The role of microvesicles derived from mesenchymal stem cells in tissue regeneration; a dream for tendon repair?

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Summary

Tendon injuries represent even today a challenge as repair may be exceedingly slow and incomplete. Regenerative medicine and stem cell technology have shown to be of great promise. Here, we will review the current knowledge on the mechanisms of the regenerative potential of mesenchymal stem cells (MSCs) obtained from different sources (bone marrow, fat, cord blood, placenta). More specifically, we will devote attention to the current use of MSCs that have been used experimentally and in limited numbers of clinical cases for the surgical treatment of subchondral-bone cysts, bone-fracture repair and cartilage repair. Based on the recently emerging role in regenerative mechanisms of soluble factors and of extracellular vesicles, we will discuss the potential of non-cellular therapies in horse tendon injuries.

Key words: horse tendinopathies, microvesicles, regenerative medicine, soluble factors, stem cells.

Introduction

Stem cells have evoked considerable excitement in veterinary medicine because of the promise that stem cell technology could deliver tissue regeneration for injuries for which natural repair mechanisms do not deliver functional recovery and for which current therapeutic strategies have minimal effectiveness. Tendon injuries have represented an area of particular interest since conventional treatments often lead to an unsatisfactory healing process that usually results in a relatively high recurrence rate. In recent years, regenerative medicine has emerged as an attractive field for new cellular and non-cellular approaches to tissue repair. Here, we will review the current knowledge on the mechanisms of the regenerative potential of mesenchymal stem cells (MSCs) obtained from different sources (bone marrow, fat, cord blood, placenta). More specifically, we will devote attention to the current use of MSCs that have been used experimentally and in limited numbers of clinical cases for the surgical treatment of subchondral-bone cysts, bone-fracture repair[1] and cartilage repair[2,3]. However, by far the most frequent clinically use has been the treatment of overstrain-induced injuries of tendons in horses. We will discuss the hypothesis that also soluble factors and extracellular vesicles, also called microvesicles (MVs), released by MSCs may have a relevant regenerative potential and may open new therapeutic perspectives.

The paracrine effect of stem cells

Increasing experimental evidence indicate that the active factors exert effects on neighbouring cells. Indeed, MSCs express high levels of transcripts of hematopoietic stem cells maintenance factors, including CXCL12 chemokine, stem cell factor, angiopoietin-1 (Ang-1), interleukin-7, vascular cell adhesion molecule 1 and osteopontin[4]. Support for the hypothesis of paracrine action of MSCs derives from *in vivo* studies indicating that, although MSCs exhibit multilineage differentiation potential and can migrate to injured sites after systemic administration, the differentiation of MSCs in cells of injured tissues contributed little to their therapeutic benefits. A growing number of evidence indicates that the *in vivo* effects of MSCs depend primarily on their capacity to secrete bioactive soluble factors. This bioactive molecules may inhibit fibrosis and apoptosis, enhance angiogenesis, stimulate mitosis and/or differentiation of tissue-intrinsic progenitor/stem cells[5] and modulate the immune response[6].
In different pre-clinical animal models, MSCs administration have been shown to improve perfusion and restore cardiac function after myocardial infarction[7]; MSCs accelerates recovery in acute kidney injury (AKI) induced by toxic agents or ischemia reperfusion and induces functional improvement in chronic kidney disease[8-13]. In addition, MSCs have been studied in several *in vivo* models

of lung disease[14,15]. For example, in the bleomycin induced lung injury and fibrosis, MSCs improve lung inflammation and survival when given intravenously. These effects are not accounted to lung engraftment rates (< 5%) but rather to a paracrine mechanism[16].
The beneficial effects of MSCs infusion in different animal models are interpreted as not dependent on a direct substitution of injured cells, but rather on paracrine effectors that facilitate endogenous repair processes. In this way, a paracrine role of MSCs in renal tissue repair has been supported by experiments showing that conditioned medium (CM) from MSCs mimics the beneficial effects of the cells of origin, when intra-peritoneal injected in mice with cisplatin induced AKI[17]. Moreover, intravenous administration of CM from MSCs induces significant survival improvement in fulminant hepatic failure[18,19].
MSCs have been also investigated as a new therapeutic strategy for graft-versus-host disease, Chron's disease and for the prevention of organ transplantation rejection. The mechanism by which MSCs modulate the immune response is still under investigation, but it is evident that it involves also the release of soluble factors and not only the cell-to-cell contact. MSCs may suppress several T-lymphocyte activities both *in vitro* and *in vivo* and may alter the cytokine expression profile of dendritic cells (DCs), naïve and effector T cells and natural killer cells (NK) to induce a more anti-inflammatory or tolerant phenotype and to increase the proportion of regulatory T (Treg) cells. Prostaglandin E2 (PGE2) is implicated in the immunomodulatory effects of MSCs. Indeed, PGE2 production is up-regulated after co-culture of human MSCs with peripheral blood mononuclear cells[20] and the inhibitors of PGE2 production diminish MSC-mediated immunomodulation *in vitro*[21]. Indoleamine 2, 3 deoxygenase (IDO), PGE2 and TGF-?1 can represent relevant mediators of MSC inhibition of NK functions[21-23]. MSCs also secrete IL-6, that is involved in the reversion of maturation of DCs to a less mature phenotype[24]. Blockade of PGE2 synthesis in MSCs reverts the inhibitory effects on DC differentiation and function. PGE2 and IL-6 can mediate the effects of MSCs on DCs, thus leading to T-cell suppression[25].

Regenerative medicine and tendinopathies

Tendon repairs are often weak and susceptible to re-injury. Given the frequency and increasing cost of these injuries, mainly in sport horse, as well as the relatively poor result of surgical intervention, it is not surprising that new and innovative strategies like tissue engineering have become more appealing.
Several lines of evidence suggest that multipotent stem cells are present also in tendons and ligaments. First, both human and mouse tendons develop fibrocartilage and ossification in response to injury[26,27]. Second, tendon-derived immortalized cell lines or human tendon derived fibroblasts express genes of adipogenic, osteogenic and chondrogenic differentiation pathways, suggesting that they possess multiple differentiation capacities *in vitro*[28,29]. Finally, postnatal stem cells capable of differentiating into adipocytes and osteoblastic cells have been identified in human periodontal ligaments[30] while human and mouse tendons harbor a unique cell population, termed tendon stem/progenitor cells (TSPCs), that has universal stem cell characteristics such as clonogenicity, multipotency and self-renewal capacity[31]. Recently, Lovati et al.[32] identified TSPCs specifically in the horse SDFT with the ability to be highly clonogenic, to grow fast and to differentiate in different induced cell lineages as well as bone marrow derived progenitor cells (BM-MSCs). The hypothesis that TSPCs possess a mesenchymal stem cell behavior opens a new prospective for tendon regenerative medicine approaches because TSPCs could represents an important tool to study basic tendon biology. The exact site for TPSCs cells within tendon is not known, but they are most likely to reside in the endotenon tissue between the collagen fascicles and adjacent to the vasculature[33]. Although this might be true in young growing tendon, mature equine tendon, however, does not appear to possess a substantial subpopulation of these cells capable of differentiating into multiple cell lines, as reported for adult tissue[34,35], and this may explain why this component of the repair process is limited and hence natural repair is inferior to normal tendon.
During the repair process, there is a large influx of cells into the lesion. Kajikawa et al.[36] showed that at 24 h after the injury, the wound contained circulation-derived cells but not tendon-derived cells. Tendon-derived cells appeared in the injured area at 3 days after the wound, and significantly increased in number with time and maintained a high level of proliferative activity until 7 days after the injury, whereas the circulation-derived cells decreased in number and are replaced by the tendon-derived cells. These findings suggest that circulation-derived and tendon-derived cells contribute to the healing of tendons in different periods as part of a biphasic process but that the cells mainly involved in the synthesis of new tissue are believed to be tendon derived cells[36,37]. For this reason some authors hypothesized that the implantation of far greater numbers of progenitor stem cells, than are present normally within tendon tissue, would have the potential of regenerating or improving the repair of the tendon. Fibroblasts derived from tendon or other sources could be used[38], but the removal of sections of tendon to recover cells leads to the formation of a secondary lesion in the horse that is unacceptably. Alternative cell sources under investigation (Tab. 1) include dermal fibroblasts, which were shown to be capable of functionally bridging a tendon defect and to have similar histological and tensile properties to the tenocyte-seeded scaffold[39] although *in vitro* these cells behave differently from tenocytes[40]. By contrast, an optimal *in vivo* regenerative response could be accomplished by MSCs of different sources (Tab. 1).

Stem cell therapies in tendons

MSCs have been implanted into surgical defects in tendons in multiple *in vivo* experiments in laboratory animals with mostly positive outcomes. Most of these models have used surgically created defects in rabbit or rat tendons and have variously shown some improvement in

Table 1 - Sources for cell therapy of tendinopathies.

Cell source		Advantages	Disadvantages	Ref
EMBRYO	**Embryonic stem cells (ESC)**	- pluripotent	- teratoma formation	[88]
EXTRA-FETAL TISSUED	**Amnion-derived cells**	no invasive collection high plasticity and proliferative capacity high number of immediately available cells for therapy well-tolerated by horses	- strict surveillance of parturition	[72]
	MSCs from umbilical cord tissue	- no invasive collection - greater multipotent than BM-MSCs - possibility to obtain more rapidly proliferating cells by cell sorting - no immune response	- strict surveillance of parturition	[89-90]
ADULT TISSUES	**Concentrated bone marrow aspirate (BMC)**	- minimal manipulation - no cell expansion	invasive aspiration procedure with risk of pneumopericaridium no reports on the use of BMC on tendonitis	[91]
	Stromal vascular fraction from adipose tissue	- minimal manipulation - no cell expansion - well-tolerated by horse	- invasive collection	[92]
ADULT STEM/PROGENITOR CELLS	**MSCs from bone marrow (BM-MSCs)**	**-** multipotenti - no immune response	- invasive aspiration procedure with risk of pneumopericaridium - limited potential than ESC in terms of expansion (delay of 2-4 weeks to obtain a sufficient number of cells to *in vivo* implant)	[93-95]
	MSCs from adipose tissue	- higher proliferative potential and less senescence of BM-MSCs - multipotent	- invasive collection	[94-97]
	Tendon stem/progenitor cells	- possible activation of this endogenous population - multipotent	- invasive collection (removal of sections of tendons leads to the formation of secondary lesion) - mature equine tendon do not posses a substantial population of these cells	[31]
ADULT DIFFERENTIATED CELLS	**Tenocytes**	- appropriate tendon matrix synthesis	- invasive collection - age-related reduction in synthesis of matrix ability	[38]
	Fibroblasts derived from tendon	**-** appropriate tendon matrix synthesis	- invasive collections	[37]
	Dermal fibroblasts	- easy to recover, with acceptable donor site lesion - similar histological and tensile properties than tenocyte	- different protein-matrix synthesis than tenocytes	[39]

structure and strength of defects implanted with MSCs in a biodegradable scaffold (collagen gel, Vicryl knitted mesh or fibrin glue) compared to controls implanted with just the scaffold, as assessed by histology or simple biochemical assays[41-45]. In other studies using a rat patellar defect model, MSCs implantation has been associated with both greater ultimate tensile stress and improved quality of reparative tissue determined by an increased collagen I/III ratio[46,47]. Thus, MSCs-seeded constructs implanted *in vivo* have shown the ability to integrate into the tissue and induce the synthesis of tissue-specific extracellular matrix. In the horse, tendon injuries are mostly located in the superficial digital flexor tendon (SDFT), which represents the strongest tendon in the equine body. The SDFT displays several similarities to the human Achilles tendon concerning anatomy, biomechanics and pathogenesis of tendinopathy. In different species, pathomorphology of tendinopathy differs in lesion size. In the horse, one typical so-called "core lesion" is usually centrally located within the tendon, extended in length and still surrounded by intact tendon tissue. The equine SDFT injury lends itself to cell therapy because provide many of the additional elements required for tendon tissue engineering. The lesion manifests within the central core of the tissue provides a natural enclosure for implantation that, at the time of stem cell implantation is filled of granulation tissue, which acts as a scaffold (Fig. 1)[48]. This enables the application of MSCs without any artificial scaffold material, merely by injecting a cell suspension directly into the lesion[49]; thereby, MSCs are exposed to a natural environment providing collagen fibers and growth factors. In addition, during rehabilitation with controlled exercise, there is an ideal mechanical stimulation allowing the newly created tissue to organize itself in the direction of the force application, hence this approach can be referred to as "*in vivo* tissue engineering"[50]. Unfortunately, in the horse, the efficacy of these treatments is difficult to determine, since the use of control animals is rarely reported and often the stem cell treatment is combined with other biological factors, such as bone marrow supernatant, autologous serum, or platelet-rich plasma. In any case, since this treatment regime was first published in 2003[49] there have been several experimental and clinical studies with encouraging results, giving evidence of the benefit and safety of MSCs application for tendon regeneration. Furthermore, unfortunately, it is still unclear whether the major contribution of the MSCs to the healing process is to differentiate into tenocytes and thus produce extracellular matrix molecules, whether it is rather to supply growth factors and thus stimulate the residing cells within the tendon[51,52] or whether a combination of the two mechanisms occurs[6,53]. Mononuclear cells could represent an exogenic stimulus for induction of pro-inflammatory mediators in tendon[54]. In addition, recent studies have suggested an anti-inflammatory role of implanted stem cells. In this context animal model studies have demonstrated that MSCs are hypo-immunogenic and inhibit the activation of T and B lymphocytes and NK cells[55,56]. The precise mechanism of the anti-inflammatory effect of these cells is largely unknown. The role of soluble factors and extracellular vesicles as effectors in paracrine effect is described below. In essence, the paracrine effect results in the combination of different, biological activities: anti-apoptosis, additional recruitment of resident multipotent stem cells, stimulation of angiogenesis, and the release of growth factors[48].

The clinical and not experimental nature of the use of MSCs for horse tendinopathies preclude the routine *post mortem* analyses but some experimental works has been carried out to monitor the fate of injected MSCs in horses and the structural aspect of the healing. Guest et al.[57] studied the fate of autologous and allogeneic MSCs transfected with green fluorescent protein (GFP) following injection into the SDFT and revealed that GFP labeled cells located mainly within injected lesions, but with a small proportion integrated into healthy tendon. Furthermore, the authors showed that both autologous and allogeneic MSCs may be used without stimulating an undesirable cell mediated immune response from the host. Other postmortem examinations have shown that MSCs application improved the extracellular matrix structure of damaged tendons. In histological sections of MSC-treated tendon lesions, compared to non-treated tendon lesions, increased tendon fiber densities, increased organization

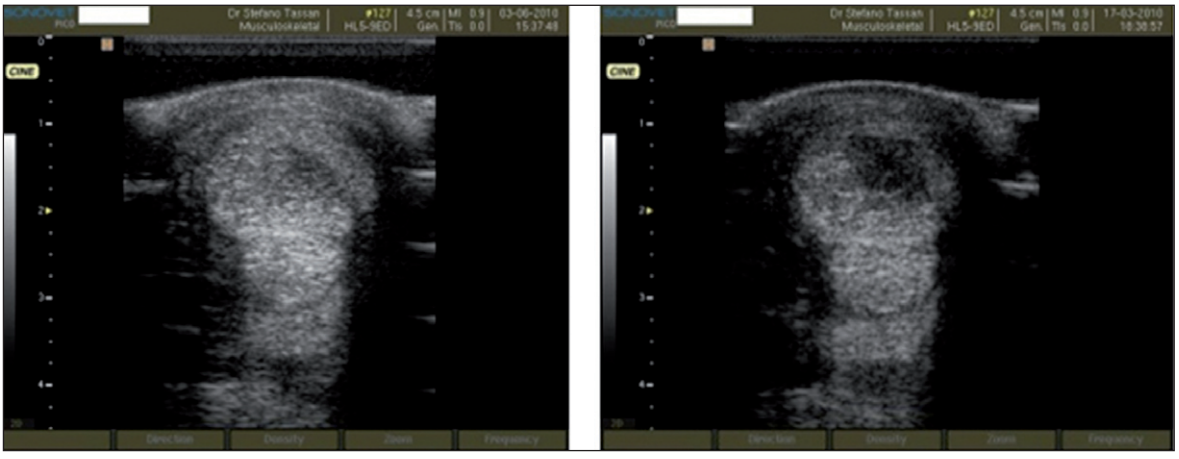

Figure 1. Severe SDFT core lesion in a forelimb SDFT. Arrows show anechoic area in transverse (A) ultrasound scans, and slightly ipoechoic area in transverse (B) ultrasound sections, respectively, in the same lesion 50 days after amniotic derived cells implant.

of the collagen fibers and a reduced vascularity have been found[58-60]. The beneficial effect of MSCs seems to be due to the improvement of structural organization rather than of matrix composition. However, it has been shown that MSCs treatment can enhance expression levels of cartilage oligomeric matrix protein (COMP)[58,59], a glycoprotein that is known to be important for tendon elasticity and stiffness[62]. Ultrasonographic follow-up examinations showed significant improvements in fiber alignment and echogenicity scores at 1, 3 and 6 months after MSCs treatment[63], supporting the histological findings in the above-mentioned studies. In these studies, autologous adult progenitor cells have been used, either expanded bone marrow-derived MSCs[60,64-66], or adipose derived MSCs[59,67] or adipose-derived mononuclear cells (ADNCs)[58,68]. Furthermore, the effects of autologous bone marrow derived expanded MSCs and bone marrow-derived mononuclear cells on tendon healing have been compared revealing a similar improvement, in both treatment groups compared to the control group, which was demonstrated by significantly improved ultrasonography and histology scores, higher COMP expressions and relatively lower type III collagen contents[61,70].

If stem cells are truly immunomodulatory, allogeneic transplantations should be possible. Safe and efficacious applications of allogeneic stem cells would imply that off - the-shelf stem cell products could be developed for increased availability and rapid implementation of stem cell therapies early in a disease course[54]. Indeed, not only autologous progenitor cells but also allogeneic bone marrow-derived MSCs[57], allogeneic adipose-derived MSCs[67] and allogeneic amniotic derived MSCs[72] have been applied for treatment of equine tendon injuries and no evidence of immune rejection were detected.

Extracellular vesicles released from MSCs as an emerging paracrine mechanism

Recent studies have shown that beside soluble factors small vesicles released from cells, named extracellular vesicles or MVs, are instrumental in cell-to-cell communication[73,74] (Fig. 2). MVs are an heterogeneous population of small vesicles constituted by a circular fragment of membrane containing cytoplasm components which are released by different cell types. The two major classes of MVs released in the extracellular environment are the exosomes and shedding vesicles[75]. Exosomes originate from inward of endosomal membrane, accumulate within multivesicular bodies, are secreted by a process of exocytosis and exhibit a 30-120 nm size. At variance, shedding vesicles take place from direct budding of plasma membrane surface and are more heterogeneous in size ranging from 80nm to <1mm depending from the cell of origin and on stimuli[75]. The released MVs can be up-taken by neighbouring cells either as result of surface receptor mediated interaction or by a process of membrane fusion. After interaction MVs can be internalized by the recipient cells and deliver their content[73,74]. Therefore, MVs have been uncovered as a new mechanism of inter-cellular communication that involves direct receptor mediated stimulation of the target cells and delivery of bio-active lipids, proteins and nucleic acids. The content of MVs and their biological action not only depends on the cell of ori-

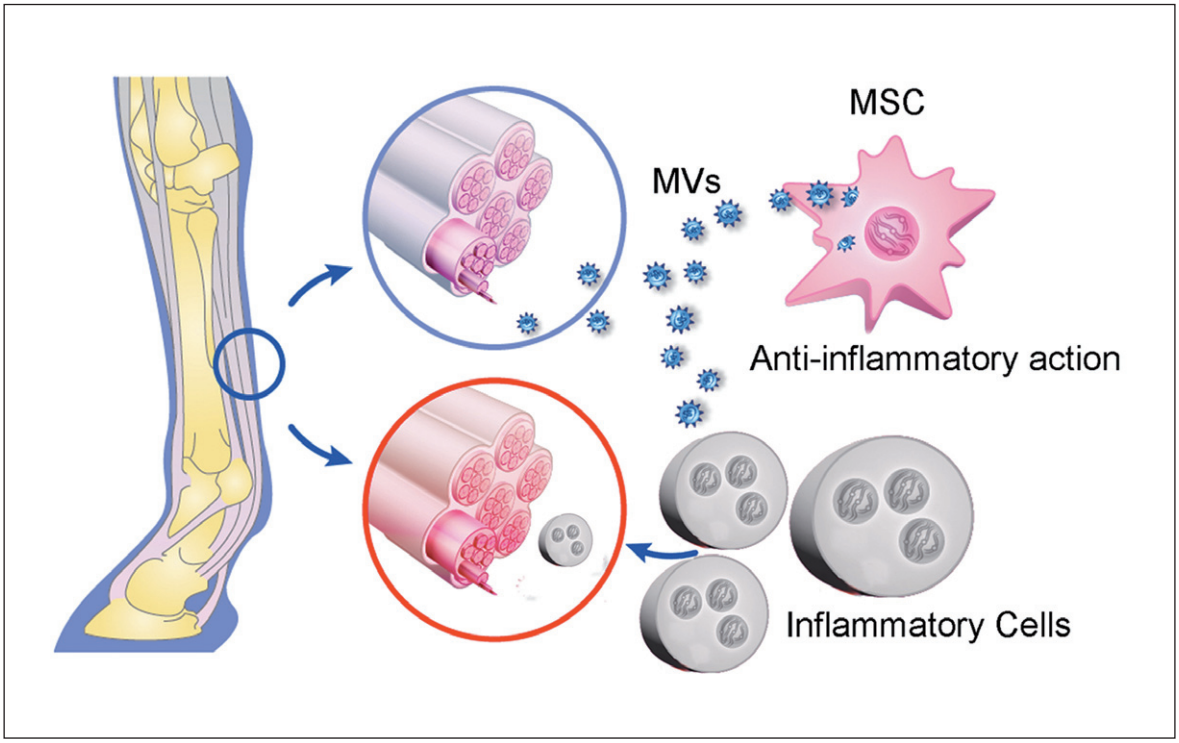

Figure 2. Schematic representation of the potential anti-inflammatory action of microvesicles (MVs) released by mesenchymal stem cells (MSC) on horse tendon.

gin, but also on the metabolic state of the cells. Therefore, different stimuli may modify not only the amount of MVs release, but also their content. One of the most exiting findings is that MVs were found to be a vehicle for exchange of genetic information capable to induce transient or permanent phenotypic changes in the recipient cells[73,74]. This observation has deep implications in different physiological and pathological conditions. In the contest of stem cell biology it has been suggested that signals shuttled by MVs are an integral component of the stem cell niche and may be critical in the differentiation decision of stem cells[76]. In particular, the signals between injured cells and stem cells are bi-directional[73]. Indeed, MVs derived from injured cells are able to induce tissue specific differentiation of bone marrow cells and MVs derived from stem cells are capable to activate regenerative programs in cells survived to injury. The first possibility is proved by the observation that MVs released from injured lung cells induce expression of specific lung transcripts and phenotypic changes in bone marrow cells[77]. The horizontal transfer of genetic information from stem/progenitor cells to differentiated cells was firstly shown for MVs derived from human endothelial progenitors (EPC). These MVs shuttle mRNA to quiescent endothelial cells *via* interaction with specific adhesion molecules (?4- and ?1-integrins) and activate an angiogenic program[78]. The molecular analysis of mRNA indicate that MVs derived from EPC contain specific subset of cellular mRNA, including mRNA associated with pathways relevant for angiogenesis such as the *PI3K/AKT* and eNOS signalling pathways[78]. This mRNA are functional as are they are translated into proteins within the recipient cells. Besides mRNA, MVs may transfer microRNAs (miRNAs) to target cells[79]. Since miRNAs are naturally occurring regulators of protein translation, this observation opens the possibility that stem cells can alter the expression of gene products in neighbouring cells by transferring miRNAs contained in MVs[80].

Concerning the regenerative potential of MSC-derived MVs experiments have been performed in different animal models of tissue injury[81-85]. In models of acute renal injury MSC-derived MVs were found to be able to mimic the beneficial effects of the cells. In particular MVs accelerate the recovery in models of toxic and ischemia-reperfusion injury of the kidney and significantly enhance survival in a lethal model of cisplatin induced acute renal injury[81,82]. The mechanism was related to the delivery of mRNA derived from the MSCs and to its translation in the recipient cells. Through this mechanism MSC-derived MVs can limit the injury by inhibiting apoptosis and stimulate regeneration by inducing cycle re-entry of injured tubular epithelial cells. Therefore, the recovery for acute renal injury promoted by MSCs, mainly take place from the renal resident cells that undergo transient de-differentiation, proliferation to reconstitute the loss cell mass and finally re-differentiation. Similar results were observed in a model of ischemic hearts treated with MVs derived from embryonic MSCs[84,85].

Based on these observations, we can speculate that MVs released from MSCs may act also in different context of regenerative medicine such as the tendinopathies (Fig. 2). MVs, released by MSCs, may interact with and stimulate tendon-resident cells to initiate an anti-inflammatory, anti-apoptotic and angiogenic response, and to reprogram somatic cells toward a regenerative response. In particular, MVs derived from MSCs may counteract the action of inflammatory cells accumulated at the site of injury.

Perspectives

In recent years, regenerative medicine has emerged as an attractive field for new cellular and non-cellular approaches to tissue repair. The current knowledge on the mechanisms of the regenerative potential of MSCs put attention on the role of soluble components released by cells in the conditioned media. Soluble components, or growth factors, are used indirectly in equine medicine, as before discussed, in cases where stem cells are combined with platelet rich plasma, bone marrow supernatant, or autologous serum.

Growth factors are peptide signaling molecules that regulate many aspects of cellular metabolism including the cell cycle, cell growth and differentiation, and the production and destruction of extracellular matrix products. Their effects are mediated primarily via autocrine and paracrine mechanisms, which provides the rationale for local administration of exogenous growth factors to influence cellular metabolism[59]. Of the growth factors influencing tendon metabolism, platelet derived growth factor, insulin-like growth factor-I (IGF-I), and transforming growth factor show the most promise for enhancing tendon healing[86]. Although exogenous IGF-I has been shown to stimulate tendon healing *in vivo* in an equine model[86] it has a short half-life, which necessitates repeated dosing, making clinical application challenging and costly. For this reason Schnabel et al.[59] examined the effects of MSCs, as well as IGF-I gene enhanced MSCs (AdIGF-MSC) on tendon healing *in vivo* showing that both MSC and AdIGF-MSC injection resulted in significant histological tendon healing with minimal added value of IGF-I gene-enhanced MSC implantation compared to native MSC injection. This minimum added value would confirm the hypothesis that in itself the stem cells secrete growth factors and that the therapeutic effects of MSCs are mediated by paracrine factors secreted by the cells to stimulate the residing cells within the injured tissue rather than differentiate themselves. These paracrine factors could be exploited to extend the therapeutic possibilities of MSCs for the treatment of a variety of diseases. In this context MVs have a potential therapeutic application, as they mimic several of the biological actions of stem cells and may limit the concern of using of active replicating cells that may undergo mal-differentiation or mutation. In addition, MVs may be engineered to express and deliver molecules that favor reprogramming of resident cells toward regeneration.

Conclusions

Use of the cells and technologies presented here in the horse are likely to continue and expand in the near future. The horse has been advocated as an animal model of

tendon and ligament injuries, since many of the spontaneous injuries seen in horses are similar to those seen in human athletes but other equine tissues and diseases, such as recurrent airway obstruction (asthma) and various hypoxic ischemic injuries, seem like straightforward candidates for equine stem cell research.

It is hoped that experience gained from treating naturally-occurring tendon injury in horses will provide sufficient supportive data to encourage the translation of this technology into the human field where large randomized controlled trials will lead to a higher level of clinical evidence[87].

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