博士論文（要約）

Exploration of a novel mesenchymal stem cell in canine bone marrow and its therapeutic effects for acute spinal cord injury

（犬の新規骨髄間葉系幹細胞の探索と急性期脊髄損傷に対する治療効果の検討）

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General introduction

Section 1. Spinal cord injury in dogs and stem cell based therapy

1-1 Spinal cord injury in dogs

Spinal cord injury (SCI) is a relatively common neurological disease. The most common causes of SCI in dogs are intervertebral disc disease (IVDD) and trauma caused by motor vehicle accident [1,2]. Ability of walk recovers by surgical or medical treatment in most dogs with mild to moderate SCI [3-5]. However, in case of dogs with severe SCI accompanied by loss of nociceptive perception, the recovery percentage of neurological function is only up to 50% by conventional treatments [6]. Severe SCI is usually not lethal, but it may impair the quality of life of the dogs and their owners seriously [7]. Therefore, it is strongly desired to develop an innovative and effective treatment for dogs with severe SCI.

1-2 Pathophysiology and therapeutic approach of spinal cord injury

It is important to understand the pathophysiology of SCI to develop a novel treatment. Spinal cord parenchyma is compressed by IVDD or trauma with various amounts of the materials, velocity and duration. These forces damage spinal cord by primary and secondary injury [8]. Primary injury is caused by physical force on the spinal cord and results in laceration, contusion, compression, and traction of the spinal cord tissue [9]. Subsequently a cascade of cellular, molecular and biochemical events occurs and results in progressive destruction of spinal cord tissue, termed as "secondary injury or damage". Secondary injury induces disruption of spinal cord vasculature followed by ischemia and/or congestion, glutamatergic excitotoxicity, oxidative cell stress, lipid peroxidation, inflammation and induction of extrinsic and intrinsic apoptosis [10-12]. Changes of microenvironment initiate the activation and proliferation of astrocytes in the lesion site [13] and contribute to the formation of glial scar. Although the role of glial scar remains controversial, it has been reported that glial scar secretes inhibitory extracellular matrix molecules which

inhibits axonal regeneration [14] Therefore, the strategy for development of a novel therapeutic approach for severe SCI is divided into two large ways. One of them is to prevent progression of secondary injury and another is to regenerate damaged spinal cord tissue. To achieve these two goals, stem cell based therapy is expected to be most promising.

1-3 Stem cell based therapy for SCI

Stem cell based therapies for SCI target several SCI pathological processes. It has been reported that transplantation of various kinds of stem cells protected the host neurons, axons, and myelin by preventing apoptosis [15], promote axonal or myelin regeneration accompanied with synapse formation [16,17], and replace the damaged neurons in the spinal cord [18]. A variety of stem cells have been proposed as sources for cellular treatment of SCI on the basis of these therapeutic strategies.

Pluripotent stem cells such as embryonic stem cells (ES cells) and induced pluripotent cells (iPS cells) are well known to have a prominent ability to differentiate into almost of all types of cells including neural lineage [19-21]. In

humans, these cells have been recognized as the most promising cells for tissue reconstruction of spinal cord after injury. However, clinical studies of ES cells are not currently in progress because of ethical issues and risk of malignant transformation [22]. Although iPS cells have few ethical concerns, they have a risk of uncontrolled proliferation or even tumor formation due to the use of viral vectors or transcription factors [23,24]. Therefore, to date, clinical use of iPS cells is still challenging and careful screening of oncogenic capacity prior to transplantation should be required [25]. In dogs, several researches have already reported the establishment of canine iPS cells [26,27], however, it seems to be highly difficult to standardize and apply the generating method of canine iPS cells for the clinical settings.

On the other hand, mesenchymal stem cells (MSCs), which are multipotent, have been known as an alternative stem cell source for treatment for SCI. MSCs were first identified from bone marrow in 1970 [28], and then isolated from various origins, such as bone marrow, adipose tissue, and umbilical cord matrix or blood. MSC is defined as a rare population of multipotent progenitor cell with the capacity of self-renewal and differentiation into a mesenchymal lineage [29].

The practical advantages of MSCs are the ease of isolation, low cost of expansion, low immunogenicity [30-33], low tumorigenic risk [34], and ability to migrate to the site of injury or inflammation [35,36]. Although MSCs are supposed to differentiate into mesodermal and neural lineages, the ability of differentiation into neurons remains controversial [37]. However, evidence obtained in experimental animals suggest that transplanted MSCs are most likely to provide favorable effects for tissue repair after SCI through the secretion of cytokines or trophic factors [38]. Due to the ease of application for clinical settings and its favorable effects expected, MSCs are currently considered to be the most promising cell source of treatment for SCI in dogs.

Section 2. Canine MSCs for the treatment of SCI in dogs: therapeutic mechanisms, and potential and limitation for clinical applications

2-1 Characterization of canine MSCs

MSCs are somatic stem cells, which have been widely studied for more than 40 years. To date, canine MSCs have been isolated from various tissues such as bone marrow, adipose tissue, umbilical cord, dental pulp, and amniotic membrane [39-43]. In dogs, bone marrow mesenchymal stem cells (BMMSCs) and adipose-derived mesenchymal stem cells (ADMSCs) have been well characterized because of the ease of availability. Canine BMMSCs are usually isolated by adherent culture of mononuclear cells from bone marrow [44-46]. Canine ADMSCs are isolated by adherent culture of the stromal vascular fraction, which can be obtained by centrifugation of collagenase-digested adipose tissue [47,48]. These cells generally show fibroblast-like morphology in vitro which is same as human MSCs. The minimal criteria required to define the cells obtained as MSCs in human have been documented by the International Society for

Cellular Therapy (ISCT) as follows: (1) plastic-adherent property when maintained in standard culture conditions, (2) positive expression of CD105, CD73 and CD90, and absence of expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR surface antigen, and (3) capacity of trilineage (osteocytes, chondrocytes and adipocytes) differentiation *in vitro* [29,49]. However, so far, there have been no uniform characterization criteria available for canine origin regarding to the expression of cell surface antigens. The cell surface marker profile of canine MSCs is variously reported depending on the source of the cell and there remain absolute disagreements among the researchers. As the definitive cell markers of canine MSCs remain unknown, their properties as MSCs are exclusively validated by self-renewal and differentiation ability. Therefore, specific antibodies absolutely detect the canine cell surface antigens should be developed for more accurate definitive characterization of canine MSCs.

2-2 Potential and limitation of clinical application of canine MSCs for SCI in dogs

2-2-1 Therapeutic mechanisms of MSC transplantation for SCI

Canine MSCs have already been applied for experimental studies or clinical trials of canine SCI which successfully promoted lesion repair and improve the neurological function in transplanted dogs [50-53]. However, the details of its therapeutic mechanism of MSC transplantation remain unclear and cellular therapy for SCI using canine MSCs have not yet been accepted as a treatment based on scientific evidence. Therefore, it is important to understand the therapeutic mechanisms of canine MSCs transplantation and to establish a therapeutic strategy based on the scientifically verified mechanism.

The mechanism of MSCs transplantation for the treatment of SCI have been investigated in many studies [54]. The possible mechanisms of improvement reported in these studies include immunomodulation, scar reduction, cell rescue, promotion of directed progenitor/precursor cell migration, differentiation and remyelination, angiogenesis and trophic effect. These reports also suggest that

tissue repair and functional recovery is mainly due to the trophic and protective properties of the canine MSCs but not to differentiation and regeneration by transplanted cells [55].

Trophic effects of MSCs depend on the local environment and may be modulated by dynamic extracellular matrix–cytoskeletal interactions, cell–cell contacts, and soluble and transcription factor signaling [56]. The secretion of trophic factors from MSCs may be modified by various factors. Several studies have documented that MSCs exposed to pro-inflammatory cytokines, even for as short as a few hours, change their gene and protein expression for days later [57]. MSCs have been reported to be able to secrete growth factors and cytokines, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), transforming growth factor-beta (TGF-β) and hepatocyte growth factor (HGF). Recently, Nakano et al. demonstrated that conditioned medium for BMMSCs included IGF-1, HGF, VEGF, and TGF-β resulted in higher levels of neuronal survival and neurite outgrowth in vitro study [58]. In addition, other studies also

showed that the conditioned medium of BMMSCs promoted neuronal and glial survival in vitro [59,60].

2-2-2 Hepatocyte growth factor (HGF)

Among the trophic factors secreted from MSCs described above, hepatocyte growth factor (HGF) is one of the most potent factors for tissue repairing [61]. HGF is a paracrine growth factor of which primary function is tissue repair. Phosphorylation of c-Met, which is a HGF receptor tyrosine kinase induces growth, proliferation, morphogenesis and migration of epithelial and endothelial cells [61]. In nervous system, HGF stimulates proliferation of glial cells such as Schwann cells and oligodendrocyte progenitor cells and improves neuronal survival [62-64]. HGF has also been shown to reduce astrocytic scar formation and promote axonal growth beyond glial scar after SCI. Exogenous administration of HGF has also been reported to preserve axons and myelinated area and promote functional recovery in rodent and primate models of SCI [65,66]. According to these results, phase I/II clinical trials of administration of

human recombinant HGF has already started in human medicine in 2014. However, the effect of recombinant HGF was limited due to its short half-life of elimination in vivo, difficulty of continuous supply of HGF to the target area, and excessive cost.

After the injury, pro-inflammatory cytokines such as TNF-α and IL-1β are upregulated at the lesion site during acute to subacute phase. As these factors are known to induce HGF secretion by MSCs [67], therapeutic effects of transplanted canine MSCs could be evaluated by the secretion of HGF with or without pro-inflammatory cytokines such as TNF-α and IL-1β. In addition, MSCs originally possess the ability to migrate to the site of injury or inflammation [35,36]. Therefore, acute to subacute phase after SCI is considered as the appropriate period to induce HGF secretion from MSCs. In case the ability of HGF secretion from canine MSCs is verified, it is indicated that MSCs which are successfully delivered and survived at lesion site under inflammation contribute to continuous supply of HGF to the injured tissue.

2-2-3 Limitation of existing canine MSCs in clinical application for SCI in dogs

Both canine BMMSCs and ADMSCs can be harvested readily, and transplantation of these cells was safe and effective for locomotion recovery in experimental models and clinical cases of canine SCI [50,52,53,68-71]. However, clinical application of these cells has some limitations. Some experimental studies reported that allogenic canine BMMSCs showed limited tissue preservation and functional recovery as compared with autologous cells [51], hence autologous transplantation seems to be superior in clinical use. When considering the best period to induce HGF secretion from MSCs is acute to subacute phase after SCI, transplantation of autologous MSCs should be performed as fast as possible after SCI. However the proportion of canine BMMSCs among mononuclear cells in bone marrow is very low [72,73], therefore a longer expansion time is needed to prepare sufficient numbers of BMMSCs for clinical application and the proper period for transplantation may be missed. On the other hand, ADMSCs generally consist of a heterogeneous cell population contaminated by endothelial cells, smooth muscle cells, pericytes, and blood cells

such as monocytes and lymphocytes even though proliferation ability is superior to BMMSCs [48,74,75]. The heterogeneous property of canine ADMSCs can lead instable ratio of MSCs in transplanted cells and may lead to decreased success rate of treatment. Thus, it is favorable to develop a novel method to obtain purified MSCs with a high proliferative capacity for higher success rate of stem cell based therapy using canine MSCs.

Recently, some researchers have shown that mature adipocytes derived from subcutaneous or omental adipose tissues of humans, mice, pigs, and cats dedifferentiate into fibroblast-like cells called dedifferentiated fat (DFAT) cells when cultured with the ceiling culture method [74,76-78]. DFAT cells exhibit high proliferation capacity and multipotency, with higher homogeneity and colony-forming efficiency than ADMSCs. Since DFAT cells are considered to be composed of purer multipotent cells than ADMSCs, they are one of the promising candidates for stem cell therapies for various tissues [74,76,79].

In another study, Shigematsu et al. showed that adipocytes in calf bone marrow could dedifferentiate into fibroblast-like cells after ceiling culture and showed proliferation ability and spontaneous re-differentiation into an

adipogenic lineage after confluence [80]. Although the multipotent property of these cells is unknown, adipocytes in bone marrow may generate multipotent cells like DFAT cells and provide enough number of MSCs in a shorter period.

Section 3. Objective of the study

Therefore, the objective of this study was to explore a new novel mesenchymal stem cell in canine bone marrow using ceiling culture method and compare its MSC potential with that of conventional BMMSCs (Chapter 1). In chapter 1, small cells adhering to adipocytes in canine bone marrow, which proliferated quickly and showed multipotency were explored. I named these cells as “bone marrow peri-adipocyte cells” (BM-PACs) and evaluated the potential of HGF secretion with or without the stimulation of pro-inflammation cytokines, TNF-α and IL-1β in order to investigate whether BM-PACs would be a novel source for cell transplantation therapy for SCI (Chapter 2). Finally, I transplanted BM-PACs, which were labeled with red fluorescence to trace the distribution, into an mouse severe SCI model in acute phase intralesionally or intravenously to estimate the clinical efficacy and safety of BM-PACs transplantation for SCI in dogs (Chapter 3).

Chapter 1

Exploration of a novel mesenchymal stem cell derived from peri-adipocyte cells in canine bone marrow / Comparison of mesenchymal stem cell potential with BMMSCs

Introduction

Stem cell based therapy is one of the most promising therapies for various refractory diseases including spinal cord injury (SCI). Mesenchymal stem cells (MSCs) are thought to be more suitable than ES cells and iPS cells in veterinary medicine because of their safety, less ethical problems and low cost. BMMSCs and ADMSCs are common MSC sources, but have some limitation for clinical use. They need long time to obtain enough number of stem cells for clinical application because of their low proliferation or heterogeneous characteristics. Dedifferentiated fat (DFAT) cells obtained from mature adipocytes in adipose tissue by ceiling culture method have favorable characteristics as stem cells compared with adipose tissue-derived mesenchymal stem cells (ADMSCs) because of much higher homogeneity, proliferation ability and colony-forming efficiency [74,76,79].

As described before, Shigematsu et al. showed that adipocytes in calf bone marrow could dedifferentiate into fibroblast-like cells after ceiling culture and showed proliferation and spontaneous re-differentiation into an adipogenic lineage after confluence [80]. These results indicated MSCs obtained from bone

marrow-derived adipocytes may generate multipotent cells like or superior to DFAT cells.

Therefore, the purpose of this study was to culture adipocytes derived from canine bone marrow to explore a novel mesenchymal stem cell superior to conventional MSCs.

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Chapter 2

Secretion of hepatocyte growth factor (HGF) by BM-PACs and its bioactivity

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Chapter 3

Evaluation of transplantation of BM-PACs for acute spinal cord injury in a mouse model

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Conclusion

Spinal cord injury (SCI) is a common cause of neurological diseases in dogs [1,2]. In case of severe SCI, the conventional therapies such as surgical or medical treatments have failed to lead sufficient recovery. To date, no therapies which are scientifically proven to be effective for severe SCI in dogs have been established. Stem cell based therapy has recently been recognized as one of the promising therapeutic approach for severe SCI. Mesenchymal stem cells (MSCs) are attractive candidates for cell based therapy in veterinary medicine due to their availability. Several studies have been documented transplanted MSCs are most likely to provide therapeutic effects through secreted trophic factors [38]. However, the details of their trophic effect remain unclear and there are some problems for application of existing canine MSCs for clinical use. Therefore, in this study, a novel canine MSC was explored to resolve the limitations of conventional canine MSCs and its trophic effect was investigated. Finally, its therapeutic effect was evaluated using mice severe SCI model to establish effective therapy based on the scientific evidences for severe SCI in dogs.

In chapter 1, isolation and characteristics of novel MSCs, named as BM-PACs were demonstrated. BM-PACs can be easily obtained by ceiling culture of

adipocytes in canine bone marrow and showed superior MSC properties such as proliferation and multilineage differentiation ability compared with conventional canine BMMSCs. Significant differences of the expression of CD73 were detected in between the two types of cells. It was suggested that CD73 is an important cell surface marker to identify canine MSCs in bone marrow.

Hepatocyte growth factor (HGF), which is one of the most powerful trophic factors for tissue repair have been recently attracted attention as a therapeutic agent for SCI. Therefore, in chapter 2, trophic effect of BM-PACs through HGF secretion was investigated. BM-PACs showed significant higher expression of HGF mRNA than BMMSCs and secretion of HGF protein from BM-PACs extremely increased in response to pro-inflammatory cytokines, TNF-α and IL-1β. Although BM-PACs were likely to release not only HGF but also various trophic factors, supernatant of BM-PACs stimulated by inflammatory cytokines induced bioactivities on MDCK cells similar to recombinant HGF protein. These results indicated that transplantation of BM-PACs into the lesion of spinal cord where inflammation occurred was expected to supply sufficient amount of HGF and provide therapeutic effects.

In chapter 3, the feasibility and the safety of transplantation of BM-PACs to mice severe SCI model was investigated to estimate the impact of BM-PACs transplantation on clinical application for SCI in dogs. BM-PACs were intralesionally or intravenously transplanted in acute phase when the highest expression of pro-inflammatory cytokine was expected. Intralesional transplantation of BM-PACs successfully exhibited significant functional recovery and preservation of myelin was likely to be an underlying cause of functional recovery. Although it remains unclear whether HGF secreted from BM-PACs contributed to these favorable effects, it was suggested that trophic effect of BM-PACs played an important role in healing effect on SCI in acute phase. It was a novel discovery that intravenous transplantation could deliver BM-PACs to the injured spinal cord, even though most of cells were entrapped in the lung immediately after transplantation. However, intravenous transplantation failed to induce functional recovery. Therefore, the number of cells delivered to the lesion was considered to be important to exert healing effects.

In conclusion, this study revealed that novel canine MSCs, BM-PACs were

easily harvested from canine bone marrow and had therapeutic effects for acute phase of severe SCI by intralesionally transplantation. When considering clinical application of BM-PACs to severe SCI in dogs, their superior proliferation ability is helpful to prepare sufficient number of cells as quickly as possible. Although it remains unclear how HGF secreted from BM-PACs contribute to functional and histological repair after SCI, trophic effect through HGF secretion can be useful for other diseases with inflammation such as osteoarthritis and inflammatory bowel disease in dogs. Further studies should be needed to optimize the transplantation strategy of BM-PACs in clinical settings.

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References

1. McKee WM. (1990). Spinal trauma in dogs and cats: a review of 51 cases. Vet Rec 126:285-9.
2. Fluehmann G, MG Doherr and A Jaggy. (2006). Canine neurological diseases in a referral hospital population between 1989 and 2000 in Switzerland. J Small Anim Pract 47:582-7.
3. Ruddle TL, DA Allen, ER Schertel, MD Barnhart, ER Wilson, JA Lineberger, NW Klocke and TW Lehenbauer. (2006). Outcome and prognostic factors in non-ambulatory Hansen Type I intervertebral disc extrusions: 308 cases. Vet Comp Orthop Traumatol 19:29-34.
4. Davis GJ and DC Brown. (2002). Prognostic indicators for time to ambulation after surgical decompression in nonambulatory dogs with acute thoracolumbar disk extrusions: 112 cases. Vet Surg 31:513-8.
5. Aikawa T, H Fujita, S Kanazono, M Shibata and Y Yoshigae. (2012). Long-term neurologic outcome of hemilaminectomy and disk fenestration for treatment of dogs with thoracolumbar intervertebral disk herniation: 831 cases (2000-2007). J Am Vet Med Assoc 241:1617-26.
6. Griffin JFt, J Levine, S Kerwin and R Cole. (2009). Canine thoracolumbar invertebral disk disease: diagnosis, prognosis, and treatment. Compend Contin Educ Vet 31:E3.
7. Schulz KS, M Walker, M Moon, D Waldron, M Slater and DE McDonald. (1998). Correlation of clinical, radiographic, and surgical localization of intervertebral disc extrusion in small-breed dogs: a prospective study of 50 cases. Vet Surg 27:105-11.
8. Dumont RJ, DO Okonkwo, S Verma, RJ Hurlbert, PT Boulos, DB Ellegala and AS Dumont. (2001). Acute spinal cord injury, part I: pathophysiologic mechanisms. Clin Neuropharmacol 24:254-64.
9. Webb AA, S Ngan and JD Fowler. (2010). Spinal cord injury I: A synopsis of the basic science. Can Vet J 51:485-92.
10. Eftekharpour E, S Karimi-Abdolrezaee and MG Fehlings. (2008). Current status of experimental cell replacement approaches to spinal cord injury. Neurosurg Focus 24:E19.
11. Nashmi R and MG Fehlings. (2001). Mechanisms of axonal dysfunction

after spinal cord injury: with an emphasis on the role of voltage-gated potassium channels. Brain Res Brain Res Rev 38:165-91.

12. Tator CH and MG Fehlings. (1991). Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms. J Neurosurg 75:15-26.
13. Lukovic D, M Stojkovic, V Moreno-Manzano, P Jendelova, E Sykova, SS Bhattacharya and S Erceg. (2015). Concise review: reactive astrocytes and stem cells in spinal cord injury: good guys or bad guys? Stem Cells 33:1036-41.
14. Pekny M and M Nilsson. (2005). Astrocyte activation and reactive gliosis. Glia 50:427-34.
15. Oliveri RS, S Bello and F Biering-Sorensen. (2014). Mesenchymal stem cells improve locomotor recovery in traumatic spinal cord injury: systematic review with meta-analyses of rat models. Neurobiol Dis 62:338-53.
16. De Feo D, A Merlini, C Laterza and G Martino. (2012). Neural stem cell transplantation in central nervous system disorders: from cell replacement to neuroprotection. Curr Opin Neurol 25:322-33.
17. Cusimano M, D Biziato, E Brambilla, M Donega, C Alfaro-Cervello, S Snider, G Salani, F Pucci, G Comi, JM Garcia-Verdugo, M De Palma, G Martino and S Pluchino. (2012). Transplanted neural stem/precursor cells instruct phagocytes and reduce secondary tissue damage in the injured spinal cord. Brain 135:447-60.
18. Stenudd M, H Sabelstrom and J Frisen. (2015). Role of endogenous neural stem cells in spinal cord injury and repair. JAMA Neurol 72:235-7.
19. Murry CE and G Keller. (2008). Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. Cell 132:661-80.
20. Yu J, MA Vodyanik, K Smuga-Otto, J Antosiewicz-Bourget, JL Frane, S Tian, J Nie, GA Jonsdottir, V Ruotti, R Stewart, Slukvin, II and JA Thomson. (2007). Induced pluripotent stem cell lines derived from human somatic cells. Science 318:1917-20.
21. Takahashi K and S Yamanaka. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663-76.

22. Li JY, NS Christophersen, V Hall, D Soulet and P Brundin. (2008). Critical issues of clinical human embryonic stem cell therapy for brain repair. Trends Neurosci 31:146-53.
23. Miura K, Y Okada, T Aoi, A Okada, K Takahashi, K Okita, M Nakagawa, M Koyanagi, K Tanabe, M Ohnuki, D Ogawa, E Ikeda, H Okano and S Yamanaka. (2009). Variation in the safety of induced pluripotent stem cell lines. Nat Biotechnol 27:743-5.
24. Tsuji O, K Miura, Y Okada, K Fujiyoshi, M Mukaino, N Nagoshi, K Kitamura, G Kumagai, M Nishino, S Tomisato, H Higashi, T Nagai, H Katoh, K Kohda, Y Matsuzaki, M Yuzaki, E Ikeda, Y Toyama, M Nakamura, S Yamanaka and H Okano. (2010). Therapeutic potential of appropriately evaluated safe-induced pluripotent stem cells for spinal cord injury. Proc Natl Acad Sci U S A 107:12704-9.
25. Nori S, Y Okada, A Yasuda, O Tsuji, Y Takahashi, Y Kobayashi, K Fujiyoshi, M Koike, Y Uchiyama, E Ikeda, Y Toyama, S Yamanaka, M Nakamura and H Okano. (2011). Grafted human-induced pluripotent stem-cell-derived neurospheres promote motor functional recovery after spinal cord injury in mice. Proc Natl Acad Sci U S A 108:16825-30.
26. Nishimura T, S Hatoya, R Kanegi, K Sugiura, V Wijewardana, M Kuwamura, M Tanaka, J Yamate, T Izawa, M Takahashi, N Kawate, H Tamada, H Imai and T Inaba. (2013). Generation of functional platelets from canine induced pluripotent stem cells. Stem Cells Dev 22:2026-35.
27. Luo J, ST Suhr, EA Chang, K Wang, PJ Ross, LL Nelson, PJ Venta, JG Knott and JB Cibelli. (2011). Generation of leukemia inhibitory factor and basic fibroblast growth factor-dependent induced pluripotent stem cells from canine adult somatic cells. Stem Cells Dev 20:1669-78.
28. Friedenstein AJ, RK Chailakhjan and KS Lalykina. (1970). The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet 3:393-403.
29. Dominici M, K Le Blanc, I Mueller, I Slaper-Cortenbach, F Marini, D Krause, R Deans, A Keating, D Prockop and E Horwitz. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8:315-7.
30. Bartholomew A, C Sturgeon, M Siatskas, K Ferrer, K McIntosh, S Patil, W Hardy, S Devine, D Ucker, R Deans, A Moseley and R Hoffman. (2002).

Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. Exp Hematol 30:42-8.

31. Corcione A, F Benvenuto, E Ferretti, D Giunti, V Cappiello, F Cazzanti, M Risso, F Gualandi, GL Mancardi, V Pistoia and A Uccelli. (2006). Human mesenchymal stem cells modulate B-cell functions. Blood 107:367-72.
32. Di Nicola M, C Carlo-Stella, M Magni, M Milanesi, PD Longoni, P Matteucci, S Grisanti and AM Gianni. (2002). Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood 99:3838-43.
33. Jiang XX, Y Zhang, B Liu, SX Zhang, Y Wu, XD Yu and N Mao. (2005). Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. Blood 105:4120-6.
34. Bernardo ME, N Zaffaroni, F Novara, AM Cometa, MA Avanzini, A Moretta, D Montagna, R Maccario, R Villa, MG Daidone, O Zuffardi and F Locatelli. (2007). Human bone marrow derived mesenchymal stem cells do not undergo transformation after long-term in vitro culture and do not exhibit telomere maintenance mechanisms. Cancer Res 67:9142-9.
35. Kidd S, E Spaeth, JL Dembinski, M Dietrich, K Watson, A Klopp, VL Battula, M Weil, M Andreeff and FC Marini. (2009). Direct evidence of mesenchymal stem cell tropism for tumor and wounding microenvironments using in vivo bioluminescent imaging. Stem Cells 27:2614-23.
36. Sasaki M, R Abe, Y Fujita, S Ando, D Inokuma and H Shimizu. (2008). Mesenchymal stem cells are recruited into wounded skin and contribute to wound repair by transdifferentiation into multiple skin cell type. J Immunol 180:2581-7.
37. Nishimura S, A Yasuda, H Iwai, M Takano, Y Kobayashi, S Nori, O Tsuji, K Fujiyoshi, H Ebise, Y Toyama, H Okano and M Nakamura. (2013). Time-dependent changes in the microenvironment of injured spinal cord affects the therapeutic potential of neural stem cell transplantation for spinal cord injury. Mol Brain 6:3.
38. Tolar J, K Le Blanc, A Keating and BR Blazar. (2010). Concise review: hitting the right spot with mesenchymal stromal cells. Stem Cells 28:1446-55.
39. Vieira NM, V Brandalise, E Zucconi, M Secco, BE Strauss and M Zatz.

(2010). Isolation, characterization, and differentiation potential of canine adipose-derived stem cells. Cell Transplant 19:279-89.

40. Kisiel AH, LA McDuffee, E Masaoud, TR Bailey, BP Esparza Gonzalez and R Nino-Fong. (2012). Isolation, characterization, and in vitro proliferation of canine mesenchymal stem cells derived from bone marrow, adipose tissue, muscle, and periosteum. Am J Vet Res 73:1305-17.
41. Dissanayaka WL, X Zhu, C Zhang and L Jin. (2011). Characterization of dental pulp stem cells isolated from canine premolars. J Endod 37:1074-80.
42. Seo MS, YH Jeong, JR Park, SB Park, KH Rho, HS Kim, KR Yu, SH Lee, JW Jung, YS Lee and KS Kang. (2009). Isolation and characterization of canine umbilical cord blood-derived mesenchymal stem cells. J Vet Sci 10:181-7.
43. Filioli Uranio M, L Valentini, A Lange-Consiglio, M Caira, AC Guaricci, A L'Abbate, CR Catacchio, M Ventura, F Cremonesi and ME Dell'Aquila. (2011). Isolation, proliferation, cytogenetic, and molecular characterization and in vitro differentiation potency of canine stem cells from foetal adnexa: a comparative study of amniotic fluid, amnion, and umbilical cord matrix. Mol Reprod Dev 78:361-73.
44. Bruder SP, KH Kraus, VM Goldberg and S Kadiyala. (1998). The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. J Bone Joint Surg Am 80:985-96.
45. Crovace A, A Favia, L Lacitignola, MS Di Comite, F Staffieri and E Francioso. (2008). Use of autologous bone marrow mononuclear cells and cultured bone marrow stromal cells in dogs with orthopaedic lesions. Vet Res Commun 32 Suppl 1:S39-44.
46. Fortier LA and AJ Travis. (2011). Stem cells in veterinary medicine. Stem Cell Res Ther 2:9.
47. Black LL, J Gaynor, D Gahring, C Adams, D Aron, S Harman, DA Gingerich and R Harman. (2007). Effect of adipose-derived mesenchymal stem and regenerative cells on lameness in dogs with chronic osteoarthritis of the coxofemoral joints: a randomized, double-blinded, multicenter, controlled trial. Vet Ther 8:272-84.
48. Zuk PA, M Zhu, H Mizuno, J Huang, JW Futrell, AJ Katz, P Benhaim, HP Lorenz and MH Hedrick. (2001). Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7:211-28.

49. Calloni R, GS Viegas, P Turck, D Bonatto and JA Pegas Henriques. (2014). Mesenchymal stromal cells from unconventional model organisms. Cytotherapy 16:3-16.
50. Jung DI, J Ha, BT Kang, JW Kim, FS Quan, JH Lee, EJ Woo and HM Park. (2009). A comparison of autologous and allogenic bone marrow-derived mesenchymal stem cell transplantation in canine spinal cord injury. J Neurol Sci 285:67-77.
51. Lim JH, YE Byeon, HH Ryu, YH Jeong, YW Lee, WH Kim, KS Kang and OK Kweon. (2007). Transplantation of canine umbilical cord blood-derived mesenchymal stem cells in experimentally induced spinal cord injured dogs. J Vet Sci 8:275-82.
52. Nishida H, M Nakayama, H Tanaka, M Kitamura, S Hatoya, K Sugiura, Y Harada, Y Suzuki, C Ide and T Inaba. (2012). Safety of autologous bone marrow stromal cell transplantation in dogs with acute spinal cord injury. Vet Surg 41:437-42.
53. Nishida H, M Nakayama, H Tanaka, M Kitamura, S Hatoya, K Sugiura, Y Suzuki, C Ide and T Inaba. (2011). Evaluation of transplantation of autologous bone marrow stromal cells into the cerebrospinal fluid for treatment of chronic spinal cord injury in dogs. Am J Vet Res 72:1118-23.
54. Wright KT, W El Masri, A Osman, J Chowdhury and WE Johnson. (2011). Concise review: Bone marrow for the treatment of spinal cord injury: mechanisms and clinical applications. Stem Cells 29:169-78.
55. Jang BJ, YE Byeon, JH Lim, HH Ryu, WH Kim, Y Koyama, M Kikuchi, KS Kang and OK Kweon. (2008). Implantation of canine umbilical cord blood-derived mesenchymal stem cells mixed with beta-tricalcium phosphate enhances osteogenesis in bone defect model dogs. J Vet Sci 9:387-93.
56. Hofer HR and RS Tuan. (2016). Secreted trophic factors of mesenchymal stem cells support neurovascular and musculoskeletal therapies. Stem Cell Res Ther 7:131.
57. Czekanska EM, JR Ralphs, M Alini and MJ Stoddart. (2014). Enhancing inflammatory and chemotactic signals to regulate bone regeneration. Eur Cell Mater 28:320-34.
58. Nakano N, Y Nakai, TB Seo, Y Yamada, T Ohno, A Yamanaka, Y Nagai, M Fukushima, Y Suzuki, T Nakatani and C Ide. (2010). Characterization of

conditioned medium of cultured bone marrow stromal cells. Neurosci Lett 483:57-61.

59. Wilkins A, K Kemp, M Ginty, K Hares, E Mallam and N Scolding. (2009). Human bone marrow-derived mesenchymal stem cells secrete brain-derived neurotrophic factor which promotes neuronal survival in vitro. Stem Cell Res 3:63-70.
60. Ribeiro CA, AJ Salgado, JS Fraga, NA Silva, RL Reis and N Sousa. (2011). The secretome of bone marrow mesenchymal stem cells-conditioned media varies with time and drives a distinct effect on mature neurons and glial cells (primary cultures). J Tissue Eng Regen Med 5:668-72.
61. Matsumoto K and T Nakamura. (1993). Roles of HGF as a pleiotropic factor in organ regeneration. Exs 65:225-49.
62. Maina F and R Klein. (1999). Hepatocyte growth factor, a versatile signal for developing neurons. Nat Neurosci 2:213-7.
63. Krasnoselsky A, MJ Massay, MC DeFrances, G Michalopoulos, R Zarnegar and N Ratner. (1994). Hepatocyte growth factor is a mitogen for Schwann cells and is present in neurofibromas. J Neurosci 14:7284-90.
64. Ohya W, H Funakoshi, T Kurosawa and T Nakamura. (2007). Hepatocyte growth factor (HGF) promotes oligodendrocyte progenitor cell proliferation and inhibits its differentiation during postnatal development in the rat. Brain Res 1147:51-65.
65. Kitamura K, K Fujiyoshi, J Yamane, F Toyota, K Hikishima, T Nomura, H Funakoshi, T Nakamura, M Aoki, Y Toyama, H Okano and M Nakamura. (2011). Human hepatocyte growth factor promotes functional recovery in primates after spinal cord injury. PLoS One 6:e27706.
66. Kitamura K, A Iwanami, M Nakamura, J Yamane, K Watanabe, Y Suzuki, D Miyazawa, S Shibata, H Funakoshi, S Miyatake, RS Coffin, T Nakamura, Y Toyama and H Okano. (2007). Hepatocyte growth factor promotes endogenous repair and functional recovery after spinal cord injury. J Neurosci Res 85:2332-42.
67. Tamura M, N Arakaki, H Tsubouchi, H Takada and Y Daikuhara. (1993). Enhancement of human hepatocyte growth factor production by interleukin-1 alpha and -1 beta and tumor necrosis factor-alpha by fibroblasts in culture. J Biol Chem 268:8140-5.
68. Ryu HH, BJ Kang, SS Park, Y Kim, GJ Sung, HM Woo, WH Kim and OK

Kweon. (2012). Comparison of mesenchymal stem cells derived from fat, bone marrow, Wharton's jelly, and umbilical cord blood for treating spinal cord injuries in dogs. J Vet Med Sci 74:1617-30.

69. Ryu HH, JH Lim, YE Byeon, JR Park, MS Seo, YW Lee, WH Kim, KS Kang and OK Kweon. (2009). Functional recovery and neural differentiation after transplantation of allogenic adipose-derived stem cells in a canine model of acute spinal cord injury. J Vet Sci 10:273-84.
70. Park SS, YJ Lee, SH Lee, D Lee, K Choi, WH Kim, OK Kweon and HJ Han. (2012). Functional recovery after spinal cord injury in dogs treated with a combination of Matrigel and neural-induced adipose-derived mesenchymal Stem cells. Cytotherapy 14:584-97.
71. Tamura K, Y Harada, N Nagashima, T Itoi, H Ishino, T Yogo, Y Nezu, Y Hara, Y Suzuki, C Ide and M Tagawa. (2012). Autotransplanting of bone marrow-derived mononuclear cells for complete cases of canine paraplegia and loss of pain perception, secondary to intervertebral disc herniation. Exp Clin Transplant 10:263-72.
72. Kamishina H, JP Farese, JA Storm, JA Cheeseman and RM Clemmons. (2008). The frequency, growth kinetics, and osteogenic/adipogenic differentiation properties of canine bone marrow stromal cells. In Vitro Cell Dev Biol Anim 44:472-9.
73. Bertolo A, P Schlaefli, C Malonzo-Marty, M Baur, T Potzel, F Steffen and J Stoyanov. (2015). Comparative characterization of canine and human mesenchymal stem cells derived from bone marrow. Int. J. Stem Cell Res.Ther 2:1-6.
74. Matsumoto T, K Kano, D Kondo, N Fukuda, Y Iribe, N Tanaka, Y Matsubara, T Sakuma, A Satomi, M Otaki, J Ryu and H Mugishima. (2008). Mature adipocyte-derived dedifferentiated fat cells exhibit multilineage potential. J Cell Physiol 215:210-22.
75. Yoshimura K, T Shigeura, D Matsumoto, T Sato, Y Takaki, E Aiba-Kojima, K Sato, K Inoue, T Nagase, I Koshima and K Gonda. (2006). Characterization of freshly isolated and cultured cells derived from the fatty and fluid portions of liposuction aspirates. J Cell Physiol 208:64-76.
76. Kono S, T Kazama, K Kano, K Harada, M Uechi and T Matsumoto. (2014). Phenotypic and functional properties of feline dedifferentiated fat cells and adipose-derived stem cells. Vet J 199:88-96.

77. Sakuma T, T Matsumoto, K Kano, N Fukuda, D Obinata, K Yamaguchi, T Yoshida, S Takahashi and H Mugishima. (2009). Mature, adipocyte derived, dedifferentiated fat cells can differentiate into smooth muscle-like cells and contribute to bladder tissue regeneration. J Urol 182:355-65.
78. Yagi K, D Kondo, Y Okazaki and K Kano. (2004). A novel preadipocyte cell line established from mouse adult mature adipocytes. Biochem Biophys Res Commun 321:967-74.
79. Miyazaki T, Y Kitagawa, K Toriyama, M Kobori and S Torii. (2005). Isolation of two human fibroblastic cell populations with multiple but distinct potential of mesenchymal differentiation by ceiling culture of mature fat cells from subcutaneous adipose tissue. Differentiation 73:69-78.
80. Shigematsu M, H Watanabe and H Sugihara. (1999). Proliferation and differentiation of unilocular fat cells in the bone marrow. Cell Struct Funct 24:89-100.