

The study of used of adult stem cells differentiated into the hematopoietic cells for use in the therapy of marrow ablated horse

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Abstract:

Hematopoietic stem cells because of ready accessibility of the system and by the ease with which fractions of cells from bone marrow could be used to rescue hematopoietic systems whose marrow had been ablated by chemotherapy or x-ray irradiation, driven attention of researchers by the use of bone marrow for transplantation in patients and encouraged us to investigate multipotent adult progenitor cells in marrow ablated horse. multipotent adult progenitor cells was derived from cultures of adherent cells from bone marrow of a horse has a great potential for differentiation into hematopoietic cells, acquired functional characteristics of tissues following differentiation in-vitro. It was engrafted into the marrow-ablated compromised of the same horse. The cells promote tissue repaired either by proliferating into the phenotype of the damaged cells or by creating a milieu that promotes regeneration by endogenous cells of the tissues. The obvious barrier from an unrelated donors and thresholds for events such as malignant transformation likely was due to provoke immune reaction.

Key words: Hematopoietic stem cells, Marrow ablated, Transplantation, Immune reaction, Horse

Introduction:

The most common human cell-based therapy applied today is hematopoietic stem cell (HSC) transplantation. HSCs can be defined by two essential properties: self-renewal and multilineage hematopoietic differentiation. These combined HSC properties allow them to differentiate into all blood cell types (multilineage) in a sustained manner for the lifetime of the animal, which requires their ability to make cellular copies of themselves (self-renewal). These features can be tested by transplantation from donor to recipient and provide a functional basis to define and identify HSCs. Currently, human bone marrow (BM), represent the major sources of transplantable HSCs, but their availability for use is limited by both quantity and compatibility. Although increasing evidence suggests that somatic HSCs can be expanded to meet current needs, their *in vivo* potential is concomitantly compromised after *ex vivo* culture. Bone marrow transplantation (BMT), also called hematopoietic stem cell transplant or hematopoietic cell transplant, is a type of treatment for cancer and a few other conditions as well. In bone marrow transplantation, a patient receives very high doses of chemotherapy or radiation therapy, which kills cancer cells and destroys all the normal cells developing in the bone marrow, including the critical stem cells. After the treatment, the patient must have a healthy supply of hematopoietic stem cells reintroduced, or transplanted. The transplanted cells then reestablish the blood cell production process in the bone marrow (Langston, 2005). BMSC have been seen, under specific conditions, to differentiate into neuronal cells (Sanchez-Ramos et al., 2000) and improve cardiac function in patients with myocardial infarction (Mathur & Martin, 2004). Beyond these discoveries, SC research still has more milestones to overcome. If SC are to become the cell therapy people hope for, methods of harvesting, enrichment, and storage must be developed (Rossi & Cattaneo 2002). Currently the signaling devices to compel SC to specialized are not consistent in every study and this greatly inhibits the progression of SC research (Berná, et al., 2001). It was once thought that myocardial infarction was irreversible, but studies have shown that injection or mobilization” of bone marrow SC does recover cardiac function. SC not only distinguished into cardiac myocytes, but they stimulated dwelling cardiac stem cells as well (Kang, Kim, & Park, 2004; Lee & Makkar, 2004). When bone marrow SC were directly introduced in the infarct area of a mouse, the cells migrated, differentiated and improved heart functioning. In one study, ten patients treated with SC had smaller infarcted areas as well as improved stroke indexes (Lee & Makkar, 2004). While SC transplantation for myocardial infarction is still young, it carries great possibilities because most of the clinical studies and experiments held show some degree of improvement in the functioning of the heart and in the reduction of the infarct region (Mathur, A. & Martin, J. F., 2004; Lee & Makkar, 2004).

Mesenchymal stem cells (MSCs), (Fridenshtein, 1982; Pittenger et al., 1999)¹ which can be isolated from bone marrow, adhere to plastic *in vitro* and expand in tissue culture, with a finite lifespan of 15–50 population doublings (PDs), (Pittenger et al., 1999). Under proper inductive stimuli, MSCs differentiate *in vitro* and *in vivo* into adipocytes (Wakitani et al., 1995), chondrocytes (Pittenger and Martin, 2004; Wakitani et al., 1995), osteoblasts (Gronthos et al., 1994; Reyes et al., 2001), and smooth and skeletal myoblasts (Jaiswal et al., 2000; Pittenger et al., 1999). Recently, we demonstrated that human, mouse, and rat postnatal bone marrow (BM) contains primitive progenitors termed multipotent adult progenitor cells (MAPCs), (Reyes et al., 2001; Schwartz et al., 2002). MAPCs can be expanded under defined low-serum conditions for more than 100 (human) or 400 (rat) PDs without telomere shortening or

karyotypic abnormalities. MAPCs not only differentiate into mesenchymal cell types (osteoblasts, chondrocytes, adipocytes, and smooth and skeletal myoblasts), (Reyes et al., 2001 ; Jiang et al., 2002) but also into cells with phenotypic and functional characteristics of endothelial cells(Reyes et al., 2001), hepatocytes(Schwartz et al., 2002), and neural cells(Keene et al., 2003). Although many studies have tested the effect of different stem cell populations in rodent models, results seen in rodents can often not be duplicated when applied to human and animal cells, or in human and animal clinical trials. For instance, there are extensive data from the hematopoietic stem cell literature showing that significant differences exist when comparing human and murine hematopoietic stem cells (Horn et al.,2004). Likewise, more recent studies demonstrating that grafting of BM-derived cells in a cardiac infarct model in mouse results in significant levels of engraftment and functional improvement, have not been replicated in human clinical trials (Orlic et al., 2001). Such differences may be due to the remarkable anatomical and physiological differences between the mouse and human heart. In addition, the small size of the mouse heart makes it difficult to evaluate the functional consequences of cell transplantation with good spatial differentiation. These studies demonstrate therefore that a number of observations made in rodent models will need to be confirmed in larger animal models prior to applying to human clinical trials. Hence, the rationale for isolating MAPCs from horse, which would allow, at least in some disease models, results from rodent models to be confirmed and extended preclinically to perhaps more relevant models.

In this study, we demonstrate that MAPCs can be isolated from horse BM by methods similar to those used for mouse, rat, and human with minor modifications. These horse MAPCs (horseMAPCs) have the same phenotype as human, rat, and mouse MAPCs; differentiate in vitro into cells with phenotypic and functional characteristics of hematopoietic cells for use in the therapy of marrow ablated horse.

Materials and Methods:

Experimental Horses:The present study was conducted on 10Thoroughbred horses, between 2-5 years of age . Horses were kept under Uniform International Health Condition. Each of them carry health certificate and kept with proper management and care with proper feed and water without stress. To help minimize the problems that can be caused by the expected immune response, a donor who has similar genetic makeup to the patient was preferred. The patient's cells will seem "less foreign" to the transplanted donor cells. Siblings (ie, Off springs who share the same parents as the patient) are typically tested first, as they have the greatest chance of sharing genetic characteristics with the patient. Each sibling has a one in four chance of being matched to the patient. In general, parents, Off springs and relatives are not suitable donors since they do not share the same parents, and therefore do not have the same genetic material. Before undergoing BMT, horses checked a complete evaluation of their health. The horse complete health history was reviewed by the transplant team, including blood testing for liver and kidney function, an examination of the mouth and teeth (a common site of infection after transplant), an examination of the heart (usually including an electrocardiogram (ECG) and/or echocardiogram (ultrasound of the heart)), and a bone marrow biopsy or PET scan to determine the status of the disease. Horses care taker met with a transplant coordinator to discuss the transplant process. Because horses who received donor bone marrow were hospitalized for several weeks to months, it was important that we have a clear understanding of what will happen and what services were available. A number of medications were required before, during, and after BMT. To avoid the need for multiple

intravenous lines and needle sticks, most horses had a central line placed before treatment begins. This requires a short surgical procedure to insert a thin, flexible plastic tube into a large vein in the neck. The line usually has two ports, which can be used to infuse medications or blood products (including the stem cell product), as well as to withdraw blood samples. After the central line was placed, horses watched for signs and symptoms of infection (pain, redness, swelling, or fluid drainage from the site, fever or chills).

Harvesting horse stem cells: The horses were having an autologous transplant, stem cells was a removed from the horse before intensive chemotherapy or radiation begins. The most common sources for stem cells were bone marrow and blood, we used bone marrow sources. If the patient's bone marrow has been invaded with cancer cells, stem cell removal may be preceded by one or more courses of chemotherapy. Removal (called harvest) of bone marrow stem cells is done while the patient is under general (completely asleep) or epidural anesthesia, and involves removing a sample of bone marrow fluid from the patient's pelvic and hip bones with a long needle. Electromyography was used as an aid to the diagnosis of equine motor neuron disease in a conscious horse while it was under caudal epidural anaesthesia. A muscle biopsy was taken to confirm the diagnosis which was then supported by a postmortem examination.

RESULTS:

Isolation of hMAPCs: In initial studies ($n = 10$ postnatal horse BM samples), we attempted to isolate MAPCs from BM aspirates immediately following harvesting, as we have described for human MAPCs (Reyes et al., 2001). However, we were unsuccessful. After 2–3 days colonies of large flat cells appeared, which were $CD44^+$, $CD45^-$, $MHC-I^+$, $MHC-II^-$, $CD90^+$, consistent with an MSC phenotype (Liu et al., 2004). Such cells could only be passaged for ~ 30 PDs, and they differentiated as classic MSCs only in mesodermal cell lineages (Liu et al., 2004). Initially we thought that this could be due to the age of the donor animals, as animal and human MAPCs are more frequent in marrow of younger animals and humans

(Reyes et al., 2001). We therefore tested whether MAPCs could be isolated from the BM of younger, that is, prenatal, animals. From the first attempt, cells with morphological and phenotypic features of MAPCs could be isolated from BM harvested ~ 48 hours after the death of a fetal animal. Interestingly, in contrast with cultures initiated with BM from postnatal animals, we did not see cell colonies appear until approximately 7 days after initiation of the culture. These cells were morphological smaller and did not have the typical flat and elongated MSC appearance. Rather, they were small cells, like mouse and rat MAPCs (Jiang et al., 2002). We hypothesized that a number of differences in the samples used in the initial 20 attempts from postnatal animals and the successful attempt from fetal BM might contribute to the different outcome, including the age of the animal, time of plating after the death of the animal (immediately after harvesting in postnatal samples and 48 hours after the death of the animal for the fetal sample), and method of collecting the sample (aspiration in postnatal animals and flushing from the bone in fetal animals). To discriminate among these possibilities, we next evaluated the ability to isolate MAPCs from fetal horse BM immediately after the death of the animal or 48 hours after death ($n = 8$ experiments). As we had seen during our initial attempts at isolating MAPCs, when BM obtained immediately after the death of the fetal animal was used to initiate cultures, colonies of relatively large and flat cells appeared by day 3. By day 7, the larger cells grew to near confluence, and we could not detect colonies of the smaller cells. On the other hand, when BM collected 48 hours after the death

of the fetal animal was used, smaller cells appeared by day 7. These studies suggested thus that MSC-like cells may proliferate faster than MAPC-like cells and outcompete the latter when both of them are plated together. The fact that BM harvested 48 hours after the death of the animal did not lead to outgrowth of the larger and flatter cells suggests that MSC-like cells may be selectively lost when horse BM is stored at 4°C. As there are no definitive cell surface determinants that can discriminate between the two cell populations, it is not possible to definitively prove this hypothesis. We re-evaluated the ability to isolate MAPCs from postnatal horse BM and found that keeping the bone for 36–48 hours at 4°C also decreased the frequency of MSC-like colonies appearing early after plating and allowed for the outgrowth of cells with MAPC morphology, phenotype, and functional characteristics (described below).

The second difference between the initial experiments performed with postnatal horse BM and the isolation of MAPCs from fetal horse BM was the manner of harvesting (aspiration in postnatal horse and flushing in fetal horse). We also compared in three experiments the two methods of harvesting cells prior to MAPC culture. These studies were done by maintaining the horse bone at 4°C for 36–48 hours, followed by flushing or aspirating the BM. BM obtained by flushing generated more readily MAPCs than when BM was aspirated, possibly indicating that hMAPCs are attached more strongly to bone spiculae, similar to other stem cells within BM (Calvi et al., 2003), and are dislodged more efficiently when the marrow cavity is flushed than when the BM is aspirated. In subsequent studies, we demonstrated that homogenous hMAPCs populations could be culture-isolated and expanded for more than 100 PDs from three of four fetal horse BM samples and one of three postnatal horse BM samples harvested by flushing from the bone 48 hours after the death of the animal. Hence, aside from the manner in which BM cells are obtained to initiate MAPC cultures, age may also be a factor in the ability to isolate MAPCs.

Characterization of hMAPCs: Culture isolation and subsequent expansion involved initial plating of the total BMMNC for 2–3 passages, as indicated in Materials and Methods, followed by single-cell subcloning. In all cases, cells were plated at ~1 cell per well in four 48-well plates. Of these, approximately 10 wells contained a single colony of cells with the typical small MAPC morphology (supplemental online Table 2). Approximately 80% of clones could be expanded for >30 PDs. These clones were then evaluated by Q-RT-PCR for Oct3a transcript levels, as MAPCs from other species express Oct3a. We used primers designed against human ESC-specific Oct3a, which also identifies horse Oct3a (determined using testicular horse tissue and confirmed by sequencing). Of these individual clonal populations, 64% had Oct3a transcript levels between 1% and 50% of hESCs, 21% had levels between 0.1% and 1% of hESCs, and 15% had levels <0.1% of hESCs. From each individual BM, one clone with levels of Oct3a mRNA >20% of hESCs was maintained in culture beyond 60 PDs. All other clones were cryopreserved and not evaluated further. For the four clones, one each from three fetal and one postnatal horse BM isolations that were maintained in culture, levels of Oct3a were between 5% and 20% of those identified in hESCs and remained stable for >90 PDs. The expression of Oct3a in hMAPCs was confirmed by immunocytochemistry. It should be noted that Oct3a mRNA could not be detected in horse MSCs or all clonal MAPC populations, cell doubling time was 24 hours for the initial 30–40 PDs and 36–48 hours when cultures reached >40 PDs. hMAPCs are round or triangular, lightly adherent, less than 10 µm in diameter, and exhibit a very high nucleus-to-cytoplasm ratio. hMAPCs have an instinctive ability to separate from each other following cell divisions, even when cells reach higher density (~4,000 cells per cm², just before they are passed). When cultures were allowed to grow to very high densities (>6,000 cells per cm²),

proliferation slowed down, demonstrating contact inhibition. Phenotypic analysis after 50 PDs indicated a homogenous population of cells that is negative for CD44, CD45, MHC class I, and MHC class II whereas cells analyzed prior to 50 PDs were negative for CD45 and MHC class II but were mixed for expression of MHC class I and CD44 (Fig. 1).

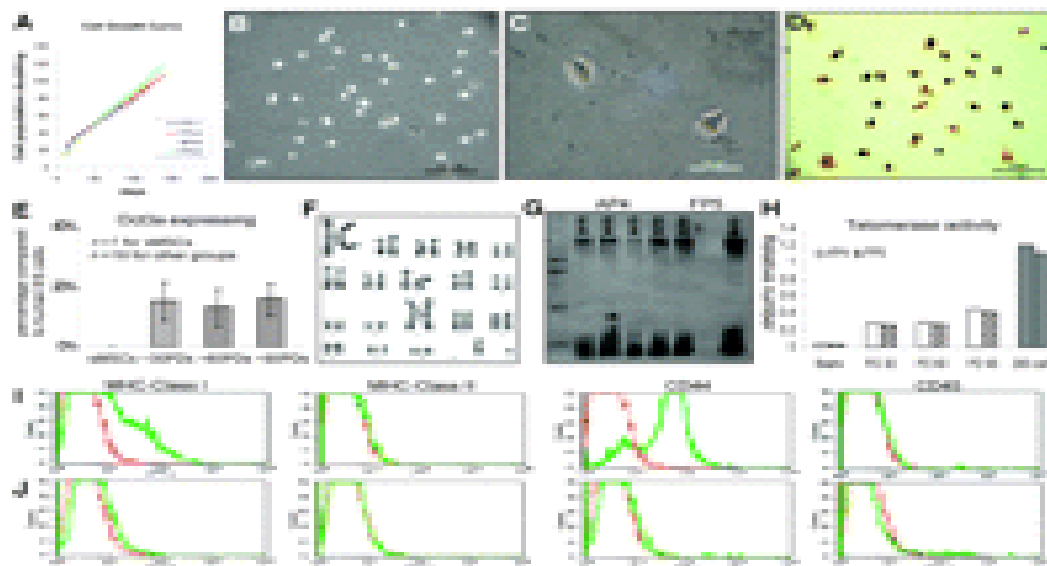


Figure1. Horse multipotent adult progenitor cells (hMAPCs) morphology and phenotype. horseBM MNCs were plated with epidermal growth factor and platelet-derived growth factor-BB in FN-coated flasks. After passing in bulk for 2–3 passages, hMAPCs were subcloned at 1 cell per well in fibronectin-coated 48-well plates in the same medium and expanded at 500 cells per cm^2 . (A): Growth curve. Cells were enumerated at each passage under a hemocytometer. Data shown are for three fetal clonal cell lines (FP-1, FP-4, and FP-5) and one postnatal clonal cell line (AP4). (B): Colony generated from a single sMAPC was replated and expanded at 500 cells per cm^2 until a density of $\sim 4,000$ cells per cm^2 was reached. (C): High-power light microscopic view of hMAPCs morphology at 80 PDs. (D, E): hMAPCs express Oct3a evaluated by both immunohistochemistry (D) and quantitative-reverse transcription-polymerase chain reaction (E). Mean \pm SD of Oct3a transcript levels at different PDs compared with hECs and MSCs. mRNA levels were normalized using glyceraldehyde-3-phosphate dehydrogenase as a housekeeping gene. (F): Karyotype of hMAPCs at 90 PDs (G): Telomere length of hMAPCs: AP4 at PD 30 (lane 2), PD 60 (lane 3), and PD 90 (lane 4); FP-5 at PD 30 (Lane 5), PD 60 (lane 6), and PD 90 (lane 7). Lane 1 is a ladder. (H): Telomerase activity of hMAPCs cultured for 30, 60, and 90 PDs for both AP4 and FP-5 cell line. (I, J): hMAPCs cultured for both ~ 30 PDs (I) and ~ 80 PDs (J) were labeled with fluorescein isothiocyanate-coupled antibodies against MHC class I, MHC class II, CD44, or CD45 or IgG isotype control antibodies. Cells were analyzed using a FACSCalibur. Red line, control IgG; green line, specific antibody. Scale bar = 100 μm (B), 10 μm (C), 50 μm (D). Abbreviations: AH, adult horse; FH, fetal horse; MHC, major histocompatibility complex; PD, population doubling; HMCC, horse mesenchymal stem cells.

Telomere lengths were evaluated at ~ 30 , ~ 60 , and ~ 90 PDs in the three fetal hMAPC lines and one postnatal hMAPC line. Telomere lengths did not differ between MAPCs isolated from fetal or postnatal horse, and telomeres did not shorten following extensive passaging. Significant levels of telomerase activity could be measured in the two hMAPCs populations tested, again irrespective of the age of the donor animal. Cytogenetic analysis of the cultured cells at multiple PDs showed that cells were diploid and contained no cytogenetic abnormalities (based on 20 cells in metaphase analyzed per sample at < 90 PDs; (Fig. 1), although some cells evaluated at PDs beyond 90 showed polyclonal karyotypic abnormalities. When established populations of hMAPCs were replated at high density ($> 2,000$ cells per cm^2), and passaged every 4 days when they reached cell densities of $> 8,000$ cells per cm^2 for 3–6 passages, they became morphologically larger. This was associated with an acquisition of CD44 and MHC class I antigens on the cell membrane and a loss of Oct3a

expression determined by Q-RT-PCR and immunohistochemistry (data not shown). When cultures were subsequently replated at low cell densities (100–500 cells per cm^2) for >20 PDs, they did not reacquire the typical small MAPC morphology, and they remained CD44- and MHC class I-positive and Oct3a-low (<0.001% of hESCs). Moreover, we found that telomeres shortened in cells allowed to grow at high density and replated for 20 and 40 PDs at low density (Fig. 2).

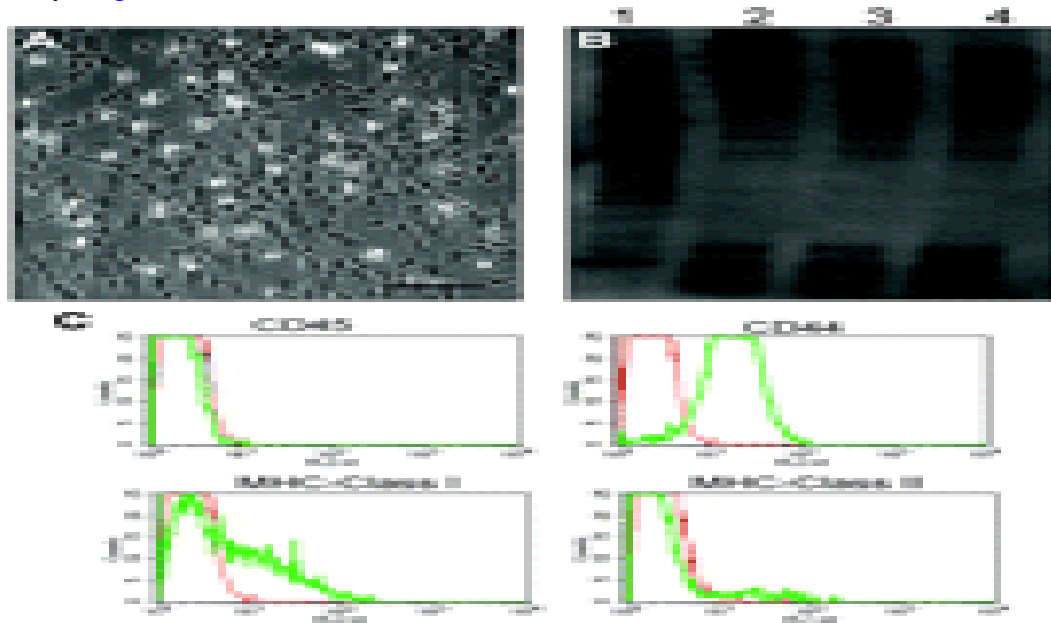


Figure 2. Loss of multipotent adult progenitor cell (MAPC) phenotype upon maintenance of MAPCs at higher density. When established populations of horse multipotent adult progenitor cells (hMAPCs) were grown at high confluence (>2,000 cells per cm^2) (A), hMAPCs became morphological larger and began to attach to each other. (B): Telomere lengths of hMAPCs shorten even upon replating at low density for >20 population doublings (PDs). Lane 1, ladder; Lane 2, hMAPCs maintained at 500 cells per cm^2 ; Lanes 3 and 4, hMAPCs maintained at >2000 cells per cm^2 and replated at 500 cells per cm^2 for 20 PDs (lane 3) and 40 PDs (lane 4). (C): hMAPCs maintained at >2,000 cells per cm^2 and replated at 500 cells per cm^2 express CD44 and MHC class I antigens on their cell membrane. Scale bar = 100 μm (A). Abbreviation: MHC, major histocompatibility complex.

In Vitro Differentiation of hMAPCs: The in vitro differentiation capabilities of hMAPCs to mesoderm, neuroectoderm, and endoderm were evaluated by the addition of cytokines on the basis of previous differentiation studies of ESCs (Li et al., 2005) and MAPC differentiation (Reyes et al., 2001). Studies were done using two fetal MAPC lines and one postnatal MAPC line, and each differentiation was performed multiple times at population doublings 60–100. Not surprisingly, we demonstrate that hMAPCs can differentiate into osteoblasts, chondroblasts, adipocytes, and SMCs, which typical MSCs can do. When hMAPCs were treated with osteogenesis differentiation medium, a significant increase in alkaline phosphates activity and mineral deposition by Alizarin red staining were seen at day 15. In response to TGF- β 1 and BMP4, hMAPCs take on an early chondrocyte phenotype. Differentiating cells expressed aggrecan and collagen mRNA and generated Alcian Blue staining matrix (Fig. 3). When the differentiation ability of hMAPCs allowed to grow at high density for 3–6 passages or hMAPCs grown at high density for 3–6 passages and then replated at 100–500 cells per cm^2 for an additional 20 PDs was tested, we demonstrated that although SMC differentiation could be induced, they no longer differentiated to osteoblast-like cells (data not shown). These observations are consistent with the MSC-like phenotype and the absence of Oct3a.

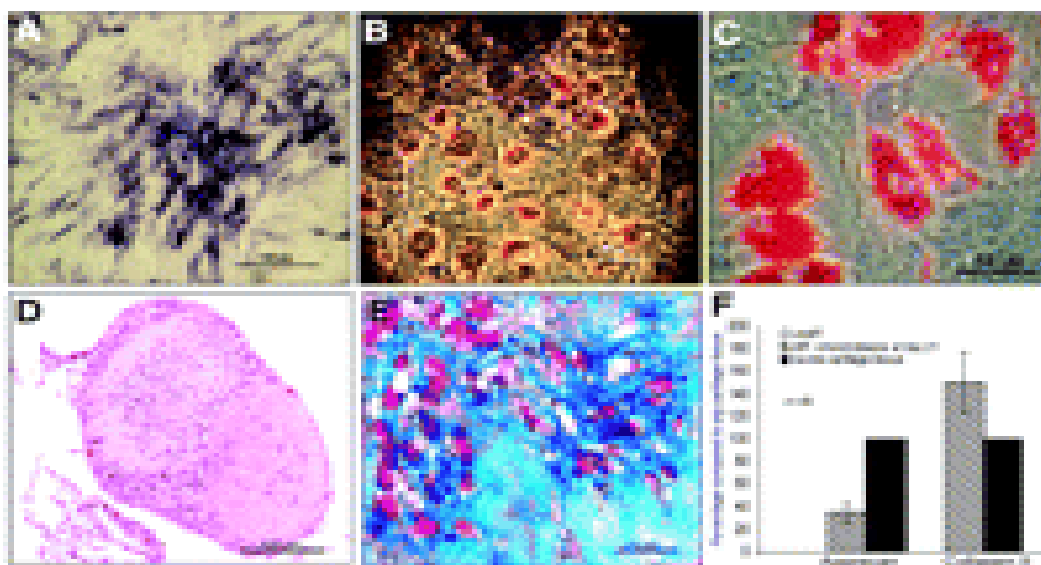


Figure 3: horse multipotent adult progenitor cells (hMAPCs) differentiate into osteoblasts, chondroblasts, and adipocytes. hMAPCs from three donors were induced to differentiate to osteoblasts as described in Materials and Methods. (A, B): Osteogenesis was demonstrated by the increase in alkaline phosphatase (A) and calcium deposition stained by alizarin red (B). Undifferentiated hMAPC groups were tested as a negative control. All control groups were negative, except that very weak alkaline phosphatase was found in some undifferentiated hMAPC control group (data not shown). (C): Adipogenesis was demonstrated by the accumulation of neutral lipid vacuoles stained by oil red O. Undifferentiated MAPCs were negative for oil red O staining (data not shown). (D–F): Chondrogenesis was shown by H&E staining of sectioned micromasses showing cartilaginous morphology (D) and Alcian Blue staining of a section showing a blue staining matrix, consistent with cartilage (E). (F) Mean \pm SD of aggrecan and collagen type II transcript levels at day 21 of differentiation ($n = 8$), compared with primary horse cartilage and undifferentiated MAPCs. The mRNA levels were normalized using glyceraldehyde-3-phosphate dehydrogenase as a housekeeping gene. Scale bar = 100 μ m (A–C), 250 μ m (D, E). Shown are representative examples from three donors. Abbreviation: diff, differentiation.

Discussion:

The primary source of early human stem cells was adult bone marrow, the tissue that makes red and white blood cells. Since scientists realized that bone marrow was a good source of stem cells, early transplants were initiated in the early 1970's to treat diseases that involved the immune system (genetic immunodeficiencies and cancers of the immune system). Bone marrow-derived stem cell therapy has been extremely successful, with dozens of diseases being treated and cured through the use of these adult stem cells. However, because the donor tissue type must be closely matched to the patient, finding a compatible donor can be problematic. According to many stem cell researchers, embryonic stem cells are the preferred stem cells for cell-based therapies. Although they tend to be more versatile than adult stem cells, other sources (including umbilical cord stem cells) have proven to be just as versatile (Kögler et al., 2004). The same properties that make embryonic stem cells so versatile are also the properties that make them unusable for therapy. Unless completely differentiated prior to use in patients, these cells will migrate throughout the body to produce tumors. Experiments performed in mice and rats have shown that spontaneous tumor formation is a persistent problem (Bjorklund et al., 2002). Maintaining and growing embryonic stem cell lines has also been problematic. Some of these lines have mutated, making them unusable in patients (Draper et al., 2003). The main problem with embryonic stem cell research is the tissue incompatibility (Phimister and Drazen, 2004). Millions of lines must be established in order to serve a significant percentage of potential patients. The use of autologous adult stem

cells (cells from the patient) eliminates the problems with tumorigenesis, mutation, and tissue incompatibility. Since millions of lines would be required to serve all the different tissue types of patients, the goal of therapeutic cloning is to produce pluripotent stem cells with the nuclear genome of the patient and induce the cells to differentiate into replacement cells, for example, for use in the therapy of marrow ablated horse. Reports on the generation of pluripotent stem cells or histocompatible tissues by nuclear transplantation, and on the correction of a genetic defect in cloned ESCs, suggest that therapeutic cloning could, in theory, provide a source of cells for regenerative therapy. Recent evidence on the efficacy of human therapeutic cloning, however, underscores the difficulties associated with the generation of human ESC lines for therapeutic purposes. Moreover, a number of limitations may hinder the strategy of therapeutic cloning for future clinical applications. Extremely low efficiency of SNT is a major concern. Analysis of the literature on mouse SNT-derived ESC lines raises concerns about the feasibility and relevance of therapeutic cloning, in its present embodiment, for human clinical practice. This limitation may be alleviated with oocytes from other species, but mitochondrial genome differences between species are likely to pose a problem. It is, therefore, desirable to develop alternative strategies to oocyte-dependent autologous stem cell generation.

In this study, our data indicating mESC extract-mediated reverse dedifferentiation of terminally differentiated horse fibroblasts and multilineage redifferentiation of these reprogrammed cells not only support the feasibility of such an approach but, more importantly, provide evidence that the stem-like cells obtained using this methodology are functionally competent for tissue repair. When hMAPCs are cultured at low densities (250–500 cells per cm^2) they express high levels of Oct3a transcript and protein, maintain telomere lengths as a result of significant telomerase activity, and are capable of trilineage differentiation.

In this study, we noted stable karyotypes for many population doublings, even though up to 20% of metaphases became abnormal by PD 120. Such late acquisition of cytogenetic abnormalities is also seen for murine ESCs (Nagy et al., 1993) and human ESCs (Ludwig et al., 2006) and may be inevitable, as cell expansion requires many sequential symmetrical self-renewal cell divisions. As for human MAPCs and unlike mouse or rat MAPCs, hMAPCs can be isolated without the addition of leukemia inhibitory factor, a cytokine needed for murine ESCs but not hESCs (Williams et al., 1988). hMAPCs also differentiate into mesodermal, endodermal, or neuroectoderm cell types, as shown by Q-RT-PCR. Importantly,

In this study, we demonstrated that differentiation of hMAPCs to -like cells occurred as would be expected during development. Consistent with MAPCs from rodents and humans, when hMAPCs were cultured at a higher density ($>2,000$ cells per cm^2) for even a short period of time (3–6 passages), the morphology of the cells began to change. This was associated with the acquisition of CD44 and MHC-I on the cell surface, loss of Oct3a transcript levels and protein, and loss of differentiation ability, with only differentiation to typical mesenchymal cell types, but not endothelium, hepatocyte-like cells, or neural-like cells. In numerous studies, when cells maintained for 3–6 passages at high cell density, were replated at 100–500 cells per cm^2 for 20 PDs, Oct3a was not re-expressed, and cells maintained an MSC morphology, cell surface phenotype, and differentiation ability. This strongly suggests, but does not prove, that the MAPC phenotype is not an *in vitro* phenomenon, since one would expect that the phenotype should be reinduced when cultures conditions favorable to MAPC induction are re-established. The mechanism underlying the loss of MAPC phenotype when cells are cultured

at higher density is currently not yet clear. This phenomenon is true to a greater or lesser extent for rodent and human MAPCs. Possibilities that are being evaluated are direct cell-cell-mediated interactions; secretion of differentiation-inducing cytokines by MAPCs, which may accumulate faster when cells are maintained at higher densities; or both. The establishment of multiple hMAPCs cell lines may now make it possible to re-evaluate observations made with horseMAPCs as animal model in human.

After engraftment occurs, blood cell counts continue to rise and the immune system becomes stronger. The horse usually remains under the care of the transplant team and was monitored closely for complications. Non-myeloablative (mini) transplants has done on an outpatient basis, allowing the patient to rest at stable . Other types of transplantation required the horse to stay in the clinic for two to three weeks following transplantation. In all cases, frequent visits to the health care provider's office were needed following discharge, and horse,s who live a distance from their provider should arrange to live in a place within reasonable distance to the treatment center until at least 100 days have passed since the transplant.Horse,s who undergo BMT were at an increased risk of infection for many months following transplantation. Horse should be monitor for signs and symptoms of infection, including fever, pain, or chills. Horses should be given antibiotics to prevent infections.

In conclusion, studies have indicated that most horses who undergo transplant and remain free of cancer have a good quality of life. Most horses were able to be active, , and in reasonably good health. Quality of life usually continues to improve in the months following transplant. A clinical trial was carefully controlled to study the effectiveness of new treatments or new combinations of known therapies, and horse care taker was asked to participate. Ask a healthcare provider for more information about clinical trials.

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