential for ultimately determining which strain may be most beneficial for transgenic applications.

In conclusion, we have shown that there are strain differences in efficiency to generate iPSCs during the early stages of reprogramming and that these strain differences are correlated with those observed in proliferation of parent MEFs. We have also shown that there are strain differences in pluripotent stability as far as ability to expand iPSC lines and achieve doxycycline independence. These findings have important implications both for future iPSC applications as well as for future investigation into determining the genes responsible for reprogramming efficiency and stability. It is possible that the Collaborative Cross, of which three of the strains examined in this study are progenitors, could be used to identify such genes.

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CHAPTER 3

IMMUNOGENIC AND IMMUNOMODULATORY PROPERTIES OF MURINE INDUCED PLURIPOTENT STEM CELLS COMPARED TO BONE MARROW-DERIVED MESENCHYMAL STEM CELLS

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Abstract

The immunogenic properties of induced pluripotent stem cells (iPSCs) are currently being investigated. Clinically, banked iPSCs will be necessary for most applications due to the extended time period needed to generate iPSCs and to screen them for efficacy and safety. In addition, autologous iPSC therapy may not be possible for some individuals because of the influence of genetic background on iPSC generation. The immunogenic and immunomodulatory properties of mesenchymal stem cells (MSCs) are investigated for many of the same reasons and it has been demonstrated that they possess significant immunomodulatory properties. The purpose of this study was to evaluate the in vitro immunogenic and immunomodulatory properties of iPSCs compared to adult bone marrow-derived MSCs using modified mixed leukocyte reactions. Our comparisons revealed that iPSCs generated through both lentiviral and *piggyBac* reprogramming methods had similar immunogenic properties as MSCs but had more potent immunomodulatory effects than MSCs. Co-culture of MHC-mismatched leukocytes with MHC-matched iPSCs resulted in significantly less responder T cell proliferation than observed for MHC-mismatched leukocytes alone and at more responder leukocyte concentrations tested than was observed for co-culture of MHC-mismatched leukocytes with MHC-matched MSCs. In addition, MHC-mismatched iPSCs were able to significantly reduce responder T cell proliferation when co-cultured with MHC-mismatched leukocytes, while MHC-mismatched MSCs were not. These results provide important information when considering the use of iPSCs in place of MSCs in both regenerative and transplantation medicine.

Introduction

Upon their creation in 2006, induced pluripotent stem cells (iPSCs) were regarded as the most promising stem cell candidate for the clinical application of regenerative therapies [1-3]. iPSCs are pluripotent, unlike mesenchymal stromal cells (MSCs), and can be used autologously, unlike pluripotent embryonic stem cells (ESCs). Additionally, iPSCs avoid the ethical concerns surrounding the isolation and use of human ESCs [4]. Since that time, however, many concerns have been raised over the safety of iPSCs in terms of genetic instability, tumorigenic potential, and immunogenic potential [4-9]. It has become evident that iPSC lines must be thoroughly screened for stability, safety, and efficacy prior to clinical application [7]. Such screening, after an already lengthy generation process, makes autologous iPSC use impractical for many of diseases that would potentially benefit from stem cell therapy. In addition, it has been demonstrated that genetic background affects iPSC generation, suggesting that autologous iPSC therapy may not be feasible for some patient regardless of timing issues [10]. For these reasons, the immunogenicity of iPSCs is of particular concern as the need for having a bank of previously screened cells has become a reality [7, 11].

In a similar interest of having cells available at the time of diagnosis for immediate treatment, the immunogenic and immunomodulatory properties of MSCs have been, and continue to be investigated [12-18]. It has also been demonstrated that both genetic background and age of the patient affect proliferation and differentiation rates of MSCs, suggesting that allogeneic MSC therapy may be required for some patients as is possible for iPSCs [19-21]. Adult MSCs have low immunogenicity when used autologously and possess significant immunomodulatory properties [13, 15, 22-25]. Many mechanisms for the immunosuppressive effects of MSCs have been described including inhibition of T cell proliferation, alteration of

dendritic cell maturation, induction of regulatory lymphocytes, and apoptosis of CD8 positive T cells [15, 22, 26-28]. While it was initially believed and demonstrated that allogeneic MSCs are immune privileged due to these immunosuppressive properties [29-32], immune rejection of allogeneic MSCs has also been reported [33-38]. The finding that MSCs are capable of alterations in their MHC class I and II expression profiles is likely to blame for these conflicting results. While adult MSCs generally display the phenotype of high MHC class I expression and low or negative MHC class II expression, MSCs from mice, humans, and more recently horses with high MHC II expression levels have also been described [33, 34, 39, 40]. Additionally, both MHC class I and class II expression levels on MSCs can be upregulated by proinflammatory cytokines such as IFN-ɣ [41, 42].

Investigation into the immunogenic properties and immune plasticity of iPSCs has just recently begun [7, 11, 43-45]. It is known that undifferentiated iPSCs, like ESCs, express low or absent levels of MHC class I and are negative for MHC class II expression [46]. Interestingly, MHC class II expression is not upregulated by differentiation or by stimulation with IFN-ɣ [46-48]. The extent to which MHC class I expression can change upon iPSC differentiation or stimulation with proinflammatory cytokines, however, is incompletely understood. Several studies have shown that MHC class I expression increases upon iPSC differentiation and with IFN-ɣ stimulation, but often only to a level still much less than that of somatic cells [44, 46]. The consequence of such a change in MHC class I expression is complex as a high expression level of MHC class I could lead to T cell activation while a continued lack of MHC class I expression could potentially lead to iPSCs being targeted by natural killer (NK) cells in vivo [6]. Conflicting results have been reported for ESCs on this subject, with some groups reporting ESCs as susceptible to NK cell lysis, and others reporting that ESCs are neither susceptible to

NK cell lysis nor capable of eliciting T cell responses [6, 49]. It is likely that culture conditions or differences in ESC lines could have affected these results.

It is not surprising that conflicting results have also been reported on the immunogenicity of iPSCs, as iPSCs are in many ways more variable than ESCs, particularly due to discrepancies in reprogramming methods including viral vs. non-viral and integrating vs. non-integrating [43-45, 47, 50, 51]. While the first report on the immunogenicity of iPSCs revealed that undifferentiated autologous (syngeneic) mouse iPSCs were immune rejected in a teratoma model study [43], two other reports since then have shown that both undifferentiated and differentiated syngeneic mouse iPSCs are non-immunogenic in vitro and in vivo [44, 45]. To date, no studies have examined the immunomodulatory properties of iPSCs even though it is known that ESCs are capable of immunosuppression through multiple mechanisms including expression of arginase I [47, 52], prevention of dendritic cell maturation [53], and upregulation of regulatory T cells [47, 54]. When considering the use of iPSCs as an alternative for MSC therapy, this information is critical. The purpose of this study, therefore, was to evaluate the in vitro immunogenic and immunomodulatory properties of iPSCs compared to adult bone marrow-derived MSCs using modified mixed leukocyte reactions. Our hypothesis, based on prior ESC knowledge, was that undifferentiated iPSCs would have similar immunogenic and immodulatory properties as MSCs.

Materials and Methods

***Mice*:** Male and female mice of the C3HeB/FeJ (MHC H*2* haplotype *k*) and C57BL/6J (MHC H*2* haplotype *b*) inbred strains were purchased from The Jackson Laboratory (Bar Harbor, ME). For each strain, mice were bred to produce offspring needed to harvest different cell types and

perform experiments. NOD.CB17-*Prkdc*^{scid}/J mice, used for teratoma formation assays, were also purchased from The Jackson Laboratory (Bar Harbor, ME). The use of mice in this study was approved by the Institutional Animal Care and Use Committee of Cornell University.

***Mouse embryonic fibroblast (MEF) isolation*:** Embryonic day 13.5 C3HeB/FeJ and C57BL/6J embryos were collected and processed to generate mouse embryonic fibroblasts (MEFs) from each strain as previously described [10]. MEFs were cultured in MEF media (high glucose Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum (FBS), penicillin (100 units/mL), and streptomycin (100μg/mL)) and cryopreserved at passage 1 (P1) to be used for iPSC generation and at P2 to be used as controls in MLR experiments. MEFs to be used as feeder cells from each strain were culture expanded, irradiated with 30 Gy from a Cs-137 source, and cryopreserved.

***Lentiviral reprogramming of MEFs*:** Lentiviral supernatant generation and reprogramming of MEFs was performed as previously described by our laboratory [10] using vectors for doxycycline-inducible transgene expression of the mouse factors *Oct4*, *Sox2*, *Klf4* and *c-Myc*. All plasmids were purchased from Addgene (Cambridge, MA). Briefly, P1 MEFs from each strain were thawed and cultured in MEF media for 24-48 hours, after which they were trypsinized and counted. The P2 MEFs were seeded onto gelatin-coated 6-well tissue culture plates at a density of $6.75x10^3$ cells/cm^2 in MEF media and allowed to adhere for 24 hours. The culture media was then replaced with fresh MEF media supplemented with viral supernatant for an additional 24 hours. Following incubation with viral media, MEFs were trypsinized and passaged onto 60mm tissue culture plates seeded with feeder cells of the same strain. Culture media at this time was changed to ESC media (KnockOut™ DMEM (Gibco, Grand Island, NY) supplemented with 15% KnockOut™ Serum Replacement (Gibco, Grand Island, NY),

recombinant LIF, MEM non-essential amino acids solution (100µm), 2mM GlutaMAX™ (Gibco, Grand Island, NY), 0.1 mM 2-Mercaptoethanol, penicillin (100 units/mL), streptomycin (100µg/mL), and doxycycline (2µg/mL; Sigma, St. Louis, MO)). ESC media was refreshed daily during reprogramming.

***PiggyBac reprogramming of MEFs*:** P2 MEFs were transfected with the Nucleofector® II electroporation device (Amaxa Biosystems, Gaithersburg, MD) set on program A-023. Each electroporation was performed in a 2mm cuvette (Amaxa Biosystems, Gaithersburg, MD) with $2x10^6$ cells and a DNA mixture of 1µg each of the *piggyBac* plasmids PB-TET-MKOS, PB-CAG-rtTA, and PB-CAG-GFP (kindly provided by the laboratory of Dr. Nagy [55]) as well as 1µg of the transposase expression vector pCyL43 (Wellcome Trust Sanger Institute, Cambridge, UK) in a total volume of 100µL Ingenio® electroporation solution (Mirius Bio, Madison, WI). Following electroporation, cells from each cuvette were seeded onto a 100mm tissue culture plate in MEF media. After 24 hours, culture media was changed to ESC media.

***iPSC line generation*:** Lentiviral and *piggyBac* iPSC colonies were picked with pipette tips and culture expanded on feeder cells in ESC media as previously described [10]. Lentiviral iPSC colonies were picked on day 7-11 of reprogramming, while *piggyBac* iPSC colonies were picked on day 17-22 post-transfection. Doxycycline was removed from the media around P7 and doxycycline-independent cell lines were then further expanded (P10-P12) in order to reach the cell numbers necessary for teratoma formation assays and cryopreservation of stock from each strain. In preparation for MLR experiments, iPSC cell lines from each strain were then further cultured in modified RPMI 1040 media containing 10% FBS, 0.1 mM 2-Mercaptoethanol, penicillin (100units/mL), streptomycin (100µg/mL), and ESGRO® LIF (1uL/mL; Millipore,

Billerica, MA). Following this transition to modified RPMI 1040 media, teratoma assays were again performed.

Teratoma formation and histological analysis: iPSC lines from each strain were trypsinized, pelleted and suspended at $1x10^7$ cells/mL in a 1:3 solution of Matrigel™ (BD Biosciences, San Jose, CA) to MEF media. 150µl of the cell suspension ($1.5x10^6$ cells) was injected subcutaneously into the flank of a NOD.CB17-*Prkdc*^{scid}/J mouse. For each cell line, a total of 2-4 injections were performed. Four to 5 weeks post injection, tumors were surgically dissected, fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. All histologic sections were reviewed by a board certified veterinary pathologist (T.L.S.) for teratoma formation.

***Bone marrow harvest and isolation of MSCs*:** Ten female mice 3-6 weeks of age from each strain were euthanized, prepared with ethanol, and processed for bone marrow harvest according to a protocol kindly provided by the laboratory of Dr. Rocky S. Tuan. Briefly, the hindlimbs were skinned, disarticulated from the pelvis, and placed in a petri dish with MEMα media with nucleosides and L-glutamine (Gibco, Grand Island, NY) containing 10% FBS, penicillin (100units/mL), streptomycin (100µg/mL), and Fungizone® (0.25µg/mL; Gibco, Grand Island, NY). All muscle and tissue was removed from the bone using a scalpel blade and placed in a second petri dish with phosphate buffered saline (PBS). Next, the ends of the long bones were cut off so that the marrow cavity was exposed. The marrow cavity of each bone was then flushed with MEMα media using a 27g needle and 12mL syringe into an empty petri dish. The bone marrow cell suspension was then passed through a 70µm cell strainer (BD Biosciences, San Jose, CA), pellets, resuspended in red blood cell lysis buffer (0.84% NH4Cl), and incubated for 2 minutes on ice. Following the incubation, cells were washed with MEMα media, counted, and

seeded at $25x10^6$ cells/mL onto 100mm tissue culture plates with MEMα media. After 5 hours in the incubator, the media was removed, the plates gently washed to remove non-adherent cells, and new MEMα media containing FGF-2 (5ng/mL) added. For the next 72 hours, media exchange occurred every 12 hours; afterwards media exchange occurred every 72 hours. When the cells were approximately 80% confluent, they were trypsinized at room temperature for 2 minutes, counted, and seeded at 5,000 cells/cm^2 onto T-75 flasks with MEMα media containing FGF-2 (5ng/mL). The cells were then expanded to P2 and P3 and stocks were cryopreserved for immunophenotyping and MLR experiments.

***Immunophenotyping of MEFs, iPSCs, and MSCs*:**

MEFs, iPSCs, and MSCs were immunophenotyped for expression levels of MHC class I and MHC class II. MSCs were additionally phenotyped for a panel of positive (CD4, CD29) and negative (CD45, CD117) markers using flow cytometry [56-58]. MHC class I (rat anti-mouse; PE-conjugated), MHC class II (rat ant-mouse; PE-Cy5-conjugated), and CD29 (hamster anti-mouse; PE-conjugated) antibodies were purchased from eBioscience (Affymetrix, Santa Clara, CA). CD44 (rat anti-mouse; FITC-conjugated), CD45 (rat anti-mouse; PerCP-Cy5.5), and CD117 (rat anti-mouse; APC-conjugated) antibodies were purchased from BD Biosciences (San Jose, CA). Cells were pelleted in aliquots containing approximately $1x10^6$ cells on 96-well V-bottom plates and treated with a 10 min blocking step using anti-mouse CD16/CD32 (Fc Block™; BD Biosciences, San Jose, CA) at 1:100 in PBS. The cells were pelleted and resuspended in conjugated primary antibody and incubated for 45 min at 4°C. Cells were then washed, resuspended in PBS and analyzed on a FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) flow cytometer equipped with 488 μm argon and 635 μm red diode lasers and BD Cell Quest™ analysis software (BD Biosciences, San Jose, CA).

Cells exposed to appropriately conjugated rat or hamster IgG was used as negative isotype controls. Data were collected on $2x10^4$ cells for each sample.

***Splenocyte isolation and leukocyte purification*:** Spleens were aseptically harvested from C3HeB/FeJ and C57BL/6J female mice 6-12 weeks of age and dissociated in RPMI 1040 media (Gibco, Grand Island, NY) using a cell dissociation sieve equipped with a 40 mesh screen (Sigma-Aldrich, St. Louis, MO). The resultant splenocyte suspension was then passed through a 100μm cell strainer (BD Biosciences, San Jose, CA), pelleted, resuspended in red blood cell lysis buffer (0.84% NH_4Cl) and incubated for 5 minutes at room temperature with rocking. Following red blood cell lysis, the suspension was washed with PBS, pelleted, and purified using Lympholyte®-M density gradient centrifugation (Cedarlane Laboratories, Burlington, NC) according to the manufacturer directions to obtain leukocytes. Cells destined to be stimulator leukocytes in mixed leukocyte reactions were aliquoted at this time. The remaining leukocyte suspension was then plated onto 100mm tissue culture plates in RPMI 1040 medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS), 0.1 mM 2-Mercaptoethanol, penicillin (100 units/mL) and streptomycin (100units/mL). After 2 hours, the non-adherent cells were removed from the plates, pelleted, and counted. Non-adherent leukocytes then underwent positive selection for CD62L, a naïve T cell marker [59-62], using MACS CD62L microbeads and LS columns (Miltenyi Biotec, Auburn, CA) according to the manufacturer directions. Adherent leukocytes (containing antigen presenting cells (APCs)) were dissociated using Accumax® cell dissociation solution (Innovative Cell Technologies Inc, SanDiego, CA), counted, and aliquoted. All leukocytes were used fresh in mixed leukocyte reactions.

***Modified one-way mixed leukocyte reactions (MLRs)*:** Modified one-way mixed leukocyte reactions (MLRs) were performed in duplicate on 24-well tissue culture plates using MHC-

matched and mismatched C3HeB/FeJ responder leukocytes and C3HeB/FeJ and C57BL/6J stimulator leukocytes, MEFs, iPSCs, and MSCs. In order to assess the immunogenic potential of the cells, MEFs, iPSCs, and MSCs were used as stimulator cells for responder leukocytes. In order to assess the immunomodulatory properties of the cells, MEFs, IPSCs, and MSCs were cultured in the presence of stimulator and responder leukocytes. MHC-matched stimulator leukocytes were used to establish baseline T cell proliferation and MHC-mismatched stimulator leukocytes were used as positive MLR controls. MEFs were considered the negative control in the immunomodulatory potential studies. C3HeB/FeJ responder leukocytes were labeled with 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE (0.13μg/ml, Sigma-Aldrich, St. Louis, MO) and examined at 4 different concentrations ($2x10^5$, $4x10^5$, $8x10^5$, and $1.2x10^6$ cells/well). Proliferative ability of responder cells was verified via mitogen stimulation with phytohemaglutinin (PHA-P (5μg/ml, Sigma-Aldrich, St. Louis, MO)). Stimulator MEFs, iPSCs, and MSCs were plated 24 hours prior to the addition of responder leukocytes in their appropriate media such that all cells would be approximately 80% confluent by the end of the experiment. MEFs were seeded at $1x10^4$ cells/well, iPSCs (removed from feeders) at $7.5x10^4$ cells/well, and MSCs at $3x10^4$ cells/well. Stimulator leukocytes were irradiated with 9 Gy from a Cs-137 source to inhibit proliferation and plated at $1.6x10^6$ cells/well. Responder APCs (adherent cells during isolation) were plated at $1x10^5$ cells/well. Importantly, responder leukocytes and APCs were mixed with stimulator leukocytes prior to plating due to concern that the 3-dimensional nature of the iPSC colonies could interfere with responder and stimulator cell contact. The resultant ratios of responder:stimulator cells was based on previously published experimental protocols and determined to be optimal for these studies in preliminary experiments [63-65]. Cultures were maintained for 5 days with modified RPMI 1040 media (1.5 mL/well) containing

10% FBS, 0.1mM 2-Mercaptoethanol, penicillin (100units/mL), streptomycin (100μg/mL), and ESGRO® LIF (1uL/mL; Millipore, Billerica, MA). Media were not exchanged over the 5 days. Following culture, leukocytes were aspirated from the wells and stained with a hamster anti-mouse APC-conjugated CD3 antibody (Abcam, Cambridge, MA). The antibody staining process for flow cytometry analysis was performed as described above for immunophenotyping.

Proliferation of gated CFSE-labeled CD3-positive responder T cells was evaluated via CFSE attenuation using flow cytometry. Cells were first gated on FL4 so that only the CD3-positive cells (T cells) were then examined on FL1 for CFSE attenuation. Non-stimulated responder T cells were used to set the boundary of non-proliferating cells such that all cells to the left (lower fluorescence intensity on FL1) of that boundary were determined to be proliferating. Because the number of cell counts in the proliferating T cell gate was measured, data was collected on the entirety of each sample.

MLRs were performed in a total of 2 experiments. MEFs from 2 different embryos of each strain were tested in addition to 2 iPSC lines (1 lentiviral and 1 *piggyBac*) from each strain and batched MSCs from each strain. Due to naturally occurring variation in leukocyte responses between mice and experiments, the relative T cell proliferation was reported as the fold change from that of the MHC-matched MLR for the immunogenic potential experiments (i.e. looking for an increase from baseline T cell proliferation potential if immunogenic) and as the fold change from that of the MHC-mismatched MLR for the immunomodulatory potential experiments (i.e. looking for a decrease from positive control T cell proliferation towards the baseline T cell proliferation if immunomodulatory). For the immunomodulatory potential experiments, data were also reported as the percentage proliferation of that of the MHC-mismatched MLR.

Statistical analyses: MLR data for lentiviral and *piggyBac* iPSC lines were first compared using two-sample t-tests. All MLR data were normalized by long transformation and analyzed with analysis of covariance (ANCOVA), with experiment as a covariate, followed by the Tukey test for multiple comparisons. All analyses were performed using Statistix 9 software (Analytical Software, Tallahassee, FL) and significance was set at $P < 0.05$.

Results

***iPSC line generation and validation*:** Multiple doxycycline-independent lentiviral and *piggyBac* iPSC lines were established from each strain and early passage stocks were cryopreserved. One doxycycline-independent lentiviral iPSC line and 1 doxycycline *piggyBac* iPSC line from each strain was tested after expansion in ESC media and then again after expansion in modified RPMI 1040 media. These lines were capable of producing teratomas in NOD.CB17-*Prkdc*^{scid}/J mice by 5 weeks post injection, thereby confirming pluripotency and lack of alteration due to the change in media.

Immunophenotyping: MEFs had a phenotype of MHC class I positive (low) and MHC class negative, while iPSCs had a phenotype of both MHC class I and class II negative. MSCs were positive for expression of MHC class I, CD44, and CD29 and negative for expression of MHC class II, CD45, and CD117.

***Modified one-way mixed leukocyte reactions (MLRs)*:** There were no significant differences in responder T cell proliferation when stimulated by lentiviral iPSCs or *piggyBac* iPSCs, or when stimulated by MHC-mismatched leukocytes in the presence of lentiviral iPSCs or *piggyBac* iPSCs (as determined by two-sample t-tests with significance set at $P < 0.05$). The cells were therefore considered one group and are collectively referred to as iPSCs for the remainder of the results.

As predicted based on MHC class II expression, all of the cell types tested (MEFs, iPSCs, and MSCs) had low immunogenicity when either MHC-matched or MHC-mismatched with the responder leukocytes (**Figures 3.1 and 3.2**). MSCs resulted in the highest levels of responder T cell proliferation compared to MEFs and iPSCs, particularly when MHC-mismatched, but these levels were not significantly different from MEFs or iPSCs at any of the responder leukocyte concentrations tested, even when statistically equivalent to the positive control of MHC-mismatched leukocytes (**Figures 3.2A and 3.2G**).

Both iPSCs and MSCs cultured in the presence of MHC-mismatched responder and stimulator leukocytes (MHC-mismatched MLR) resulted in reduction of responder T cell proliferation from that observed for the MHC-mismatched MLR alone towards the baseline proliferation value observed for the MHC-matched MLR (**Figures 3.3 and 3.4**). This reduction of responder T cell proliferation was greatest when the iPSC and MSCs were MHC-matched with the responder leukocytes compared to when MHC-mismatched with responder leukocytes. MHC-matched iPSCs resulted in significantly decreased responder T cell proliferation compared to both the MHC-mismatched MLR and the negative control of MEFs at the 3 highest responder leukocyte concentrations (**Figures 3.4B - 3.4D**). While MHC-matched MSCs resulted in statistically equivalent responder T cell proliferation compared to iPSCs for the same 3 responder leukocyte concentrations, mean T cell proliferations were greater than for iPSCs and in some cases also equivalent to the MHC-mismatched MLR and/or the negative control of MEFs (**Figures 3.4B - 3.4D**). When MHC-mismatched cell types were evaluated, only iPSCs were able to reduce responder T cell proliferation significantly from the MHC-mismatched MLR at the responder leukocyte concentration of $8x10^5$ cells (**Figure 3.4G**). Once again, while MHC-mismatched MSCs resulted in statistically equivalent responder T cell proliferation

Figure 3.1. Immunogenicity of MHC-matched (A) and MHC-mismatched (B) MEFs, iPSCs, and MSCs as determined by responder T cell proliferation in modified one-way mixed leukocyte reactions. Data are presented as the log fold change of the MHC-matched MLR which was considered the baseline responder T cell proliferation value. Bars represent mean ± S.D. from a total of 2 separate experiments performed with multiple cell lines. MHC-M = MHC-matched; MHC-MM = MHC-mismatched. None of the cell types tested approached the responder T cell proliferation of that of the positive control of MHC-mismatched leukocytes (MHC-MM MLR). Statistical data is shown in **Figure 3.2**.

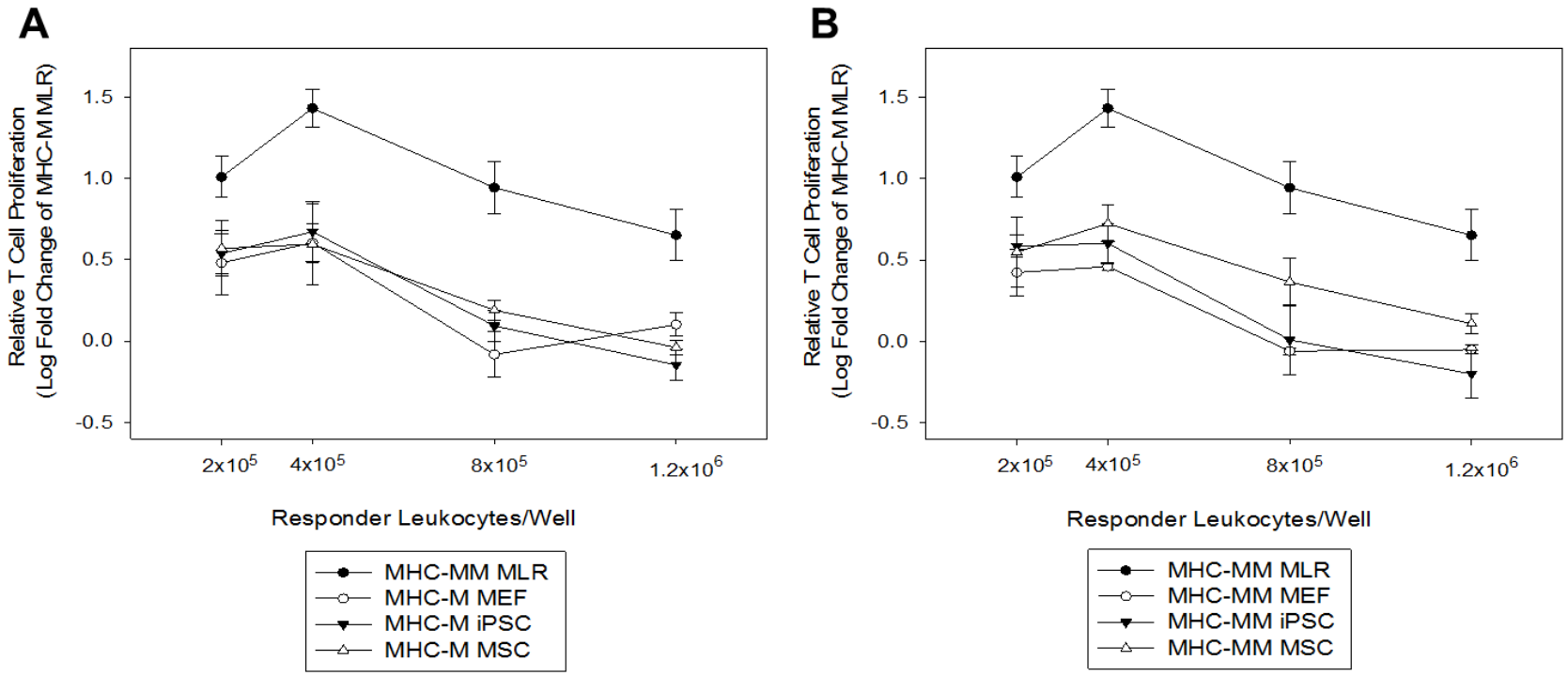

A
Relative T Cell Proliferation
(Log Fold Change of MHC-M MLR)
1.5
1.0
0.5
0.0
-0.5
2x10^5
4x10^5
8x10^5
1.2x10^6
Responder Leukocytes/Well
MHC-MM MLR
MHC-M MEF
MHC-M iPSC
MHC-M MSC
B
Relative T Cell Proliferation
(Log Fold Change of MHC-M MLR)
1.5
1.0
0.5
0.0
-0.5
2x10^5
4x10^5
8x10^5
1.2x10^6
Responder Leukocytes/Well
MHC-MM MLR
MHC-MM MEF
MHC-MM iPSC
MHC-MM MSC

Figure 3.2. Modified one-way mixed leukocyte reaction (MLR) results for MHC-matched cells (A-D) and MHC-mismatched cells (E-H) at the different responder leukocyte concentrations tested. Bars represent mean ± S.D. from a total of 2 separate experiments performed with multiple cell lines. Superscript letters indicate significant differences between groups by ANCOVA, with experiment as a covariate, followed by the Tukey test for multiple comparisons, $P < 0.05$. MHC-M = MHC-matched; MHC-MM = MHC-mismatched. MSCs resulted in the highest levels of responder T cell proliferation compared to MEFs and iPSCs, particularly when MHC-mismatched, but these levels were not significantly different from MEFs or iPSCs at any of the responder leukocyte concentrations tested, even when statistically equivalent to the positive control of MHC-mismatched leukocytes (A and G).

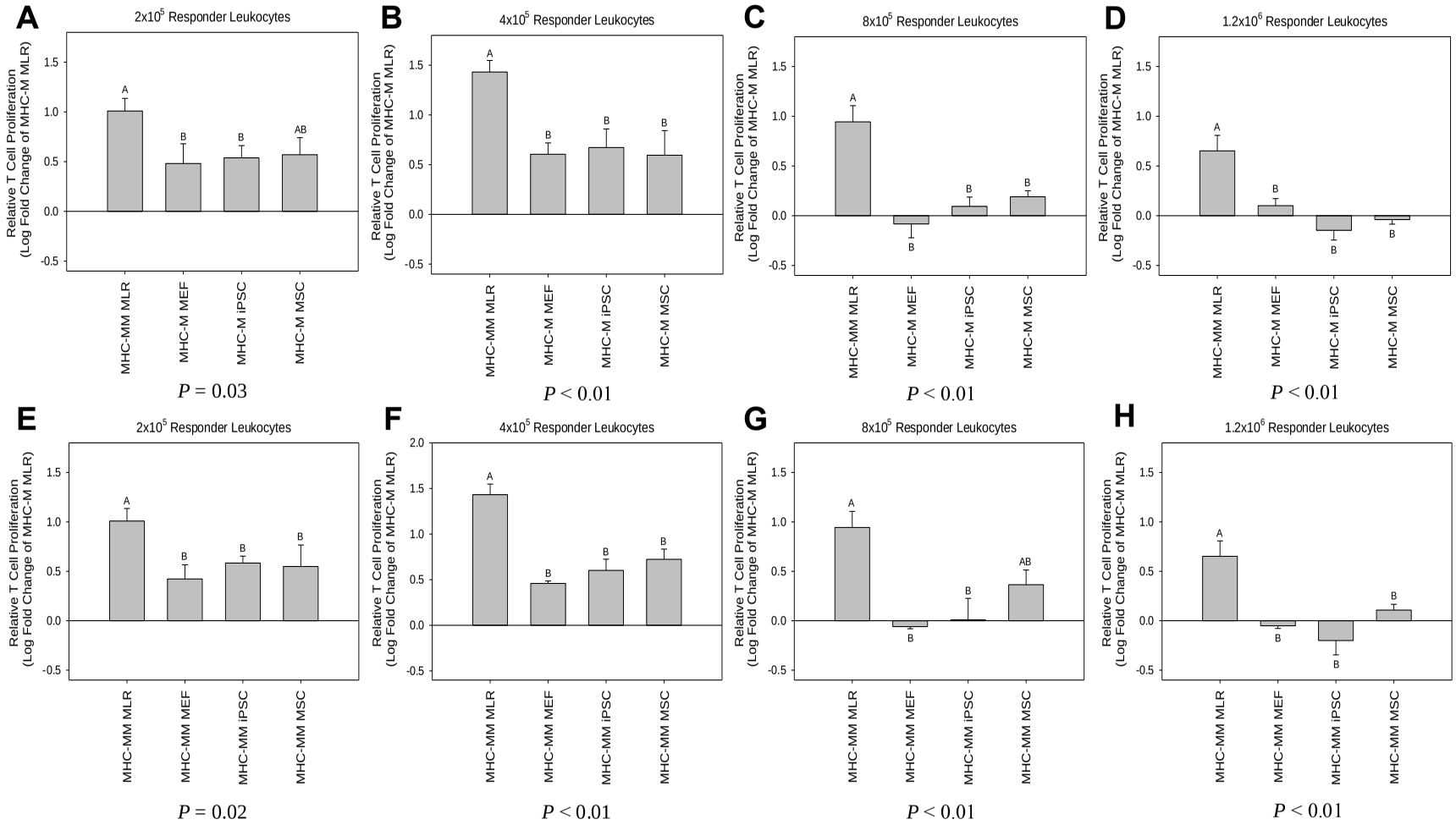

A
2x10^5 Responder Leukocytes
Relative T Cell Proliferation (Log Fold Change of MHC-M MLR)
MHC-MM MLR
MHC-M MEF
MHC-M iPSC
MHC-M MSC
P = 0.03
B
4x10^5 Responder Leukocytes
P < 0.01
C
8x10^5 Responder Leukocytes
P < 0.01
D
1.2x10^6 Responder Leukocytes
P < 0.01
E
2x10^5 Responder Leukocytes
MHC-MM MEF
MHC-MM iPSC
MHC-MM MSC
P = 0.02
F
4x10^5 Responder Leukocytes
P < 0.01
G
8x10^5 Responder Leukocytes
P < 0.01
H
1.2x10^6 Responder Leukocytes
P < 0.01

Figure 3.3. Immunomodulatory ability of MHC-matched (A) and MHC-mismatched (B) MEFs, iPSCs, and MSCs as determined by responder T cell proliferation in modified one-way mixed leukocyte reactions with the cell types in the presence of MHC-mismatched responder and stimulator leukocytes. Data are presented as the log fold change from that of the MHC-mismatched MLR. Bars represent mean ± S.D. from a total of 2 separate experiments performed with multiple cell lines. MHC-M = MHC-matched; MHC-MM = MHC-mismatched. Both iPSCs and MSCs were able to reduce responder T cell proliferation towards that of the MHC-matched MLR, while MEFs (negative control) did not. Statistical data is shown in **Figure 3.4**.

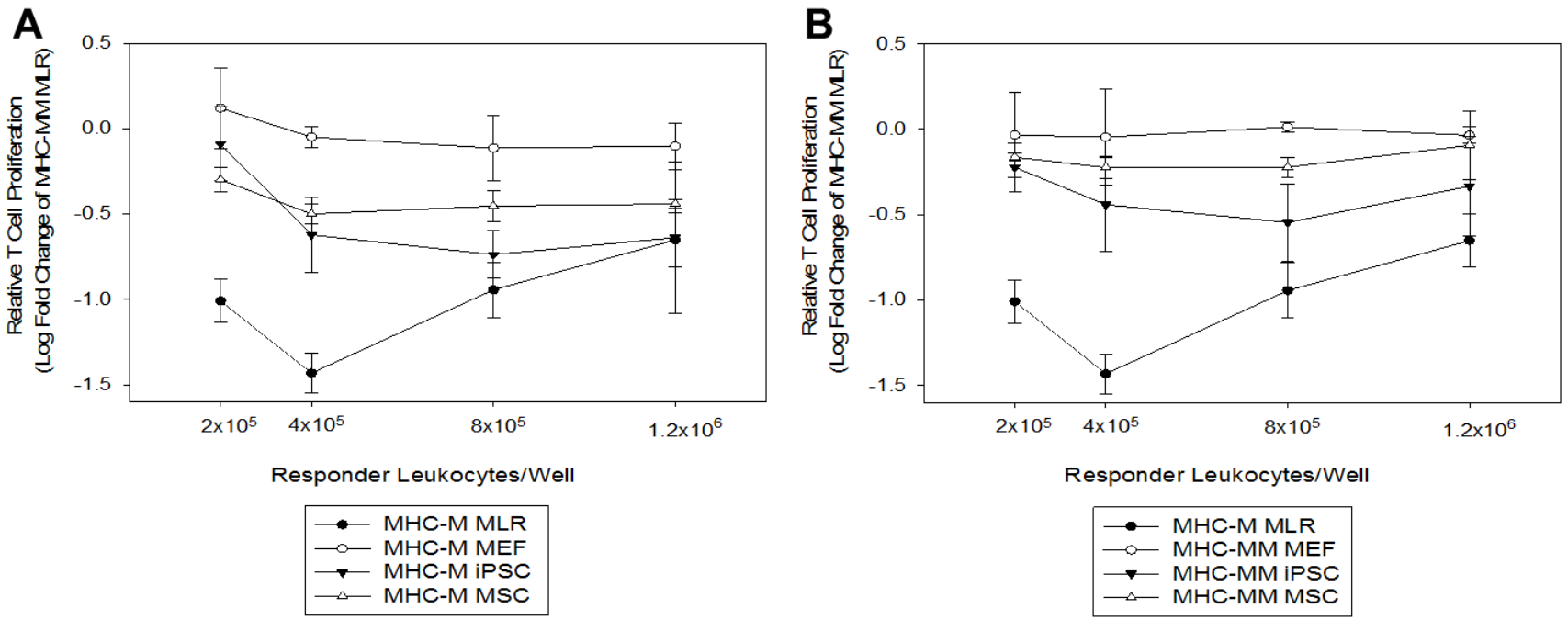
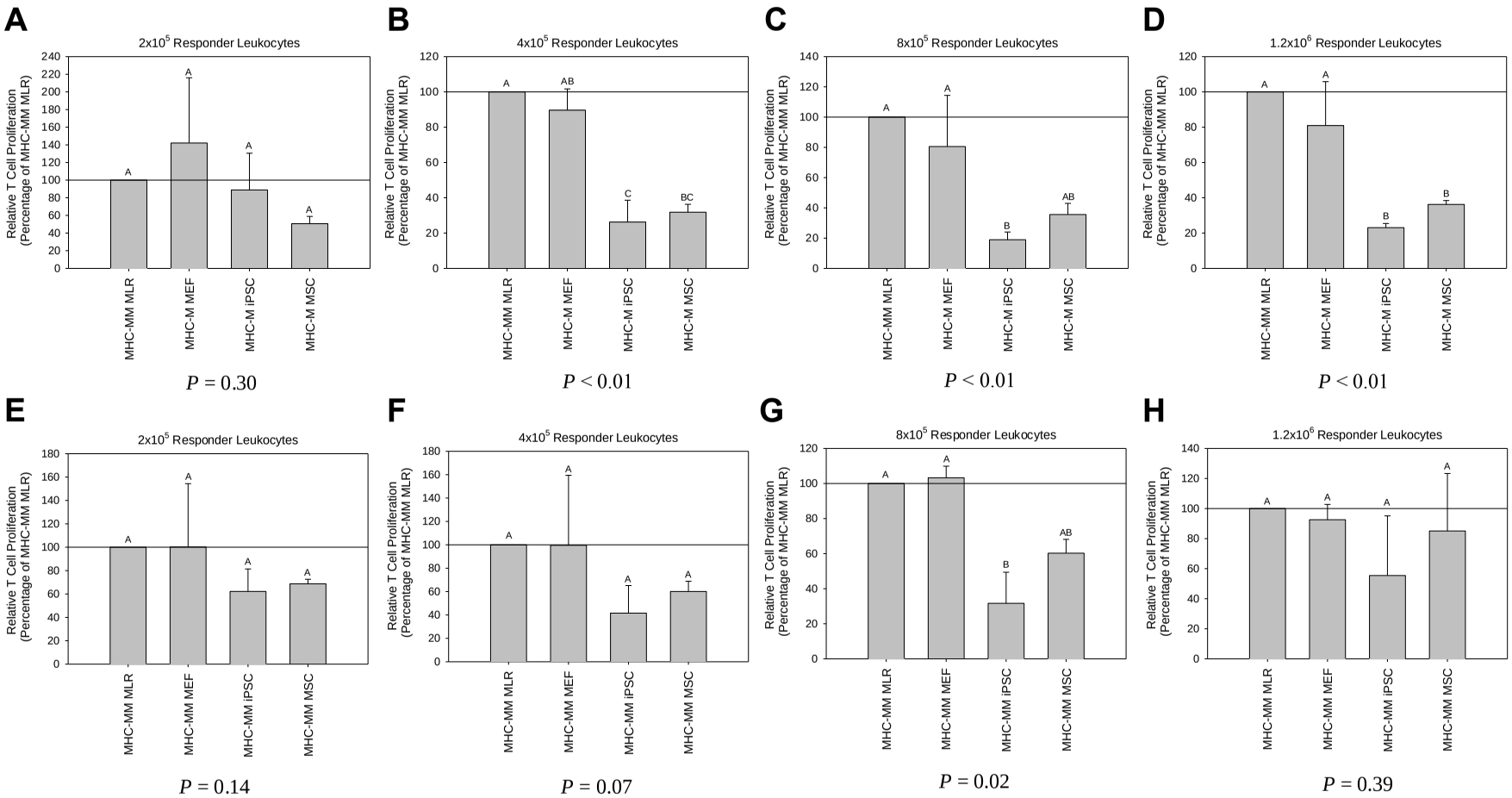

A
Relative T Cell Proliferation
(Log Fold Change of MHC-MMMLR)
0.5
0.0
-0.5
-1.0
-1.5
2x10^5
4x10^5
8x10^5
1.2x10^6
Responder Leukocytes/Well
MHC-M MLR
MHC-M MEF
MHC-M iPSC
MHC-M MSC
B
Relative T Cell Proliferation
(Log Fold Change of MHC-MMMLR)
0.5
0.0
-0.5
-1.0
-1.5
2x10^5
4x10^5
8x10^5
1.2x10^6
Responder Leukocytes/Well
MHC-M MLR
MHC-MM MEF
MHC-MM iPSC
MHC-MM MSC

Figure 3.4. Modified one-way mixed leukocyte reaction (MLR) results for MHC-matched cells (A-D) and MHC-mismatched cells (E-H) cultured in the presence of MHC-mismatched responder and stimulator leukocytes at the different responder leukocyte concentrations tested. Data are presented as the percentage proliferation of that of the MHC-mismatched MLR alone. Bars represent mean ± S.D. from a total of 2 separate experiments performed with multiple cell lines. Superscript letters indicate significant differences between groups by ANCOVA, with experiment as a covariate, followed by the Tukey test for multiple comparisons, $P < 0.05$. MHC-M = MHC-matched; MHC-MM = MHC-mismatched. MHC-matched iPSCs resulted in significantly decreased responder T cell proliferation compared to both the MHC-mismatched MLR and the negative control of MEFs at the 3 highest responder leukocyte concentrations (B-D). Only iPSCs were able to reduce responder T cell proliferation significantly from the MHC-mismatched MLR at the responder leukocyte concentration of $8x10^5$ cells (G).


A
2x10^5 Responder Leukocytes
Relative T Cell Proliferation (Percentage of MHC-MM MLR)
MHC-MM MLR
MHC-M MEF
MHC-M iPSC
MHC-M MSC
P = 0.30
B
4x10^5 Responder Leukocytes
P < 0.01
C
8x10^5 Responder Leukocytes
P < 0.01
D
1.2x10^6 Responder Leukocytes
P < 0.01
E
2x10^5 Responder Leukocytes
MHC-MM iPSC
P = 0.14
F
4x10^5 Responder Leukocytes
P = 0.07
G
8x10^5 Responder Leukocytes
P = 0.02
H
1.2x10^6 Responder Leukocytes
P = 0.39

compared to iPSCs at this concentration, the mean T cell proliferation was greater than for iPSCs and also equivalent to that of MEFs (**Figures 3.4G**).

Discussion

In this study we directly compared iPSCs to MSCs in terms of immunogenicity and immunomodulatory capability using mixed leukocyte reactions. Our comparisons revealed that iPSCs generated through both lentiviral and *piggyBac* reprogramming methods have similar immunogenic properties as MSCs and may possess more potent immunomodulatory properties than MSCs in vitro. Co-culture of MHC-mismatched leukocytes with MHC-matched iPSCs resulted in significantly less responder T cell proliferation than observed for MHC-mismatched leukocytes alone at more responder leukocyte concentrations tested than was observed for co-culture of MHC-mismatched leukocytes with MHC-matched MSCs. In addition, MHC-mismatched iPSCs were able to significantly reduce responder T cell proliferation at the responder leukocyte concentration of $8x10^{\wedge 5}$ cells when co-cultured with MHC-mismatched leukocytes while MHC-mismatched MSCs were not.

None of the cells (MEFs, iPSCs, or MSCs) tested in this study were irradiated for use in MLRs due to the fact that iPSCs died following even very low doses (100-200 rads) of gamma irradiation. This finding suggests that iPSCs undergo p53-independent apoptosis in response to DNA damage as described for ESCs [66-68] rather than p53-mediated cell cycle arrest as is well described for somatic cells [69]. For this reason, preliminary experiments were performed to determine the proper seeding density of all cell types such that they were approximately 80% confluent on the 5^{th} (final) day of MLR culture. This method is different than most previously described for MSC immunology studies examining MSC effects in MLRs in which MSCs are

irradiated and then plated at different ratios to responder leukocytes [25, 41]. It is possible that because the cells in our experiments were growing that we could have either not reached or surpassed the optimal cell:leukocyte ratio for immunomodulation in some instances. The fact that both MHC-matched iPSCs and MSCs were able to significantly downregulate responder T cell proliferation to as much as 25-30% of that of the MHC-mismatched MLR, however, suggests that we covered a broad enough range of responder leukocyte cell concentrations to confidently determine if the cells were causing immunomodulation.

Due to the nature of our MLRs, it must be considered that T cell proliferation may have been falsely diminished due to T cell competition with the growing cells for nutrients in the media. At no point during the experiments, however, did the media appear exhausted in color. More importantly, co-culture with control MEFs, which were rapidly growing and just as confluent as the other cell types by the end of the experiments, did not result in reduced T cell proliferation, arguing against nutrient competition or depletion as a reason for the reduced responder T cell proliferation observed in MLR co-cultures with iPSC and ESCs. Another potential concern is that our leukocyte media included leukemia inhibitory factor (LIF), which has been shown to have a role in MSC-mediated immunosuppression [23]. LIF is commonly used in iPSC media to maintain pluripotency and prevent differentiation [1, 2, 10]. It was used in these experiments for that reason and also to avoid the use of feeder cells with iPSCs culture, which would have further complicated experimental design and interpretation [70]. As the same leukocyte media with LIF was used for all MLR experiments, this should have prevented any biases between cell type comparisons and against MHC-matched and mismatched MLR controls. Again, our finding that control MEFs did not cause significant downregulation of responder T cell proliferation argues against this concern.

The finding that iPSCs and MSCs that were MHC-matched with respect to the responder leukocytes resulted in a greater reduction in responder T cell proliferation compared to iPSCs and MSCs that were MHC-mismatched is an interesting one. Engraftment studies evaluating the effect of MSCs have previously demonstrated similar findings, with only syngeneic (MHC-matched) MSCs resulting in enhanced engraftment [16, 37]. Previous MLR studies evaluating the immunosuppressive effects of ESCs, however, have found no difference in using MHC-matched or mismatched ESCs with the responder leukocytes [71]. The reasons for this discrepancy between iPSCs and MSCs is unclear, but could be due to specific immunosuppressive mechanisms employed by the cells or due to the differences in MHC antigen expression between the cell types with iPSC expressing very low or negligible levels of MHC class I and MSCs expressing high levels of MHC I. Further studies must be performed to elucidate this finding, including examination of soluble factors potentially expressed by iPSCs and MSCs into the media during MLRs such IL-10, TGF-β, PGE-2, IDO, and LIF [12, 13, 15, 23, 31, 72].

A very important question raised by this study is whether or not iPSCs can retain their immunogenic and immunomodulatory properties upon differentiation. Additional follow up studies will focus on differentiating these same iPSC cell lines into specific cell types and then re-evaluating their MHC class I and II expression in addition to their immunogenic and immunomodulatory properties in MLRs. Soluble factor release into the media by these cells during MLRs will also be re-assessed and compared to levels pre-differentiation. As it is unlikely that undifferentiated iPSCs will be used in human clinical applications due to concerns of teratoma formation, these follow-up studies will be critical for consideration of iPSC use in the place of MSCs for both regenerative medicine and transplant medicine [7, 11, 50].

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CHAPTER 4

EQUINE BONE MARROW-DERIVED MESENCHYMAL STEM CELLS ARE HETEROGENEOUS IN MHC CLASS II EXPRESSION AND CAPABLE OF INCITING AN IMMUNE RESPONSE IN VITRO

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Abstract

The horse is a valuable species to assess the effect of allogeneic mesenchymal stem cells (MSCs) in regenerative therapy treatments. There are no studies to date that have examined donor response to major histocompatibility (MHC)-mismatched equine MSCs. The purposes of this study were to immunophenotype bone marrow-derived MSCs from horses of known MHC haplotype and to compare the immunogenicity of MSCs with differing immunophenotypes, particularly in regards to MHC class II expression through modified one-way mixed leukocyte reactions (MLRs). Our hypotheses were that early passage MSCs would be heterogeneous in MHC class II expression and that MHC-mismatched MHC class II negative MSCs would have low immunogenicity in vitro while those that were positive would be immunogenic. MSCs (n=10 horses, 13 bone marrow aspirates) varied widely in MHC class II expression through cell passages. MSCs from 11 of 13 aspirates were positive for MHC II expression at P2 and 6 of those 11 remained positive through P8. MSCs from only one horse were MHC class II negative from P2 through P8, and results were repeatable on a second aspirate. MLR results revealed that MHC-mismatched MHC class II positive MSCs caused significantly increased responder T cell proliferation compared to MHC-mismatched MHC class II negative and MHC-matched MSCs, and equivalent to that of the positive control of MHC-mismatched peripheral blood leukocytes. These results suggest that MHC-mismatched MSCs should be immunophenotyped and confirmed as MHC class II negative prior to potential application in order to reduce the risk of allogeneic reactions.

Introduction

The immune status and immunosuppressive properties of adult bone marrow-derived mesenchymal stem cells (MSCs) have been investigated in multiple species over the past decade with conflicting results [1-4]. While MSCs are commonly thought of and referred to as immunoprivileged in the literature, multiple studies in both mice and humans have demonstrated that allogeneic adult bone marrow-derived MSCs are capable of eliciting immune responses both in vitro and in vivo [1, 5-9]. In these studies, the immunosuppressive effects of MSCs were unable to prevent an immunogenic response in vitro, or to prevent MSC rejection in vivo. Complicating our understanding of the immune status of MSCs is the fact that not all MSCs described in the literature have the same major histocompatibility complex (MHC) class II expression profile [5, 6, 10], and some studies did not include MSC immunophenotyping and/or proper experimental controls [1].

Mesenchymal stem cells are increasingly used in regenerative therapies for equine patients [11-15]. The use of allogeneic MSCs would be advantageous in cases where treatment is indicated at the time of diagnosis. Bone marrow-derived MSCs isolated from young adult horses have been previously phenotyped during mid-late passage (P3-P7) as MHC class II negative [16-18], and equine allogeneic MSCs have been reported to be both immunoprivileged and immunosuppressive in vitro [18], as well as non-immunogenic in vivo [19]. No studies have examined the immunophenotype of equine MCSs isolated from horses of varying age or sequentially over early to late passages. In addition, no studies to date have used MSCs and leukocytes isolated from horses of known MHC haplotype, which is essential for performing MHC-matched and MHC-mismatched studies. As the horse is a valuable species for assessing the effect of MSC treatment on musculoskeletal disorders such as tendonitis, cartilage damage,

and osteoarthritis [13, 20-25], it is critical to understand the immune status of equine MSCs prior to evaluating the use of allogeneic MSCs for "off the shelf" therapy in such models.

The purposes of this study were to: (1) phenotype P2-P8 bone marrow-derived MSCs from horses of known MHC haplotype; and (2) compare the immunogenicity of MSCs with differing immunophenotypes, particularly in regards to MHC class II expression, through modified one-way mixed leukocyte reactions (MLRs). This is the first equine study that evaluates the immune response elicited by MHC-matched and MHC-mismatched MSCs, including controls of MHC-matched and mismatched peripheral blood leukocytes (PBLs). Our first hypothesis, based on previous human early passage MSC immunophenotyping [10], was that early passage equine MSCs would be heterogeneous in MHC class II expression. Our second hypothesis was that MHC-mismatched MHC class II negative MSCs would have low immunogenicity in vitro while those that were positive would be immunogenic.

Materials and Methods

***Horses*:** Thoroughbred horses of known MHC haplotype belonging to the Equine Genetics Center at the James A. Baker Institute for Animal Health were used in these studies in accordance with the guidelines of the Institutional Animal Care and Use Committee of Cornell University. All horses were MHC homozygotes of equine leukocyte antigen (ELA) haplotypes ELA-A2, ELA-A3, or ELA-A9 as previously determined by ELA serotyping, direct MHC gene sequencing, and microsatellite typing [26-29].

***Peripheral blood leukocytes*:** Blood was collected via jugular venipuncture with extension sets (Baxter Healthcare, Deerfield, IL) and 16 gauge needles into 500mL evacuated containers (Baxter Healthcare, Deerfield, IL) containing 7,500 units of heparin (Sigma-Aldrich, St. Louis,

MO) each. Plasma was allowed to separate for 20 minutes at room temperature and peripheral blood leukocytes (PBLs) were then isolated from the plasma via carbonyl iron (Sigma-Aldrich, St. Louis, MO) granulocyte depletion and Ficoll-Paque Plus (Amersham Biosciences, Piscataway,NJ) gradient centrifugation. PBLs were resuspended in RPMI 1040 medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS), 0.1 mM 2-Mercaptoethanol, penicillin (100 units/mL) and streptomycin (100μg/mL) and fresh cells used for all experiments.

***Bone marrow aspirate collection and isolation of MSCs*:** Bone marrow aspirate was collected aseptically from the sternum of 10 horses using 11-gauge Jamshidi bone marrow biopsy needles under standing sedation with local anesthesia. For each harvest, a total of 120mL of aspirate was collected into 60mL syringes containing 25,000 units of heparin each. Three horses underwent a second aspirate collection 2 months after the first for a total of 13 aspirates (6 ELA-A2, 6 ELA-A3, 1 ELA-A9 haplotypes). Bone marrow aspirates were purified via Ficoll-Paque Plus (Amersham Biosciences, Piscataway,NJ) gradient centrifugation as previously described [30] and plated onto 100mm tissue culture plates in low glucose (1g/dL) DMEM media (Gibco, Grand Island, NY) containing 10% FBS, 2 mM l-glutamine, penicillin (100units/mL), streptomycin (100μg/mL), and basic fibroblastic growth factor (bFGF, 1ng/mL). MSCs were expanded over 1 passage such that cryopreserved passage 2 (P2) stocks were obtained for each aspirate. P2 MSCs were later thawed and cultured through P8. At each passage, MSC stocks were cryopreserved for immunophenotyping and mixed leukocyte reactions (MLRs). Throughout culturing, media were exchanged every 48 hours. Cells were passaged 1:3 at approximately 80% subconfluency using Accumax® cell dissociation solution (Innovative Cell Technologies Inc, SanDiego, CA) and plated at a density of approximately $1x10^4$ cells/cm^2.

***Immunophenotyping of MSCs*:** MSCs were immunophenotyped at each passage (2 to 8) for expression levels of MHC class I, MHC class II, and a panel of positive (CD44, CD29, CD90) and negative (CD11a/CD18, CD45RB) markers using flow cytometry. Antibodies for these markers were previously validated for the horse and described by our laboratory [30]. PBLs and dermal fibroblasts were used as controls. MSCs from each horse were directly compared to their own PBLs for expression levels of MHC class II to ensure that any variability in MSC MHC class II expression was not due to individual horse variability in MHC class II antibody binding. Cells were pelleted in aliquots containing approximately $1x10^6$ cells on 96-well V-bottom plates and treated with a 20 min blocking step using 10% normal goat serum in phosphate-buffered saline (PBS). The cells were pelleted and resuspended in unconjugated primary monoclonal antibody and incubated for 45 min at 4°C. Cells were then washed, a secondary fluorescent-conjugated goat anti-mouse IgG antibody (fluorescein isothiocyanate (FITC; read on FL1) or allophycocyanin (APC; read on FL4), BD Biosciences, San Jose, CA) was applied to the unconjugated antibodies, and the samples incubated for an additional 45 min at 4°C. Cells were washed and then resuspended in PBS and analyzed on a FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) flow cytometer equipped with 488 μm argon and 635 μm red diode lasers and BD Cell Quest™ analysis software (BD Biosciences, San Jose, CA). Cells exposed to mouse antiparvovirus antibody and FITC or APC-conjugated secondary antibodies were used as negative isotype controls. Cells were gated as previously determined for cultured MSCs by our laboratory [30] and data were collected on $2x10^4$ cells for each sample.

***IFN-γ stimulation of MSCs*:** Recombinant equine IFN-ɣ (R&D Systems Inc., Minneapolis, MN) was used to stimulate MSCs in culture, following which expression levels of MHC class II were assessed via flow cytometry. MSCs were plated on 100mm tissue culture plates at a density of

$1x10^4$ cells/cm^2 in MSC media as described above and allowed to adhere to plates. At 24 hours in culture, media on control plates were exchanged with fresh MSC media, while media on treated plates were exchanged with fresh MSC media containing 100ng/mL of IFN- ɣ [16, 31, 32]. At 72 hours, media on control and treated plates were again exchanged in the same manner. At 96 hours, cells were dissociated from the plates and analyzed for MHC class II expression using flow cytometry as described above for immunophenotyping.

***MHC class II antibody comparison*:** Most previously published studies examining MHC class II expression levels of equine MSCs have used a commercially available MHC class II antibody (clone CVS20, AbD Serotec, Raleigh, NC), we compared this antibody to the one used in this study for immunophenotyping (cz11, clone 130.8E8D9, Laboratory of Dr. D. Antczak, Cornell University, Ithaca, NY) . Both antibodies were generated from mouse hybridomas and are of the IgG1 isotype. PBLs, MSCs, and MSCs stimulated with IFN-ɣ were used to make comparisons in MHC class II expression levels using the 2 antibodies. For both primary mouse anti-horse MHC class II antibodies, a secondary goat anti-mouse APC antibody (BD Biosciences, San Jose, CA) was used. Cells exposed to mouse antiparvovirus antibody with the same secondary antibody were used as negative isotype controls.

Modified one-way mixed leukocyte reactions: To assess the ability of MSCs to stimulate an immune response, modified one-way mixed leukocyte reactions (MLRs) were performed in duplicate on 24-well tissue culture plates using MHC-matched and mismatched responder PBLs and stimulator MSCs. MHC-matched stimulator PBLs were used as negative MLR controls (baseline T cell proliferation) and MHC-mismatched stimulator PBLs were used as positive MLR controls. Responder PBLs were labeled with 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (0.25μg/mL of cell solution,CFSE, Sigma-Aldrich, St. Louis, MO) and

examined at 2 different concentrations ($1.5x10^6$ and $2.5x10^6$ cells/well). Proliferative ability of responder cells was verified via mitogen stimulation with concanavalin A (ConA (5 μg/ml, Sigma-Aldrich, St. Louis, MO)). Stimulator MSCs were plated at $5x10^4$ cells/well in MSC media 24 hours prior to the addition of responder PBLs such that MSCs would be approximately 80% confluent by the end of the experiment. Stimulator PBLs were irradiated with 9 Gy from a Cs-137 source to inhibit proliferation and plated at $1.2x10^6$ cells/well immediately prior to the addition of responder PBLs. The resultant ratios of responder:stimulator PBLs was based on previously published equine MLR experiments [33] and determined to be optimal for these studies in preliminary experiments. Cultures were maintained for 5 days with modified RPMI 1040 media (1mL/well) containing 10% FBS, 0.1 mM 2-mercaptoethanol, penicillin (100units/mL), streptomycin (100μg/mL), and basic fibroblastic growth factor (bFGF, 1ng/mL). Media were not exchanged over the 5 days. Following culture, PBLs were aspirated from the wells and stained with a primary mouse anti-horse CD3 antibody (clone UC F6G-3.3; Laboratory of Dr. J. Scott, University of California Davis, Davis, CA) and a secondary goat anti-mouse APC antibody (BD Biosciences, San Jose, CA). The antibody staining process for flow cytometry analysis was performed as described above for immunophenotyping.

Proliferation of gated CFSE-labeled CD3-positive responder T cells was evaluated via CFSE attenuation using flow cytometry. Cells were first gated on FL4 so that only the CD3-positive cells (T cells) were then examined on FL1 for CFSE attenuation. Non-stimulated responder T cells were used to set the boundary of non-proliferating cells such that all cells to the left (lower fluorescence intensity on FL1) of that boundary were determined to be proliferating. The number of cell counts in the proliferating T cell gate was measured as well as the CFSE Geometric Mean Fluorescence Intensity (GMFI) of all T cells to reflect the extent of

proliferation. Data was collected on the entirety of each sample because cell numbers were being measured.

MLRs were performed in a total of 5 experiments using responder PBLs from 4 different horses (2 ELA-A2, 1 ELA-A3 and 1 ELA-A9 haplotypes) and stimulator PBLs from 3 different horses (1 ELA-A2 and 2 ELA-A3 haplotypes). In each experiment, T cell proliferation in response to MHC-matched MSCs, MHC-mismatched MHC class II negative MSCs, and MHC-mismatched MHC class II positive MSCs was assessed. As stated above, T cell proliferation in response to MHC-matched PBLs (MHC-matched MLR) was used as a negative control and set as the baseline proliferation value, while T cell proliferation in response to MHC-mismatched PBLs (MHC-mismatched MLR) was used as a positive control. Due to naturally occurring variation in PBL responses between horses and experiments, the relative T cell proliferation and relative GMFI in each experiment was reported as the fold change from that of the MHC-matched MLR.

Statistical Analyses*:* Immunophenotyping data were analyzed with Pearsons correlations. MHC class II expression data obtained by the two different antibodies were analyzed with paired t-tests. MLR data were normalized by log transformation and analyzed with analysis of covariance (ANCOVA), with horse as a covariate, followed by the Tukey test for multiple comparisons. All analyses were performed using Statistix 9 software (Analytical Software, Tallahassee, FL) and significance was set at $P < 0.05$.

Results

***MSC isolation and immunophenotyping*:** MSCs were isolated and expanded from 13 of 13 bone marrow aspirates. The percentage of P2 MSCs positive for MHC class II expression varied

widely amongst horses despite fairly consistent results for PBL MHC class II expression indicating that the MSC variation observed was not due to differences in antibody binding (Fig. 1). P2 MSCs from 11 of the 13 aspirates were positive for MHC class II expression with broad and diffuse expression peaks as opposed to a well-defined narrow peak or 2 or more peaks suggestive of different but limited cell populations (**Figure 4.1**). P2 MSCs from only one horse (horse 5, ELA-A3 haplotype) were negative for MHC class II expression. P2 MSCs from all 13 aspirates had a phenotype of MHC I^{hi}, $CD44^{hi}$, $CD29^{hi}$, and $CD45RB^{lo}$ (**Table 4.1**). Consistent with previous studies between 17-21 days of culture, there was variable expression of CD90 and CD11a/CD18 surface molecules [30] with the majority of the P2 MSCs having a phenotype of $CD90^{hi}$ and $CD11a/CD18^{lo}$ (**Table 4.1**). Variability was also observed in P2 MSC morphology in that some MHC class II positive MSCs had the classic long spindle shaped morphology equivalent to that of the MHC class II negative MSCs while other MHC class II positive MSCs were more triangular to polygonal in shape and smaller in size (**Figure 4.2**). All MSCs isolated from older horses (≥ 10 years of age) displayed some degree of atypical morphology.

MSCs from 6 of the 11 aspirates positive for MHC class II expression at P2 remained positive through P8, while MSCs from the other 5 aspirates became negative over time in culture, generally by P4 or P5 (**Table 4.2**). Later passage MHC class II positive MSCs either maintained a broad and diffuse expression peak or converted to a narrower peak indicating upregulation of MHC class II in some cases (**Figure 4.3**). P2 MSCs negative for MHC class II expression (2 aspirates from horse 5, ELA-A3 haplotype) remained negative through P8. MSCs from all 13 aspirates each maintained their morphology exhibited in P2, regardless of whether or not they converted from MHC class II positive to MHC class II negative. Only in this single ELA-A3 haplotype horse with MHC class II negative MSCs from P2-P8 could a correlation be

made between MHC haplotype and MSC MHC class II expression. Interestingly, of the MSCs isolated from the 4 older horses in this study ≥ 10 years of age (2 ELA-A3, 1 ELA-A2, and 1 ELA-A9 haplotypes), all were strongly positive for MHC class II at P2 and only those of the ELA-A9 haplotype became negative later in the culture period at P7 and P8 (**Table 4.2**).

Over the culture period through P8, all MSCs maintained the phenotype of MHC I^{hi}, $CD44^{hi}$, $CD29^{hi}$, and $CD45RB^{lo}$ as well as the previously observed variability in expression of CD90 and CD11a/CD18 surface molecules. The median percentage of cells positive for CD90 was 89.75% (range 3.58 – 99.64%) and the median percentage of cells positive for CD11a/CD18 was 1.53% (range 0.12 to 70.85%). A weak but significant negative correlation was found between CD90 and MHC class II expression ($r = -0.23$; $P = 0.03$) and a moderate positive correlation was found between CD11a/CD18 and MHC class II expression ($r = 0.40$; $P < 0.01$).

IFN-ɣ stimulation of MSCs and MHC class II antibody comparison: Stimulation of MHC class II negative MSCs with IFN-ɣ resulted in markedly increased expression of MHC II (**Figure 4.4**) in both the percentage of cells positive for MHC class II and the fluorescence intensity of the cells. No significant differences were found between MHC class II expression levels (percentage of cells positive) obtained by the two different antibodies for PBLs ($n = 3$; $P = 0.83$), MSCs ($n = 4$; $P = 0.41$), or IFN-ɣ stimulated MSCs ($n = 3$; $P = 0.20$). Some variability was observed, however, in the fluorescence intensity of the MSCs stained with the different antibodies, with the commercially available antibody (Antibody 2, clone CVS20) generating a more diffuse intensity than the antibody used in this study (Antibody 1, cz11, clone 130.8E8D9) (**Figure 4.4**).

Figure 4.1. Flow cytometric histogram analyses of MHC class II expression in peripheral blood leukocytes (PBLs) and passage 2 bone marrow-derived mesenchymal stem cells (P2 MSCs) for horses 1-10. The open lines represent negative isotype control staining and the shaded curves represent MHC class II staining. The percentage of positive cells is in the upper right-hand corner of each histogram. Note the relatively minor variation in PBL MHC class II expression between horses as compared to the major variation in P2 MSC MHC class II expression.

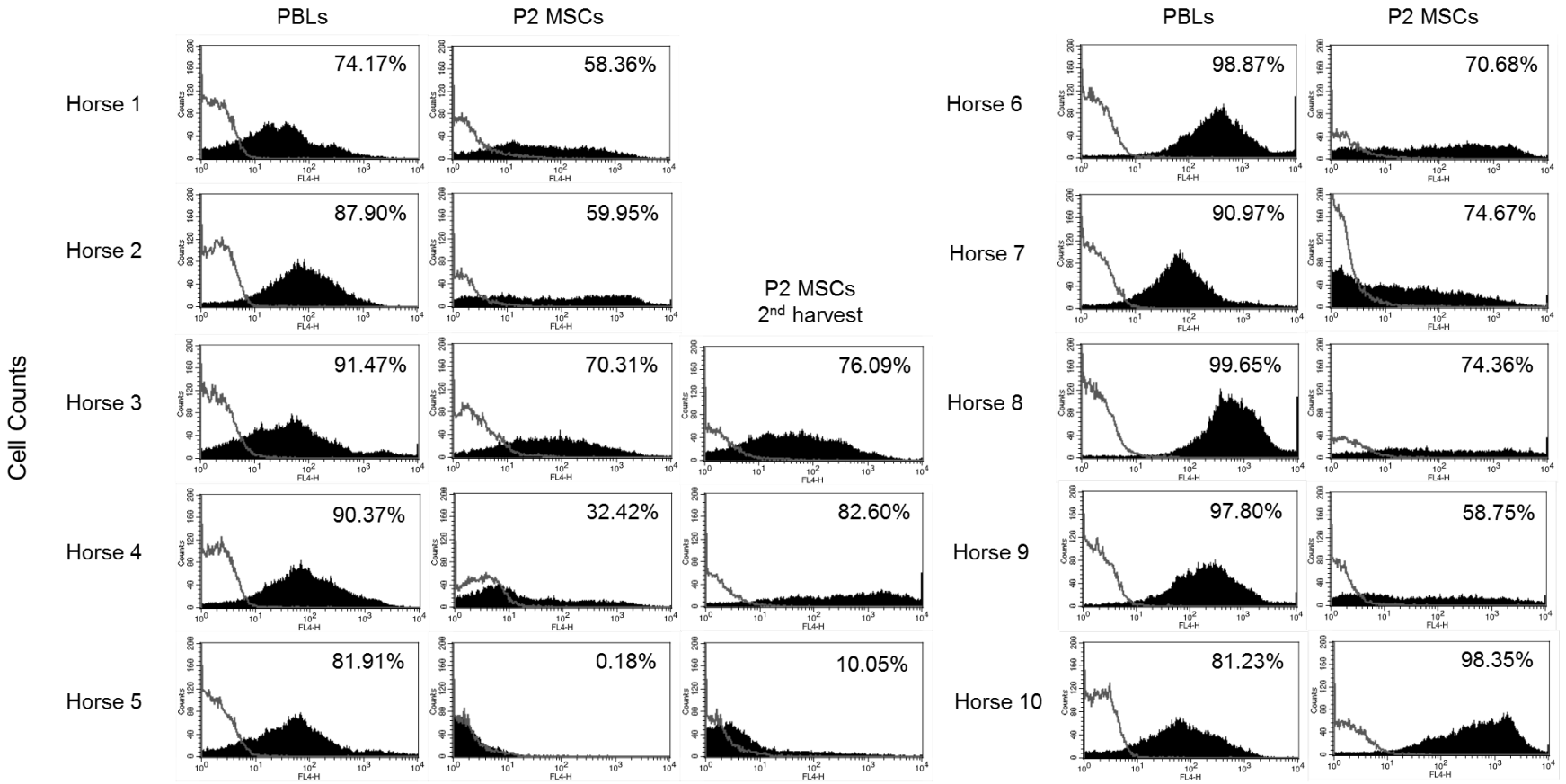

PBLs
P2 MSCs
P2 MSCs
2nd harvest
Horse 1
74.17%
58.36%
Horse 2
87.90%
59.95%
Horse 3
91.47%
70.31%
76.09%
Horse 4
90.37%
32.42%
82.60%
Horse 5
81.91%
0.18%
10.05%
Horse 6
98.87%
70.68%
Horse 7
90.97%
74.67%
Horse 8
99.65%
74.36%
Horse 9
97.80%
58.75%
Horse 10
81.23%
98.35%
Cell Counts
Mean Fluorescence Intensity (FL4): MHC class II

Table 4.1. Percentage of Equine Mesenchymal Stem Cells (MSCs) Positive for Expression of Cell Surface Markers

	Percentage of cells positive						
P2 MSCs: horse (ELA haplotype; age)	MHC I	MHC II	CD44	CD29	CD90	CD11a/CD18	CD45RB
1 (A3; 20 years)	98.77	58.36	75.94	91.59	30.35	56.05	1.34
2 (A2; 12 years)	98.01	59.95	92.92	97.26	66.34	11.43	0.46
3 (A3; 3 years)	98.67	70.31	90.80	96.86	64.60	15.64	0.57
3 (A3; 3 years) *2nd harvest*	95.94	76.09	84.11	97.47	61.48	28.69	1.43
4 (A2; 3 years)	96.51	32.42	78.67	94.42	85.94	0.72	0.41
4 (A2; 3 years) *2nd harvest*	98.16	82.60	85.84	96.45	88.50	8.20	3.27
5 (A3; 6 years)	98.20	0.18	95.59	97.68	94.34	0.30	1.58
5 (A3; 6 years) *2nd harvest*	98.13	10.05	91.54	97.39	92.25	4.31	5.44
6 (A2; 5 years)	98.68	70.68	93.07	97.27	86.41	0.75	0.98
7 (A3; 19 years)	99.07	74.67	92.62	98.34	55.05	20.92	2.44
8 (A9; 11 years)	98.17	74.36	92.09	97.06	92.27	2.92	1.17
9 (A2; 6 years)	99.22	58.75	96.39	98.93	96.39	0.79	2.95
10 (A2; 5 years)	99.57	98.35	95.57	99.05	50.51	43.49	1.38

Figure 4.2. Examples of bone marrow-derived mesenchymal stem cell (MSC) morphology observed for MHC class II negative cells (**A** and **B**) and MHC class II positive cells (**C**-**F**). Note that some MHC class II negative cells (**C** and **D**) displayed the classic spindle shape morphology equivalent to that observed for MHC class II negative cells, while others displayed a less characteristic morphology (**E** and **F**). Also note that the MSCs maintained their morphology over multiple passages whether or not they converted to MHC class II negative or not. In the example shown here, MSCs from horse 9 had the same morphology at P2 (MHC class II positive; **D**) as they did at P5 (MHC class II negative; **B**).

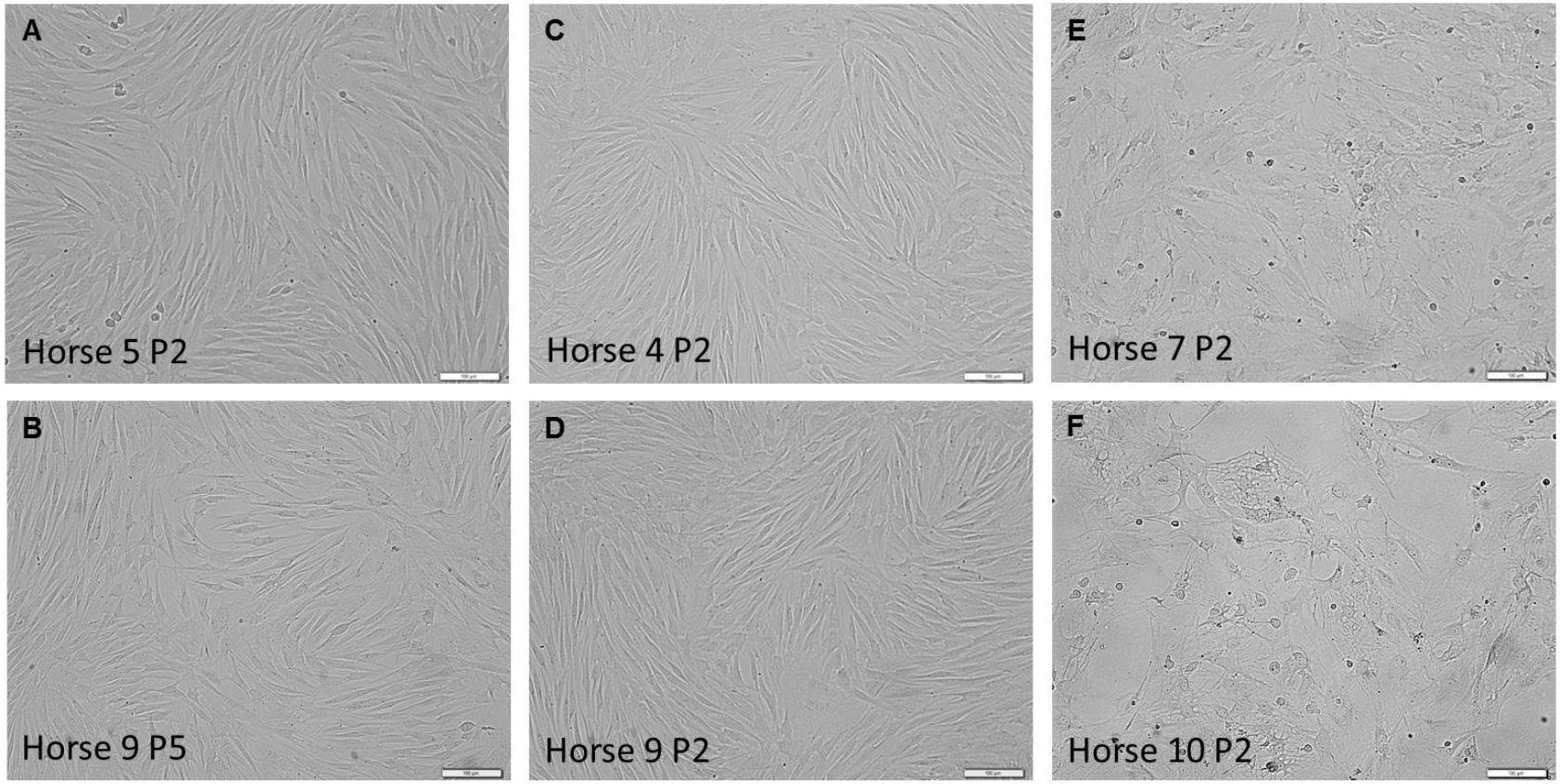
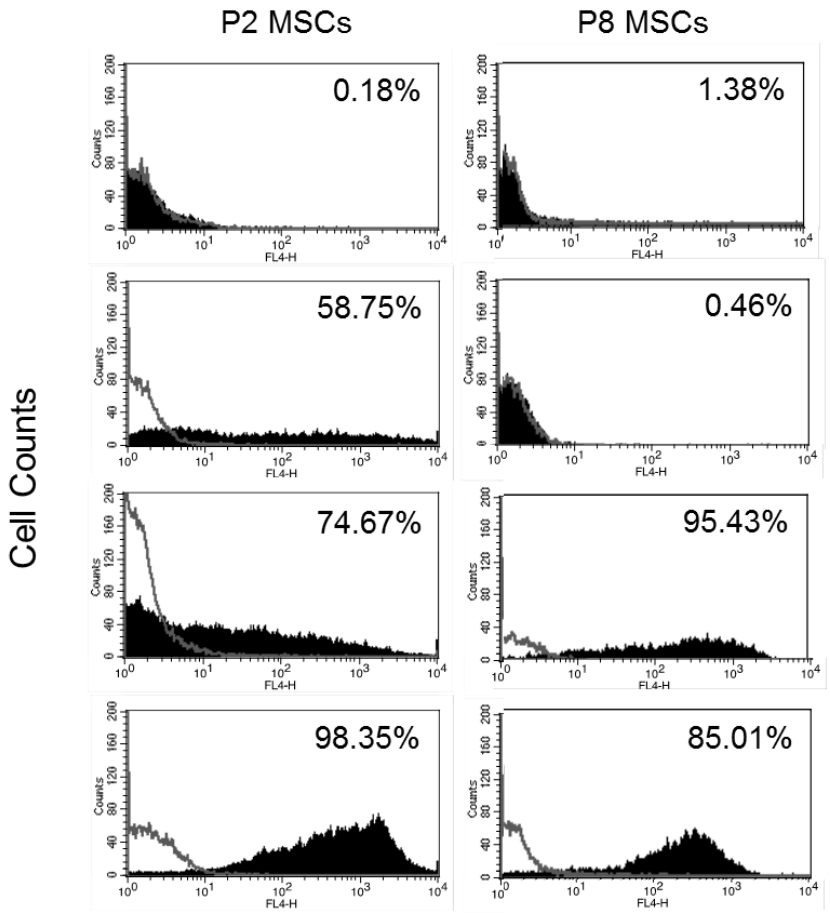

A
Horse 5 P2
C
Horse 4 P2
E
Horse 7 P2
B
Horse 9 P5
D
Horse 9 P2
F
Horse 10 P2

Figure 4.3. Flow cytometric histogram analyses of MHC class II expression in passage 2 (P2) and passage 8 (P8) bone marrow-derived mesenchymal stem cells (MSCs). The open lines represent negative isotype control staining and the shaded curves represent MHC class II staining. The percentage of positive cells is described in the upper right-hand corner of each histogram. Note the variability in both the percentage of cells positive for MHC class II expression at P8 as well as the variability in fluorescence intensity for those MSCs that remained MHC class II positive at P8 (Horses 7 and 10 in this figure).



Horse 5:
MHC II negative P2 MSCs (narrow expression peak)
MHC II negative P8 MSCs (narrow expression peak)

Horse 9:
MHC II positive P2 MSCs (broad expression)
MHC II negative P8 MSCs (narrow expression peak)

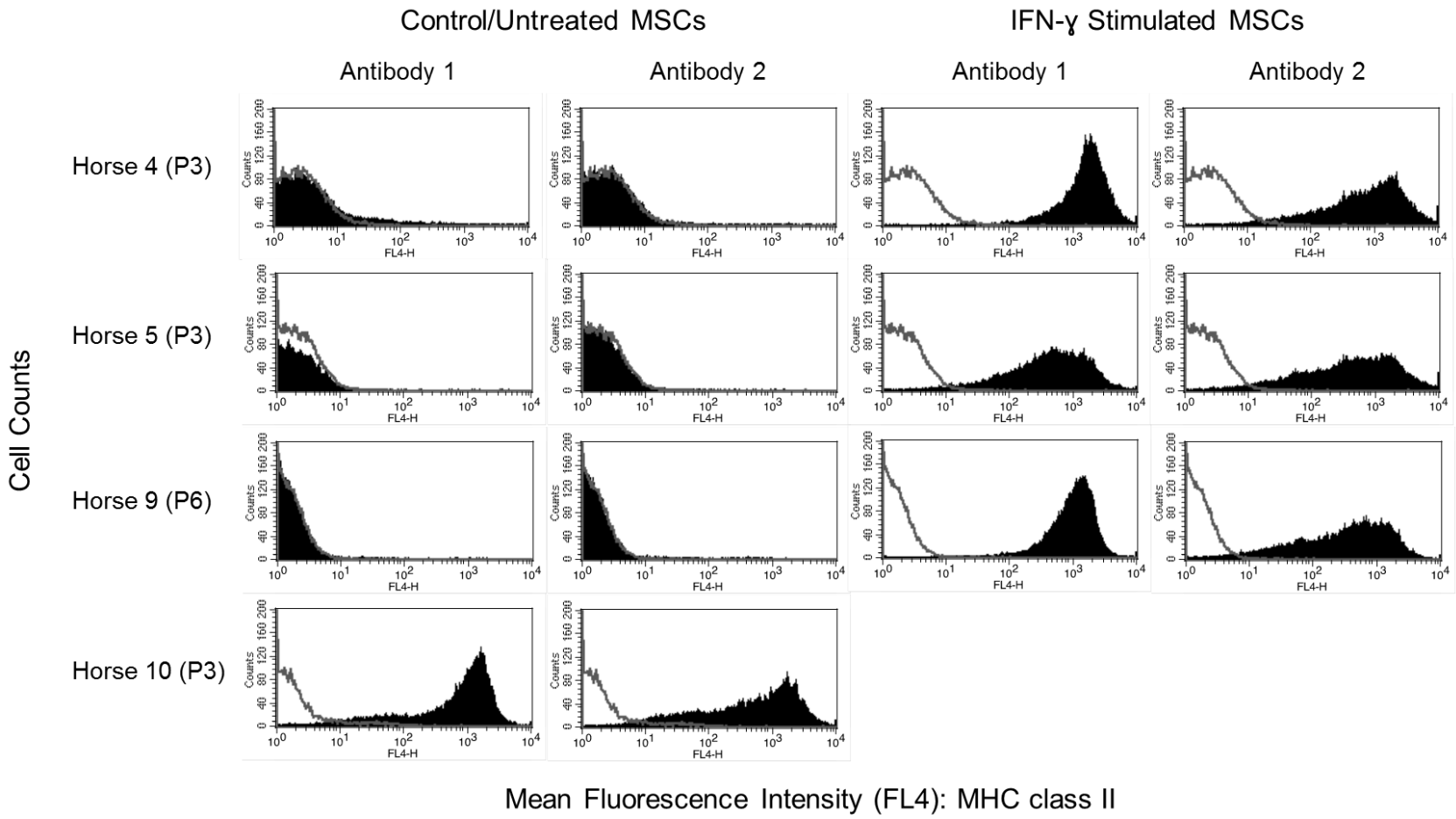
Horse 7:
MHC II positive P2 MSCs (broad expression)
MHC II positive P8 MSCs (broad expression)

Horse 10:
MHC II positive P2 MSCs (broad expression)
MHC II positive P8 MSCs (narrower expression peak)

Table 4.2. Percentage of Equine Mesenchymal Stem Cells (MSCs) Positive for Expression of MHC class II Over Multiple Passages in Culture

	Percentage of cells positive for MHC class II						
MSCs: horse (ELA haplotype; age)	P2	P3	P4	P5	P6	P7	P8
1 (A3; 20 years)	58.36	54.67	63.07	53.54	46.22	51.72	38.44
2 (A2; 12 years)	59.95	78.58	98.14	97.25	91.45	94.47	97.62
3 (A3; 3 years)	70.31	1.53	1.35	1.75	0.86	0.85	0.68
3 (A3; 3 years) *2nd harvest*	76.09	65.22	59.74	64.83	50.49	48.56	45.14
4 (A2; 3 years)	32.42	8.19	4.03	4.52	2.56	0.49	0.50
4 (A2; 3 years) *2nd harvest*	82.60	59.25	56.05	65.31	64.85	67.08	68.96
5 (A3; 6 years)	0.18	0.57	0.29	0.77	0.44	0.08	1.38
5 (A3; 6 years) *2nd harvest*	10.05	1.05	0.90	1.59	0.47	0.51	0.45
6 (A2; 5 years)	70.68	72.32	64.62	22.10	2.32	1.93	1.29
7 (A3; 19 years)	74.67	66.49	93.13	94.54	93.68	93.33	95.43
8 (A9; 11 years)	74.36	71.32	67.04	41.82	25.97	8.16	0.90
9 (A2; 6 years)	58.75	41.79	4.92	0.78	0.76	0.48	0.46
10 (A2; 5 years)	98.35	91.92	95.69	96.15	94.89	91.39	85.01

Figure 4.4. Flow cytometric histogram analyses of MHC class II expression in control or untreated bone marrow-derived stem cells (MSCs) and in IFN-ɣ stimulated MSCs used to compare the antibody used in this study (Antibody 1) to the commercially available antibody (Antibody 2). The open lines represent negative isotype control staining and the shaded curves represent MHC class II staining. Note the consistency observed between antibodies for all cell types, with the exception of the fact that MSC class II positive MSCs generally displayed a more diffuse fluorescence intensity when stained with Antibody 2 compared to Antibody 1.


Control/Untreated MSCs
IFN-ɣ Stimulated MSCs
Antibody 1
Antibody 2
Antibody 1
Antibody 2
Horse 4 (P3)
Horse 5 (P3)
Horse 9 (P6)
Horse 10 (P3)
Cell Counts
Counts
FL4-H
Mean Fluorescence Intensity (FL4): MHC class II

***Modified one-way mixed leukocyte reactions*:** MHC-mismatched MHC class II positive MSCs caused a significant increase in responder T cell proliferation compared to MHC-mismatched MHC class II negative MSCs at the lower responder PBL concentration of 1.5x10^6 cells (**Figure 4.5A**; $P < 0.01$) and compared to both MHC-mismatched MHC class II negative and MHC-matched MSCs at the higher responder PBL concentration of 2.5x10^6 cells (**Figure 4.5B**; $P < 0.01$). At both responder T cell concentrations, proliferation caused by MHC-mismatched MHC class II positive MSCs was statistically equivalent to that caused by the positive control of MHC-mismatched PBLs. MHC-mismatched MHC class II negative MSCs used in experiments were Horse 5 (ELA-A3) P2 MSCs and Horse 9 (ELA-A2) P5 MSCs. MHC-mismatched MHC class II positive MSCs used in experiments were Horse 7 (ELA-A3) P2 MSCs and Horse 5 (ELA-A3) P2 IFN-ɣ stimulated MSCs. Responder T cell proliferation results for individual experiments are shown in **Figure 4.6**.

Stimulation of responder PBLs with MHC-mismatched MHC class II positive MSCs resulted in lower responder T cell GMFI, indicative of an increased number of cell divisions, compared to MHC-mismatched MHC class II negative MSCs (**Figure 4.5C** and **Figure 4.5D**). This result was significant at the higher responder PBL concentration of 2.5x10^6 cells (**Figure 4.5D**; $P = 0.02$). At both responder PBL concentrations, stimulation of responder PBLs with either MHC-mismatched MHC class II negative MSCs or MHC-mismatched MHC class II positive MSCs resulted in responder T cell GMFI statistically equivalent to that of both MHC-matched MSCs and MHC-mismatched PBLs.

Figure 4.5. Modified one-way mixed leukocyte reaction (MLR) results at the lower responder cell concentration of $1.5x10^6$ leukocytes (**A** and **C**) and at the higher responder cell concentration of $2.5x10^6$ leukocytes (**B** and **D**) as measured by relative responder T cell proliferation (**A** and **B**) and CFSE geometric mean fluorescence intensity (GMFI; **C** and **D**). Bars represent mean ± S.D. of n = 5. Superscript letters indicate significant differences between groups by ANCOVA, with horse as a covariate, followed by the Tukey test for multiple comparisons, $P < 0.05$. MHC-M = MHC-matched; MHC-MM = MHC-mismatched. MHC-mismatched MHC class II negative MSCs caused significantly less responder T cell proliferation compared to both the positive control of MHC-mismatched PBLs (MHC-MM MLR) and MHC-mismatched MHC class II positive MSCs at both responder leukocyte concentrations (**A;** $P < 0.01$ and **B**; $P < 0.01$). MHC-mismatched MHC class II negative MSCs resulted in a significantly greater CFSE GMFI, indicative of fewer responder T cell divisions, compared to MHC-mismatched MHC class II positive cells at the higher responder leukocyte concentration (**D**; $P = 0.02$).

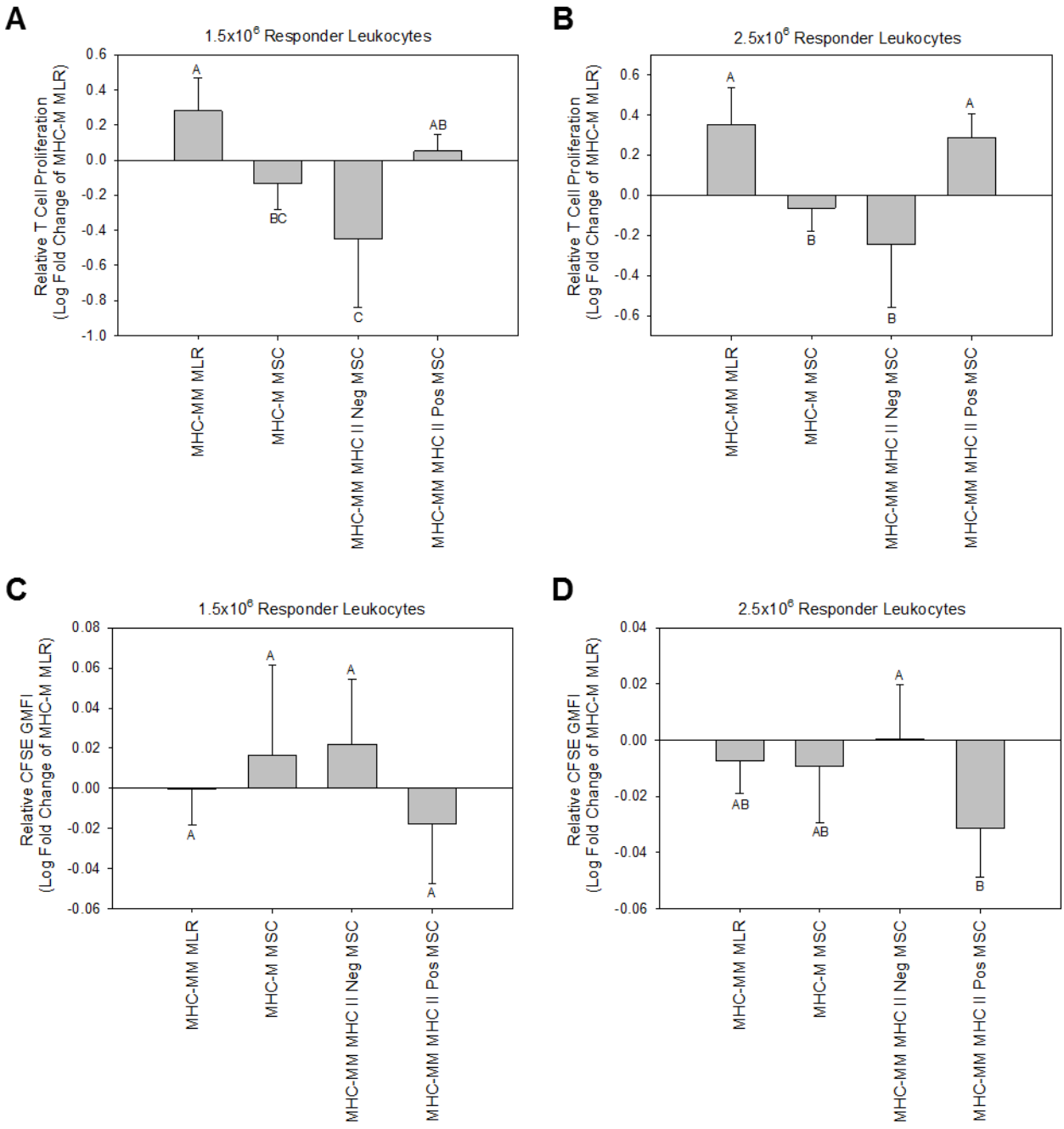
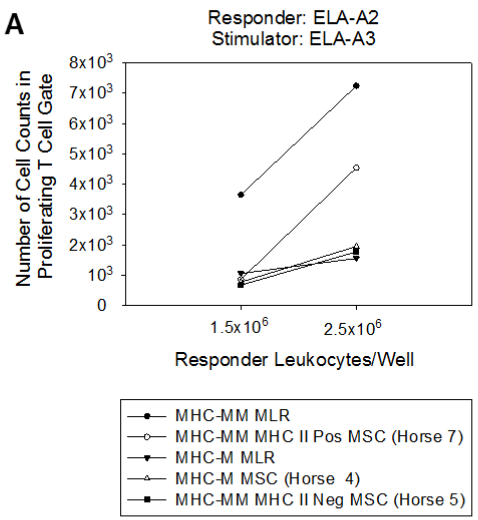
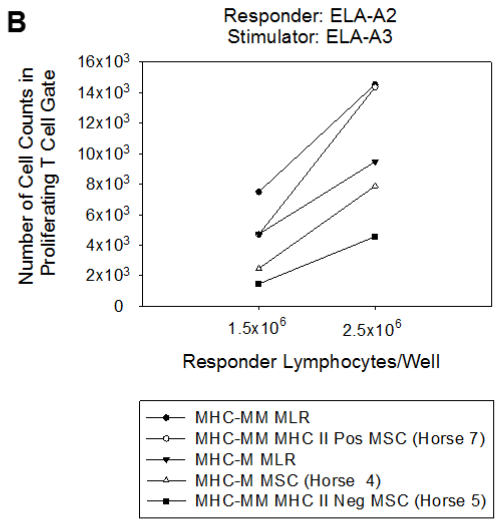
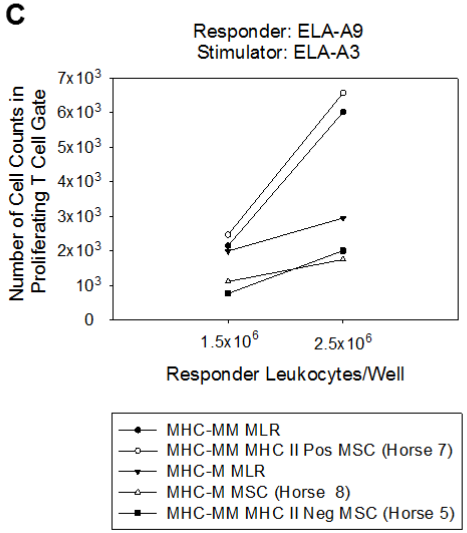
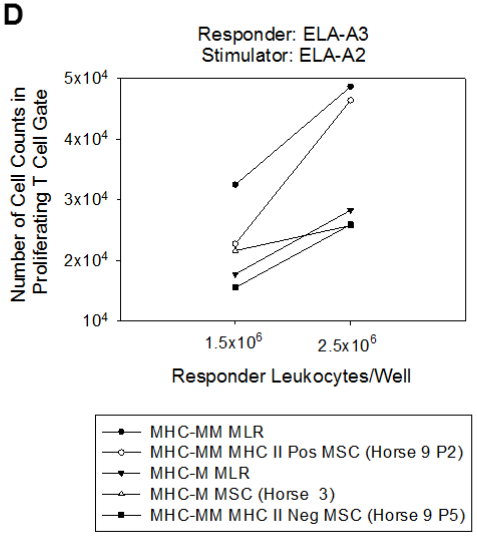

A
1.5x10^6 Responder Leukocytes
Relative T Cell Proliferation
(Log Fold Change of MHC-M MLR)
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
A
BC
C
AB
MHC-MM MLR
MHC-M MSC
MHC-MM MHC II Neg MSC
MHC-MM MHC II Pos MSC
B
2.5x10^6 Responder Leukocytes
Relative T Cell Proliferation
(Log Fold Change of MHC-M MLR)
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
A
B
B
A
MHC-MM MLR
MHC-M MSC
MHC-MM MHC II Neg MSC
MHC-MM MHC II Pos MSC
C
1.5x10^6 Responder Leukocytes
Relative CFSE GMFI
(Log Fold Change of MHC-M MLR)
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
-0.06
A
A
A
A
MHC-MM MLR
MHC-M MSC
MHC-MM MHC II Neg MSC
MHC-MM MHC II Pos MSC
D
2.5x10^6 Responder Leukocytes
Relative CFSE GMFI
(Log Fold Change of MHC-M MLR)
0.04
0.02
0.00
-0.02
-0.04
-0.06
AB
AB
A
B
MHC-MM MLR
MHC-M MSC
MHC-MM MHC II Neg MSC
MHC-MM MHC II Pos MSC

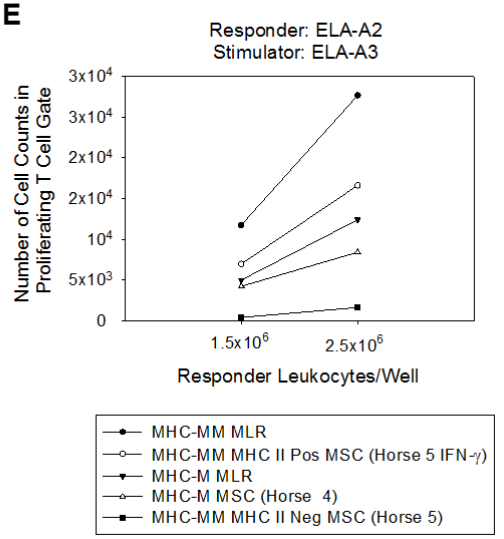
Figure 4.6. Responder T cell proliferation results for individual experiments (**A**-**E**) used to generate **Figure 4.5A** and **Figure 4.5B**. MHC-M = MHC-matched; MHC-MM = MHC-mismatched. Note that for every experiment the responder T cell proliferation in response to MHC-mismatched MHC class II positive MSCs was greater than that observed for the negative/baseline control of MHC-matched PBLs (MHC-M MLR), MHC-mismatched MHC class II negative MSCs, MHC-matched MSCs.


A
Responder: ELA-A2
Stimulator: ELA-A3
Number of Cell Counts in Proliferating T Cell Gate
8x10^3
7x10^3
6x10^3
5x10^3
4x10^3
3x10^3
2x10^3
10^3
0
1.5x10^6
2.5x10^6
Responder Leukocytes/Well
MHC-MM MLR
MHC-MM MHC II Pos MSC (Horse 7)
MHC-M MLR
MHC-M MSC (Horse 4)
MHC-MM MHC II Neg MSC (Horse 5)


B
Responder: ELA-A2
Stimulator: ELA-A3
Number of Cell Counts in Proliferating T Cell Gate
16x10^3
14x10^3
12x10^3
10x10^3
8x10^3
6x10^3
4x10^3
2x10^3
0
1.5x10^6
2.5x10^6
Responder Lymphocytes/Well
MHC-MM MLR
MHC-MM MHC II Pos MSC (Horse 7)
MHC-M MLR
MHC-M MSC (Horse 4)
MHC-MM MHC II Neg MSC (Horse 5)


C
Responder: ELA-A9
Stimulator: ELA-A3
Number of Cell Counts in Proliferating T Cell Gate
7x10^3
6x10^3
5x10^3
4x10^3
3x10^3
2x10^3
10^3
0
1.5x10^6
2.5x10^6
Responder Leukocytes/Well
MHC-MM MLR
MHC-MM MHC II Pos MSC (Horse 7)
MHC-M MLR
MHC-M MSC (Horse 8)
MHC-MM MHC II Neg MSC (Horse 5)


D
Responder: ELA-A3
Stimulator: ELA-A2
Number of Cell Counts in Proliferating T Cell Gate
5x10^4
4x10^4
3x10^4
2x10^4
10^4
1.5x10^6
2.5x10^6
Responder Leukocytes/Well
MHC-MM MLR
MHC-MM MHC II Pos MSC (Horse 9 P2)
MHC-M MLR
MHC-M MSC (Horse 3)
MHC-MM MHC II Neg MSC (Horse 9 P5)


E
Responder: ELA-A2
Stimulator: ELA-A3
Number of Cell Counts in Proliferating T Cell Gate
3x10^4
3x10^4
2x10^4
2x10^4
10^4
5x10^3
0
1.5x10^6
2.5x10^6
Responder Leukocytes/Well
MHC-MM MLR
MHC-MM MHC II Pos MSC (Horse 5 IFN-γ)
MHC-M MLR
MHC-M MSC (Horse 4)
MHC-MM MHC II Neg MSC (Horse 5)

Discussion

This study reports the heterogeneous immunophenotypes of bone marrow-derived MSCs isolated from horses of varying age and MHC haplotype. We hypothesized that MSCs would variably express MHC class II during early passages as has been previously shown for human and mouse MSCs [5-6,10], and that this expression would decrease over time in culture such that MSCs would be negative for MHC class II expression at later passages. While this turned out to be the case for MSCs isolated from certain horses, others remained strongly positive for MHC class II expression through P8. This variability observed in MHC class II expression was not due to differences in antibody binding between horses as determined by comparison of PBL MHC class II expression and also was not due to differences in antibody binding between the antibody primarily used in this study (Antibody 1) and the antibody used in previous studies (Antibody 2) as demonstrated in **Figure 4.2**. Interestingly, even MSCs isolated from different bone marrow aspirate harvests from the same horse were found to differ in MHC class II expression. These results suggest that a combination of factors including genetics, bone marrow aspirate quality, immunologic background at a given point in time, and culture conditions are responsible for the extreme heterogeneity of MHC class II expression observed.

Similar to the majority of the equine MSCs in this study, human MSCs found to be MHC class II positive displayed a diffuse or broad MHC class II expression peak on flow cytometry histogram analysis suggestive that the individual MSCs themselves varied in terms of the number of MHC class II molecules expressed on their cell surface [6]. Both mouse and human MSCs in these studies otherwise had the expected profile of positive and negative markers for "stemness" and were capable of tri- lineage differentiation as typical for MSCs. While tri-lineage differentiation assays were not performed in this study, equine MSCs cultured in our

laboratory using the same methods have previously been shown to be capable of in vitro adipogenic, osteogenic, and chondrogenic differentiation [30]. Although it is possible, or perhaps even likely, that the cultured cells collectively referred to as MSCs are not all stem cells, at no time during flow cytometric analysis of MSCs from any horses in this study did there appear to be two or more distinct cell types or cells with distinct marker profiles. All MSCs remained with the previously determined MSC gate and consistently expressed the $CD44^{hi}$, $CD29^{hi}$, and $CD45RB^{lo}$ phenotype [30]. This suggests that MSCs themselves are capable of extreme variation in MHC class II expression.

The variation observed in expression of the cell surface markers CD90 and CD11a/CD18 (LFA-1) is difficult to interpret as it has previously been shown that equine bone marrow-derived MSCs express variable levels of these markers, and that their expression changes over time in culture such that cells are $CD90^{lo}$ and $CD11a/CD18^{hi}$ early on but become $CD90^{hi}$ and $CD11a/CD18^{hi}$ over time. [30]. Such variability may be expected when using examining cells from different donors. A weak negative correlation was found between CD90 and MHC class II expression when analyzing the phenotype data as a whole, but this correlation was not consistent for the same MSCs over multiple passages. There were numerous examples of MSCs that remained strongly positive for MHC class II over several passages but whose expression of CD90 increased over those same passages. Similarly, while a moderate positive correlation was found between CD11a/CD18 and MHC class II expression, there were numerous examples of MSCs that remained MHC class II positive over several passages but whose expression of CD11a/CD18 decreased. These findings suggest that such correlations may be more of a temporal finding than a defining finding as the greatest number of MSCs were MHC class II

positive during early passages, when we would expect CD90 expression to be low and CD11a/CD18 expression to be high [30].

The finding that the majority of passage 2-4 MSCs in this study were positive for MHC class II expression has not been previously described. Equine studies examining MHC class II expression and immunogenic properties of MSCs to date have largely focused on later passage cells [17, 18, 34], even though most MSCs used in experimental models and in clinical applications are early passage in order to maintain proliferative ability [20, 21, 35, 36]. This knowledge is critical for potential allogeneic applications, as MHC-mismatched MHC class II positive MSCs incited significant proliferation of T cells from responder horses of all ELA haplotypes (A2, A3, and A9) examined in this study. This underscores the caution that must be taken when using allogeneic MSCs. Thoroughbreds were used in the present study, but most breeds of horses can have multiple ELA haplotypes [29, 37]. It is therefore not safe to assume that donor MSCs from any breed of horse can be used in a recipient horse of the same breed without the potential for an immune reaction.

The results of this study suggest that MSCs should be immunophenotyped and confirmed as MHC class II negative prior to allogeneic application. As demonstrated, examination of MSCs for classic morphologic characteristics cannot be used alone to assess the potential for MHC class II expression. While all MSCs in this study that displayed less desirable morphologic characteristics were MHC class II positive, some MSCs displaying the classic morphology were also found to be MHC class II positive. Both the quantity and quality of MSCs decreases with advanced donor age [38-40], so younger horses are sought as donors. However, in the present study, even MSCs isolated from the younger horses that had the classic morphology were MHC class II positive. Additionally, it must be considered that even MHC class II negative MSCs

could potentially upregulate their MHC class II expression if implanted into an area of active inflammation as was demonstrated upon in vitro stimulation with IFN-ɣ.

In summary, we have shown that equine MSCs are heterogeneous in MHC class II expression and that MHC-mismatched MHC class II positive MSCs are capable of inciting an immune reaction in vitro. It is essential that all potential donor MSCs be immunophenotyped and screened for MHC class II expression. Further studies are warranted to determine the in vivo response to MHC-mismatched MSCs that are either MHC class II negative or MHC class II positive as well as the effect of multiple injections of these cells on a recipient's immune response. Further research is also required to determine the effect of MHC class II expression on equine MSC immunosuppressive properties.

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CHAPTER 5

GENERAL DISCUSSION

Application of Findings to Future Studies

The results of the studies presented in this dissertation have led to the formulation of many important biologic questions and to the design of future studies as discussed below. Importantly, before we can proceed to a comparison between equine MSCs and iPSCs for future in vivo studies to examine both the immunogenic, immunomodulatory, and regenerative properties of the cells, some unanswered questions concerning equine MSCs remain.

Can we control culture conditions to decrease MHC class II expression by equine MSCs?

There are a large number of mediators known to affect MHC class II expression. These mediators can be cell type dependent as well as species specific [1-3]. Mediators of particular interest to the regulation of MHC class II expression by equine MSCs during the culture period are bFGF (FGF-2) and TGF-β1. In human MSC MHC class II expression studies, it has been demonstrated that bFGF in the culture media starting at P0 can cause markedly increased expression of MHC class II by MSCs [3, 4]. Interestingly, the same effect is not observed for mouse MSCs, which have previously been shown to have a different MHC class II regulation pathway than humans [5]. The upregulation of human MSC MHC class II expression by bFGF was consistent in all MSCs evaluated in a study by Bocelli-Tyndall et al. and was dose (0.1ng/mL to 10ng/mL) and donor dependent only in the degree of upregulation [3]. As we routinely use bFGF (1ng/mL) in our equine MSC media throughout the culture period, it would be very interesting to repeat bone marrow aspirates on a few of our MHC haplotyped horses

whose MSCs were previously MHC class II positive and to then isolate and culture expand their MSCs both with and without bFGF in the media to compare MHC class II expression.

It has also been demonstrated that TGF-β1 is able to reduce IFN-ɣ-induced MHC class II expression both in mice and human [5]. As we have already observed that IFN-ɣ stimulation of equine MSCs results in marked upregualtion of MHC class II expression, it would be logical as a next step to then determine if this upregulation can be reduced by the addition of TGF-β1 to the culture media. The TGF-β1 used could be the recombinant protein or of even greater clinical interest would be to use platelet rich plasma (PRP) as a source of TGF-β1 [6]. As discussed in the "Current Status of Clinical Stem Cell Use in the Horse" section of chapter 1, it is common in equine practice to implant MSCs with PRP [7]. Could this be of potential benefit to downregulate MHC class II expression when implanting the MSCs clinically in area of active inflammation? This is certainly an intriguing question that could be investigated preliminarily in vitro by culturing MSCs with IFN-ɣ alone, IFN-ɣ in addition to recombinant TGF-β1, and IFN-ɣ in addition to PRP to compare MHC class II expression.

In addition to soluble mediators, cell density has also been observed to affect MHC class II upregulation following IFN-ɣ stimulation [5]. Opposite effects were observed for human and mouse MSCs, with low-density human MSCs being more permissive of IFN-ɣ-induced MHC class II upregulation and high-density mouse MSCs being more permissive [5]. Equine MSCs in our study presented in chapter 4 were all passaged at approximately the same rate and density. It would be interesting, therefore, to plate the MSCs at different densities and observe if there is any effect on MHC class II expression both with and without IFN-ɣ stimulation. If certain cell culture conditions were able to prevent MHC class II expression by MSCs, this would alleviate a lot of our concern over a potential immunogenic response to allogeneic MSCs in vivo.

Does equine MSC MHC class II expressions affect their immunomodulatory properties in vitro?

Further in vitro studies are necessary to determine if equine MSC MHC class II expression affects their immunomodulatory properties. While a preliminary study by Carrade et al. has demonstrated that equine bone marrow-derived MSCs are capable of immunomodulation in vitro, the MSCs used in that study were late passage MHC class II negative cells and the leukocytes used for the MLRs were not from haplotyped horses [8]. Therefore, in future studies we would use MSCs and PBLs isolated from our MHC haplotyped horses and test their immunomodulatory capability as described for mice in our study presented in chapter 3.

What is the in vivo response to equine MSCs that are either MHC class II positive or negative prior to implantation?

While in vitro studies are necessary to understand basic biologic properties of MSCs and to gain preliminary data, of ultimate importance is the question of whether or not equine MSCs that are either MHC class II positive or negative prior to implantation can incite an immune response in vivo. In order to assess this, a subdermal transplant model developed in Dr. Antczak's laboratory will be used [9]. MSCs will be transplanted into the neck region of a MHC-mismatched horse and serial blood and injection-site biopsy samples will be collected from the recipient horse once weekly for 4 weeks. Lymphocyte microcytotoxicity assays will be used to measure the antibody response to the transplanted cells. Peripheral blood leukocytes (PBLs) from the MSC-donor horse will be isolated and tested against antisera from the recipient horse. The cytotoxic antibody titer will be determined by the highest dilution of each serum (N) that

results in the death of at least 80% of the donor cells [9]. Cytotoxic antibody titer results will be reported as (1:N), where a higher N is indicative of a stronger cytotoxic antibody response and will be compared between MHC class II positive and negative MSCs.

Injection-site biopsy sections will be cryopreserved, sectioned and stained with monoclonal antibodies to equine leukocyte and MHC antigens. Morphometric analysis will be performed on 4 random fields of view as previously described by the Atczak laboratory to quantify mean T cell (CD3) and macrophage (CZ2.2) numbers between recipient horses for the different cell types [9]. Sections from MSC recipients will also be stained with monoclonal antibodies to equine MSCs (CD44 and CD29) for identification of MSCs.

Importantly, all of these in vitro and in vivo studies can also be used to compare the immunogenic and immunomodulatory properties of equine MSCs to equine iPSCs once we have generated iPSCs from our MHC haplotyped horses. We have become proficient in the in vitro studies as described in this dissertation for both mice and horses, and our collaborator, Dr. Douglas Antczak, has extensive experience with the in vivo studies described above. In addition, these in vivo studies are already partially funded by my National Institutes of Health Mentored Clinical Scientist Research Career Development Award (K08).

Can implantation of MSCs with PRP reduce an immune response in vivo?

If we were to discover in our in vitro studies that PRP could downregulate MHC class II expression and if we were to find that allogeneic MSCs incited an immune response in vivo, we would then test whether or not implanting the MSCs with PRP in vivo results in abolishment of an immune reaction. This information would be critical to future clinical application of MSCs and is quite relevant as that is how MSCs are being currently being implanted into tendon and

other musculoskeletal lesions by many practitioners [7] PRP is also routinely used in human sports medicine and could be applied in the same manner for human patients treated with MSCs [10-13].

If necessary, is it possible to prevent MHC class II expression by targeting CIITA, the master regulator of MHC class II gene expression?

Another intriguing idea for MHC class II modulation is to target class II transactivator (CIITA), the master regulator of MHC class II gene expression, either through transcriptional or post-translational modification [1, 2]. Naturally occurring epigenetic regulation of CIITA through promoter hypermethylation and histone deacetylation has been implicated as a cause of placental immune tolerance [1], and it has been demonstrated that treatment of trophoblasts with methylation and histone deacetylation inhibitors in addition to IFN-ɣ results in removal of CIITA transcriptional repression [1]. It is also known that CIITA is post-translationally modified by acetylation, phosphorylation and ubiquitylation [1]. The broad effects of treating MSCs with synthetic methylation, histone, or post-translational modifiers are unknown and likely complex but could potentially be of great value for manipulating the immune status of the cells.

Clinical Relevance

As discussed throughout this dissertation, the field of regenerative medicine is in need of banked and screened stem cells that would essentially be "off the shelf" and ready for use upon a patient's diagnosis. It has previously been demonstrated that genetic background can affect MSC proliferation and differentiation rates [14]. In this dissertation research, we have also demonstrated that genetic background affects the ability to generate iPSCs. It therefore may not

be feasible to use autologous MSCs or iPSCs from all patients. Even more importantly, the time it takes to isolate MSCs or to generate iPSCs and screen them for efficacy and safety is not practical in a clinical setting. In order to advance the field by being able to treat our patients at an appropriate time, we need to have previously cultured and screened allogeneic cells available to us.

In the studies described in chapter 3, we have demonstrated that undifferentiated iPSCs have similar immunogenic and even more potent immunomodulatory properties than MSCs in vitro. This is an important first step towards determining if these cells could be used in an allogeneic fashion. As discussed, however, it is imperative to determine if iPSCs retain these properties upon differentiation for potential future use in regenerative or transplantation medicine.

In the studies described in chapter 4, we have reported for the first time the extreme heterogeneity that exists in MHC class II expression by equine MSCs and that MHC class II positive equine MSCs are capable of inciting an immune response in vitro. This knowledge is critical for the treatment of our equine patients as well as for future studies using the horse as an animal model for human diseases. Our next experiments to determine if we can modulate this MHC class II expression in culture will be of great interest prior to our in vivo studies to examine the immune response to allogeneic MSCs.

Through completion of these dissertation studies we have obtained the skills and knowledge needed to move forward and complete further studies which will aid in the understanding of the safety and efficacy of allogeneic stem cell therapy. We are very fortunate to have the expertise of our collaborators and the generous use of the MHC haplotyped horses belonging to our collaborator, Dr. Douglas Antczak. With such aid, we are making significant

progress toward our long-term goal of comparing not only the in vivo immunogenic and immunomodulatory properties of allogeneic equine MSCs and iPSCs, but also their regenerative potential in an equine tendonitis model which will benefit both equine and human patients.

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