CHARACTERIZATION OF CANINE BONE MARROW-DERIVED STROMAL CELLS: A POTENTIAL CELL SOURCE FOR TREATMENT OF NEUROLOGICAL DISORDERS

By

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ALCAM	activated leukocyte cell adhesion molecule
BBB	Basso-Beattie-Bresnahan
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
BHA	butylated hydroxyanisole
BME	β-mercaptoethanol
BMP-2	bone morphogenic protein-2
BMSC	bone marrow-derived stromal cell
BNP	brain natriuretic peptide
BSA	bovine serum albumin
CFU-F	colony-forming unit fibroblast
CNS	central nervous system
DAPI	4',6-diamino-2-phenylindole
dbcAMP	dibutyryl cyclic AMP
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DRG	dorsal root ganglion
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EG cell	embryonic germ cell
EGF	epidermal growth factor
ES cell	embryonic stem cell
FACS	fluorescence activated cell sorting

FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GDNF	glial cell line–derived neurotrophic factor
GFAP	glial fibrillary acidic protein
HGF	hepatocyte growth factor
HSC	hematopoietic stem cell
IBMX	isobutylmethylxanthine
ICAM	intercellular adhesion molecule
LIF	leukemia inhibitory factor
MAPC	multipotent adult progenitor cell
MBP	myelin basic protein
MHC	major histocompatibility complex
MNC	mononucleated cell
MPC	mesodermal progenitor cell
NCAM	neural cell adhesion molecule
NeuN	neuronal nuclear antigen
NGF	nerve growth factor
NICD	notch intracellular domain
NSE	neuron-specific enolase
NT-3	neurotrophin-3
OEC	olfactory ensheathing cell
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PNS	peripheral nervous system
PPAR-γ	peroxisome proliferator-activated receptor-γ

RA	retinoic acid
RMS	rostral migratory stream
RT	room temperature
RUNX-2	runt-related transcription factor 2
SCI	spinal cord injury
SVZ	subventricular zone
TBS	tris buffered saline
TGF-β	transforming growth factor-beta
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth facto

Abstract of Dissertation Presented to the Graduate School
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CHARACTERIZATION OF CANINE BONE MARROW-DERIVED STROMAL CELLS – A POTENTIAL CELL SOURCE FOR TREATMENT OF NEUROLOGICAL DISORDERS

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Bone marrow-derived stromal cells (BMSCs) represent a promising cell source for treatment of traumatic and ischemic injury of the central nervous system (CNS). Increasing evidence suggests that BMSCs hold multiple modes of action in promoting repair process of various CNS injuries. Based on these findings, initial clinical studies of autologous BMSC transplantation in human spinal cord injury patients are being conducted. The potential therapeutic value of BMSCs is certainly not limited to human applications. For example, dogs can sustain traumatic spinal cord injuries at relatively high incidence. A need thus exists for developing novel treatments and cell-based therapies for veterinary practice. These canine patients also afford an important animal-to-human translational opportunity.

Our study first systematically characterized adult canine BMSCs, in an attempt to understand the frequency of BMSCs in canine bone marrow, growth kinetics in culture, phenotypic profile, and differentiation potentials. Next, neural differentiation properties of canine BMSCs were studied *in vitro* and *in vivo*. Finally, the effects of canine BMSCs on neurite extension were studied *in vitro*. Our data suggest that adult canine bone marrow contains approximately 1 BMSC/2.38 × 10^4 bone marrow mononucleated cells. Under standard culture

techniques, canine BMSCs grow rigorously to generate morphologically heterogeneous populations of plastic-adherent cells. The flow cytometric profile of canine BMSCs was similar to those of rodent and human counterparts. Standard protocols for osteogenesis and adipogenesis induced differentiation of primary canine BMSCs into respective lineages. Canine BMSCs intrinsically express neuronal and glial markers *in vitro*, and upon transplantation into a neonatal mouse brain, a small portion of canine BMSCs isolated from young donors, but not from adult donors, migrated into the subventricular zone as well as the olfactory bulb where they exhibited neuronal phenotypes. When co-cultured with dorsal root ganglion neurons, canine BMSCs promoted neurite extension via production of extracellular matrix molecules. We conclude that BMSCs can be isolated from adult canine bone marrow and expanded *ex vivo*. Canine BMSCs have the potential to differentiate into osteoblasts and adipocytes *in vitro* although the standard culture method does not support expansion of osteogenic cell populations in passaged cultures. Bone marrow of young dogs contains neurogenic cells; however, it is not known whether cells with similar properties exist in adult canine bone marrow. Nonetheless, adult canine BMSCs have the potential for promoting neuritic outgrowth in tissue culture.

CHAPTER 1
INTRODUCTION AND BACKGROUND INFORMATION

There have been a number of breakthroughs in the field of cell biology that have led to our current knowledge of cell plasticity, including the demonstration of the incredible plasticity of the adult somatic nucleus giving birth to the cloned sheep Dolly (Wilmut et al., 1997). Development of the novel technique by Thomson (1998) for isolation and culture of human embryonic stem (ES) cells has been a huge stride as well. Even more intriguing idea, derived from these two findings, is "therapeutic cloning" where a nucleus from a patient's somatic cell may be used to create patient-specific ES cells, thus avoiding immune rejection of transplants. More recently, adult stem cells have gained considerable attention on the grounds that the use of adult stem cells may circumvent logistical and ethical issues posed by ES and fetal-derived stem cells. Bone marrow-derived stromal cells (BMSCs) are a particularly promising cell source, and recent evidence suggests that these cells may be effective in treating diseases of the central nervous system (CNS). In this chapter, I present a brief overview of stem cell research, in general and BMSCs in particular. I also present scientific rationales for the use of BMSCs in treatment of CNS injury, as well as some of the controversies surrounding the authenticity of the plasticity of BMSCs. In the following chapters, I present our findings on characterization of canine BMSCs and their neural transdifferentiation properties *in vitro* and *in vivo*, as well as their stimulatory effects on neuritic extension *in vitro*.

Definition and Terminology of Stem Cells

A stem cell is defined as an undifferentiated cell that is capable of both replicating itself (a process termed self-renewal) and producing multipotent daughter cells (a process termed differentiation). Daughter cells produced from stem cells are precursor cells that usually proliferate before giving rise to fully differentiated cells. Therefore, these precursor cells are

also called transit amplifying cells. The terms precursor cell and progenitor cell are used interchangeably; however, some progenitor cells are considered to have more developmental potential than other precursor cells. There is inconsistency as to how to define the self-renewal ability of stem cells; some definitions require stem cells be able to self-renew indefinitely, whereas others do not. Multipotentiality (or multipotency) refers to the ability to give rise to multiple cell types and has been used as another definition of stem cells; however, this is not a required definition of a stem cell. For example, spermatogonial stem cells in the testis produce only spermatozoa (Meachem et al., 2001).

Classification of Stem Cells

Stem cells can be operationally classified based on their developmental potential. Pluripotent stem cells refer to those that can generate all the cells in the body including germ cells. Embryonic stem cells (ES cells) and embryonic germ cells (EG cells) are produced in culture from the epiblast cells of a blastocyst and primordial germ cells of an early embryo, respectively. Because ES cells and EG cells cannot produce extra-embryonic tissues that are necessary for embryonic development, these cells are not totipotent. Most of the stem cells in animal organs are multipotent, but there are also unipotent stem cells as in the case of spermatogonial stem cells.

Stem cells can also be categorized by their embryonic, fetal, or adult origin. Fetal and adult stem cells can be further divided according to their tissue of origin and referred to as organ-specific or tissue-specific stem cells; those that are found in adult organs are specifically called adult stem cells.

Adult Stem Cells

It was originally thought that adult stem cells were only present in certain organs that have high cell turnover rates such as blood, gut, skin, testis, and the respiratory tract. These adult stem

cells are responsible for life-long replenishment of damaged and lost cells in the organs in which they reside. The hematopoietic stem cell (HSC) in the bone marrow is a classical example of adult stem cell and the most extensively studied adult stem cell. A single HSC can reconstitute the entire hematopoietic system in an irradiated mouse by producing all the blood cells and the immune system. HSCs were the first adult stem cells to be used clinically and their therapeutic potentials have been fully recognized for numerous hematologic and immune diseases (Kroger et al., 2002).

In recent years, it has become increasingly clear that most, if not all, adult organs contain stem cells. The discovery of stem cells in the adult mammalian central nervous system (neural stem cells) was particularly unexpected because the adult central nervous system has a limited regenerative capacity (Gritti et al., 1996; Lois and Alvarez-Buylla, 1993; Morshead et al., 1994). It is now known that neural stem cells reside in specific areas in the adult brain including the hippocampus and olfactory bulb where adult neurogenesis takes place (Altman and Das, 1966). However, the identity of neural stem cells *in vivo* is still not fully understood (Laywell et al., 2000).

Much of the interest in adult stem cell research relates to its great therapeutic potentials. There are three major rationales for advancing adult stem cell research in the context of cell therapy. First, the use of adult stem cells for therapy avoids ethical problems related to the use of embryos and fetuses for isolation of ES cells and fetal stem cells. Second, it is possible to isolate adult stem cells from the patient requiring the treatment, allowing autologous transplantation to be performed. This will avoid immunological rejection of transplants and the use of immunosuppressive therapy. Third, because adult stem cells are thought to be more restricted in their lineage specification than ES cells and fetal stem cells, the risk of forming tumors is

believed to be reduced. This idea may conflict in one way to the ability of adult stem cells to transdifferentiate into multiple cell types. Among various types of adult stem cells, BMSCs have generated tremendous attention because of their accessibility and multipotency. In particular, the perspectives of the use of BMSCs for neurological diseases have expanded considerably during the last decade (Dezawa et al., 2004). Although promising, many fundamental questions about basic biology of BMSCs remain, such as their true identity and differentiation properties that are clinically meaningful.

Bone Marrow-Derived Stromal Cells (BMSCs)

Historical Background of BMSC Research

Discovery of the presence of bone-forming cells in bone marrow came from early transplantation studies performed in 1950s and 1960s. In these studies, whole bone marrow was transplanted in ectopic sites (e.g. anterior chamber of the eye or under the renal capsule) and shown to form an osseous tissue (Petrakova et al., 1963; Tavassoli and Crosby, 1968; Urist and Mc, 1952) and marrow stromal microenvironment for hematopoiesis (Tavassoli and Crosby, 1968). Initial attempts to identify and isolate these osteogenic cells in bone marrow were made by Friedenstein and colleagues in 1960s and 1970s. They found characteristic fibroblastic colony-forming cells (CFU-F) from the bone marrow of guinea pig, which could be isolated by using their plastic adherent property, cultured *in vitro*, and shown to have osteogenic potential *in vivo* (Friedenstein et al., 1970; Friedenstein et al., 1968; Friedenstein et al., 1966). Thus, adherent, fibroblastic, colony-forming cells in the bone marrow were recognized to form bone.

In the early 1980s, Ashton et al. (1980) showed that rabbit bone marrow stromal cells consistently generated bone and cartilage in diffusion chambers implanted into the peritoneal cavity of the host animals. It was not clear, however, whether generation of bone and cartilage represented different stages of a skeletal developmental process or separate activities by distinct

cell populations. Friedenstein et al. (1987) later reported that a portion of CFU-F colonies have an extensive proliferative capacity after passaging and proposed that precursor cells with stem cell characteristics reside within the CFU-F compartment. At around this time, the stromal stem cell hypothesis was proposed in which there exists a hierarchy of cellular organization with different developmental stages supported by a small number of self-renewing stem cells, analogous to the organization of the hematopoietic system.

It was only in the late 1990s when the first detailed description of multipotential mesenchymal stem cells was published by Pittenger et al. (1999). This group performed extensive characterization of human BMSCs by means of flow cytometric analysis, gene expression analysis, and multi-lineage differentiation assays. It was clearly shown that clonal BMSCs derived from single cells demonstrated multipotentiality by differentiating into osteoblasts, chondrocytes, and adipocytes, indicating unequivocally the presence of multipotent mesenchymal stem cells in the adult human bone marrow. They also noted that some of the isolated colonies had limited differentiation potential; thus, adherent colonies obtained from adult human bone marrow are composed of mixed cell populations of multipotential BMSCs and more lineage restricted progenitor cells.

Recent studies suggested the presence of a rare cell population with more primitive stem cell characteristics within the BMSC compartment in adult bone marrow. Jiang et al. (2002a) reported that in murine bone marrow, there are populations of cells with extensive proliferative and differentiation capacity. These cells were termed multipotent adult progenitor cell (MAPC). Murine MAPCs express transcription factors important in maintaining undifferentiated ES cells such as Oct-4, Rex-1, and SSEA-1, and interestingly, require leukemia inhibitory factor (LIF) for expansion, a feature found in murine ES cells but not in human ES cells (Odorico et al., 2001;

Williams et al., 1988). Murine MAPCs differentiated *in vitro* not only into mesenchymal cells but also visceral mesodermal, neuroectodermal, and endodermal cells, and upon transplantation into an early blastocyst, contributed to most, if not all, somatic cell types. Reyes et al. (2001) suggested that human bone marrow also contains a primitive cell population, termed mesodermal progenitor cells (MPCs), that have extensive proliferative and differentiation potential. These studies provided evidence that BMSCs are mixed populations of stem and progenitor cells, which also include pluripotent stem cells.

Isolation, Expansion, and Cell Surface Markers of BMSCs

Currently used technique for isolation of BMSCs relies on the adhesive property of BMSCs to tissue culture plastic, a technique similar to the original one described by Friedenstein (1970) and later optimized by Caplan et al. (1991a). In rodents, bone marrow is typically harvested by flushing excised long bones (e.g. femurs, tibiae, and humeri). In humans and large animals including dogs, bone marrow can be harvested from long bones or the iliac crest of the pelvis by aspiration. After bone marrow collection, the marrow is often subjected to fractionation via density gradient centrifugation using Percoll or Ficoll to isolate mononucleated cells. These cells can be cultured in a standard medium such as Dulbecco's modified Eagle's medium (DMEM), containing 10-20% fetal bovine serum (FBS). Primary cells form symmetric colonies after low-density plating or single-cell sorting (Brockbank et al., 1985; Colter et al., 2000; Javazon et al., 2001; Kuznetsov et al., 1997), an important feature of the BMSC. As demonstrated by Owen and Friedenstein (1988) and DiGirolamo et al. (1999), these colonies are, however, heterogeneous in both appearance (morphology and size) and differentiation potential. Initially, culture is consisted of heterogeneous cell populations, but becomes morphologically homogeneous over time by depletion of non-adhesive hematopoietic cells (Bruder et al., 1997). Primary cultures are usually maintained for 10-14 days, and are then detached by trypsinization

followed by sub-culturing. Passaged cells are spindle-shaped and fibroblast-like in their undifferentiated state, which grow in uniform monolayer.

Although commonly used isolation and expansion techniques are easy and do not require special equipments, hematopoietic contaminations remain problematic in some species, particularly in the mouse (Phinney et al., 1999). Therefore, many attempts have been made to develop techniques for isolation and expansion of a pure population of BMSCs. Most of these techniques are dependent on utilizing cell surface antigen specific antibodies combined with either cell sorting technique or immunological selection methods. However, due to a lack of unique cell markers on BMSCs (Majumdar et al., 2003), most of these selection techniques are designed to eliminate unwanted cell types, mainly hematopoietic cells, from the starting materials (Tropel et al., 2004), although attempts have been made to purify BMSCs using an antibody Stro-1 (Gronthos et al., 1994; Simmons and Torok-Storb, 1991).

Investigation of cell surface marker profiles of BMSCs has been carried out for the purpose of characterizing BMSCs and developing better purification methods. This task has been difficult since BMSCs do not express unique cell surface markers (Digirolamo et al., 1999; Haynesworth et al., 1992a), in a similar manner to CD34 positive hematopoietic stem cells. Therefore, BMSCs are identified by a combination of immunophenotypic profiles. These include negative profiles against hematopoietic lipopolysaccharide receptor CD34, CD14, and the leukocyte common antigen CD45, as well as endothelial markers such as CD31, von Willebrand factor, and P-selectin (Pittenger et al., 1999). Human and murine BMSCs express a number of receptors for cytokines (IL-1, IL-3, IL-4, IL-6, IL-7), adhesion molecules (ICAM, VCAM, ALCAM, integrins), and growth factors (bFGF, PDGF) (Majumdar et al., 2003). The expression profiles of some of these cell surface molecules are not static and influenced by their

developmental stage and other cytokines (Barry, 2003; Majumdar et al., 2003). This may explain significant variations of the cell surface marker profiles of BMSCs reported from different laboratories (Majumdar et al., 1998; Peister et al., 2004).

Haynesworth et al. (1992a) developed the monoclonal antibody SH-2 raised against human BMSCs, which reacts with an epitope present on the transforming growth factor-beta (TGF-β or CD105) and was later used in immunomagnetic selection methods (Barry et al., 1999). The antibodies SH-3 and SH-4 were also raised against human BMSCs, which recognize distinct epitopes on CD73 (membrane-bound ecto-5'-nucleotidase) (Barry et al., 2001a).

Multipotentiality of BMSCs: Mesengenesis

As first shown by Pittenger et al. (1999), *in vitro* differentiation into three major mesenchymal cell types (osteoblasts, chondrocytes, and adipocytes) has been the widely accepted and perhaps the most reliable single requirement to identify BMSC populations. To induce differentiation into each cell type, different combinations of reagents are used, which slightly vary among different species. Differentiation along osteoblastic cells typically requires β-glycerolphosphate, ascorbic acid-2-phosphate, and glucocorticoids such as dexamethasone (Jaiswal et al., 1997). BMP-2 has been reported to further stimulate osteogenic differentiation of BMSCs isolated from rodents and dogs (Volk et al., 2005), but not to the equivalent degree in humans (Einhorn, 2003; Govender et al., 2002). When cultured in monolayer in the presence of these stimulators, BMSCs acquire osteoblastic (cuboidal) morphology with concomitant up-regulation of related genes (alkaline phosphatase, osteocalcin, osteopontin, RUNX-2, etc). Chondrogenic differentiation can be induced in a three dimensional culture in the absence of FBS and in the presence of the TGF-β superfamily (Mackay et al., 1998). Under these conditions, BMSCs lose their fibroblastic morphology and initiate expressing cartilage-specific extracellular matrix molecules (Barry et al., 2001b). Differentiation into adipocytes can be

induced in monolayer in the presence of isobutylmethylxanthine (IBMX) via activation of the ligand-induced transcription factor peroxisome proliferator-activated receptor-γ (PPAR-γ) (Suzawa et al., 2003). Differentiated cells are easily identified by their morphology and the presence of large lipid-filled vacuoles.

In vivo mesengenic differentiation of BMSCs has been demonstrated in a number of studies by their contribution to a tissue repair process in osseous and cartilaginous tissues upon transplantation. Transplantation of BMSCs loaded on porous ceramics in a bone defect led to formation of copious amounts of bone and facilitated bone healing in the canine model (Arinzeh et al., 2003; Bruder et al., 1998; Kadiyala et al., 1997). In initial clinical trials of allogenic BMSC transplantation in children with osteogenesis imperfecta, a genetic disorder in which osteoblasts produce defective type I collagen, leading to osteopenia and multiple fractures, new dense bone formation and increases in the total body bone mineral content were reported (Horwitz et al., 2002; Horwitz et al., 1999). Ponticiello et al. (2000) transplanted scaffolds loaded with human BMSCs in an osteochondral defect in the rabbit femoral condyle and showed filling of defective lesion with cartilaginous tissues.

In vivo differentiation of BMSCs into cardiomyocytes has also been reported and attracted many investigators as a potential source for cellular cardiomyoplasty. Toma et al. (2002) reported that human BMSCs, when delivered by infusion to an immunocompromised mouse, could engraft to the normal myocardium and differentiate into cardiomyocytes. Engrafted cells could persist in the heart and displayed normal cellular organization of cardiomyocytes. Preclinical studies of autologous BMSC (Vulliet et al., 2004) or bone marrow cell (Li et al., 2003; Memon et al., 2005) transplantation for cardiomyoplasty have been performed in the

canine mode of ischemic myocardium and successfully translated into clinical studies on patients with myocardial infarction (Li et al., 2003; Stangel and Hartung, 2002).

Neural Transdifferentiation of BMSCs

Evidence of *in vitro* Neural Transdifferentiation of BMSCs

Early *in vitro* studies (Sanchez-Ramos et al., 2000; Woodbury et al., 2000) reporting differentiation of BMSCs along neuronal phenotypes have aroused considerable interest. This phenomenon was considered to represent "trans" differentiation based on the fact that BMSCs (mesoderm origin) differentiated into neurons (ectoderm origin), crossing the developmental germ layer boundaries. In these studies, human or mouse BMSCs were treated with different combinations of reagents (e.g. β-mercaptoethanol [BME], dimethylsulfoxide [DMSO], and butylated hydroxyanisole [BHA]) and growth factors (e.g. epidermal growth factor [EGF], retinoic acid [RA], and BDNF). Neuronal differentiation was confirmed by their morphological changes and expression of a panel of neuronal markers such as nestin, neuron-specific enolase, TrkA, and βIII-tubulin. Following these studies, Deng et al. (2001) reported that human BMSCs differentiated into neurons *in vitro* when treated with isobutylmethylxanthine (IBMX) and dibutyryl cyclic AMP (dbcAMP), two reagents known to increase the intracellular concentration of cAMP. They reported based on the expression pattern of neuronal markers that this treatment induced human BMSCs to commit to early neuronal cells but not mature neurons.

Several independent groups re ported that native neuronal cells can instruct BMSCs to differentiate into neurons via cell-to-cell contact in addition to their trophic effects. BMSCs were co-cultured with various types of neurons such as fetal mesencephalic cells (Sanchez-Ramos et al., 2000), neonatal hippocampal neurons (Abouelfetouh et al., 2004), and neonatal cerebellar granule neurons (Wislet-Gendebien et al., 2005). Wislet-Gendebien et al. (2005) was the first to report that when co-cultured with cerebellar granule neurons, rat BMSCs

differentiated into neuron-like cells that sequentially expressed voltage-gated potassium and sodium channels and became excitable in response to depolarizing voltage steps via whole-cell patch clamp.

Modification of the gene expression pattern has been used to generate electrophysiologically excitable neurons from BMSCs. Kohyama et al. (2001) transfected mouse BMSCs with Noggin and subsequently cultured them in a medium supplemented with NGF, NT-3, and BDNF. This treatment gradually induced BMSCs to differentiate into mature neurons which had developed voltage-gated ion channels and the ability to uptake calcium in response to potassium and neurotransmitters (e.g. acetylcholine and glutamate). Because Noggin is known to play a role in neural development during early embryogenesis (Smith and Harland, 1992) and adult neurogenesis (Lim et al., 2000) by antagonizing bone morphogenic protein signaling (Zimmerman et al., 1996), it was suggested that Noggin may inhibit osteogenic activities of BMSCs and induce neural differentiation.

Dezawa et al. (2004) transfected human and rat BMSCs with Notch intracellular domain (NICD) and subsequently treated them with bFGF, forskolin, and ciliary neurotrophic factor. Upon activation by neighboring cells through Delta/Serrate/Lag-1 ligands, NICD is cleaved and enters the nucleus where it influences the expression of transcription factors related to progenitor pool maintenance, cell fate decision, and, in case of nervous system, terminal specification of cells as neurons and glial cells (Gaiano et al., 2000; Lundkvist and Lendahl, 2001; Morrison et al., 2000). They reported that transfection of NICD was highly efficient and specific in generating neuronal cells with electrophysiological properties from both human and rat BMSCs, without generation of glial cells. Further treatment with glial cell line–derived neurotrophic factor (GDNF) increased the proportion of tyrosine hydroxylase-positive and dopamine-

producing cells, which upon intrastriatal implantation, improved behavior outcomes in a rat model of Parkinson disease.

Evidence of *in vivo* Neural Transdifferentiation of BMSCs

Initial evidence that BMSCs produce neural cells *in vivo* was provided by Pereira et al. who implanted systemically wild-type mouse BMSCs into osetogenesis imperfect transgenic mice (Pereira et al., 1998). In the study, donor-derived DNA was detected in numerous non-hematopoietic tissues including the brain, although the engraftment rate was low and phenotypes of engrafted cells were not described. Azizi et al. (1998) injected human BMSCs directly into the adult rat brain striatum and reported that BMSCs migrated through the host brain tissue in a manner similar to that of implanted neural stem cells. They reported that transplanted BMSCs lost immunoreactivity against fibronectin and collagen-I, putative BMSC markers in culture, but expression of neuronal markers were not examined. Kopen et al. (1999) transplanted murine BMSCs in the lateral ventricle of neonatal mouse brains and reported that BMSCs migrated throughout the forebrain, cerebellum, and brain stem where engrafted cells expressed GFAP or neurofilament. More recently, Deng et al. (2006) transplanted murine BMSCs into the lateral ventricle of neonatal mouse brains and found that these cells migrated and differentiated into olfactory bulb granule cells and periventricular astrocytes. These events were independent on cell fusion, evaluated by sex chromosome-specific in situ hybridization. These previous studies indicate that BMSCs may be capable of responding to environmental cues provided by the developing and adult CNS and adopting neuronal phenotypes *in vivo*. It remains, however, to be confirmed whether engrafted BMSC-derived neuronal cells possess electrophysiological properties and become integrated into an existing or newly established neural circuitry.

Controversies in Neural Transdifferentiation of BMSCs

Findings of previous *in vitro* and *in vivo* studies, as described above, appeal that under certain conditions adult stem cells may be reprogrammed and generate cells of other germ layer origins including neuroectodermal derivatives. Consequently, neuronal transdifferentiation of BMSCs has generated great excitement since cellular therapy using autologous BMSCs may become possible for diseases of the CNS. However, this concept has been repeatedly challenged during the last few years and suggested for a need of more careful interpretations. Some of the findings that led to this controversy are described below.

Two independent groups reported that adult stem cells can fuse with ES cells and adopt the phenotypes of other cell types. Terada et al. (2002) showed that murine BMSCs spontaneously fused with ES cells in culture under the presence of interleukin-3. Ying et al. (2002), in the same year, reported that co-cultured neural stem cells from transgenic mice fused with ES cells and produced non-neural derivatives. Generated tetraploid hybrid cells had full pluripotent character and contributed to multilineage chimera formation. Following these studies, several groups have described *in vivo* cell fusion after transplantation of BMSCs. Vassilopoulos et al. (2003) implanted transgenic murine BMSCs into livers of mice that lacked fumarylacetoacetate hydrolase. Transplanted BMSCs fused with recipient hepatocytes, which resulted in restoration of normal liver function. Therefore, this study pointed to the therapeutic potential of cell fusion. Fusion of bone marrow-derived cells with cerebellar Purkinje cells and cardiomyocytes were also reported (Alvarez-Dolado et al., 2003; Weimann et al., 2003a; Weimann et al., 2003b). These studies suggest that cell fusion may explain some of the early observations that were interpreted as transdifferentiation or cell plasticity and prompt to include genotyping of potentially transdifferentiated cells for careful evaluation of such observations.

Contamination of other cell types is another concern in investigation of transdifferentiation. This concern may be particularly important for BMSCs because, as mentioned earlier, adult bone marrow is suggested to contain pluripotent stem cells (Jiang et al., 2002a; Reyes et al., 2001) (e.g. MAPC and MPCs) which may be within the BMSC fraction. These pluripotent cells are thought to be quiescent, but under certain conditions, may be triggered to differentiate into a wide range of cell types, some of which could be cells of other germ layer origins. It was further suggested that, in addition to bone marrow, adult tissues such as brain and muscle also contain quiescent pluripotent stem cells (Jiang et al., 2002b). If the fraction of bone marrow contain MAPCs and was transplanted into the brain, neuronal transdifferentiation could result from differentiation of MAPCs. If the whole bone marrow is to be used for transplantation, hematopoietic stem cells may also contribute to neural transdifferentiation of donor cells as suggested previously (Cogle et al., 2004; Mezey et al., 2003). Therefore, these studies highlight the importance of proper identification of transplanted cells and the origin of differentiated cells.

Early *in vitro* findings (Sanchez-Ramos et al., 2000; Woodbury et al., 2002; Woodbury et al., 2000) claiming neural transdifferentiation of BMSCs have been questioned as to whether the observed morphological and phenotypic changes represented true neural differentiation or perhaps cytotoxic changes caused by induction medium. In these studies, when BMSCs were cultured in neural induction medium, conversion of fibroblastic morphology into neuron-like morphology occurred rapidly (within a few hours). Lu et al. (2004) reported that using various chemicals (e.g. BME, MHA, DMSO) similar morphological changes can be induced on fibroblasts, HEK293 cells (human embryonic kidney cell line), and PC-12 cells (cell line derived from a pheochromocytoma). They noted that the morphological changes primarily resulted from

shrinkage of cytoskeleton rather than genuine neurofilament extension. Further, immunoreactivity against neuronal markers (NSE and NeuN) was detected on these induced cells, which was, however, not detectable as changes in corresponding mRNA levels with RT-PCR. Similar results were also reported by Neuhuber et al. (2004). Rismanchi et al. (2003) found that the initial neural differentiation protocol reported by Woodbury (2000) resulted in significantly increased cell death of differentiated cells and that maintenance of neuronal morphology required continued exposure to the induction medium. It is thus unlikely that morphological changes into neuron-like cells resulted from tightly regulated process of changes in the gene expression pattern and cellular organization in early studies showing rapid neural transdifferentiation of BMSCs. As such, interpretation of neural differentiation of BMSCs *in vitro* needs great caution and systematic approaches including assays at the molecular level.

Potential Mechanisms of Action

There has been convincing evidence that transplantation of BMSCs in experimental models of CNS injury leads to significant improvement in functional as well as electrophysiological measures. Although these studies suggest therapeutic potentials of BMSC transplantation, mechanisms underlying their effects are still largely unknown. A few studies addressing this question propose potential modes of action of transplanted BMSCs, which could be divided into four categories as follows; 1) Neuronal replacement, 2) Production of soluble factors, 3) Axonal regrowth stimulatory effects, and 4) Generation of myelinating cells. Many think that these effects play roles in combination, potentially synergistically to one another.

Neuronal Replacement by BMSCs

The idea of neuronal replacement by BMSCs is based on early studies showing neuronal transdifferentiation of rat and human BMSCs *in vitro* and *in vivo* as described above. However, this topic is still under intensive controversy. Even if BMSCs can generate functional neurons *in*

vivo, a practical problem still exists on the grounds that BMSC-derived neurons may need to be integrated in the host neural circuitry in order to produce functional improvement. This mode of action itself is therefore unlikely to be sufficient to explain significant functional improvements observed in previous studies with experimental CNS injury. On the other hand, it can be said that neuronal transdifferentiation and replacement by BMSCs may not be the essential requirement for functional recovery to occur after CNS injury.

Production of Soluble Factors by BMSCs

Recent studies have revealed that BMSCs naturally produce various cytokines, neurotrophic factors, and angiogenic growth factors, including nerve growth factor (NGF) (Chen et al., 2005; Chen et al., 2002a; Chen et al., 2002b; Crigler et al., 2006; Garcia et al., 2004; Li et al., 2002), glial cell-derived neurotrophic factor (GDNF) (Chen et al., 2005), brain-derived growth factor (BDNF) (Chen et al., 2002a; Chen et al., 2002b; Crigler et al., 2006; Li et al., 2002), vascular endothelial growth factor (VEGF) (Chen et al., 2002a; Chen et al., 2002b), hepatocyte growth factor (HGF) (Chen et al., 2002a; Chen et al., 2002b), and brain natriuretic peptide (BNP) (Song et al., 2004). Neurotrophic factors such as NGF and BDNF support survival of damaged neurons (DeKosky et al., 1994; Goss et al., 1998; Hammond et al., 1999; Lu et al., 2001) and stimulate neurite regrowth (Mocchetti and Wrathall, 1995), whereas vasoactive peptides such as VEGF (Chen et al., 2003b; Chen et al., 2002a; Chen et al., 2002b) and BNP (Song et al., 2004) induce angiogenesis and reduce secondary edema thereby participate in the reparative processes after CNS injury. In addition, soluble factors secreted by BMSCs may stimulate differentiation of neural progenitor cells (Wu et al., 2003).

A previous observation that transplanted BMSCs lack voltage-gated ion channels necessary for the generation of action potentials while promoting functional recovery of paralyzed rats supports the hypothesis based on the growth factor-mediated effects (Hofstetter et

al., 2002). Fibroblasts genetically engineered to express neurotrophin-3 (NT-3) (Grill et al., 1997), or to secrete NGF (Nakahara et al., 1996) or BDGF (Schwartz et al., 2003) have been shown to be of therapeutic benefit in rat SCI models. Therefore, these observations further corroborate the effects of neural growth factors secreted by transplanted cells on the CNS repair.

Another possibility is that BMSCs may have the ability to degrade nerve-inhibitory molecules present in the site of CNS injury thereby allow regenerating axons to grow. Human BMSCs have been shown to produce membrane type I matrix metalloproteinase and matrix metalloproteinase-2 (Son et al., 2006) which degrade nerve-inhibitory molecules (d'Ortho et al., 1997; Fosang et al., 1992; Passi et al., 1999).

Axonal Regrowth Stimulatory Effects of BMSCs

Perhaps the most plausible mechanism of BMSCs in promoting recovery from CNS injury is mediated by the ability of BMSCs to stimulate axonal regrowth. This effect is thought to be mediated by several distinct functions of BMSCs, which are likely to work in combination. First, as descried above, BMSCs produce an array of neurotrophic factors, some of which are known to enhance axonal regrowth *in vitro* and *in vivo* (Auffray et al., 1996; Chen et al., 2002a; Chen et al., 2002b; Crigler et al., 2006; Li et al., 2002). Second, BMSCs produce extracellular matrix and adhesion molecules that are known to have strong influence on the attachment and extension of neuronal bodies and their axons. Among these are fibronectin and laminin, which human BMSCs are shown to produce (Grayson et al., 2004; Hofstetter et al., 2002), are potent neurite extension-promoting substrates. Adhesion molecules such as E-cadherin (Oblander et al., 2007), N-cadherin (Puch et al., 2001; Schense et al., 2000), and neural cell adhesion molecule (NCAM) (Crigler et al., 2006; Kimura et al., 2004) have all been implicated to play a role in axonal regeneration. Through production of these molecules BMSCs are thought to provide permissive microenvironment for regenerating axons (Hofstetter et al., 2002). Third, it was

proposed that BMSCs may guide regenerating axons by acting as a "cellular bridge" and "towing" axons (Wright et al., 2007). In a rat contused spinal cord, BMSCs were shown to form bundles to which regenerating axons were intimately associated (Hofstetter et al., 2002). It has been further suggested that BMSCs may provide a nerve-permissive environment by degrading nerve-inhibitory molecules (such as neural proteoglycans, myelin associated glycoprotein, and Nogo-A) with metalloproteinase and matrix metalloproteinase-2 (d'Ortho et al., 1997; Fosang et al., 1992; Passi et al., 1999; Son et al., 2006). Thus, BMSCs may modify nerve-inhibitory molecules and provide physical, nerve-permissive cellular bridges for axons to regenerate and guide axons to cross the lesion site that is otherwise severely hostile to regenerating axons.

Remyelination by Transplanted BMSCs

It has been known that peripheral myelinating cells, Schwann cells, can myelinate demyelinated axons and enhance axonal regeneration in the spinal cord when transplanted into a lesion site (Blakemore, 1977; Honmou et al., 1996). Olfactory ensheathing cells (OECs) are another type of peripheral myelinating cells which are found in the olfactory nerve and the outer layer of the olfactory bulb, which normally do not myelinate axons but can remyelinate demyelinated axons when transplanted into the CNS lesion (Franklin et al., 1996; Imaizumi et al., 1998). Although attracting as transplants, harvest of a sufficient number of autologous Schwann cells or OECs poses practical difficulty. There are, however, interesting studies demonstrating that bone marrow cells may act like peripheral myelinating cells when transplanted in the CNS and restore conduction properties of the damaged spinal cord. Sasaki et al. (2001) transplanted an acutely isolated mononuclear cell fraction of rat bone marrow into rat spinal cords that were demyelinated by x-irradiation followed by microinjection of ethidium bromide. They found that donor cells remyelinated demyelinated host axons with predominately a peripheral myelination pattern. When CD34+ cells were transplanted no remyelination was

observed, indicating that BMSCs remyelinated axons. Remyelination of the rat demyelinated spinal cord was also reported following systemic delivery of the mononuclear cell fraction of rat bone marrow, which showed both CNS and PNS patterns of myelination and resulted in restoration of conduction velocity (Akiyama et al., 2002a). The same group later demonstrated that rat BMSCs have the ability to form both central and peripheral myelin around demyelinated CNS axons and restore conduction properties of the spinal cord (Akiyama et al., 2002b).

An alternative approach may be to coax BMSCs in myelinating cells *in vitro* prior to transplantation in order to maximize the myelination efficacy. Several studies suggest that this may be possible by inducing BMSCs to differentiate into Schwann cells. Dezawa et al. (2001) reported that when rat BMSCs were treated with beta-mercaptoethanol followed by retinoic acid and cultured in the presence of forskolin, bFGF, PDGF and heregulin, these cells differentiated into cells resembling morphologically and immunophenotypically Schwann cells. Upon transplantation into the cut ends of the sciatic nerve, differentiated cells remyelinated and enhanced axon regeneration. Using the same protocol, Chen et al. (2006) reported that rat BMSC-derived Schwann cells contributed to reconstruction of the sciatic nerve and improvement of functional analyses. Kamada et el. (2005) demonstrated that transplantation of rat BMSC-derived Schwann cells resulted in enhanced axonal regeneration and functional recovery in rats with completely transected spinal cord.

BMSC Transplantation for CNS Injury

Experimental Studies in Animal Models of CNS Disorders

Following early *in vitro* studies of BMSC plasticity, the clinical efficacy and safety of BMSC transplantation for various CNS disorders have been investigated. Most of these studies are performed in preclinical models of CNS injury, primarily in rodent models, but some have been translated in human clinical trials. Intensive research has been undertaken in the fields of

spinal cord injury (SCI) and brain stroke models, but neurodegenerative disorders have also been investigated.

Spinal Cord Injury

Functional recovery after SCI is very limited because of the limited ability of damaged axons and neurons to regenerate in the site of injury. This is largely due to the hostile microenvironment at the injury site containing inflammatory mediators, nerve inhibitory molecules, and lack of trophic factors. As discussed, it has been suggested that neuronal replacement by BMSCs is unlikely the prime mechanism underlying functional recovery after SCI. Alternatively, evidence suggests that BMSCs help overcome the inhibitory barriers by providing a permissive environment for regenerating axons.

The efficacy of BMSC transplantation on functional recovery has been reported in rat contusion SCI models. Chopp et al. (2000) transplanted BMSCs intramedullary in rats with SCI 1 week after injury and observed functional recovery, as measured by the Basso-Beattie-Bresnahan (BBB) scores, over a 5-week period. Wu et al. (2003) showed that rat BMSCs promoted differentiation of neurosphere cells *in vitro*, and upon transplantation in rats with contusion SCI immediately after injury, reduced the size of cavity and promoted functional recovery. In both studies, authors speculated that functional recovery was primarily mediated by trophic support by BMSCs because only a small portion of BMSCs showed neuronal phenotypes. In contrast, Hofstetter et al. (2002) reported that significant larger numbers of surviving cells and functional improvement were achieved in rats with contusion SCI when BMSCs were given 1 week after injury compared to immediate treatment. They found 5 weeks after injury that BMSCs formed bundles associated with longitudinally arranged immature astrocytes, bridging the epicenter of the injury. Robust bundles of neurofilament-positive fibers and some 5-hydroxytryptaminepositive fibers were found mainly at the interface between the

graft and scar tissues. Functional recovery from SCI has also been reported after BMSC administration in a mouse model with (Lee et al., 2003) and without (Koda et al., 2005) neuronal differentiation of BMSCs.

The effects of BMSCs on chronic SCI are less known; so far there are 2 studies addressing this question. Zurita et al. (2004) transplanted BMSCs into rats with contusion SCI 3 month after the injury and evaluated functional improvement over a 4 weeks period. They reported that progressive functional recovery was clearly observed in animals treated with BMSCs. Histological sections of the spinal cord revealed that grafted BMSCs formed cellular bridges within the centromedullary cavity and expressed neuronal as well as astrocytic phenotypes. They also noted marked ependymal cell proliferation expressing nestin, a marker of neural stem cells, which might have become activated by initial injury and/or subsequent grafting of BMSCs as endogenous restorative and regenerative processes. The same group (Zurita and Vaquero, 2006) reported a one year follow-up of the same procedures in rats with contusion SCI receiving intralesional BMSC transplantation. They found that functional recovery, evaluated by BBB scores, started a few weeks after transplantation, increased over the following months, and stabilized 1 year post transplantation at which point the functional recovery was almost complete. Histochemistry revealed that donor cells formed tissue bundles bridging the central cord cavity.

Brain Injury

Two types of experimental brain injury model are commonly studied in the context of BMSC transplantation; a contusion model has been used to mimic human traumatic brain injury whereas an ischemic model has been used to mimic brain stroke.

In rats with contusion brain injury, intra-arterial and intravenous administration of rat BMSCs resulted in functional recovery (Chopp and Li, 2002). BMSCs administered

intravenously targeted damaged tissues in the brain and led to functional recovery; these effects were not further promoted by intra-arterial injection. As for the case of SCI, the effects of BMSCs on functional recovery were attributed to enhancement of endogenous reparative processes as only a small number of grafted cells adopted neural phenotypes (Chopp and Li, 2002; Mahmood et al., 2002). In a subsequent study by the group emphasized the role of NGF and BDNF in neurogenesis, synaptogenesis, and reduction in apoptotic cells in the boundary zone of the injury site (Mahmood et al., 2004a). Intracerebral administration of BMSCs was also shown to be beneficial in rats with traumatic injury, leading to long lasting functional recovery over a 3 months period (Mahmood et al., 2004b; Mahmood et al., 2006). Therapeutic effects were dose dependent and thought be mediated by proliferation of endogenous progenitor cells (Mahmood et al., 2004b; Mahmood et al., 2006). Additional studies showed that transplantation of human BMSCs also provided a similar degree of functional recovery in the rat model of traumatic brain injury (Mahmood et al., 2003; Mahmood et al., 2005).

BMSC transplantation has also provided promising results in the rat model of ischemic brain injury. It was shown that intracerebral transplantation of rat bone marrow with (Chen et al., 2000) and without BDNF (Li et al., 2000) resulted in neural differentiation of grafted cells and improved motor function. In a following study, Chen et al. (2003a) found that rat BMSCs administered intravenously preferentially migrated in the ipsilateral ischemic hemisphere where they reduced apoptosis and promoted proliferation of endogenous progenitor cells, leading to improved function recovery. Long-term effects of rat BMSC transplantation in stroke were reported by Li et al. (2005). In their study, significant improvement of neurological deficits was observed 4 months post-transplantation. BMSC treatment reduced the thickness of the scar wall and reduced the numbers of microglia/macrophages within the scar wall. Further, they found

increased expression of growth-associated protein-43 (GAP43) (a marker of axonal growth cones), among reactive astrocytes in the scar boundary zone and in the subventricular zone in the treated rats. The beneficial effects of BMSC transplantation (1 month post-injury) on a chronic model of stroke has also been reported (Shen et al., 2007). Functional recovery following intravenous transplantation of human BMSCs in the rat ischemic model has been reported by several groups. Most of these studies emphasize the importance of soluble factors released by BMSCs, including neurotrophic factors (Chen et al., 2002b), vasoactive factors (Chen et al., 2003b; Chen et al., 2002b; Song et al., 2004), and various cytokines (Kinnaird et al., 2004).

Neurodegenerative Disorders

Although the potential use of BMSCs for the treatment of neurodegenerative diseases has been implicated, there is only limited information about the prospect of BMSCs for treatment of neurodegenerative diseases. Unlike other types of CNS injury (e.g. traumatic and ischemic), neurodegenerative diseases often affect specific populations of neurons. Previous studies demonstrated that BMSCs can be induced to differentiate into neurons expressing glutamic acid decarboxylase (Li et al., 2004), choline acetyltransferase (ChAT) (Li et al., 2004), or tyrosine hydroxylase (Dezawa et al., 2004). These findings encourage researchers to pursue development of a new therapeutic strategy for neurodegenerative diseases using BMSCs.

As discussed earlier Dezawa et al. (2004) reported generation of tyrosine hydroxylase positive neurons from rat and human BMSCs by NICD transfection and GDNF treatment. These neurons produced dopamine and, upon transplantation in a 6-hydroxy dopamine rat model of Parkinson's disease, promoted functional recovery. Alternatively, BMSCs may be used as a vector to deliver therapeutic molecules to a target tissue. Schwarz et al. (1999) genetically engineered rat and human BMSCs to synthesize L-DOPA using retroviruses encoding tyrosine hydroxylase and GTP cyclohydrolase I. Although transduced BMSCs synthesized L-DOPA and,

upon transplantation into the striatum of 6-hydroxy dopamine-lesioned rats, led to functional recovery, expression of transgenes ceased at about 9 days and therapeutic effects were short-lived.

An attempt to treat Alzheimer's disease with BMSCs has also been reported recently (Wu et al., 2007). Transplantation of BMSCs in a rat model of Alzheimer's disease (induced by injection of Aβ amyloid protein in the hippocampus) increased the number of ChAT neurons in the CA1 zone of the hippocampus and improved learning and memory of the lesioned rats. Some of the transplanted GFP-positive BMSCs seemed to have differentiated into ChAT neurons and might have contributed to the functional recovery.

Human Clinical Trials

The first report on human trial of BMSCs transplantation was reported in 2005 by Park et al. (2005). In their study, five human patients with complete acute SCI received intra-lesional transplantation of autologous whole bone marrow and subcutaneous injection of granulocyte macrophage-colony stimulating factor (GM-CSF). One patient received GM-CSF only. They reported that side effects following the procedure were limited to those caused by GM-CSF injection, including transient a fever, myalgic pain, and leukocytosis, and no serious complications increasing mortality and morbidity were found. Sensory improvements were noticed immediately after the operations followed by significant motor improvements noticed 3 to 7 months postoperatively. Neurological improvement, judged by the American Spiral Injury Association Impairment Scale (AIS), was achieved in four of five patients receiving bone marrow transplantation and GM-CSF, and no worsening of neurological symptoms was found. They concluded that bone marrow transplantation and GM-CSF administration represent a safe protocol to efficiently manage SCI patients, especially those with acute complete injury. It

should be noticed, however, that since they used whole bone marrow, it is not clear which cell populations in the bone marrow primarily contributed to the observed functional recovery.

The same group reported the results of a phase I/II open-label and nonrandomized study of bone marrow transplantation and GM-CSF injection in 35 complete spinal cord injury patients (Yoon et al., 2007). They reported that the procedures were not associated with any serious adverse events increasing morbidities. The AIS grade increased in 30.4% of the acute (within 14 days postinjury) and subacute (between 14 days and 8 weeks postinjury) treated patients (AIS A to B or C), whereas no significant improvement was observed in the chronic treatment group (more than 8 weeks postinjury).

CHAPTER 2
CHARACTERIZATION OF CANINE BONE MARROW STROMAL CELLS

Background and Introduction

Recently, bone marrow stromal cells (BMSCs), also referred to as bone marrow-derived mesenchymal stem cells, have gained considerable attention as a potential source for cellular transplantation therapies for a variety of diseases due to their proliferative capacity, accessibility, and multipotentiality. Clinical application of BMSCs depends on development of preclinical animal models. Canine models have been used to evaluate various aspects of human diseases, some of which were developed to evaluate the safety and clinical efficacy of BMSC transplantation. The most widely accepted canine models that are being used in the context of BMSC research are myocardiac infarction models (Li et al., 2003; Memon et al., 2005; Vulliet et al., 2004) and bone defect models (Arinzeh et al., 2003; Bruder et al., 1998). These canine models have provided important insights into stem cell biology under a setting that is perhaps closer to human patients than many laboratory animal models are.

Previous studies on canine BMSCs have focused on osteogenic (Arinzeh et al., 2003; Bruder et al., 1998; Kadiyala et al., 1997; Volk et al., 2005) and ,to a less extent, chondrogenic (Kadiyala et al., 1997) differentiation properties because of the high prevalence of disorders in the skeletal system in dogs and the potential of such disorders to be an animal model in bone and cartilage research. These studies demonstrated proof of concept for the clinical use of BMSCs for bone and cartilage regeneration. Despite the potentials of canine models in BMSC research and their clinical relevance in veterinary medicine, information regarding the basic biology of canine BMSCs has been rather scarce compared to that of BMSCs from other species. Thus far, little is known about phenotypic properties, purification and culture expansion techniques, and differentiation abilities of canine BMSCs, all of which will become imperative to develop

preclinical studies using canine BMSCs. In human and rodent BMSC research, emphases have been put on the development of prospective identification and optimal expansion techniques in order to selectively expand authentic BMSCs. This relates to the fact that BMSCs have been classically isolated by the use of their physical property to adhere to plastic substrates (Friedenstein, 1995; Goshima et al., 1991b) which results in a high degree of hematopoietic contamination. Although there has been much progress in identifying cell surface proteins expressed on BMSCs (Haynesworth et al., 1992a), there is no specific BMSC marker that can be used to prospectively identify, isolate, and expand BMSCs in a similar way to hematopoietic stem cells. Therefore, identification of unique BMSC cell surface markers and development of culture expansion techniques have been a focus of may investigators.

While phenotypic characterization is needed to develop techniques for selective expansion of BMSCs, functional assays are used to demonstrate the multipotency of expanded cell populations. Classically, multipotency of BMSCs has been demonstrated by their ability to differentiate into three major mesodermal cell types *in vitro* and/or *in vivo*, namely osteoblasts, chondrocytes, and adipocytes (Haynesworth et al., 1992b; Jaiswal et al., 1997; Pittenger et al., 1999). Furthermore, it has been shown that in addition to these cell types, BMSCs differentiate into myoblasts (Wakitani et al., 1995), cardiomyocytes (Toma et al., 2002) and tendocytes (Jiang et al., 2002a) *in vitro*. Although demonstration of multipotency has been the gold standard to characterize BMSCs there is evidence that responses to given differentiation stimuli are noticeably diverse among different species. Interspecies variation of osteogenic differentiation properties of BMSCs has been most intensively studied to date (Diefenderfer et al., 2003a; Diefenderfer et al., 2003b; Osyczka et al., 2004; Volk et al., 2005). The differentiation capacity of BMSCs into cell types other than those of mesodermal origin has been demonstrated in recent

studies and explained in part by their tremendous plasticity. The possibility of generating various cell types from BMSCs has expanded potential applications of BMSC-based therapies, and we addressed *in vitro* and *in vivo* neural transdifferentiation properties of canine BMSCs in Chapter 3 and Chapter 4, respectively.

In the present study, we characterized phenotypic and functional properties of canine BMSCs. First, we estimated the frequency of BMSCs in canine bone marrow by use of CFU-F assay and investigated the growth kinetics *in vitro*. Next, flow cytometry was used to characterize the phenotypic properties and homogeneity of expanded canine BMSCs. Last, we evaluated the differentiation properties of canine BMSCs by observing osteogenic and adipogenic differentiation *in vitro* and comparing the results to those of rat BMSCs.

Materials and Methods

Bone Marrow Collection

A total of 5 adult mongrel canine cadavers were collected from a local animal shelter after euthanasia. Exact ages of these dogs were unknown; however, we collected only those which had a complete set of adult dentures with minimum dental calculus deposition, allowing inclusion of "young adults" in the study. The use of these animals was approved by the Institutional Animal Care and Use Committee of the University of Florida. Bone marrow was aseptically aspirated from iliac crests into sterilized 10 mL syringes containing 2,000 units of Heparin, using 16-G Jamshidi needles. All bone marrow collections were performed within 30 minutes after euthanasia.

Rat bone marrow was obtained from a total of 3 young adult male Sprague-Dawley rats (90–150 g) that were euthanized for an unrelated project. Specifically, both femurs and tibiae were removed, both ends cut, and bone marrow obtained by flushing it with complete medium through a 21-G needle inserted into the proximal end. The complete medium consisted of

Dulbecco's Modified Eagle's Medium (1g/L glucose) supplemented with 20% fetal bovine serum (FBS), 100 U/mL penicillin G, 100 µg/mL streptomycin sulfate, and 0.25 µg/mL amphotericin B.

BMSC Culture

Canine BMSCs were isolated and cultured using techniques described by Kadiyala et al. (1997) with some modifications. Briefly, the marrow was washed with Hank's balanced salt solution (HBSS) and mixed with two volumes of complete culture medium. Aliquots of the bone marrow suspension were layered over Ficoll and centrifuged at 400 × g for 30 minutes to enrich mononucleated cells. Enriched mononuclear cells were plated in T-75 culture flasks at a cell density of $1.5 \times l0^5$ cells/cm^2 in 15mL of complete medium for each animal and incubated at 37°C in a humidified 5% CO_2 environment. After 48 hours, flasks were washed with phosphate buffered saline (PBS) to remove nonadherent cells. The cultures were fed biweekly and passaged upon reaching approximately 80% confluence by releasing the cells with 0.05% trypsin/0.53 mM EDTA. Released cells were collected by centrifugation at 800 × g for 5 minutes, washed twice with HBSS, counted by use of a hematocytometer, and replated at $8 \times l0^3$ cells/cm^2 for subsequent passages. Portions of Ficoll-separated mononucleated cells from each dog were suspended in 10% dimethylsulfoxide (DMSO) and 90% FBS and frozen in aliquots in liquid nitrogen for later use.

Rat bone marrow was plated in 2 T-75 flasks and cultured in complete medium. Upon reaching semiconfluency, primary cells were detached by 0.05% trypsin/0.53 mM EDTA and frozen in liquid nitrogen for later use.

Colony-Forming Unit Assay

In order to estimate the frequency of BMSCs in the canine bone marrow, CFU-F assay was performed according to the method described by Meirelles et al. (2003) with modifications.

After Ficoll separation, mononucleated cells were plated in 60mm culture dishes with increasing cell numbers (4.2×10^5, 8.4×10^5, and 1.68×10^6). Cells were cultured for 12 days in complete medium after which colonies were fixed, stained with Diff-Quick (Dade Diagnostics, Inc.), and observed under an inverted microscope. Colonies containing more than 50 cells were scored. All assays were performed in duplicate.

Growth Kinetics

The rate of cell growth was measured in samples obtained from three bone marrow samples. Frozen Ficoll-separated mononucleated cells were thawed, counted with a hematocytometer, and seeded at $1.5 \times 10^6/cm^2$ in T-75 culture flasks. Adherent cells were expanded and first passaged (P1) BMSCs were used to measure cell proliferation. On day 0, P1 BMSCs were plated in 6-well culture plates at low (10 cell/cm^2), medium (100 cells/cm^2), and high (1,000 cells/cm^2) cell densities and cultured for 12 days in complete medium. Medium changes were performed every three days. Each day, 2 wells from each cell density group were trypsinized and the cell numbers were determined by use of a hematocytometer.

Flow Cytometric Characterization

To evaluate the cell surface markers and the homogeneity of canine BMSCs, P3 BMSCs were analyzed by flow cytometry. Culture medium was removed and attached cells were washed three times with PBS. The cells were detached from the flasks by incubation with 0.05% Trypsin/0.53mM EDTA. Detached cells were collected by centrifugation, washed with FACS buffer (PBS containing 0.5% BSA and 0.1% sodium azide), and counted. Aliquots containing 1×10^6 cells were incubated with primary antibodies for 30 minutes on ice. The primary antibodies used were R-phycoerythrin (R-PE) conjugated mouse anti-canine CD34 (BD Biosciences; 1:1), FITC-conjugated rat anti-canine CD45 (Serotec; 1:15), mouse anti-canine CD90 (Thy-1) (VMRD Inc; 1:80), mouse anti-canine MHC-I (VMRD Inc; 1:133), and mouse

anti-canine MHC-II (VMRD Inc; 1:133). APC-conjugated goat anti-mouse IgM (Caltag Laboratories; 1:5) was used to label anti-CD90 antibodies and FITC-conjugated rat anti-mouse IgG2a (BD Biosciences; 1:25) was used to label anti-MHC I and MHC II antibodies for 30 minutes on ice. Aliquots containing the equal number of cells were incubated with respective isotype controls under the same conditions. Cells without antibodies were used as a negative control. Labeled cells were then washed with FACS buffer, pelleted, and fixed in 0.5% paraformaldehyde and 0.1% sodium azide in PBS. Data were analyzed by collecting 10,000 events on FACS Calibur cytometer (Becton Dickinson Immunocytometry Systems) using Cell Quest software (BD Biosciences).

Differentiation Assays

Osteogenic and adipogenic differentiation properties of canine BMSCs were evaluated using the protocols described by Pittenger et al. (1999) with slight modifications. To maximize the possibility and rate of cell differentiation, P1 canine BMSCs were used for the assays. Frozen Ficoll-separated canine BMSCs were plated in T-75 culture flasks and expanded in complete medium until semiconfluency. Expanded primary cells were released by 0.05% trypsin/0.53 mM EDTA and subcultured at $8 \times l0^3$ cells/cm^2 as first passage BMSCs in 6-well culture plates. P0 BMSCs from 2 dogs were also subjected to osteogenic differentiation. Rat primary (Po) BMSCs were plated in 6-well culture plates and used for comparative observations.

Osteogenic induction was initiated by replacing the complete medium with medium containing 50μM ascorbate 2-phosphate (Sigma), 10mM β-glycerophosphate (MP Biomedical), 100nM dexamethasone (MP Biomedical), 10% FBS, in low-glucose (1g/L) DMEM. In selected cultures, bone morphogenic protein-2 (recombinant human BMP-2, Pepro Tech) was added at 50ng/mL as per Volk et al. (2005). Osteogenic induction media were replaced every three days and continued for 12 days at 37°C in a humidified 5% CO_2 environment. On day 12,

differentiation of BMSCs into the osteoblastic phenotype was evaluated under a phase contrast microscope and by Alizarin Red S (Gregory et al., 2004) as well as Von Kossa (Anselme et al., 2002) staining as described previously.

Adipogenic differentiation was induced by replacing the complete medium with adipogenic induction medium containing 1mM dexamethasone, 0.5mM methyl-isobutylxanthine, 10μg/mL insulin, 100mM indomethacin, and 10% FBS in DMEM (4.5g/L glucose). Cells were cultured for 3 days and the medium was changed to adipogenic maintenance medium containing 10μg/mL insulin and 10% FBS in DMEM (4.5g/L glucose) for 24 hours. This cycle of induction and maintenance was repeated three times with last maintenance cycle being extended for 7 days. After 7 days, cells were fixed in 4% paraformaldehyde and stained with Oil Red O staining.

Results

Bone marrow was aspirated from each dog and mononucleated cells were isolated as described above. The summary of the bone marrow samples used in this study is shown in Table 2-1. The average yield of mononucleated cells per 1mL of bone marrow was 2.27×10^7 cells.

Table 2-1. Summary of bone marrow samples.

Dog ID#	Dog sex	Bone marrow collected (mL)	Total MNCs yield* ($\times 10^7$)
21	Male	5	16.0
22	Female	3	3.75
23	Female	10	23.9
24	Male	9	18.0
25	Male	10	25.2

All bone marrow samples were obtained from the iliac crest of young adult canine cadavers.
* indicates the number of mononucleated cells isolated after Ficoll separation.

Morphological Observation of Canine BMSCs

Growth characteristics of plastic-adherent cells isolated from canine bone marrow were similar to BMSCs from other species as described previously. Approximately after 5 days of

plating, adherent cells could be observed in most cultures. Initially, there were morphologically two major types of cells in the culture, loosely attached rounded cells and tightly attached spindle-shape cells. Typically, loosely attached rounded cells (Fig. 2-1A, arrowheads), presumably mature leukocytes and hematopoietic stem/progenitor cells, were observed on top of the spindle-shape cells. Most of these rounded cells were washed out over time with changes in medium and almost completely removed from the culture by the time of the first passage (typically after 14 days). After 7 to 10 days of initial plating, spindle-shape cells rapidly grew, forming discrete symmetric colonies (Fig. 2-1A). Of the adherent colony-forming cells, there were various cell morphologies, including flattened fibroblastic cell (Fig. 2-1B), long spindle-shaped cells (Fig. 2-1C), and short spindle-shaped cells (Fig. 2-1D).

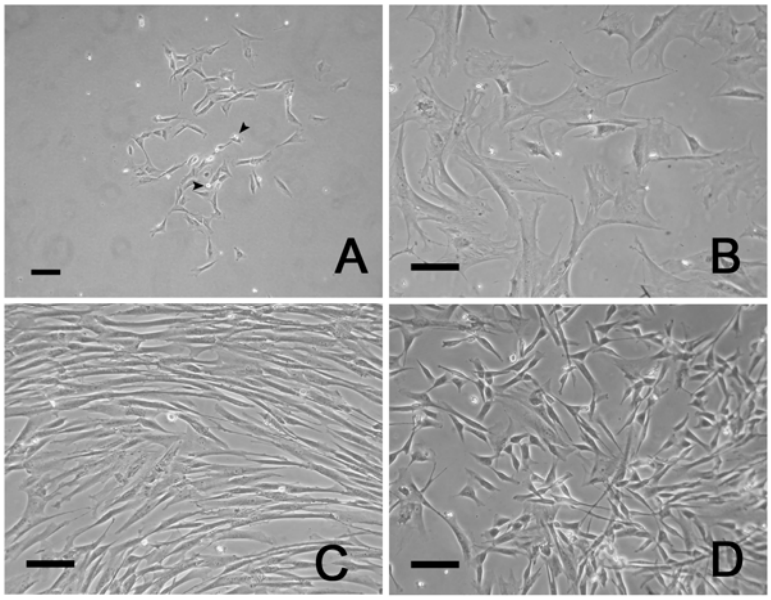

Figure 2-1. Morphological observation of canine primary BMSCs. (A) Low magnification view of primary culture on day7. Primary BMSCs grow in colonies with various cell morphologies comprising each colony. Small rounded cells are occasionally observed on top of adherent BMSCs (arrowheads). There are at least three major types of adherent cells as observed under higher magnification views on day14; (B) large flattened fibroblastic cells, (C) long spindle-shaped cells, and (D) short spindle-shaped cells with refractile soma. Scale bar = 100μM.

From the first passage culture on, the spindle-shape cells became predominant which grew in uniform monolayer and further became flattened to assume fibroblastic morphology upon further passaging.

The Frequency of Canine BMSCs

In all samples, the numbers of colonies consistently increased as a function of the number of cells seeded (Fig. 2-2). Overall, the frequency of canine BMSCs was estimated to be 0.0042 ± 0.0019 % which equals approximately 1 BMSC in every 2.38×10^4 mononucleated cells. It was noted, however, that there was variability among bone marrow samples, represented by a wide range of the frequencies of CFU-F (0.0014 - 0.0057%) (Table 2-2).

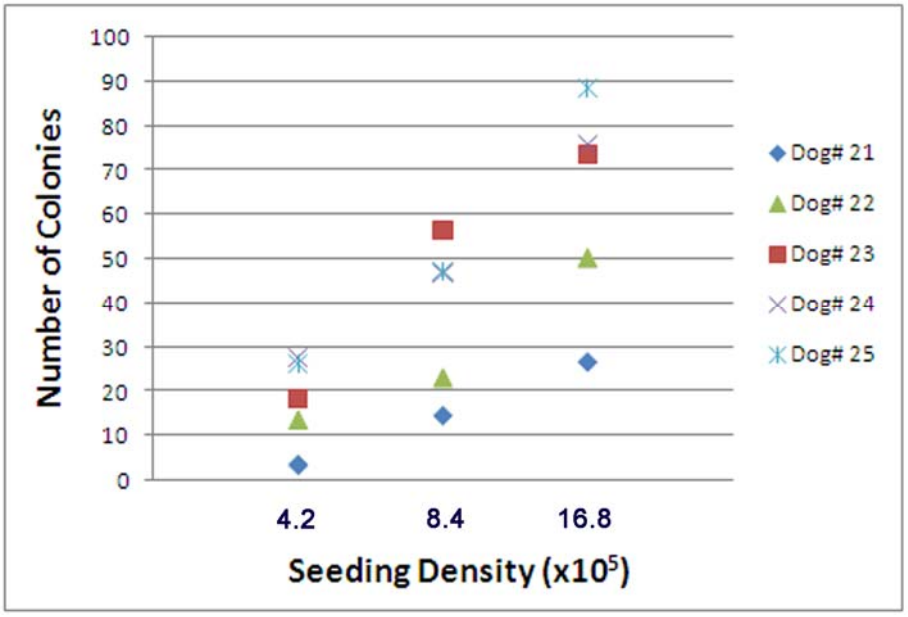

Figure 2-2. The CFU-F assays from five adult canine bone marrow samples. Primary cells were seeded at three different cell densities and cultured for 12 days. Colonies containing more than 50 cells were scored. All cultures were performed in duplicate and the average numbers of colonies were plotted for each dog for each cell density.

Growth Kinetics of Canine BMSCs

Three bone marrow samples (Dog#23, #24, #25) were used to evaluate cell growth kinetics at low (10 cells/cm^2), medium (100 cells/cm^2), and high (1,000 cells/cm^2) cell density. Cells seeded at 1,000 cells/cm^2 proliferated and produced approximately 4.6×10^5 cells over a 12-day

Table 2-2. Number and frequency of CFU-F from five bone marrow samples.

Sample #		Seeding density			Average Frequency
		4.2×10^5	8.4×10^5	1.68×10^6	
Dog 21	Plate 1	2	17	25	
	Plate 2	5	12	28	
	Average	3.5	14.5	26.5	
	Frequency	0.000833333	0.001726	0.001577	0.001379
Dog 22	Plate 1	16	28	49	
	Plate 2	11	18	52	
	Average	13.5	23	50.5	
	Frequency	0.003214286	0.002738	0.003006	0.002986
Dog 23	Plate 1	19	54	78	
	Plate 2	18	59	69	
	Average	18.5	56.5	73.5	
	Frequency	0.004404762	0.006726	0.004375	0.005169
Dog 24	Plate 1	31	54	70	
	Plate 2	24	40	82	
	Average	27.5	47	76	
	Frequency	0.006547619	0.005595	0.004524	0.005556
Dog 25	Plate 1	29	44	105	
	Plate 2	24	51	72	
	Average	26.5	47.5	88.5	
	Frequency	0.006309524	0.005655	0.005268	0.005744

Numbers of colonies for each plate and the average are shown. Average frequencies represent the frequency of CFU-F for nucleated cells in bone marrow.

culture period, whereas cells seeded at 10 cells/cm^2 produced only 7.0×10^3 cells (Fig 2-3A). However, cells seeded at 10 cells/cm^2 expanded approximately 74-fold in 12 days, whereas cells seeded at 1,000 cells/cm^2 expanded only 48-fold (Fig 2-3B). Cells seeded at 100 cells/cm^2 displayed intermediate growth kinetics patterns (Fig 2-3A and B).

Flow Cytometric Profile of Canine BMSCs

The surface marker profile of P3 BMSCs was analyzed by flow cytometry. The results indicated that the cell surface profile of canine BMSCs was comparable to those reported for human and rodent MSCs; they were positive for CD90 (mean total % ± standard deviation, 88.5% ± 3.9) and MHC-I (85.4% ± 3.7), and negative for MHC-II (0.9% ± 0.5) (Fig. 2-4). The

absence of the lipopolysaccharide receptor CD34 (1.1% ± 0.1) and the leukocyte common antigen CD45 (0.5% ± 0.1) expression indicated that cells of hematopoietic origin had been excluded during the cell expansion process.

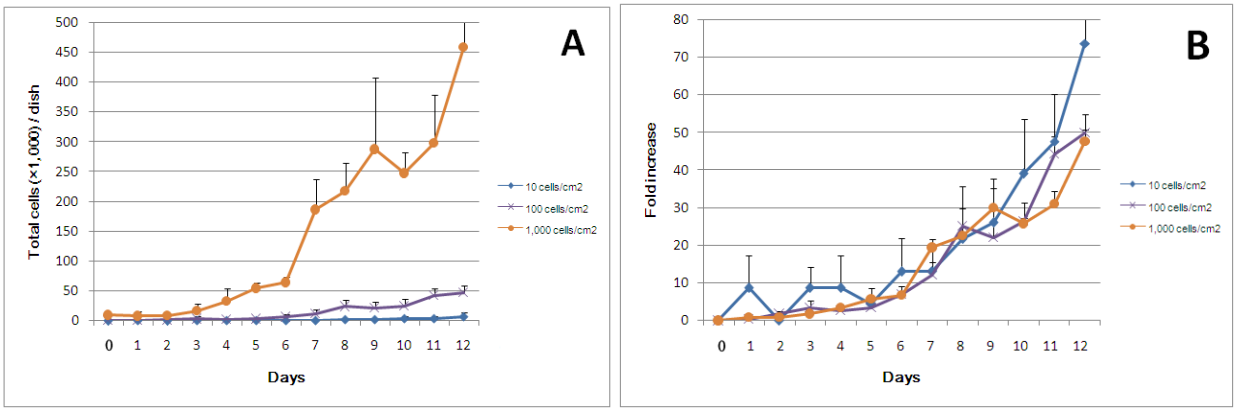

Figure 2-3. Cell growth kinetics as a function of initial cell densities. BMSCs from three dogs were plated at low (10 cells/cm^2), medium (100 cells/cm^2), and high (1,000 cells/cm^2) cell density. The average total cell counts (A) and fold increases are plotted for each cell density over a 12-day culture period. Data points represent mean values from three samples. Bars represent standard errors.

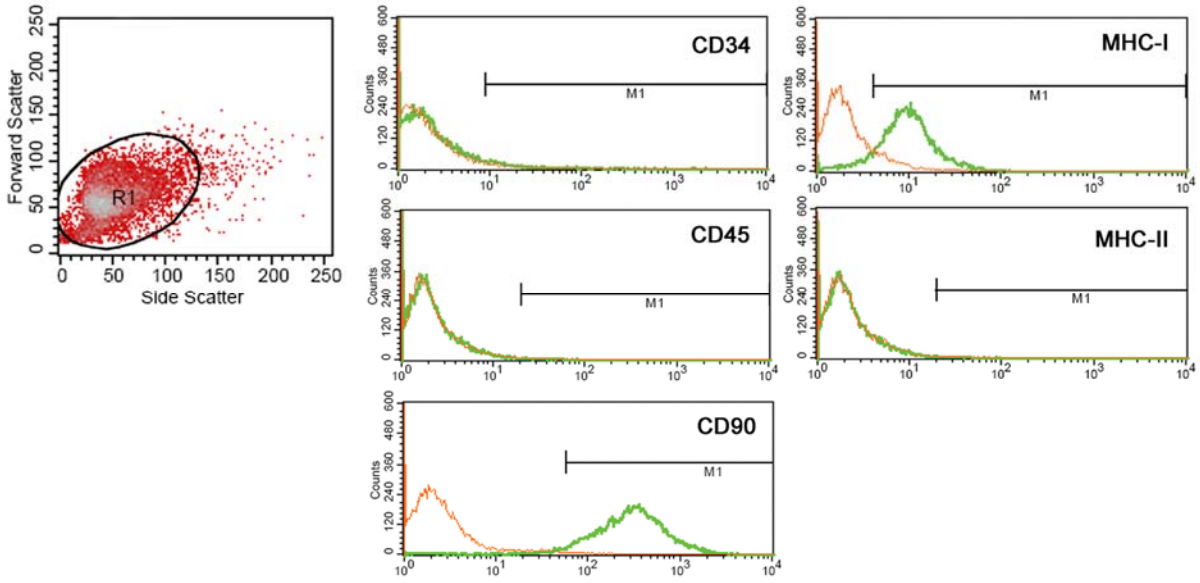

Figure 2-4. Representative results of flow cytometric analysis of canine BMSCs. Canine BMSCs are negative to CD34, CD45, MHC-II and positive to CD90 and MHC-I. Green lines represent counts of the cell population that is positive for the cell surface marker indicated in each panel. Orange lines represent corresponding isotype controls. M1 indicates the gated area.

Osteogenic and Adipogenic Differentiation of Canine BMSCs

To define the osteogenic differentiation potential of canine BMSCs, P1 BMSCs were cultured in medium containing osteogenic inducers. It was clearly noted that in the presence of osteogenic inducers, BMSCs proliferated at a much higher rate with concomitant morphological changes. Addition of BMP-2 further stimulated cell proliferation. The main morphological change appeared at the early stage was transformation from fibroblastic cells to spindle-shaped refractive cells (Fig. 2-5E and F). At later stages, proliferated cells became cuboidal, a characteristic morphology of osteoblastic cells (Fig. 2-5H, I, K, and L). However, distinct nodular aggregates were not found under any culture conditions. A significant change was not observed in BMSCs in control medium (Fig. 2-5A, D, G, and J). Neither differentiated cells nor control cells were stained against Von Kossa or Alizarin Red S staining. It was also noted that because of accelerated cell growth in the presence of osteogenic inducers, cells often detached from culture plastic around day 6, which precluded further analysis.

Then, we tested the osteogenic differentiation property of primary canine BMSCs under the same induction condition. Primary BMSCs migrated and formed nodular aggregates in osteogenic medium (Fig. 2-6B), whereas cells grew in colonies in control medium (Fig. 2-6A). Larger nodules were consistently seen in culture with BMP-2 (Fig. 2-6C) when compared to those without BMP-2 (Fig. 2-6B). The nodular aggregates in osteogenic medium were negative for Von Kossa staining, indicating that mineralization of the matrix had not occurred (Fig. 2-6E). In contrast, strong Von Kossa staining was found in nodules formed by BMSCs cultured in osteogenic medium with BMP-2 (Fig. 2-6F).

Rat P1 BMSCs were used for a comparative observation. Rat BMSCs appeared as flattened and larger polygonal cells in the control medium, which proliferated but did not

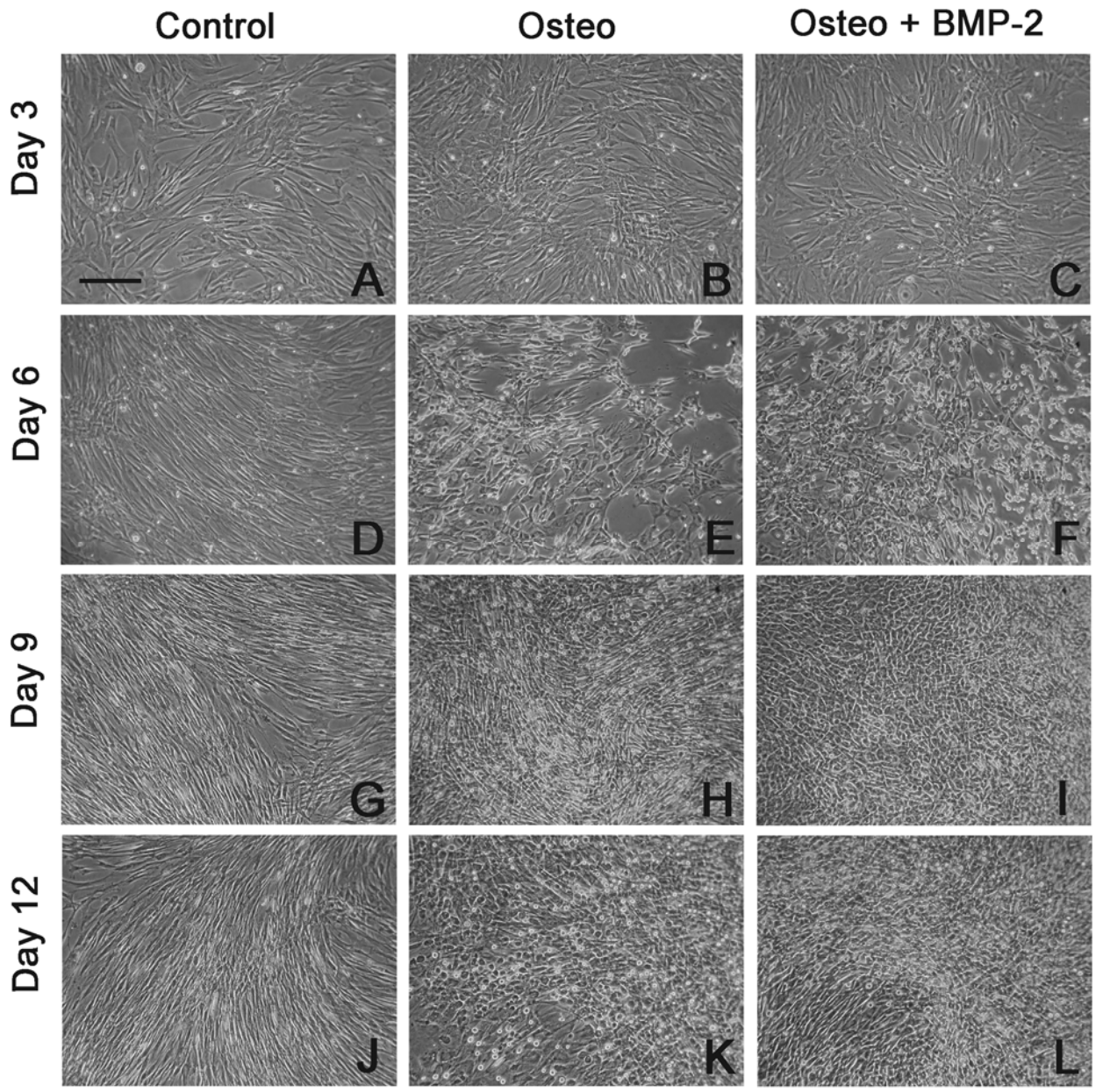

Figure 2-5. Morphological changes of canine P1 BMSCs during osteogenic induction. Significant morphological changes were not observed in BMSCs cultured in control medium (A, D, G, and J) over a 12-day culture period. Osteogenic medium stimulated cell growth and induced morphological transformation from fibroblastic cells to spindle-shaped cells between day 6 (E) and day 9 (H), and to cuboidal cells by day 12 (K). These changes were pronounced in cultures in the presence of BMP-2 (C, F, I, and L). Nodular aggregate formation was not found in any of the cultures. Scale bar = 250μM.

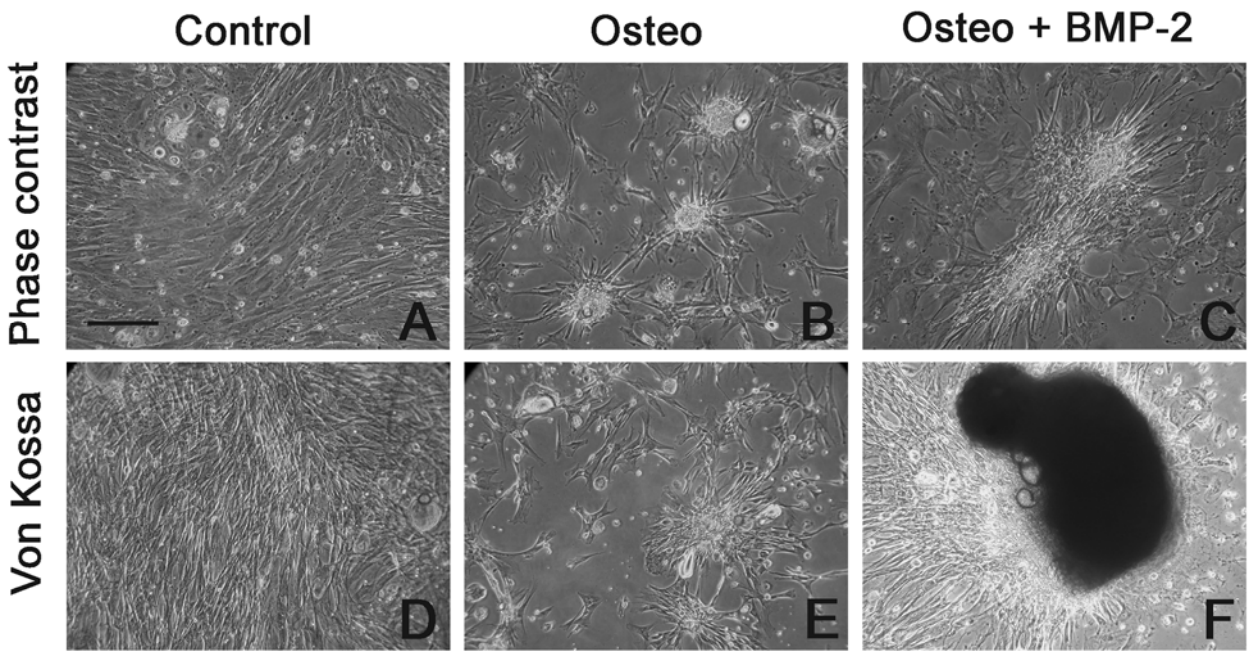

Figure 2-6. Osteogenic differentiation of canine P0 BMSCs. Primary cells grew in control medium without apparent nodule formation (A and D). In osteogenic medium, small nodules were formed (B), which were Von Kossa-negative (E). BMP-2 stimulated nodule formation (C) which was strongly mineralized as evident by Von Kossa staining (F). Scale bar = 250μM.

undergo apparent morphological changes during the 12-day culture period (Fig. 2-7A, D, G, and J). In contrast, in the osteogenic medium, cells started to migrate by day 6 and formed nodular aggregates by day 12 (Fig. 2-7B, E, H, and K). Addition of BMP-2 further stimulated cell proliferation and nodule formation (Fig. 2-7C, F, I, L). Mineralization of the cellular matrix was not found in cells grown in control medium (Fig. 2-8A and D). In the osteogenic medium, cellular aggregates showed minimal deposition of mineralized materials (Fig. 2-8B and E). In the presence of BMP-2, extensive mineralization of the cellular matrix was observed (Fig. 2-8C and F).

Next, adipogenic differentiation was induced on canine P1 BMSCs. It was consistently noticed that cells in the induction medium became flattened, losing their refractile appearance by day 3 (Fig. 2-9A). By day 6 of induction, there was a small portion of cells containing cytoplasmic vacuoles (Fig. 2-9B). These cytoplasmic vacuoles further enlarged in the induction

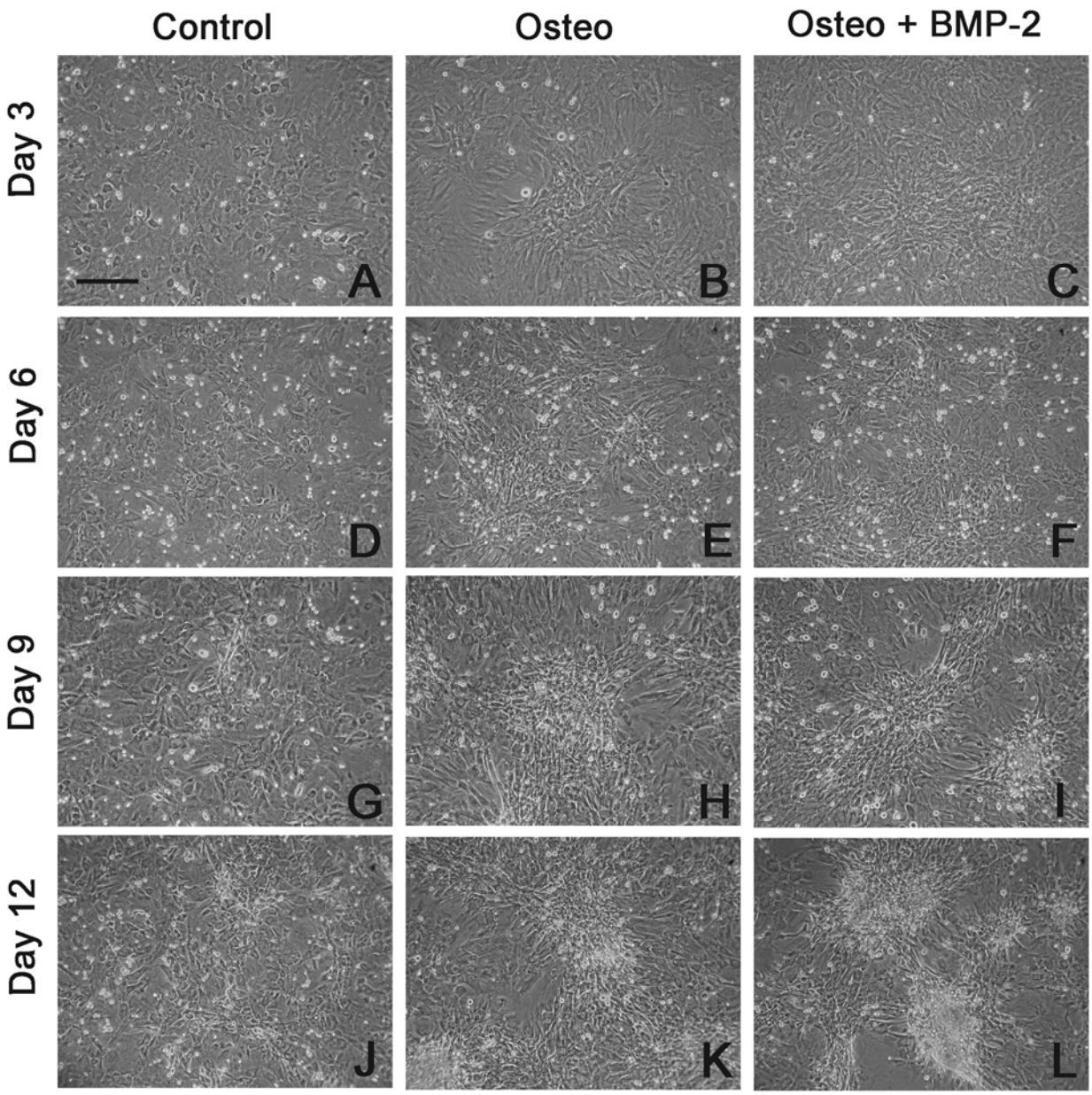

Figure 2-7. Morphological changes during osteogenic differentiation of rat P1 BMSCs. Significant morphological changes were not observed in BMSCs in control medium (A, D, G, and J) over a 12day culture period. On day3, confluent rat BMSCs were observed in osteogenic media (B and C). On day6, clear nodular aggregates were observed in BMSCs in osteogenic media (E and F). On day9, large nodules were seen in BMSCs (K), which were further stimulated by addition of BMP-2 (L). Scale bar = 250μM.

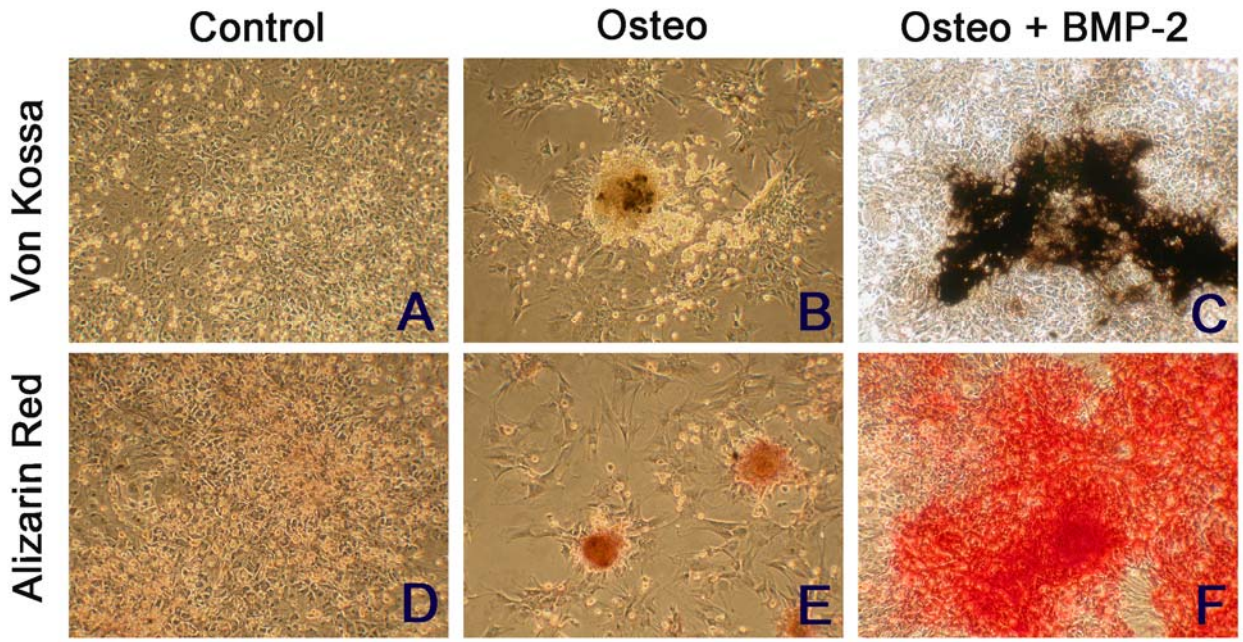

Figure 2-8. Osteogenic differentiation of rat P1 BMSCs (day12). Phase contrast photomicrographs show confluent rat P1 BMSCs in control cultures which were negative for both Von Kossa (A) and Alizarin Red (D) staining. In osteogenic induction medium, rat BMSCs migrated and formed small nodules. These nodules were partially positive for Von Kossa (B) and Alizarin Red (E). Addition of BMP-2 strongly stimulated mineralization of extracellular matrix evident by strong staining with Von Kossa (C) and Alizarin Red (F).

medium by day 9 (Fig. 2-9C) and some of them appeared to fuse by day 12 (Fig. 2-9D). Differentiated adipocytes were found dispersed across the plate, which tended to cluster as seen in Fig. 2-9C, D, and E, indicating that differentiated cells were produced from the common BMSCs. After around day-7 when some of the cells started to differentiate, a significant number of cells detached from culture plastic. These changes were not observed in cells grown in control medium. Oil Red O staining showed accumulation of lipid substances in the cytoplasmic vacuoles of differentiated cells on day 12 of induction (Fig. 2-9E).

Conclusion and Discussion

In the present study, we have characterized canine BMSCs based on their morphological, growth characteristic, phenotypic, and functional properties. Morphological characteristics of canine BMSCs were comparable to BMSCs isolated from rodents and humans as described

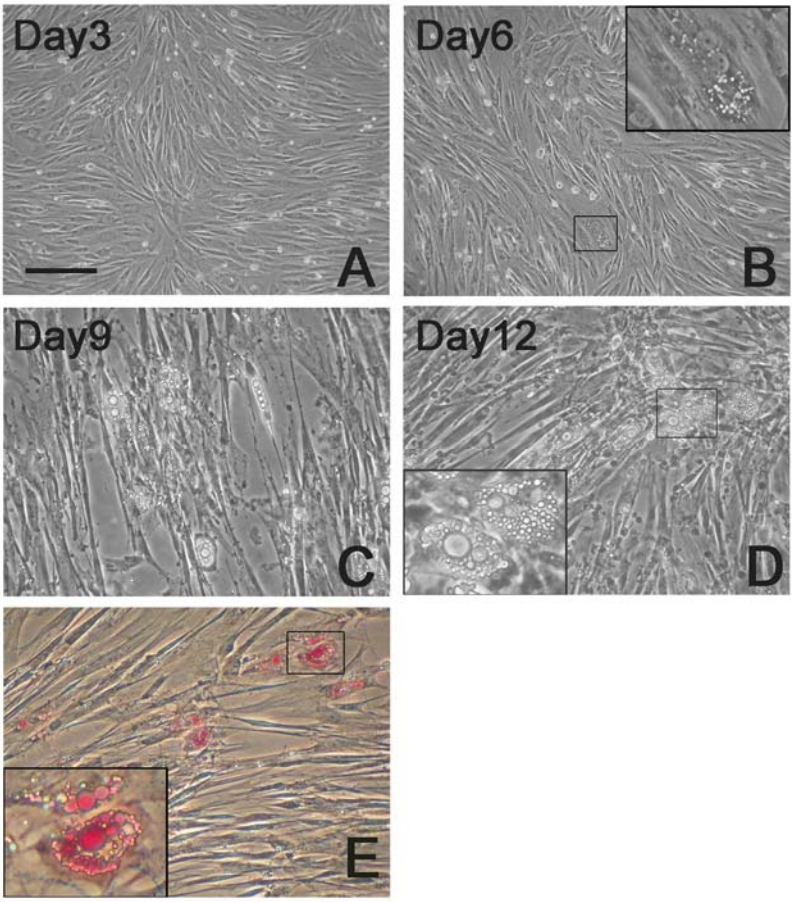

Figure 2-9. Adipogenic differentiation of canine P1 BMSCs. BMSCs cultured in adipogenic medium became flattened after day 3 (A). By day 6, a small number of cells had cytoplasmic vacuoles (B) which further enlarged by day 9 (C). By day 12, these vacuoles fused to form large lipid droplets. Oil Red O staining showed lipid substances in the cytoplasm of differentiated cells on day 12 (E). Insets in B, D, and E show higher magnification views of boxed areas. Scale bar = 250μM.

previously. Similar to BMSCs of rodents and humans, canine primary BMSCs adhered to culture plastic and grew in colonies to form CFU-F. Primary BMSCs were morphologically heterogeneous, containing at least three different types of adherent cells that could be clearly distinguishable. In passaged cultures, spindle-shaped cells progressively became the predominant cell type. These morphological features of primary and passaged canine BMSCs are similar to rodent and human counterparts. FACS analysis showed that the cell surface marker profile of passaged canine BMSCs was also comparable to those from rodents and humans. Although canine primary adherent cells were consisted of heterogeneous cell

populations, significant hematopoietic contamination was not evident; therefore, the plastic adhesion method seemed sufficient for isolation of canine BMSCs. This is in contrast to murine BMSCs in which the plastic adhesion method results in significant contamination of granulo-monocytic cells in both primary and passaged cultures (Phinney et al., 1999; Xu et al., 1983), thus requiring preceding immunodepletion using CD11b antibody (Kopen et al., 1999). It is unknown whether morphologically heterogeneous cell populations in the canine primary adherent cultures represent distinct classes of stem cells, perhaps containing rare pluripotent stem cell populations present in human and murine bone marrow (Jiang et al., 2002a; Jiang et al., 2002b; Reyes et al., 2001), or cells of the same lineage with different developmental stages. At present, the latter is likely the case as human BMSCs are also morphologically heterogeneous even when cloned from single-cell-derived colonies (Colter et al., 2000; Zohar et al., 1997).

The frequency of BMSCs in canine bone marrow has been reported by Kadiyala (1997) to be approximately 0.004% (1 per 2.5×10^4 mononucleated cells), although detailed data was not presented in their study. In the present study, CFU-F assays demonstrated that the frequency of canine BMSCs was approximately 0.0042% (1 per 2.38×10^4 mononucleated cells). This frequency of canine BMSCs is twofold and fourfold higher than the previously reported frequencies of murine (Friedenstein et al., 1976) and human BMSCs (Bruder et al., 1997), respectively. However, the number of CFU-F can be influenced by culture conditions (e.g. FBS concentration, type of basal medium, pH of medium, etc) (Xu et al., 1983); therefore, further studies are needed to optimize the culture condition for canine BMSCs. We found that the numbers of CFU-F were variable among bone marrow samples and several factors could account for this finding. Age is a known factor that has impact on the frequency of BMSCs. In the present study, we did not have information regarding the exact age of the dogs. Although the

dogs in the present study were considered to represent "young adult" based on their denture, differences in their maturity might have influenced the frequency of BMSCs. The degree of peripheral blood contamination is another factor since CFU-F is calculated based on the number of mononucleated cells in the sample. This could be significantly influenced by postmortem changes such as coagulation of peripheral blood. This problem may arise when bone marrow is collected by aspiration but not by flushing long bones which is a common technique for collection of rodent BMSCs.

Growth kinetics of canine BMSCs were evaluated under three different cell seeding densities (10 cells/cm^2, 100 cells/cm^2, and 1,000 cells/cm^2). It was clearly shown that initial cell densities have a profound influence on the proliferation rate of canine BMSCs, as the case for human BMSCs (Sekiya et al., 2002). Among the three cell densities tested, there was an inverse relationship between the total yield of BMSCs and expansion rate (fold increase). This suggests that when BMSCs are seeded at a lower density, they undergo more rapid proliferation. It also implies that, because increasing cell density is known to trigger cell differentiation (Caplan et al., 1983), there must be a compromise between the total cell yield and the content of undifferentiated cells. Further studies are necessary to define the optimal culture condition, which generates the maximal cell yield with the greatest content of multipotent canine BMSCs.

Our functional assays demonstrated that passaged canine BMSCs showed limited osteogenic differentiation ability. Although some morphological changes, which resembled osteoblastic morphology, were observed in passaged BMSCs cultured in osteogenic medium, formation of nodular aggregates was never found in passaged BMSCs, indicating that full differentiation toward osteoblasts did not occur. Osteogenic inducers stimulated cell growth, which often resulted in detachment of cell monolayer around after day 6 of induction and

precluded further culture of BMSCs. Despite previous studies (Kadiyala et al., 1997; Volk et al., 2005) describing differentiation of passaged canine BMSCs into osteoblasts, these results were not reproducible in our hands. In contrast, primary canine BMSCs differentiated into osteoblasts and formed nodules which were intensively mineralized. Primary BMSCs cultured in medium without osteogenic inducers did not show similar changes; thus, contamination of osteoblastic cells in the samples seemed unreasonable. When passaged BMSCs were grown in adipogenic inducers, a small number of cells differentiated into mature adipocytes. Upon exposure to a high concentration of dexamethasone, a significant number of undifferentiated cells detached from the culture plate. These results demonstrated that a small number of BMSCs possess adipogenic capacity in the passaged BMCS culture. All together, the results of our functional assays suggest that canine bone marrow contains inducible mesenchymal progenitors with the ability to giving rise to osteoblasts and adipocytes. Upon passaging, a significant number of these stem cells either lose their multipotency or did not survive in the standard culture condition. Further studies are needed to develop culture techniques which enable expansion of canine BMSCs in their undifferentiated state.

CHAPTER 3
IN VITRO NEURAL DIFFERENTIATION OF CANINE BONE MARROW STROMAL CELLS

Background and Introduction

A variety of cell types such as embryonic stem cells (McDonald et al., 1999; Wernig et al., 2004) and neural progenitor/stem cells (Ogawa et al., 2002; Pluchino et al., 2003), and developing tissues harboring stem cells such as fetal spinal cord tissues (Anderson et al., 1995; Houle, 1992) have been shown to promote CNS regeneration in experimental studies. However, clinical application of these sources is severely limited due to issues associated with the availability of the transplants, immunological reactions, and most importantly with ethical considerations. Cells derived from bone marrow, particularly bone marrow-derived stromal cells (BMSCs, also referred to as mesenchymal stem cells), possess potentials to circumvent many of these obstacles; therefore, may represent the most realistic cell source.

Recent *in vitro* studies demonstrated that BMSCs can generate not only mesodermal cells but also endodermal and ectodermal cell types, suggesting that these cells are less restricted than were previously thought. Neural differentiation of cells derived from rat and human bone marrow has been reported by several independent groups (Deng et al., 2001; Sanchez-Ramos et al., 2000; Woodbury et al., 2000). These studies have aroused considerable interest in the potential use of BMSCs for neurological disorders. Unlike ES cells and neural stem cells where embryos or neonatal neural tissues are necessary to obtain these cells, BMSCs are easily accessible by bone marrow aspiration. Furthermore, BMSCs are easily expandable in culture without losing their multipotentiality (Pittenger et al., 1999). Because of these advantages, BMSCs have received great attention as a promising source of cells for a variety of therapies including neuroregeneration.

Early studies on neural differentiation of bone marrow-derived stem cells were based on morphological changes and immunoreactivity against some of the neural markers (Sanchez-Ramos et al., 2000; Woodbury et al., 2000). The significance of the morphological changes observed in these studies, however, has been questioned by others and attributed to actin cytoskeleton shrinkage rather than genuine neurofilament extension (Bertani et al., 2005; Lu et al., 2004; Neuhuber et al., 2004). As such, interpretation of neural differentiation of BMSCs *in vitro* needs great caution and systematic approaches including assays at the molecular level are desirable. While it is still the subject of debate whether neurogenesis of BMSCs represents genuine "trans-differentiation" or an epiphenomenon caused by induced chemical stress, functionalities of MSC-derived neurons, such as their abilities to secrete neurotransmitters and/or fire action potentials, have been reported by several groups (Hermann et al., 2004; Kohyama et al., 2001; Tao et al., 2005; Wislet-Gendebien et al., 2005). These *in vitro* studies have raised tremendous interest that BMSCs have the potential to differentiate into functional neural cells.

Based on the previous findings in human and rodent BMSC studies, we hypothesized that canine BMSCs can be culture-expanded while maintaining their multipotentiality and that these cells bear neural properties. We induced a rapid neural differentiation on canine BMSCs as previously described by Deng et al. (2001) and evaluated expression of neuronal and glial-specific proteins, using immunocytochemical and western blotting analyses. We further evaluated the electrophysiological properties of neurally induced BMSCs using the whole-cell voltage clamp technique.

Materials and Methods

Preparation of Canine BMSCs

Bone marrow aspirates from 5 canine cadavers were used to isolate and culture canine BMSCs. Detailed descriptions of bone marrow collection, cell isolation, and culture expansion

can be found in Chapter 2. Third passage (P3) BMSCs were used for the assays described in this chapter.

Neural Differentiation of Canine BMSCs

Neural differentiation of P3 BMSCs was induced by replacing the complete medium by neural induction medium. The neural induction medium consisted of 0.5mM methyl-isobutylxanthine (IBMX) and 1mM dibutyryl cAMP (dbcAMP) in DMEM/F12 as used in neural differentiation of human bone marrow stromal cells (Deng et al., 2001). To support growth of neural cells the medium was supplemented with 1% N2 supplements. For immunocytochemistry, a total of 1×10^4 P3 BMSCs were suspended in 100 µl of complete medium and plated on coverslips placed in each well of 12-well culture plates. After 12 hours, 900 µl of complete medium was added and cells were cultured until reaching 70% confluency. Consequently, the medium was changed to 1 ml of neural induction medium and cultured at 37°C in a humidified 5% CO2 environment for 5 hours. For Western blot analysis, P3 BMSCs were plated at 8,000 cells/cm2 on 60 mm culture dishes in complete medium. Upon reaching 70% confluency, cells were washed with PBS and cultured in neural induction medium for 5 hours. Cells were also cultured in complete media and served as control. We also used canine fibroblast cultures (also from passage 3) (Coriell Institute for Medical Research) as a control for the assays described below.

Immunocytochemistry for Neural Specific Markers

We performed immunocytochemistry to evaluate whether canine express neuronal (βIII-tubulin), astrocyte-specific (GFAP), and oligodendrocyte-specific (MBP) proteins. Cells on coverslips cultured in complete medium or neural differentiation medium were gently washed with PBS and fixed in ice-cold 4% paraformaldehyde for 30 minutes at room temperature (RT). Fixed cells were permeabilized in 1.0% Triton X-100 in PBS for 10 minutes at RT. Non-specific

binding was blocked with a blocking solution (1.0% BSA in PBS) for 30 minutes at RT. Cells were then incubated with primary antibodies, including anti-ß III-tubulin (Promega Corp, 1:1,000), anti-GFAP (BD Biosciences, 1:1,000), anti-MBP (HyTest Ltd, 1:50,000), for 1 hour at RT. The primary antibodies were removed, cells washed with PBS, and cells incubated with one of secondary antibodies for 1 hour in dark at RT. The secondary antibodies (all from Jackson ImmunoResearch Laboratories Inc.) used were Cy2 conjugated goat anti-mouse IgG1 (1:400), Rhodamine conjugated goat anti-mouse IgG2b (1:100), FITC conjugated goat anti-mouse IgG2a (1:400). Cells were also incubated without primary antibodies to control for non-specific staining by secondary antibodies. Cells were washed with PBS and mounted with a mounting medium containing DAPI (Vector Laboratories). Stained cells were observed under a fluorescent microscope (Zeiss Axioplan II, Carl Zeiss MicroImaging) with appropriate filters.

Western Blotting and Densitometric Analyses

To confirm expressions of neuronal (ß III-tubulin) and glial (GFAP and MBP) markers on canine BMSCs, cultures were detached with 0.05% Trypsin/0.53mM EDTA for western blot analyses. Cells were lysed with a lysis solution (0.18M Tris-HCl, 40% Glycerol, 4% SDS, 0.04% BPB, 0.05M DTT). Canine spinal cord lysate was obtained using the same lysis solution and used as positive control. Total protein concentrations of each sample were determined by a protein assay kit (Micro BCA protein assay, Pierce). Samples containing 20 μg of protein were loaded in each lane of 12% polyacrylamide gels and electrophoretically separated. Separated proteins were transferred to nitrocellulose membranes, blocked with TBS containing 5% non-fat dry milk and 0.1% Tween20 for 1 hour at RT, and incubated with primary antibodies (βIII-tubulin; 1:500, GFAP; 1:4,000, MBP; 1:50,000) overnight at 4°C. The membranes were also probed with β-actin (Abcam Inc, 1:1,000) as loading controls. After washing the membranes with TTBS (0.05% Tween20 in TBS solution) for three times, the membranes were incubated

with alkaline phosphate conjugated secondary antibodies (Jackson ImmunoResearch Laboratories Inc., 1:5,000) for 1 hour at RT. The membranes were washed three times in TTBS and developed in a solution containing NBT and BCIP. Photographs of the membranes were taken using a molecular imager (Flour-S, Multi-imager, Bio-Rad Laboratories) and densitometric analyses performed using a software program (Quantity One, Bio-Rad Laboratories). Western blotting and densitometric analyses were duplicated in all samples.

Electrophysiological Recording

Two representative BMSC cultures were used for electrophysiological recordings. Neural differentiation was performed as described above. Spontaneous and depolarizing pulse-elicited action potentials were recorded with the whole-cell voltage clamp configuration in current clamp mode (Hamill et al., 1981). Experiments were performed at room temperature (23–24°C) with an Axopatch 200B amplifier and a Digidatal 1200B interface (Axon Instruments, Burlingame, CA). Data acquisition and analyses were performed with the use of Axoscope 7.0 and pClamp 6.2. Differentiated BMSCs were bathed in Tyrode's solution containing 140 mM NaCl, 5.4 mM KCl, 2.0 mM $CaCl_2$, 2.0 mM $MgCl_2$, 0.3 mM NaH_2PO_4, 10 mM HEPES, and 10 mM dextrose, pH adjusted to 7.4 with NaOH. BMSCs in the culture dish (volume 1.5 ml) were superfused at a rate of 2–4 ml/min. The patch electrodes (Kimax-5.1, Kimble Glass, Toledo, OH) had resistances of 3–4 MOhms when filled with an internal pipette solution containing 140 mM KCl, 4 mM MgCl2, 4 mM ATP, 0.1 mM guanosine 59-triphosphate, 10 mM dextrose, and 10 mM HEPES, pH adjusted to 7.2 with KOH. The whole-cell configuration was formed by applying negative pressure to the patch electrode.

Results

Morphological Observation during Neural Induction

The neural differentiation induction caused rapid morphological changes of canine BMSCs from fibroblastic morphology to neuron-like morphology with multiple processes seen in some cells (Fig. 3-1A and B). Initially, cells became rounded leaving some portions of the cytoplasm still attached to the original location. These changes typically appeared after around 3 hours of neural induction and almost completed after 5 hours. During the course of the neural induction, cells became less adhesive to the culture flask. As a result, some cells detached during flask manipulation. The changes in cell morphology were, however, not specific to BMSCs as similarly treated fibroblasts assumed neuron-like morphology after 5 hours of induction (Fig. 3-1C and D).

Immunocytochemical Characterization of Neurally Induced Canine BMSCs

The results of immunocytochemistry demonstrated that canine BMSCs constitutively express neuronal (βIII-tubulin) and astrocyte-specific (GFAP) proteins. We observed that almost all BMSCs showed immunoreactivity against GFAP at a relatively low level (Fig. 3-2A) whereas only subsets of these cells were strongly βIII-tubulin positive (Fig. 3-2B). Double staining of cells showed co-expression of GFAP and βIII-tubulin (Fig. 3-2C). After the neural induction, expressions of both βIII-tubulin and GFAP appeared to be pronounced, although these changes might have resulted from condensed localization of these proteins which accompanied with the morphological changes of the cells as described (Fig. 3-2D, E, F). In contrast, MBP positive BMSCs were not found under our culture condition either before or after the neural differentiation induction. Canine fibroblasts used as negative control did not show immunoreactivity against all neuronal/glial proteins.

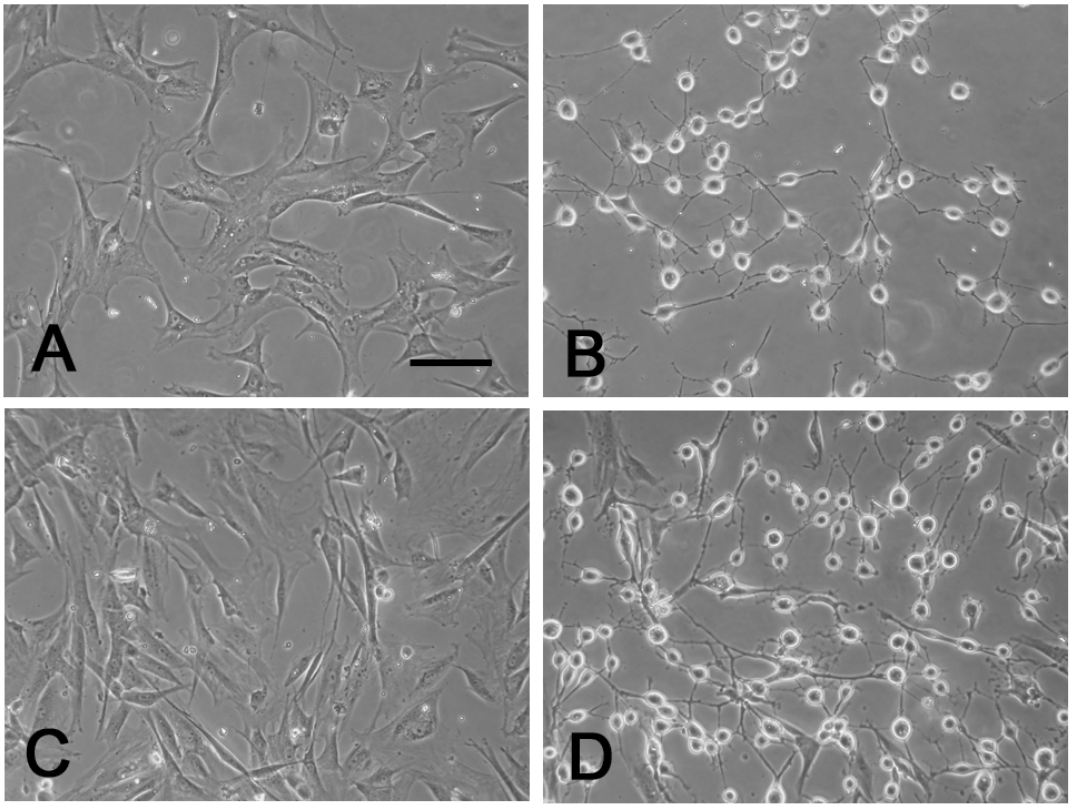

Figure 3-1. Phase-contrast photomicrographs of canine BMSCs and fibroblasts. Neural differentiation induction caused transformation from fibroblastic morphology to neuron-like morphology in both BMSCs (A, before; B, after) and fibroblasts (C, before; D, after). Scale bar = 100 μM.

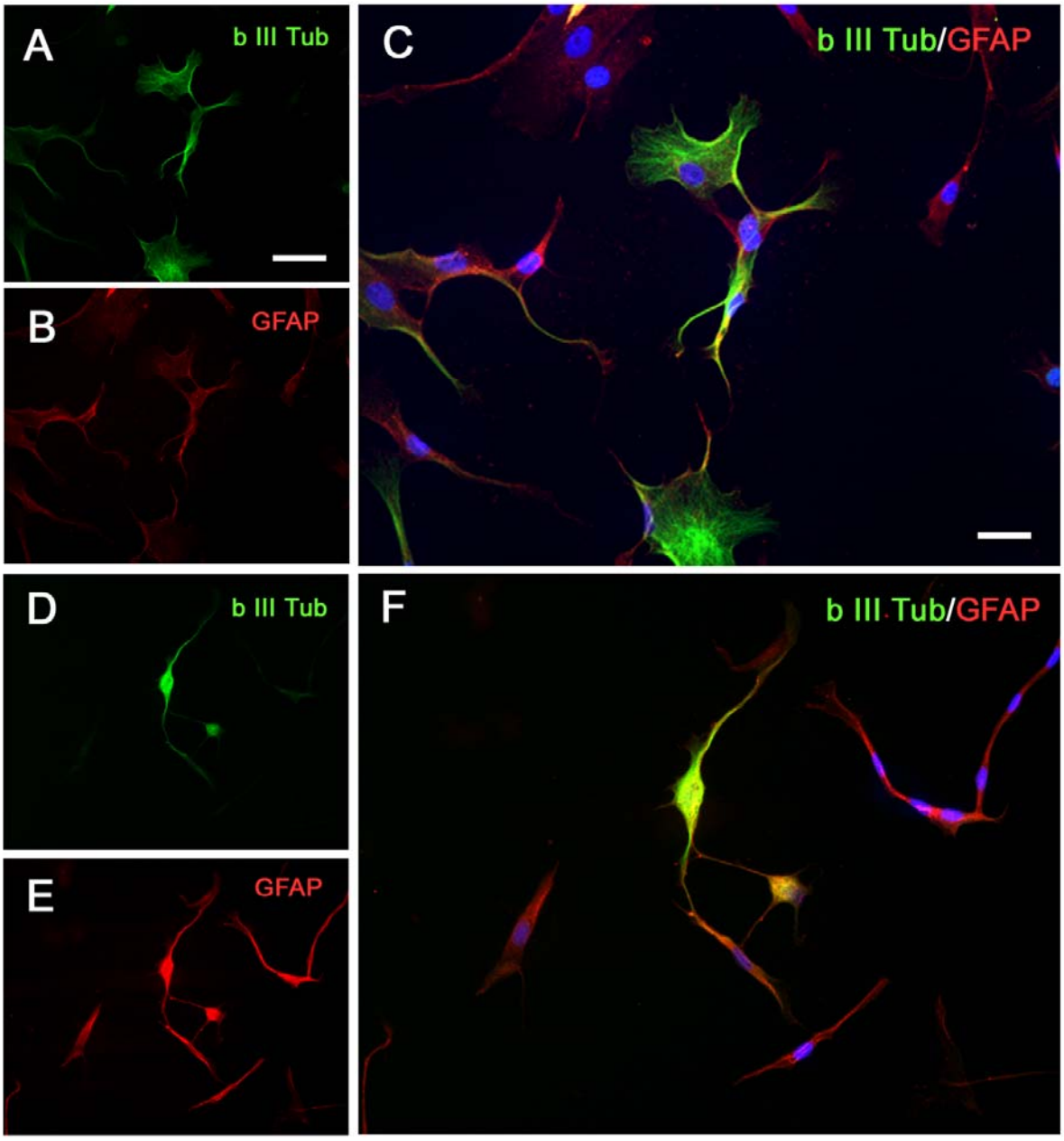

Figure 3-2. Immunofluorescent micrographs of canine BMSCs. Canine BMSCs were positively stained against ß III-tubulin (A) and GFAP (B) before neural differentiation induction. C shows merged images of A and B. Co-expression of ß III-tubulin and GFAP is shown in C as double-stained cells (yellow). The expression levels of ß III-tubulin (D) and GFAP (E) increased after neural differentiation induction, partly due to condensed protein localization. F shows merged images of D and E. Nuclei in C and F were counterstained with DAPI (blue). Scale bar in A = 100 µM, scale bar in C = 50 µM.

Western Blot Analysis of Neural Specific Proteins

We performed western blot analyses to confirm the presence of neuronal and glial protein expressions on canine BMSCs and to evaluate their expression levels before and after the neural differentiation induction. The presences of distinct bands corresponding to ß III-tubulin and GFAP were observed in untreated canine BMSCs from all samples (Fig. 3-3). Densitometric analyses confirmed that the expression level of GFAP in BMSCs consistently increased after the neural differentiation induction (39.0 ± 20.6 % increase relative to ß-actin). On the other hand, the expression level of ß III-tubulin in BMSCs did not significantly differ before and after the neural differentiation induction (9.6 ± 18.5 % increase relative to ß-actin). Bands corresponding to MBP were not detected in BMSCs before and after the neural differentiation induction. Expressions of these proteins were not detectable in fibroblasts.

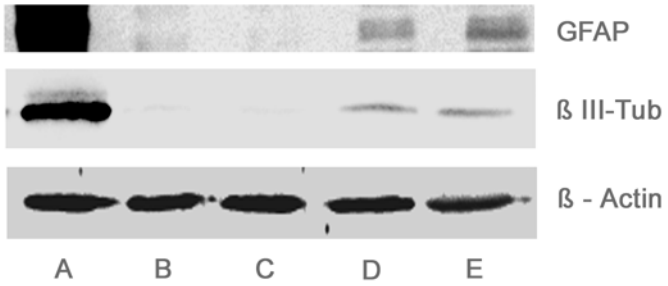

Figure 3-3. Western blotting of neuronal (ß III-tubulin) and glial proteins (GFAP). Loaded samples were obtained from (A) spinal cord lysate (positive control); (B) fibroblasts before neural induction; (C) fibroblasts after neural induction; (D) BMSCs before neural induction; (E) BMSCs after neural induction.

Electrophysiological Recording

In order to assess the electrophysiological properties of neurally induced canine BMSCs, we applied the whole-cell voltage clamp technique. We observed neither spontaneous action potentials nor depolarizing pulse-elicited action potentials from induced canine BMSCs. This suggested that although canine BMSCs constitutively expressed a neuronal marker and neurally

induced canine BMSCs assumed neuronal morphology these cells did not acquire electrophysiological properties characteristic of mature neurons.

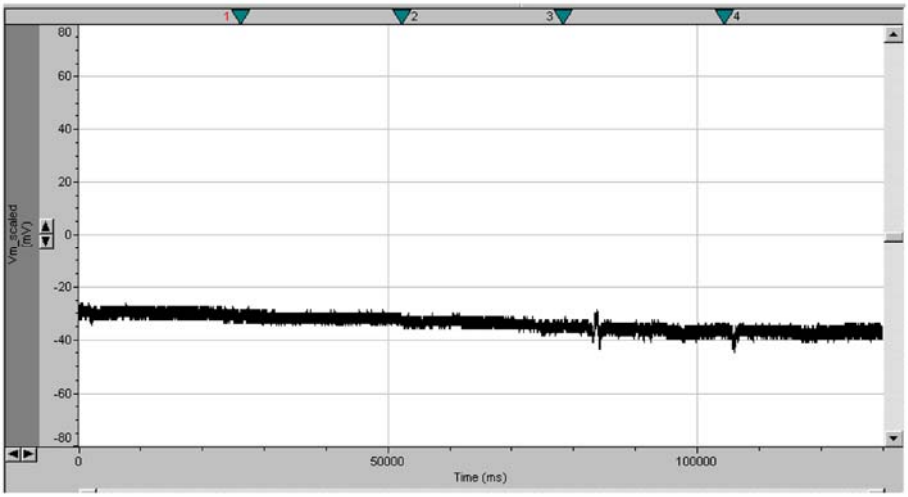

Figure 3-4. Result of whole-cell voltage clamp recording of neurally induced BMSCs. Canine BMSCs treated with dbcAMP and IBMX for 5 hours showed neither spontaneous action potentials nor depolarizing pulse-elicited action potentials.

Conclusion and Discussion

We have demonstrated, under our culture conditions, that canine BMSCs constitutively express neuronal and astrocyte-specific markers. The results of immunocytochemistry and western blotting revealed that untreated canine BMSCs strongly express βIII-tubulin. This observation was consistent with previous reports on human MSCs (Deng et al., 2001; Tondreau et al., 2004). Since βIII-tubulin has been known to be present on early neurons, βIII-tubulin positive canine BMSCs might have the potential to differentiate into neuronal cells under appropriate conditions. We found that GFAP, a marker for mature astrocytes, was also expressed on untreated canine BMSCs. Reports on constitutive expression of GFAP in untreated human BMSCs have been conflicting; for example, Deng et al. (2001) and Woodbury et al. (2000) reported the absence of the expression of GFAP whereas Sanchez-Ramos et al. (2000) and Tondreau et al. (2004) reported the expression of GFAP in untreated human BMSCs. These conflicting results from different laboratories may reflect the lack of a defined set of surface

markers for BMSCs, resulting in inclusion of unidentified subsets of BMSCs with slightly different phenotypic profiles. In our study, although expanded canine BMSCs appeared to be morphologically and phenotypically homogeneous, it was possible that several subsets of BMSCs existed in our culture. This was illustrated by discrete properties of expanded cells in their immunoreactivities against ß III-tubulin and GFAP; GFAP was expressed in nearly all BMSCs whereas ß III-tubulin expression was restricted in approximately 75% of all BMSCs. It was interesting to note that canine BMSCs expressing neuron-specific proteins also expressed astrocyte-specific proteins (approximately 75% of all BMSCs). Therefore, similar to human BMSCs (Reyes et al., 2001; Tondreau et al., 2004), canine MSCs are considered undifferentiated but may also be characterized as "multi-differentiated", which may explain their high plasticity. The absence of MBP expression on canine BMSCs was somewhat predictable as this protein is only expressed in mature oligodendrocytes and discrete molecular signals may be required to induce BMSCs to differentiate towards this lineage. Markers for earlier stages of oligodendrocytes need to be investigated to further characterize the plasticity of canine BMSCs in their neural differentiation capacity. The effects of the neural differentiation induction on cell morphology and neuronal/glial marker expressions were evaluated on canine BMSCs. The induction protocol using dbcAMP and IBMX has been previously described and known to induce a rapid neuron-like morphological change in human BMSCs as a result of elevated intracellular cyclic AMP levels (Deng et al., 2001). In our study, after the neural induction, canine BMSCs exhibited a neuron-like morphology as early as 3 hours after the induction. However, a similar morphological change was also observed in canine fibroblasts at about the same rapidity. These observations suggested that the transformation of canine BMSCs from the fibroblastic morphology to the neuron-like morphology was not a specific event associated with

the multipotentiality of canine BMSCs. Therefore, these dramatic morphological changes should not be overvalued and special care must be taken to interpret results of *in vitro* neural differentiation of BMSCs. As pointed previously by several investigators, a rapid morphological change of BMSCs after neural induction may represent cytotoxic effects of the reagents in the induction medium, leading to cell shrinkage and subsequent adoption of the neuron-like morphology (Bertani et al., 2005; Lu et al., 2004; Neuhuber et al., 2004). Commonly used reagents, solely or in combination, that have effects on BMSCs to induce neuron-like morphology by cytoskeletal shrinkage include butylated hydroxyanisole (BHA), dimethylsulphoxide (DMSO), and ß-mercaptoethanol (BME).

Based on the results of immunocytochemistry and western blot analysis, we found that neural induction caused up-regulation of the expression level of GFAP on canine BMSCs. Therefore, these results may indicate that canine BMSCs can be stimulated to differentiate along mature astrocytes. Similar results have been reported in human BMSCs (Tondreau et al., 2004) and murine BMSCs treated with dbcAMP/IBMX (Deng et al., 2006). Although strong expression of ß III-tubulin was observed on canine BMSCs before and after neural induction, the expression levels were not affected by this treatment. As mentioned, canine BMSCs became less adhesive to the culture flask and there was a significant reduction of the cell number during the neural differentiation induction. As a result, all analyses were performed 5 hours after the induction in the present study. However, it would be interesting to further investigate whether a longer induction period would cause emergence of more mature neuron markers and stable phenotypes.

Electrophysiological recordings of neurally induced canine BMSCs revealed that these cells did not acquire the ability to fire spontaneous or depolarizing pulse-elicited action

potentials. It may be the case that induced BMSCs committed to the neuronal lineage, as shown by expression of the early neuronal marker, but have not fully differentiated into a mature phenotype. Again, a longer induction protocol may be required for full maturation in neural differentiation of canine BMSCs. Alternatively, other techniques may be more useful to evaluate the process of differentiation and maturation of neurally induced BMSCs. For example, expression of voltage-gated ion channels and recordings of inward sodium currents are more sensitive methods to monitor the process of neural maturation. At current, acquisition of electrophysiological properties by BMSCs was only achieved by gene transfection (Dezawa et al., 2004; Kohyama et al., 2001) and treatment with DMSO and BHA on rat BMSCs was reported to be insufficient in inducing differentiation into mature neuron phenotypes with electrophysiological properties (Hofstetter et al., 2002).

With the advancement of our understanding of regulatory mechanisms underlying neural differentiation, it may become possible to generate a specific neural cell type from canine BMSCs. This approach has been most intensively studied in embryonic stem (ES) cells in an attempt to generate functional neural precursor cells (Zhang et al., 2001). This approach holds an advantage in that pre-induction of ES cells into neural progenitor cells may reduce the possibility of developing tumor (i.e. teratomas) in the transplantation site. Further, induction of ES cells into more specific neural cell types has been proposed; for example, generation of dopaminergic neurons from human (Brederlau et al., 2006; Perrier et al., 2004), monkey (Kawasaki et al., 2002; Takagi et al., 2005), and murine (Thinyane et al., 2005) ES cells has been reported for potential treatments for Parkinson's disease. It has also been shown that purified oligodendrocytes can be generated from human and murine ES cells (Glaser et al., 2005; Nistor et al., 2005). These ES cells-derived oligodendrocytes have extensive myelinating capacity *in*

vivo, therefore, hold promise for treatment of demyelinating diseases in humans. Dezawa et al. (2004) showed that rat and human BMSCs can be specifically induced to generate functional neurons, using gene transfection with Notch intracellular domain. *In vitro* differentiation of rat BMSCs into myelinating cells with phenotypic and functional characteristics of Schwann cells has been previously described (Dezawa et al., 2001; Keilhoff et al., 2006). Kamada et el. (2005) demonstrated that transplantation of rat BMSC-derived Schwann cells resulted in enhanced axonal regeneration and functional recovery in rats with completely transected spinal cord. These studies on BMSC differentiation into specific neural cells are particularly promising in that if stable functional phenotypes can be achieved BMSCs may become the strongest candidate for cellular therapies since autologous transplantation is clearly advantageous in a clinical setting. Induction of canine BMSCs into specific functional neural cell types may be possible by understanding the transcription factors involved in neurogenesis. Global gene expression profiling, which has become available through DNA microarray for the canine genome, would aid developing such techniques.

In conclusion, a large-scale expansion of transplants is one of the critical prerequisites for clinical applications of cellular transplantation therapies. Canine BMSCs can be readily isolated from bone marrow of the patient, thus allowing autologous transplantation. These cells can be further expanded in culture while retaining multi-differentiation capacity. Canine BMSCs hold neural differentiation properties; therefore, may have the potential for usages in treating various neurodegenerative diseases and spinal cord injuries in dogs. Further investigations on mechanisms of neurogenic abilities of canine BMSCs are warranted, which may lead to the developments of novel therapeutic strategies to target specific CNS diseases. Applying these novel therapies in canine patients will provide important insights into the safety and clinical

efficacy of the treatment, which are the essential elements in translational research of neurological disorders in human patients.

CHAPTER 4
IN VIVO NEURAL DIFFERENTIATION OF CANINE BONE MARROW STROMAL CELLS

Background and Introduction

Neurotransplantation has generated tremendous attention as a potential therapeutic approach for disorders of the central nervous system (CNS). Cells and tissues derived from fetuses have been used to treat CNS disorders such as Parkinson's disease (Kordower et al., 1995; Spencer et al., 1992) and spinal cord injury (SCI) (Thompson et al., 2001) and led to significant improvement of clinical signs in some patients. Neural stem cells (Ogawa et al., 2002; Pluchino et al., 2003; Teng et al., 2002) and embryonic stem cells (Keirstead et al., 2005; McDonald et al., 1999; Nistor et al., 2005) have also been extensively studied and shown to ameliorate CNS diseases in experimental models. However, these cell/tissue types pose serious obstacles with respect to the harvesting of donor cells/tissues and the possibility of tumorigenesis. Transplantation of bone marrow-derived cells, which has been effectively used to treat diseases of the hematopoietic system, may provide a viable alternative strategy. Bone marrow contains bone marrow stromal cells (BMSCs, also referred to as mesenchymal stem cells), nonhematopoietic cells that provide a bone marrow microenvironment and regulate hematopoiesis (Dormady et al., 2001) and also serve as a stem cell reservoir for mesenchymal cells (Pittenger et al., 1999). Due to their multipotency, BMSCs have been shown to regenerate mesenchymal tissues by differentiating into osteoblasts (Bruder et al., 1997; Kadiyala et al., 1997), chondrocytes (Kadiyala et al., 1997; Noth et al., 2007; Williams et al., 2003), or skeletal muscle cells (Dezawa et al., 2005). BMSCs are easily accessible via bone marrow aspiration, extensively expandable *ex vivo*, and amenable for gene transfection (Lu et al., 2006). BMSCs, therefore, represent a promising candidate cell source for cellular therapy for various diseases including those of the CNS.

Perspectives on BMSCs for neurotransplantation have rapidly grown during the last decade, based on several reports on BMSC transdifferentiation into neural phenotypes both *in vitro* (Sanchez-Ramos et al., 2000; Woodbury et al., 2000) and *in vivo* (Arnhold et al., 2006; Azizi et al., 1998). These studies demonstrated that adult stem cells may be capable of adopting appropriate phenotypic fates in response to environmental cues. Therapeutic benefits of BMSC transplantation has been repeatedly shown in various experimental models of CNS injuries, which include ischemic (Lee et al., 2003; Zhao et al., 2002), traumatic (Wu et al., 2003), and degenerative (Wu et al., 2007) lesions in the brain or the spinal cord. Although functional recovery from CNS injury may not result solely from neural transdifferentiation and cellular replacement by transplanted BMSCs, investigations of neural transdifferentiation properties of BMSCs provide insights into their potentials in the treatment of CNS diseases and aid in refining the design of new clinical trials.

Initial clinical trials in humans with SCI using autologous bone marrow cells have already been reported (Park et al., 2005; Yoon et al., 2007), and the potential therapeutic value of bone marrow cells is certainly not limited to human applications. For example, dogs can sustain traumatic SCI at relatively high incidence. A need thus exists for developing novel treatments and cell-based therapies for veterinary practice. Based on existing evidence, BMSCs are a reasonable candidate cell platform. Veterinary clinical applications also afford an important animal-to-human translation opportunity, especially the pathology of clinical SCI in dogs appears very similar to that documented in humans, as well as in cats and numerous examples of experimental SCI (Jeffery et al., 2006; Smith and Jeffery, 2006).

The objective of the present study was to determine by immunohistochemistry whether canine BMSCs can migrate and adopt neural phenotypes in the developing mouse brain. We

also evaluated by species-specific chromosome painting technique whether cell fusion events contributed to transdifferentiation properties of canine BMSCs.

Materials and Methods

Preparation of Canine BMSCs and Fibroblasts

Bone marrow from four adult canine cadavers, obtained from a local animal shelter, was used to isolate and culture canine BMSCs. Exact ages of these dogs were unknown; however, we collected from only those which had a complete set of adult dentures with minimum dental calculus deposition, thus meeting inclusion criteria for "young adults". Bone marrow was also collected from two young dogs (estimated age between 3 to 5 months old). The use of these animals was approved by the Institutional Animal Care and Use Committee of University of Florida. In all animals, bone marrow was collected from a femur by flushing the medullary canal immediately after euthanasia. Mononucleated cells were isolated on a Ficoll density gradient, washed in PBS, and suspended in culture medium consisting of DMEM (1g/L glucose) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B). Primary adherent cells were grown until semiconfluency after which cells were trypsinized and labeled with the fluorescent carbocyanine dye, DiI (Invitrogen). Third passage canine fibroblasts (Coriell Institute for Medical Research) were used for comparative purposes. Fluorescent labeling was performed according to a protocol of Laywell et al. (1996) with modifications. Briefly, trypsinized BMSCs or fibroblasts were washed three times in PBS and resuspended in PBS containing DiI (final concentration, 40μg/mL). The cells were incubated in the DiI-containing PBS for 5 minutes at 37°C followed by 15 minutes at 4°C before being washed three times in PBS. DiI labeled BMSCs and fibroblasts were frozen at -80°C until transplantation was performed.

Transplantation of Canine BMSCs and Fibroblasts into Neonatal Mouse Brain

Postnatal day-2 immunocompromised mice (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ, The Jackson Laboratory) were used as recipients (n=29). On the day of transplantation, DiI-labeled BMSCs or fibroblasts were thawed, washed three times in PBS, and the cell number was determined by use of a hematocytometer. Cell suspensions were made in PBS ($2.0 \times 10^5/\mu L$) and transferred on ice to the mouse facility. DiI-labeled BMSCs or fibroblasts were transplanted into the left lateral ventricle of postnatal day-2 immunocompromised mice as described previously (Deng et al., 2006). Under hypothermic anesthesia, 2.0×10^5 cells in 1μL of PBS were slowly injected into the left lateral ventricle, through a 30G needle attached to a 5μL Hamilton syringe. Ten day post-transplantation, mice were euthanized with CO_2 gas and transcardially perfused with 4% paraformaldehyde in PBS. The brains were excised and postfixed in 0.4% paraformaldehyde containing 30% sucrose for 2-3 days. Brains were sectioned with a freezing microtome into 40μM sagittal or coronal slices for immunohistochemistry or 20 μM sagittal slices for fluorescence *in situ* hybridization.

Immunohistochemistry to Evaluate Transdifferentiation of Canine BMSCs

Immunohistochemical staining of free-floating brain slices were performed, using neuron-specific βIII-tubulin (1:500; Promega, Madison, WI) and AlexaFluor 488-conjugated NeuN (1:50; Chemicon), and astrocyte-specific GFAP (1:100; BD Biosciences, Franklin Lakes, NJ) antibodies. Brain slices were washed three times in PBS, and permeabilized in 0.4% Triton X-100 in PBS for 30 minutes at 25ºC. Non-specific binding was blocked with a blocking solution (3.0% normal goat serum in PBS) for 60 minutes at 25ºC. Brain slices were then incubated overnight at 4ºC with the primary antibodies. The primary antibodies were removed, slices were washed three times in PBS, and incubated for 60 minutes in dark at 25ºC with secondary antibodies. The secondary antibodies were Cy2 conjugated goat anti-mouse IgG1 (1:400;

Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) or IgG2b (1:200; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Stained slices were observed under a fluorescent microscope (Zeiss Axioplan II, Carl Zeiss Microimaging Inc.) to evaluate engraftment and phenotypes of transplanted canine BMSCs.

Chromosome Painting to Evaluate Cell Fusion

Twenty micron sagittal sections were used for assaying possible fusion events associated with DiI-labeled donor BMSCs in the neonatal mouse brain. Brain sections were first viewed under a fluorescence microscope to identify sections with DiI-positive donor cells. Identified sections were then treated with 0.2N HCl for 30 minutes, and retrieved in 1M sodium thiocyanate (NaSCN) for 30 minutes at 85°C. The sections were digested with 4mg/mL pepsin (Sigma; diluted in 0.9% NaCl pH2.0) for 60 minutes at 37°C. After equilibrating in 2x SSC for 1 minute, the sections were dehydrated through graded alcohols. The tissue was then denatured with FITC-conjugated canine X-chromosome probes (Cambio, UK) and biotin-conjugated mouse X- chromosome probes (Cambio, UK) for 10 minutes at 60°C and hybridized at 37°C overnight. After hybridization, slides were washed first in 1:1 formamide:2x SSC, then in 2xSSC. Dual color detection was performed using a commercially available kit (Cambio, UK) to visualize mouse X-chromosomes with Cy-5 and enhance FITC signals of canine X-chromosomes. Some slides were reacted only with mouse X- chromosome probes and visualization was performed with streptavidin-conjugated Alexa Fluor® 555 (Invitrogen). Slides were then coverslipped in mounting medium containing DAPI (Vector, Burlingame, CA) and evaluated under a fluorescence microscope.

Results

Distribution and Phenotypic Fates of Adult Canine BMSCs and Fibroblasts

Of 29 recipient mice, 27 survived the 10-day survival period and were processed for analysis. Transplanted cells could be readily identified by the presence of DiI in the cytoplasm. In mice that received adult canine BMSCs (n=17), most of the engrafted cells remained around the injection site, adhering to the wall of the lateral ventricle (Fig. 4-1). Some cells were found in the underlying parenchyma of the thalamus and hippocampus; however, it was not possible to determine whether these cells had migrated into the parenchyma from the injection site or were directly injected into these locations. There were also cells sparsely dispersed in the cerebral cortex distant from the injection site, which seemed to have migrated there instead of being directly injected. Additionally, a small number of cells were widely distributed around the periphery of the brain, appearing to have attached to the pia matter but not penetrated into the tissue.

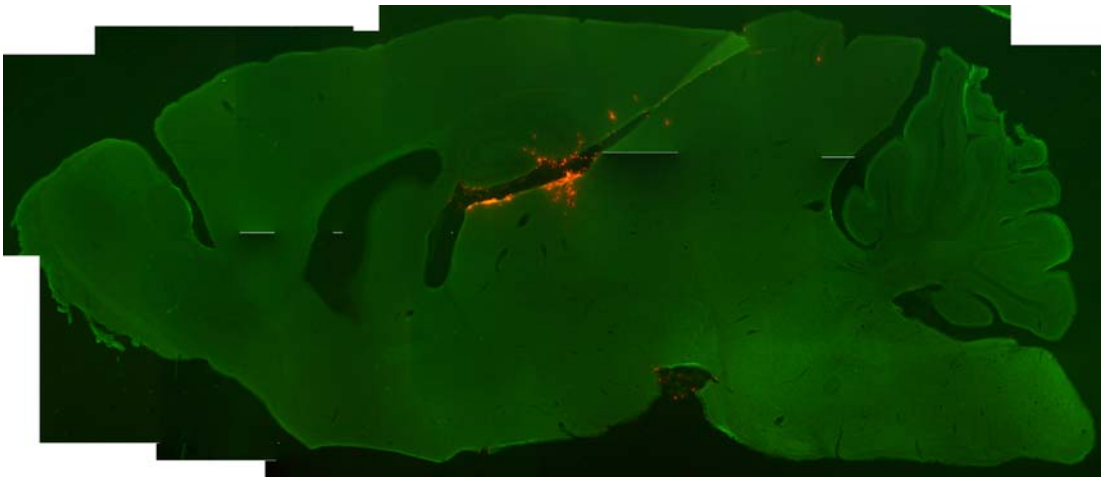

Figure 4-1. Montage immunofluorescence photomicrograph of mouse brain with engrafted adult canine BMSCs. This sagittal section was stained with neuron-specific βIII-tubulin and visualized with Cy2 (green). Most of DiI-positive BMSCs (red) were located around the injection site along the wall of the lateral ventricle while some were found around the periphery of the brain and sparsely in the cortical parenchyma.

The majority of the engrafted adult BMSCs exhibited a spindle-shaped appearance similar to their *in vitro* characteristic morphology. A small population of cells that migrated into the parenchyma underlying the ventricle showed process-bearing morphology. However, immunohistochemistry showed that engrafted cells expressed neither neuron-specific (βIII-tubulin and NeuN) nor astrocyte-specific (GFAP) markers. Engrafted canine fibroblasts (n=3) showed a similar tendency of distribution and phenotypic patterns to adult BMSCs with the exception that fibroblasts remained spindle-shaped morphology.

Distribution and Phenotypic Fates of Young Canine BMSCs

In contrast to adult BMSCs and fibroblasts, BMSCs isolated from young donors (n=7) demonstrated a different behavior. Although most BMSCs of young donors remained around the injection site in the lateral ventricle similar to adult BMSCs, a small number of cells were located in the olfactory bulb (Fig 4-2). These cells in the olfactory bulb were βIII-tubulin positive and thought to have migrated from the lateral ventricle through the rostral migratory stream (RMS), a known pathway of neuronal precursors migrating from the subventricular zone (SVZ) to the olfactory bulb. There were also DiI-positive donor cells in the RMS. In the SVZ, most of engrafted BMSCs exhibited typical fibroblastic morphology (Fig 4-3A), but some cells assumed astrocyte-like (Fig 4-3B) or bipolar neuron-like (Fig 4-3C) morphology. Immunostaining revealed that a significant number of BMSCs in the SVZ were βIII-tubulin positive (Fig 4-4). Expression of neither NueN nor GFAP was found in any region containing BMSCs (Fig 4-5).

Fluorescence *In Situ* Hybridization for Chromosome painting

We first attempted to visualize canine X-chromosomes and mouse X- chromosomes using different fluorescence-conjugated antibodies to evaluate possible fusion events between transplanted canine BMSCs and host neural cells. However, due to different required

hybridization conditions for chromosome probes for each species this was not possible. Consequently, we stained sections only with mouse X- chromosome probes and looked for co-localization of DiI and mouse X- chromosomes. Regardless of the transplanted cell types (adult BMSCs, young BMSCs, or fibroblasts) we did not find any cells containing both DiI and mouse X- chromosomes, indicating that cell fusion did not occur in any instances (Fig 4-6).

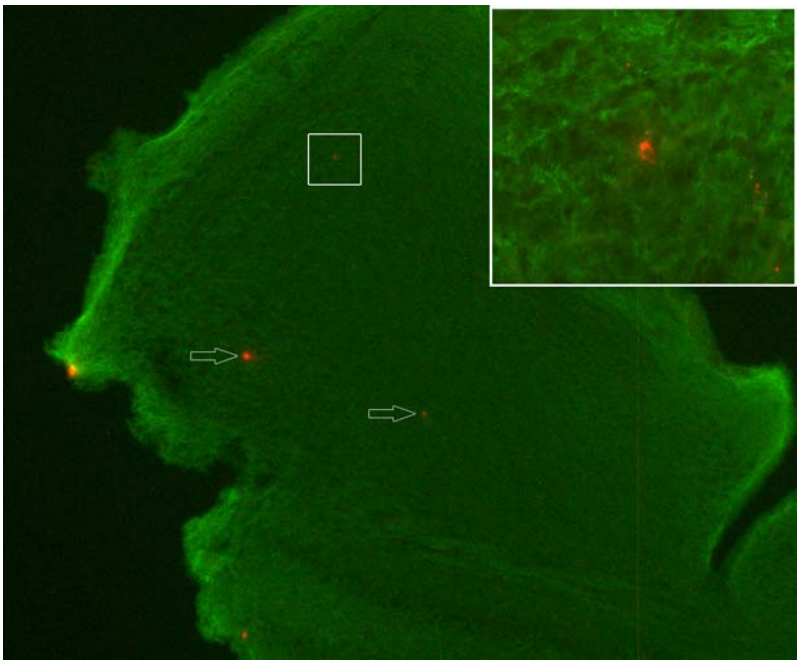

Figure 4-2. DiI-positive BMSCs isolated from young donors present in the olfactory bulb. A small number of DiI positive BMSCs were found in the olfactory bulb (arrows). Inset shows a higher magnification view of a BMSC in the boxed area.

Conclusion and Discussion

Our data suggest that canine BMSCs isolated from young donors but not from adult donors may have the capacity to undergo neural transdifferentiation *in vivo* in response to environmental cues provided by the developing mouse brain. Young canine BMSCs injected in the lateral ventricle penetrated into the SVZ and were integrated in the postnatal neurogenic pathway of the RMS/olfactory bulb system. Our observation that a small number of young canine BMSCs migrated to the olfactory bulb and expressed neuron-specific marker βIII-tubulin suggests that these cells may possess neural transdifferentiation capability. This finding also suggests that a

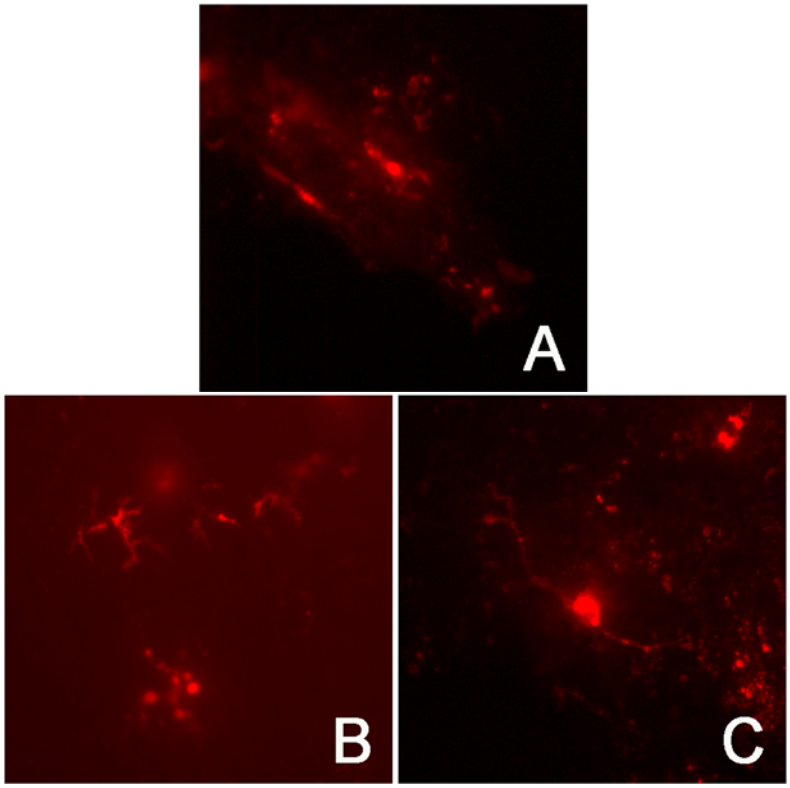

Figure 4-3. Various morphologies of BMSCs in the subventricular zone. Most of engrafted BMSCs exhibited typical fibroblastic morphology (A). Some cells assumed astrocyte-like (B) or bipolar neuron-like morphology (C) in the SVZ.

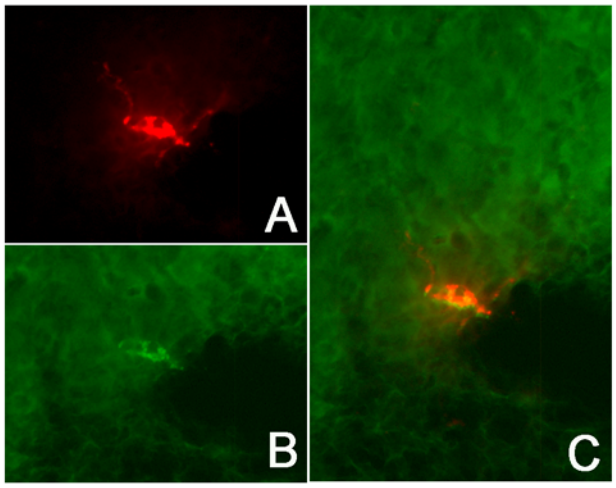

Figure 4-4. BMSC from a young donor located in the subventricular zone. DiI positive BMSC seen in the SVZ (A) is immunopositive against βIII-tubulin (B). C shows a merged image of A and B.

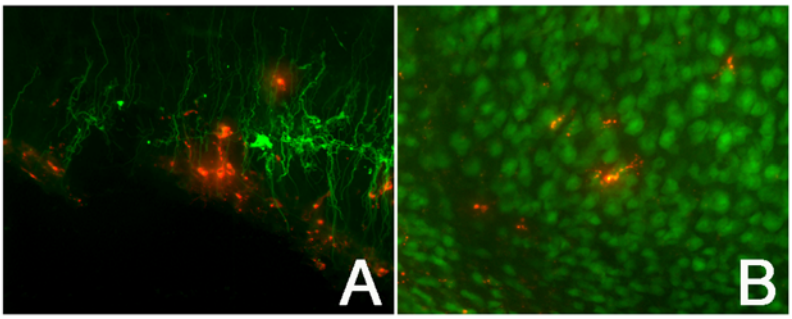

Figure 4-5. Immunostaining of BMSCs in the subventricular zone for GFAP and NeuN expression. Engrafted BMSCs isolated from young donors (red) did not express GFAP (green in A) in the SVZ or NeuN (green in B) in the cerebral cortex.

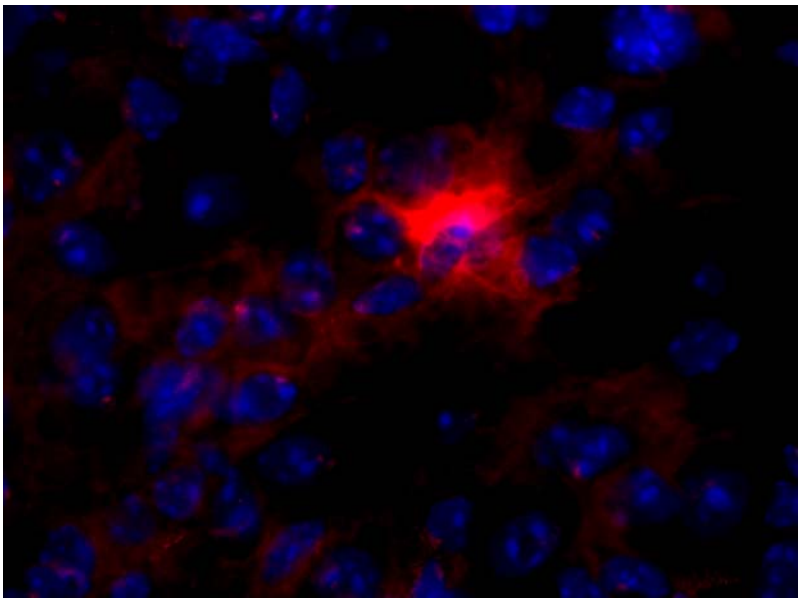

Figure 4-6. Fluorescence *in situ* hybridization for chromosome painting. A cell containing DiI (red) in its cytoplasm is a transplanted BMSC from a young donor. This DiI positive cell does not contain a mouse X-chromosome. Other cells in this view are neural cells of this male recipient mouse as they all contain a single mouse X-chromosome (pink dots in the nucleus). Some X-chromosomes cannot be seen because of the selected focal plane. Nuclei are stained with DAPI (blue).

population of young canine BMSCs may behave like neural precursor cells that contribute to postnatal neurogenesis. In contrast to young canine BMSCs, most adult canine BMSCs remained in the injection site and did not migrate to the olfactory bulb. Some adult BMCSs were found in the SVZ and distant sites and assumed process-bearing morphology; however, these cells expressed neither neuron- nor astrocyte-specific markers. Canine fibroblasts used as a control showed no migration capacity and remained as spindle-shaped cells.

Our observation of the migration and differentiation properties of young canine BMSCs is similar to those of previous studies in which murine BMSCs were engrafted into the lateral ventricle of the neonatal mouse brain (Deng et al., 2006; Kopen et al., 1999). However, the extent of migration and adopted phenotype was much more restricted in our study compared to these previous studies. For example, Kopen et al. (1999) reported that murine BMSCs injected into the lateral ventricle of neonatal mouse brains migrated extensively throughout the forebrain and cerebellum and differentiated into both neurons and astrocytes. They found that engrafted BMSCs preferentially populated neuron rich regions including the Islands of Calleja, the olfactory bulb, and the internal granular layer of the cerebellum, events similar to those occur in the ongoing developmental processes in early postnatal life. Based on these observations, they suggested that BMSCs mimic the behavior of neural progenitor cells. Deng et al. (2006) more recently showed that, upon intraventricular injection, murine BMSCs integrated into the postnatal neurogenic pathway of the RMS/olfactory bulb system by migrating appropriately and differentiating into olfactory granule cells, supporting the contention that the bone marrow derived adult stem cell indeed possesses neural transdifferentiation capability under the influence of environment cues from the brain.

We found that although most of BMSCs remained in the injection site, a small number of young canine BMSCs penetrated into the SVZ and assumed various morphologies resembling neurons or astrocytes. Immunostaining suggested that some of these cells in the SVZ express βIII-tubulin. We believe that migration of young canine BMSCs into the SVZ and differentiation toward the neuronal phenotype occurred in a specific way in response to microenvironmental cues provided by the SVZ. The SVZ forms adjacent to the lateral ventricle during embryogenesis and is known to contain multipotent neural stem cells (Doetsch et al.,

1997; Lois and Alvarez-Buylla, 1993; Weiss et al., 1996). The SVZ is also the area with active neurogenesis in the postnatal and adult brain (Altman, 1969; Altman and Das, 1966; Luskin, 1993). Our observation that migrating BMSCs were found in the RMS and also localized in the olfactory bulb further suggested that young canine BMSCs were integrated into the RMS/olfactory bulb neurogenic system. The olfactory bulb is one destination of neural progenitors generated in the SVZ (Altman, 1969; Lois and Alvarez-Buylla, 1994).

Little is currently known about what drives implanted BMSCs to differentiate into neural cells *in vivo*. Postnatal brain development occurs and this process is governed in part by various growth factors of which the expression levels are regulated precisely in a controlled spatiotemporal manner. For example, levels of fibroblast growth factor 2 (FGF-2 or bFGF) expression increase dramatically during late embryonic and early postnatal stages of development in rodent brain (Caday et al., 1990; Ford-Perriss et al., 2001). FGF-2 is known to promote proliferation and self-renewal of neural stem cells derived from the neuroepithelium of the developing cortex (Gritti et al., 1996) and the adult SVZ (Bartlett et al., 1995). FGF-2 is also a potent mitogenic factor for BMSCs and it is known that FGF-2 promotes self-renewal of BMSCs *in vitro* (Locklin et al., 1995; Oliver et al., 1990; Tsutsumi et al., 2001). Therefore, high levels of FGF-2 present in the developing brain might have stimulated proliferation and more importantly the self-renewal capacity of canine BMSCs. It is also likely that neurotrophic factors played a role in instructing BMSCs to differentiate into the neuronal phenotype. Neurotrophic factors for which BMSCs express receptors, such as NGF (Caneva et al., 1995) and BDNF (Li et al., 2007), might have induced neural differentiation of BMSCs when coupled with other cues present in the SVZ.

What contributed to the observed difference between adult donor BMSCs and young donor BMSCs in their behavior in the mouse brain? It may simply be that bone marrow from young dogs contained a larger number of stem cells, which survived transplantation, than adult bone marrow. Alternatively, it could be that transdifferentiated BMSCs were derived from a more primitive stem cell population present in the BMSC fraction of the young donors. The presence of a rare pluripotent stem cell population has been reported in adult mouse (Jiang et al., 2002a) and human (Reyes et al., 2001) bone marrow and shown to have transdifferentiation capacity. These cells can only be expanded at extremely low cell density on fibronectin and in the presence of LIF and PDGF. Existence of a similar cell population in adult bone marrow has not been reported for other species including canines. As we utilized the standard culture condition (without growth factors at standard cell density) to prepare all BMSCs for transplantation, cells with multipotent differentiation properties may not have been expanded from the adult bone marrow sample. Identification of cell populations with multipotential differentiation properties resident in canine bone marrow needs to be investigated.

In conclusion, BMSCs isolated from young donors exhibited neuronal phenotype upon transplantation into the developing mouse brain. Transplanted young canine BMSCs responded to instructive cues provided by the SVZ and migrated to the olfactory bulb via the RMS. These migratory and differentiation properties of young canine BMSCs resemble behavior of indigenous neural progenitors. Adult canine BMSCs failed to demonstrate a similar degree of migration and differentiation neither did canine fibroblasts used as a control. The results suggest that a primitive stem cell population with neural transdifferentiation capacity may exist in the BMSC compartment isolated from young dogs. Identification of the responsible cell population

in the young canine BMSCs and the development of selective culture techniques may aid further understanding of the identity of neurogenic stem cell populations in the canine bone marrow.

CHAPTER 5
EFFECTS OF CANINE BONE MARROW STROMAL CELLS ON NEURITE EXTENSION FROM DORSAL ROOT GANGLION NEURONS *IN VITRO*

Background and Introduction

Bone marrow stromal cells (BMSCs), also referred to as mesenchymal stem cells, have been known to play a key role in regulating hematopoiesis through interactions with hematopoietic stem cells within the bone marrow microenvironment (Dormady et al., 2001). More recent investigations have provided evidence indicating that BMSCs may also serve as a stem cell reservoir for mesodermal cells and thus participate in regeneration of mesodermal tissues (Pittenger et al., 1999). In addition, several lines of evidence suggest that bone marrow derived cells from rodents and humans may be capable of transdifferentiation and thereby have the capacity to generate cells *in vitro* (Deng et al., 2001; Sanchez-Ramos et al., 2000; Woodbury et al., 2000) as well as *in vivo* (Azizi et al., 1998; Cogle et al., 2004; Deng et al., 2006; Lee et al., 2003; Mezey et al., 2003) expressing various neuronal markers.

After transplantation of BMSCs, functional recovery has been reported in animal models involving ischemic (Chen et al., 2001; Lee et al., 2003; Li et al., 2002; Zhao et al., 2002) and traumatic lesions (Chopp et al., 2000; Hofstetter et al., 2002; Lee et al., 2003; Ohta et al., 2004) of the CNS. Although studies have shown the presence of transplanted BMSCs in host CNS tissues (Arnhold et al., 2006; Azizi et al., 1998; Kopen et al., 1999), which in some cases appear to have distinct neuronal features (Cogle et al., 2004), there is currently no definitive evidence that BMSCs significantly contribute directly to neuronal replacement after CNS injuries (Ohta et al., 2004). Alternatively, BMSCs have been shown to have other tissue repair properties such as promoting remyelination (Akiyama et al., 2002b) and production of various neurotrophic and angiogenic growth factors, including nerve growth factor (NGF) (Auffray et al., 1996; Chen et al., 2005; Chen et al., 2002a; Chen et al., 2002b; Crigler et al., 2006; Garcia et al., 2004; Li et al.,

2002), glial cell-derived neurotrophic factor (GDNF) (Chen et al., 2005), brain-derived growth factor (BDNF) (Chen et al., 2002a; Chen et al., 2002b; Crigler et al., 2006; Li et al., 2002), vascular endothelial growth factor (VEGF) (Chen et al., 2002a; Chen et al., 2002b), hepatocyte growth factor (HGF) (Chen et al., 2002a; Chen et al., 2002b), and brain natriuretic peptide (BNP) (Song et al., 2004). Neurotrophic factors such as NGF and BDNF support survival of damaged neurons (DeKosky et al., 1994; Goss et al., 1998; Hammond et al., 1999; Lu et al., 2001), and it has been suggested that production of these factors is increased in ischemic brain lesion models after BMSC transplantation which in turn can lead to reduced apoptosis in the penumbra, proliferation of endogenous cells in the subventricular zone, and functional recovery (Li et al., 2002). Vasoactive peptides such as VEGF (Chen et al., 2002a; Chen et al., 2002b) and BNP (Song et al., 2004) may also participate in the reparative processes after CNS injury by inducing angiogenesis and reducing secondary edema after injury.

BMSCs also appear to stimulate neuritic outgrowth which may likewise be attributed to their ability to secrete neurotrophic factors (Mocchetti and Wrathall, 1995), as well as extracellular matrix (ECM) and adhesion molecules such as fibronectin and laminin (Grayson et al., 2004; Hofstetter et al., 2002), which are known to promote attachment and extension of axons. Through production of these molecules BMSCs are thought to provide permissive microenvironment for regenerating axons (Hofstetter et al., 2002). The importance of this ECM milieu in which axonal regrowth takes place is well recognized after early studies of peripheral nerve grafts into CNS lesions (David and Aguayo, 1981; Richardson et al., 1980).

Initial clinical trials in humans using autologous BMSCs have already been reported (Park et al., 2005; Yoon et al., 2007), and the potential therapeutic value of BMSCs is certainly not limited to human applications. For example, dogs can sustain traumatic spinal cord injuries

at relatively high incidence. A need thus exists for developing novel treatments and cell-based therapies for veterinary practice. Based on existing evidence, BMSCs are a reasonable candidate cell platform. Veterinary clinical applications also afford an important animal-to-human translation opportunity, especially the pathology of clinical spinal cord injury (SCI) in dogs appears very similar to that documented in humans, as well as in cats and numerous examples of experimental SCI (Smith and Jeffery, 2006).

Previous studies (Arinzeh et al., 2003; Bruder et al., 1998; Kadiyala et al., 1997; Volk et al., 2005) of canine BMSCs have focused on their osteogenic properties because of the wide clinical applicability. The osteogenic property of canine BMSCs has also been exploited in the field of tissue engineering, particularly in an attempt to promote periodontal tissue regeneration (Hasegawa et al., 2006). Cardiomyogenic property of canine BMSCs has been studied and shown to result in improvement of cardiac function in a chronic myocardial infarction model after transplantation of autologous BMSCs (Bartunek et al., 2007). These studies have demonstrated "proof of concept" for using BMSCs with clinical benefits. However, our understanding of the basic biology of canine BMSCs is rather scarce and their potential use for treatment of neurological diseases has not been studied.

In the present study, we investigated the effects of canine BMSCs on neuritogenesis from dorsal root ganglion neurons (DRG) *in vitro*. Our results suggest that canine BMSCs promote neurite outgrowth from DRG neurons with development of complex and highly branched neuritic arborizations. These effects were pronounced in co-cultures of BMSCs and DRG neurons, indicating that production of non-diffusible molecules might be the prime attribute of BMSCs in promoting neuritogenesis.

Materials and Methods

Preparation of Canine BMSCs and Fibroblasts

Bone marrow aspirates from three canine cadavers, obtained from a local animal shelter, were used to isolate and culture canine BMSCs. Exact ages of these dogs were unknown; however, we collected from only those which had a complete set of adult dentures with minimum dental calculus deposition, thus meeting inclusion criteria for "young adults". The use of these animals was approved by the Institutional Animal Care and Use Committee of University of Florida. Detailed protocols for bone marrow collection, cell isolation, and culture expansion have been described previously (Kamishina et al., 2006). Briefly, bone marrow aspirates were obtained from the iliac crest immediately after euthanasia. Mononucleated cells were isolated on a Ficoll density gradient, washed in PBS, and suspended in culture medium consisting of DMEM (1g/L glucose) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B). Homogeneous BMSC cultures were established in the third passage cultures. Third passage canine fibroblasts were used for comparative purposes. BMSCs or fibroblasts were plated at a cell density of $8 \times 10^3/cm^2$ on 18mm glass coverslips placed in 12-well culture plates for direct co-culture experiments or in 6-well plates to yield conditioned media.

Immunocytochemical Analysis for Expression of Extracellular and Adhesion Molecules

We evaluated whether canine BMSCs express some of the putative ECM and adhesion molecules that are known to favor neurite outgrowth. Third passage canine BMSCs and fibroblasts were grown on glass coverslips until confluency and immunostained as follows. Cells were fixed in 4% paraformaldehyde for 30 minutes at 25 ºC, washed three times in PBS, and permeabilized in 1.0% Triton X-100 in PBS for 10 minutes at 25 ºC. Non-specific binding was blocked with a blocking solution (1.0% BSA in PBS) for 30 minutes at 25 ºC. Cells were

then incubated overnight at 4 ºC with primary antibodies directed against following proteins; laminin (C4 and D18, both from the Developmental Studies Hybridoma Bank at the University of Iowa [DSHB]), E-cadherin (DSHB), N-cadherin (DSHB), neural cell adhesion molecule (NCAM; DSHB), and fibronectin (Sigma). The primary antibodies were removed, cells washed with PBS, and cells incubated for 1 hour in dark at 25 ºC with secondary antibodies. The secondary antibodies were Cy2 conjugated goat anti-mouse IgG1 (1:400) or Rhodamine-Red X conjugated goat anti-mouse IgG2a (1:400). Cells were also incubated without primary antibodies to control for non-specific staining by secondary antibodies. Cells were washed with PBS and mounted with a mounting medium. Stained cells were observed under a fluorescent microscope (Olympus BX50, Olympus Corporation, Tokyo Japan) with appropriate filters.

Direct Co-culture of BMSCs and Dorsal Root Ganglion Neurons

Rat fetal DRG neurons (Cambrex Corporation) were plated on 1) monolayer of BMSCs, 2) monolayer of fibroblasts, or 3) laminin-coated (2 μg/ml, Sigma) coverslips at a cell density of $3.8 \times 10^3/cm^2$ in the same medium as above. After 48 hours, cells were fixed and processed for immunocytochemistry as described above. The primary and secondary antibodies used were anti-neurofilament 200 (1:50, Sigma) and Alexa Fluor® 350 (1:200, Invitrogen), respectively. Cells were also incubated without primary antibodies to control for non-specific staining by secondary antibodies. Stained cells were observed under a fluorescent microscope equipped with a digital camera (Retiga 1300, QImaging, Surrey, BC Canada) and pictures of DRG neurons were taken from non-overlapping fields using a ×10 objective lens. These experiments were performed in triplicate.

Culture of DRG Neurons in Conditioned Medium

Third passage BMSCs or fibroblasts were cultured in 6-well culture plates for 72 hours after which conditioned media were collected. In this experiment, 6-well plates were coated with

laminin (2 μg/mL) and poly-D-lysine (30 μg/mL) and DRG neurons were plated in each well at a cell density of $1.0 \times 10^3/cm^2$. DRG neurons were also plated in culture media that were incubated for 72 hours at 37 ºC without conditioning cells. DRG neurons were cultured in conditioned media for 48 hours after which cells were washed with PBS and fixed as described. DRG neurons were stained with SimplyBlue SafeStain (Invitrogen) for 8 hours at 25 ºC, washed three times in PBS, and examined under a phase contrast microscope as previously described (Price et al., 2006). Digital pictures of DRG neurons were acquired from non-overlapping fields using a ×10 objective lens. These experiments were performed in duplicate.

Measurements of Neurite Outgrowth

Digital images were transferred into image analysis software (NIH ImageJ ver 1.37v) for neurite morphometric analyses. All neuronal processes were considered neurites as axons and dendrites were not distinguishable from one another. Primary neurites were defined as processes directly emerging from the cell body which usually have a thicker diameter than branching neurites. All primary and branching neurites were manually traced on the digital images. The following parameters were measured as previously described (Chakrabortty et al., 2000); 1) total neurite length/neuron, 2) total primary neurite length/neuron, 3) mean length of primary neurite/neuron, 4) mean number of primary neurites/neuron, and 5) mean number of branching neurites/neuron. Data are presented as mean ± standard deviation. Statistical differences among groups were tested by One-way ANOVA followed by Tukey's HSD with p-value set at .05 (SPSS).

Results

Expression of Extracellular Matrix Molecules

After 72 hours of plating, third passage canine BMSCs formed a uniform monolayer consisting of homogenous polygonal cells. Immunocytochemistry demonstrated that canine

BMSCs produced and deposited copious amounts of fibronectin (Fig. 5-1A) and laminin (Fig. 5-1B). Expressions of E-cadherin, N-cadherin, and NCAM were not detected in our BMSC culture. Canine fibroblasts were stained against fibronectin but no expression was observed for laminin, E-cadherin, N-cadherin, and NCAM.

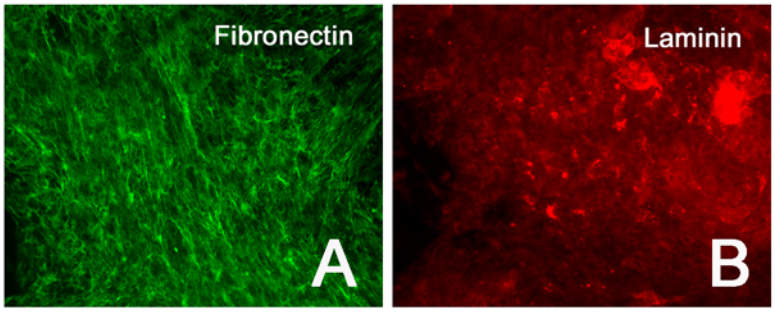

Figure 5-1. Immunofluorescent photomicrographs of canine BMSCs. Strong expression of fibronectin (A) and laminin (B) on BMSCs was observed.

Direct co-culture of DRG on BMSC Monolayer

DRG neurons were plated on BMSCs, fibroblasts, or laminin-coated coverslips and allowed to extend their axons for 48 hours. It was clearly observed that DRG neurons extended longer axons on fibroblasts and BMSCs than on laminin (Fig 2). On laminin, the conformation of DRG neurons was simple, typically having only 1 or 2 unbranched primary neurites (Fig. 5-2A). In contrast, on a fibroblast monolayer, DRG neurons extended more and longer primary neurites with a few branching neurite processes emerging from the primary neurites (Fig. 5-2B). On a BMSC monolayer, DRG neurons showed a more elaborate appearance (Fig. 5-2C). Typically, DRGs on BMSCs extended three or more primary neurites from which numerous branching neurites developed.

These features of neurite outgrowth on different substrates were reflected quantitatively in neurite measurements. The total neurite length including all primary and branching neurites per single DRG neuron was significantly longer on BMSCs (1596.5 ± 388.1μM) than those on fibroblasts (994.9 ± 225.7μM) and laminin (221.9 ± 25.0μM) (Fig. 5-3A). Similarly, the total

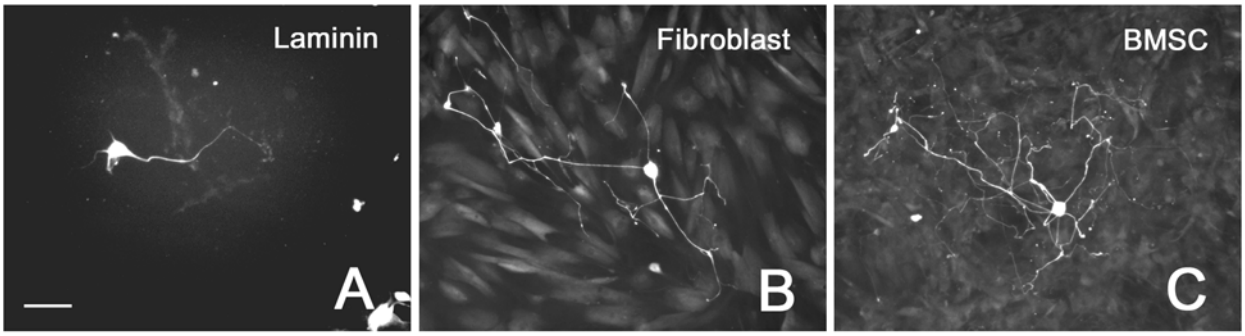

Figure 5-2. Representative photomicrographs of DRG neurons cultured on three different substrates for 48 hours. DRG neurons and neurites were indentified by immunostaining using neurofilament-200 antibody. (A) DRG neurons on laminin substrates extended short primary neurites with few branching neurites. (B) Fibroblasts stimulated extension of primary neurites with a few branching neurites. (C) BMSCs further stimulated growth of primary neurites and formation of complex arborization of branching neurites. Scale bar = 50 μM.

primary neurite length per DRG neuron on BMSCs (797.1 ± 161.8μM) was significantly longer than those on fibroblasts (560.9 ± 184.8μM) and laminin (212.4 ± 23.9μM) (Fig. 5-3B). The mean lengths of individual primary neurites on fibroblasts (233.1 ± 48.7μM) and on BMSCs (217.8 ± 35.1μM) were longer than that of laminin (117.5 ± 17.4μM) (Fig. 5-3C). The mean number of primary neurites was significantly higher on BMSCs (3.9 ± 0.7) than on fibroblasts (2.4 ± 0.2) and on laminin (1.9 ± 0.3) (Fig. 5-3D). The mean number of branching neurites was also significantly higher on BMSCs (10.4 ± 3.5) than on fibroblasts (4.3 ± 1.4) and on laminin (0.2 ± 0.0) (Fig. 5-3E). Statistical differences were found between all combinations of the three culture conditions in the total neurite length and total primary neurite length. The mean primary neurite lengths were statistically different between laminin and fibroblasts and between laminin and BMSCs. The mean numbers of primary neurites and the mean numbers of branching neurites were statistically different between laminin and BMSCs and between fibroblasts and BMSCs.

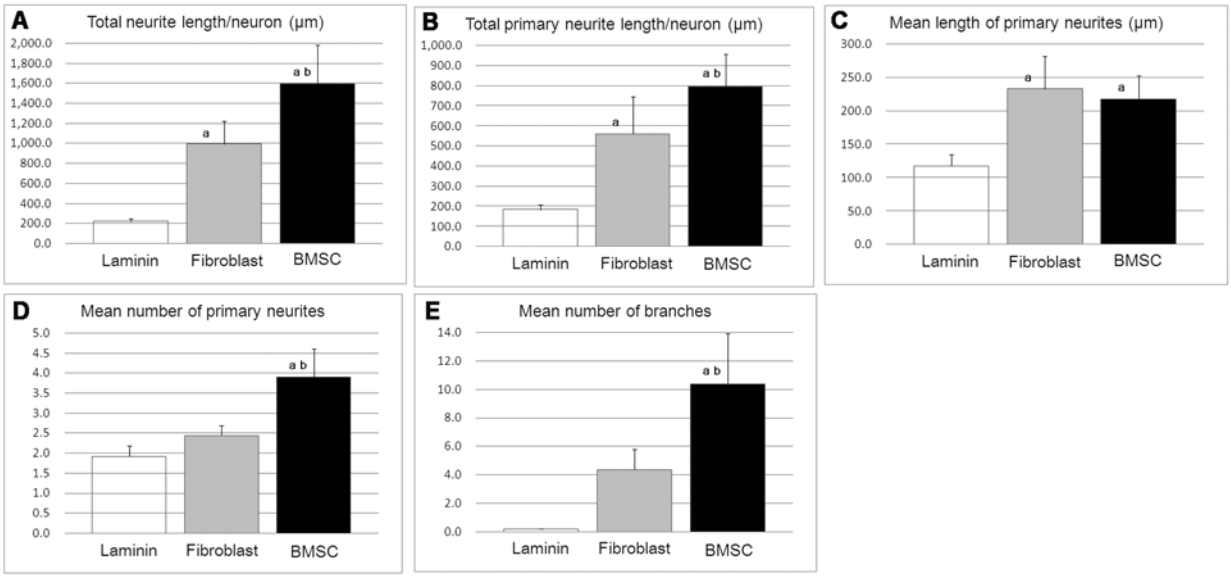

Figure 5-3. Quantitative neurite measurements of neurite outgrowth from DRG neurons cultured on laminin, fibroblasts, or BMSCs. Data are shown as the mean ± standard deviation. a indicates a significant ($p < 0.05$) difference against laminin. b indicates a significant ($p < 0.05$) difference against fibroblasts.

DRG Cultured in Conditioned Medium

The effects of BMSCs on neuritogenesis may also be mediated by soluble factors secreted in the culture medium. When DRG neurons were cultured in conditioned media, we found that the patterns of neurite outgrowth among three groups were similar to those observed in direct co-culture but the magnitude was much diminished. It was also noticed that there was a wide range in the observed measurements in all groups, particularly in the length measurements. The total neurite lengths of DRG neurons were not statistically different between groups (control, 997.7 ± 684.9 μM; fibroblast, 1370.6 ± 1096.4 μM; BMSC, 1510.3 ± 1253.0 μM) (Fig. 5-4A). The total primary neurite length was longer in BMSC-conditioned media (749.0 ± 468.2 μM) compared to that in control media (490.0 ± 393.8 μM) but not to that in fibroblast-conditioned media (647.2 ± 423.7 μM) (Fig. 5-4B). The mean lengths of individual primary neurites were not statistically different between groups (control, 217.5 ± 102.3 μM; fibroblast, 262.3 ± 114.2 μM; BMSC, 255.5 ± 112.0 μM) (Fig. 5-4C). The mean number of primary neurites was higher in BMSC-

conditioned media (3.0 ± 1.1) compared only to control media (2.3 ± 0.9) but not to fibroblast-conditioned media (2.7 ± 1.6) (Fig. 5-4D). The mean numbers of branching neurites were not statistically different between groups (control, 6.4 ± 3.5; fibroblast, 7.5 ± 6.6; BMSC, 7.9 ± 8.4) (Fig. 5-4E).

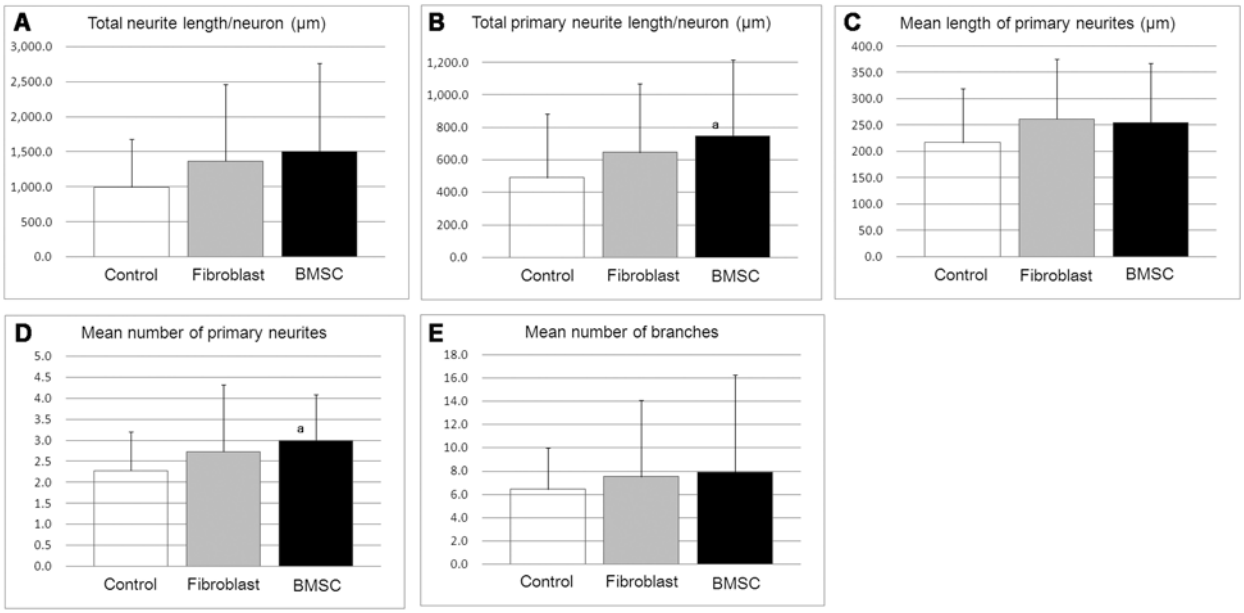

Figure 5-4. Quantitative neurite measurements of neurite outgrowth from DRG neurons cultured in control, fibroblast-conditioned, or BMSC-conditioned media. a indicates a significant ($p < 0.05$) difference against laminin.

Conclusion and Discussion

Bone marrow cells are suggested to participate in adult neurogenesis by transdifferentiation (Cogle et al., 2004; Mezey et al., 2003). Several studies claim that BMSCs can also generate neural cells *in vivo* (Azizi et al., 1998; Deng et al., 2006; Lee et al., 2003). Transplantation of BMSCs has been shown to promote functional recovery in various CNS injuries models (Chen et al., 2001; Hofstetter et al., 2002; Li et al., 2002; Mahmood et al., 2005; Wu et al., 2007; Zhao et al., 2002). However, precise mechanisms responsible for functional recovery have not been clearly demonstrated. Our data suggest that canine BMSCs have the ability to promote axonal regrowth from DRG neurons *in vitro* and that the effects are most

prominent via direct contact with neurons. The ability of BMSCs to produce ECM molecules such as fibronectin and laminin appeared to be the prime attribute for neurite outgrowth-promoting effects of canine BMSCs.

In the present study, direct co-cultures showed that DRG neurons extended substantially longer and more complex neurites on BMSCs than on laminin or fibroblasts. The total neurite length of DRG neurons on BMSCs was about 1.5 times and 7 times longer than those on fibroblasts and laminin, respectively. The total primary neurite length was also significantly longer on BMSCs than on fibroblasts or laminin; the difference between BMSCs and fibroblasts was due to the increased number of primary neurites on BMSCs and not to the increased length of individual primary neurites. The mean number of branching neurites was also significantly higher on BMSCs compared to other substrates. These findings suggest that BMSCs promote neurite outgrowth from DRG neurons by stimulating emergence of both primary and branching neurites.

We speculate that neurite outgrowth-promoting effects of BMSCs are primarily mediated by the production of ECM and adhesion molecules. Our results of immunocytochemistry demonstrated that canine BMSCs produce fibronectin and laminin similar to human BMSCs (Grayson et al., 2004; Hofstetter et al., 2002). Fibronectin and laminin are expressed by a variety of cell types and known to enhance neurite extension (Kimura et al., 2004; Orr and Smith, 1988; Smith and Orr, 1987). A previous *in vitro* study demonstrated that these ECM molecules promote adhesion of neurons and neurite extension and the effects are most potent when used in combination (Orr and Smith, 1988). Our observation that fibroblasts also produce fibronectin but not laminin points to a significant involvement of laminin in promoting neurite outgrowth. This view is in line with earlier studies indicating that cell surface factors associated with

astrocytes and Schwann cells but not with fibroblasts are primarily responsible for inducing active neurite outgrowth (Fallon, 1985a; Fallon, 1985b). Nevertheless, *in vivo* studies showed that fibronectin may provide a scaffold for invading cellular elements including macrophages and Schwann cells which in tern stimulate axonal regeneration at the site of spinal cord injury (King et al., 2003; King et al., 2006). In addition, we examined whether canine BMSCs express other adhesion molecules that are known to stimulate neurite extension, including E-cadherin (Oblander et al., 2007), N-cadherin (Puch et al., 2001; Schense et al., 2000), and neural cell adhesion molecule NCAM (Crigler et al., 2006; Kimura et al., 2004). However, these molecules were not expressed on canine BMSCs under our culture condition.

A recent *in vitro* study showed that human BMSCs promote neurite extension from DRG explants over nerve-inhibitory molecules such as neural proteoglycans, myelin associated glycoprotein, and Nogo-A (Wright et al., 2007). In the study, BMSCs reduced the inhibitory effects of these molecules and thereby permitted extension of DRG neurites. The authors described that DRG explants extended neurites when co-cultured with BMSCs that acted as "cellular bridges" and also "towed" neurites over the nerve-inhibitory molecules. In contrast, BMSC-conditioned medium stimulated neurite extension over nerve-permissive substrate (i.e., type I collagen) but not over nerve-inhibitory substrates. Therefore, axon-BMSC interactions appear to be important for BMSCs to promote neurite extension, particularly in injured nervous tissues where nerve-inhibitory molecules are present. Another possibility is that BMSCs may degrade nerve-inhibitory molecules thereby allowing regenerating axons to grow in and across the CNS injury site. Human BMSCs have been shown to produce membrane type I matrix metalloproteinase and matrix metalloproteinase 2 (Son et al., 2006) which degrade nerve-

inhibitory molecules (d'Ortho et al., 1997; Fosang et al., 1992; Passi et al., 1999). Whether canine BMSCs hold similar enzymatic properties remained to be investigated.

Although our results suggest that ECM molecules are the prime contributors in promoting neurite outgrowth, involvement of soluble factors cannot be entirely excluded. In fact, some parameters (i.e., the total primary neurite length/neuron and the number of primary neurites/neuron) were significantly greater in BMSC-conditioned media compared to control media. The candidate factors responsible for these changes might be neurotrophic factors (e.g., NGF, BDNF, and NT-3) since production of an array of neurotrophic factors has been shown in human BMSCs (Auffray et al., 1996; Chen et al., 2002a; Chen et al., 2002b; Crigler et al., 2006; Li et al., 2002). However, there were some uncertainties present in our study, including the cross-species reactivity of canine neurotrophic factors to rat derived neurons and local concentrations of soluble factors secreted in the conditioned media. In addition, a soluble form of laminin has been shown to have effects on neurite outgrowth (Kohno et al., 2005), indicating the possibility of laminin to play dual roles as an ECM molecule and a soluble factor. Investigations into identification and quantitation of candidate neurite outgrowth-promoting soluble factors produced by canine BMSCs are currently underway in our laboratory, using a range of neutralizing antibodies.

In conclusion, we demonstrated that canine BMSCs have the ability to promote neurite extension and branching from DRG neurons *in vitro*, primarily through production of ECM molecules. Because of their accessibility BMSCs may represent the most promising source for cellular treatment of CNS injuries. In order to translate our findings to clinical application of canine BMSCs, further investigations are needed to address several questions. In particular, although DRG neurons have both CNS and PNS components *in vivo*, effects of canine BMSCs

on pure CNS neurites need to be elucidated. Effects of BMSCs on neuritogenesis are also donor-dependent (Neuhuber et al., 2005) and there is even a considerable variation in the ability to produce neurotrophic factors among different populations of BMSCs from the same donor (Crigler et al., 2006). These previous reports highlight the need for thorough characterization of canine BMSCs to facilitate the development and refinement of prospective cellular based therapy for dogs with CNS injuries. Finally, xenotransplantation studies in rodent models of CNS injury will allow assessment of procedural as well as biological safety associated with transplantation of canine BMSCs and behavioral benefits *in vivo*.

CHAPTER 6
SUMMARY AND CONCLUSION

This dissertation presents a series of studies conducted to understand the biology of canine BMSCs. Chapter 2 represents an initial effort to systematically define the growth kinetics, phenotypic profile, and *in vitro* differentiation properties of canine BMSCs. It was also our goal to explore the potential of canine BMSCs for cell therapy for CNS disorders. Consequently, *in vitro* and *in vivo* neural differentiation properties of canine BMSCs were presented in chapter 3 and 4, respectively. Finally, in chapter 5, the effects of BMSCs on neurite extension were described.

We showed that canine BMSCs share many characteristics with BMSCs of humans and rodents. First, canine primary BMSCs can be readily isolated by use of their adhesive properties to culture plastic and expanded as CFU-F under the standard culture condition. From classical CFU-F assay, we estimated the frequency of canine BMSCs to be approximately 0.0042% (1 BMSC in every 2.38×10^4 mononucleated cells). Passaged canine BMSCs grow as monolayer and the growth kinetics are dependent on the initial cell density with the low cell density producing the maximal fold increase of cell expansion. Second, the cell surface marker profile of canine BMSCs evaluated by flow cytometry is similar to human and rodent counterparts. Flow cytometry also revealed that expanded cell populations did not contain a significant degree of contamination of other cell lineages. Third, similar to human and rodent BMSCs, canine BMSCs consist of heterogeneous cell populations with varying degrees of differentiation capacities. We found that canine BMSCs do contain a population of cells with the ability to differentiate into osteoblasts and adipocytes *in vitro*. However, we speculate that the standard culture method does not support expansion of multipotent stem cells present in the canine BMSC

fraction as passaged canine BMSCs demonstrated limited differentiation along osteoblastic and adipogenic pathways.

Interestingly, canine BMSCs spontaneously express neuron- and astrocyte-specific proteins *in vitro*. The significance of this finding is unknown but it may suggest that canine BMSCs are not only undifferentiated but also multi-differentiated as has been suggested for human BMSCs. Elevation of intracellular cyclic AMP levels caused rapid transformation of canine BMSCs into neuron-like morphology, but the morphological change per se was interpreted as a result of cytoskeletal shrinkage rather than genuine neurite extension.

Our xenotransplantation study suggested that BMSCs isolated from young canine donors may have the capacity of neural transdifferentiation in the neonatal mouse brain. Young donor BMSCs injected into the lateral ventricle of neonatal mouse brains migrated into the SVZ and assumed neuronal or astrocytic morphology. A small number of BMSCs further migrated in the RMS and reached the olfactory bulb where they expressed neuron-specific marker. Cell fusion events did not contribute to these observations of young donor BMSCs. Adult canine BMSCs, however, did not exhibit a similar degree of migration and differentiation. The results imply that neurogenic stem cells are present in the BMSC compartment of young dogs, whereas, in the adult canine BMSC compartment, cells of similar properties either do not exist or are lost during *ex vivo* expansion.

Finally, we evaluated the ability of canine BMSCs in promoting neurite outgrowth from DRG neurons *in vitro*. When DRGs neurons were co-cultured with canine BMSCs, neurite outgrowth was strongly promoted. This observation was attributed to the stimulatory effects of canine BMSCs on development of primary and branching neurites from DRG neurons. When DRG neurons were cultured in BMSC-conditioned medium, the magnitude of neurite outgrowth

was suppressed compared to direct co-culture. We showed that canine BMSCs express copious amounts of laminin and fibronectin, two potent neurite extension-promoting proteins. Consequently, canine BMSCs were thought to stimulate neurite outgrowth primarily via production of ECM molecules.

In summary, canine BMSCs are easily accessible and expandable *in vitro*; thus, represent a promising source for various cell therapies. However, there are still a lot of works to be done in order to better understand the biology of canine BMSCs. Future investigations should emphasize the development of culture techniques that allow maximum expansion of multipotent canine BMSCs. In spite of controversies surrounding neural transdifferentiation of bone marrow derived cells, canine bone marrow (at least from young donors) is likely to contain neurogenic cells. Upon revealing the identity of these cells and refining culture techniques, it may become possible to isolate and expand similar cell populations from adult canine bone marrow. We showed that, with the current standard culture technique, canine BMSCs have the ability to promote neurite outgrowth from DRG neurons. It is thus warranted to investigate *in vivo* effects of canine BMSCs in rodent models of CNS injury. Ultimately, BMSC transplantation in spontaneous CNS injury in dogs will provide valuable insights into the safety as well as clinical efficacy associated with this procedure.

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BIOGRAPHICAL SKETCH

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