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Canine Mesenchymal Stem Cells Sources, Properties, and Regulations for Clinical Use

1.1 Introduction

The stem cell concept is fundamental to biology that tries to understand the development of life and the formation and function of various tissues and organs. This concept stands at the forefront of 21st-century research and holds promise for groundbreaking advancements in biology and medicine. Stem cell research is anticipated to open new horizons, offering insights into the intricate processes of life and presenting innovative solutions in healthcare. The stem cell concept sees its inception in embryology (Gugjoo 2020). An individual, right from its initial developmental phase as a zygote to its maturity, harbors varied stem cell types. In dogs, prenatal development occurs in three phases/periods, namely, ovum period (2–17 days), embryo period (19–35 days), and fetal period (35th day to birth). In the ovum period, fertilization is followed by the development of the blastocyst that attaches to the uterus. The embryo period begins with blastocyst implantation till organogenesis is completed. Finally, characteristic features of an individual and its growth occur in the fetal period (Phemister 1974). In the early developmental stage, the potential of the primitive cell (zygote) up to the morula stage, is to develop the whole organism along with the fetal membranes (totipotent) (Fig. 1.1). As the developmental stages progress, this differentiation potential invariably decreases and the cells either become restricted in their differentiation potential or are terminally differentiated. Blastocyst that comprises inner cell mass and trophoblast has the potential to form an individual supported by fetal membranes. At this stage, specification is well established as the inner cell mass (harbors embryonic stem cells, ESCs) gives rise to an individual without its fetal membranes (pluripotent) while the trophoblast develops into fetal membranes (multipotent) (Gugjoo 2020). Contrary to this, zygote or cells in the morula give rise to the whole organism and its supporting membranes without any

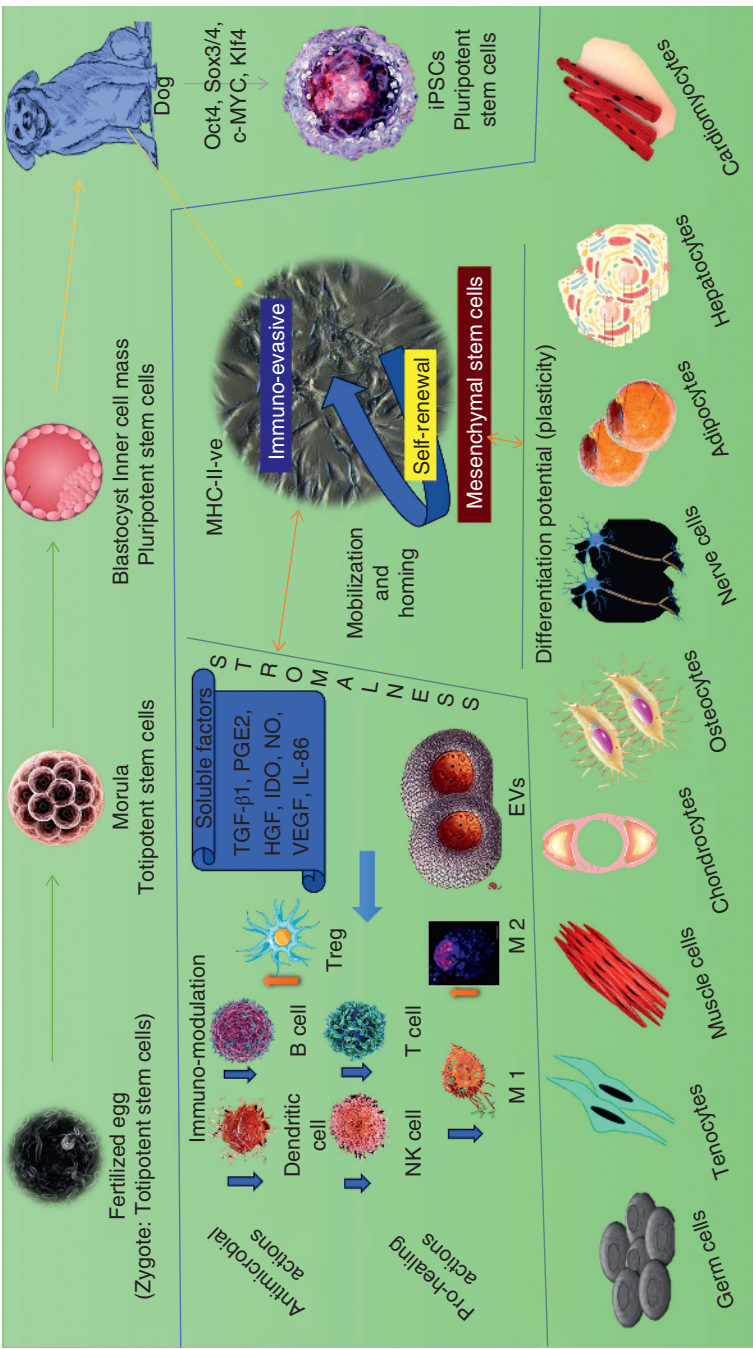


Fig. 1.1 Sources of stem cells from initial fertilization to the adulthood of dogs. The stem cells demonstrated are totipotent (zygote/morula), pluripotent (embryonic stem cells/induced pluripotent stem cells), and multipotent (mesenchymal stem cells). The image also depicts stromalness and differentiation properties of mesenchymal stem cells.

specification. Once the individual is born, there is a reserve cell pool in each tissue/organ that has the potential to maintain homeostasis. These adult stem cells are mostly multipotent and, in some cases, show features of pluripotency. In a particular organ/tissue, these adult stem cells get destined toward a particular lineage and the progenitor cells developed thereafter are oligopotent, which further get destined to a particular cell type (unipotent) and finally differentiate into a specialized somatic cell. Apart from the naturally available stem cells, somatic cells are also dedifferentiated into the stem cells through incorporation of the specific self-renewal and pluripotency maintenance markers (Oct4, Sox3/4, c-MYC, and Klf4). These adult cells induced to have pluripotency features are termed as induced pluripotent stem cells (iPSCs) (Gugjoo et al. 2020a, b, c) (Fig. 1.1).

Stem cells are being used for drug testing, understanding the development of an individual (embryology), and more recently for therapeutics. Because the focus of this book is on clinical applications of stem cells in canine practice, this chapter shall focus on the therapeutic properties of mesenchymal stem cells (MSCs) vis-à-vis available dog literature. ESCs or iPSCs have teratogenic potential as these cells tend to differentiate spontaneously. Additionally, ESCs have ethical issues of embryo destruction, and above all, the clear understanding of their physiological processes is still limited. Despite the challenges, there is decent literature on the utilization of ESCs or iPSCs for therapeutics in humans. However, in dogs such literature is very limited. This may be due to the above-cited reasons; besides, the supporting infrastructure is limited and the cost of treatment with such cells remains less economical. Contrarily, adult stem cells have numerous sources, easy isolation processes, and cultivation. Besides, the teratogenic potential and ethical issues are less. Among these adult stem cell types, MSCs are seen as the most promising stem cells due to their broader sources, ease of isolation and proliferation, limited chances of teratogenicity, and ethical issues, besides their therapeutic properties (Zuk et al. 2002; Cardoso et al. 2017). As such from here on focus shall remain on MSCs.

1.2 Understanding the Stem Cell

The stem cell is an unspecialized cell of a multicellular organism that has stemness property characterized by an ability to develop its identical copy through the “self-renewal” and a specialized destined cell through the “differentiation” (Till et al. 1964; Ab Kadir et al. 2012; Gugjoo et al. 2020d, e). Therefore, the cell that self-renews its pool and differentiates into particular lineage as per the microenvironment/niche is a stem cell. Self-renewal property differentiates stem cells from progenitor cells as the latter cells are unable to self-renew but do differentiate. Self-renewal is maintained through divisional symmetry or asymmetry. Symmetric division involves the production of identical copies in which daughter cells copy the entire

genome and epigenetic changes of the parent cell. The asymmetric division produces nonidentical daughter cells through divisional asymmetry or environmental asymmetry (Wilson and Trumpp 2006; He et al. 2009). Divisional asymmetry produces one similar/identical and another nonidentical copy. This occurs when the cell fate determinants get reorganized prior to the cell division. In environmental asymmetry, microenvironment/niche-based signals induce cell division producing identical copies. One of the identical daughter cells undergoes asymmetric cell division producing nonidentical daughter cells that function as per the demand of the local tissue (Spradling et al. 2001; Ohlstein et al. 2004). Such tissue-specific cell production is actually the differentiation process (pluripotency/multipotency) of stem cells. The microenvironment/niche maintains cell self-renewal and simultaneously as per the signals activates cell division for differentiation as well. With the loss of microenvironment, stem cells lose their self-renewal properties (Li and Neaves 2006; Lilly et al. 2011).

The self-renewal potential of the cells varies with ESCs/iPSCs able to maintain growth and proliferation for longer periods while MSCs tend to have limited self-renewal properties. Cancer cell lines are considered to grow indefinitely due to their continuous self-renewal ability. There is little understanding in this regard, although some of the known features of the cells are attributed toward such a property. Telomerase, an enzyme that maintains telomeres (DNA regions at chromosomal ends) activity, remains higher in stem cells including MSCs and might be responsible for self-renewal (Greenwood and Lansdorp 2003). Nonetheless, all these stem cells express elements of various pluripotency signaling pathways such as Wnt/fzd/beta-catenin, sonic, and Hedgehog and overexpress proteins such as homeobox 4 (Hoxb4) and caudal type homeobox 4 (Cdx4) (Watt and Hogan 2000; Kyba et al. 2002; Willert et al. 2003). It is worth noting that the self-renewal and differentiation properties are exhibited by tumor cells as well. The possible reasons for pluripotent ESCs to self-renew for indefinite periods may be due to their specific origin and developmental stage, tightly regulated genetics and epigenetics, and highly controlled environment. Apart from these two above-mentioned features, numerous other specific features make stem cells including MSCs a key-note topic for research and therapeutics. The subsequent part of this chapter shall specifically focus on various aspects of MSCs.

1.2.1 MSCs Stemness or Stromalness?

Stemness of stem cells pertains to their ability to self-renew and differentiate, while simultaneously preserving crucial differentiating genes in a state of quiescence and equilibrium. In the case of MSCs, which are a type of adult stem cell, the stemness is characterized by the capacity to undergo self-renewal and differentiate into multiple cell lineages such as adipocytes, chondrocytes, and osteoblasts.

These cells lack spontaneous differentiation as commonly seen with ESCs. Despite being recognized, the understanding of these properties is limited, and there is skepticism about the existence of true stemness properties in the cells. MSCs cell cycle is regulated in such a way as to balance self-renewal with differentiation. Compared to pluripotent stem cells, MSCs genetic and epigenetic regulation involve use of different transcription factors (Notch, Hedgehog, and bone morphogenetic protein, BMP, pathways) and growth factors [fibroblast growth factor (FGF) and transforming growth factor-beta (TGF- β)] to regulate self-renewal and differentiation. In adult tissues, the microenvironment is less tightly controlled as numerous physiological processes are undertaken and wide ranges of external stimuli arise. MSCs reside in an environment that supports their tissue repair and regeneration function. Exposure to varied stimuli eventually directs them to a state of senescence after a certain number of divisions. Stem cell-based therapeutics occur through a multitude of processes including self-renewal and differentiation. However, the higher the rate of their doubling, the earlier they lose these properties. In order to make maximum use of MSCs, it is important to understand their stemness and establish new techniques that can preserve multipotency for long.

MSCs stemness markers are supported by only a few genes while the molecular basis, especially the key transcription factors of their stemness, is poorly understood. Unlike ESCs, where pluripotent genes such as Oct4, Nanog, klf4, and Sox2 are well-established, MSCs lack a single key transcription factor. To understand factors involved in stemness, highly expressed genes in undifferentiated MSCs are compared with the trilineage differentiated cells. By knocking down each gene individually, no single transcription factor is involved in the complete blockade of the differentiation process and maintaining the stemness (Kubo et al. 2009). There is progressive research that tries to understand the stemness basis of MSCs. One of the studies shows that human MSCs positive for CD271 (low-affinity nerve growth factor receptor, LNGFR or p75 neurotrophin receptor, and p75NTR), CD90 (Thy1), and CD106 (vascular cell adhesion molecule-1, VCAM1) retains propensity of self-renewal and multipotency (Mabuchi et al. 2013). Two novel mechanisms, namely, scrapie responsive gene 1 (SCR1)/bone marrow stromal cell antigen-1 (BST1) ligand-receptor combination and cell-cell adhesion through N-cadherin, are considered to establish these properties (Chosa and Ishisaki 2018).

MSCs differentiate into various lineages to the extent of pluripotency in a specific *ex vivo* differentiation system (extended multipotency). However, *in vivo* studies in numerous cases show MSCs suboptimal performance with a limited engraftment rate raising concerns over the use of the word “stem” to these cells (D’souza et al. 2015). There are well-established biological functions that contribute to their therapeutic role and are attributed to their stromal functions rather than the multipotent differentiation (Phinney and Prockop 2007; Horwitz and

Dominici 2008; Murphy et al. 2013; Gugjoo et al. 2020a, c, d). MSCs stemness and proliferation properties were initially evaluated in the 1960s and 1970s that reached maturity in the 1980s and peaked in 2000 beyond which focus on such studies was reduced. Over a period of time, the consensus over the MSCs stromalness increased with simultaneous reduction in their stemness properties for their therapeutics (Fig. 1.1). Initially, the stromalness of MSCs is realized with the study demonstrating hematopoietic supportive role through release of various growth factors, chemokines, cytokines, extracellular vesicles (EVs), or microvesicles (Matthay et al. 2017; Lykhmus et al. 2019; Spano et al. 2019; Witwer et al. 2019). Down the line, these cells are termed as medicinal signaling cells changing the paradigm but keeping the acronym (Caplan and Correa 2011). Presently, the acronym of stemness and stromalness coexist although the latter property is preferably attributed to MSCs *in vivo* therapeutic benefits (Mastrolia et al. 2019).

Stromalness of MSCs is achieved through their secretome harboring diverse soluble factors and EVs (Maia et al. 2017). Soluble factors of MSCs secretome contain factors such as TGF- β 1 and prostaglandin E2 (PGE2), hepatocyte growth factor (HGF), indoleamine 2,3-dioxygenase (IDO), nitric oxide (NO), vascular endothelial growth factor (VEGF), and interleukin-68 (IL-68). These factors arise either constitutively or after priming with the pro-inflammatory mediators. MSCs priming with the specific microenvironment enhances their resistance and specific expression potential (Barrachina et al. 2017; de Cássia Noronha et al. 2019; Gugjoo et al. 2023). There is good evidence supporting tissue and species-specific variation in MSCs secretome (Carrade et al. 2012). There may or may not be comparable immunomodulation and the pathways involved may also vary. Canine BM-MSCs and AD-MSCs show comparable immunomodulation; however, involved pathways are different (Russell et al. 2016; Chow et al. 2017). EVs are classified as per their size, namely, exosomes (50–100 nm diameter) or microvesicles (0.1–1 μ m diameter). EVs carry a diverse range of factors ranging from proteins, organelles to genetic materials (DNA and RNA) (Manzoor et al. 2023). Presently the focus is shifting toward the use of these EVs as they remain devoid of stem cell-based limitations (immunogenicity, teratoma, and limited action in harsh environments). Besides, EVs are unaffected by the freeze-thaw process, do not require preservatives during cryopreservation, and are economical (Abraham and Krasnodembskaya 2020).

1.2.2 MSCs Plasticity

MSCs show limited ability to trans-differentiate in comparison to the ESCs/iPSCs. Nevertheless, their trans-differentiation occurs across the germ layers and is demonstrated in numerous studies from varied tissue sources in humans and animals including dogs (Fig. 1.1). MSCs trans-differentiation occurs when kept in specific

differentiation media, scaffolds, or mechanical forces. MSCs are at least evaluated for their tri-lineage differentiation in line with the International Society for Cellular and Gene Therapy (ISCT) for their therapeutic purposes (Dominici et al. 2006). However, the differentiation potential goes beyond to other lineages such as myocytes/cardiomyocytes, neural-like cells, and germ cell-like cells. MSCs trans-differentiation as per the available microenvironment/niche shows that their fate is not defined but rather changeable/plastic. It is worth mentioning that true trans-differentiation remains questionable as the cells down the lineage do not always undergo characteristic ontologic processes that are involved in the formation of a particular cell lineage (Pelagalli et al. 2018). MSCs not only are plastic in their differentiation potential but also show plasticity in immunomodulation and immunoevasion as per the available microenvironment (Wang et al. 2014; Russell et al. 2016; Wang et al. 2019). Pro-inflammatory mediators such as interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), and interleukin-2 (IL-2) variably activate dog MSCs immunomodulatory activities (Liu et al. 2006; Poncelet et al. 2007; Russell et al. 2016).

1.2.3 MSCs Mobilization and Homing

MSCs due to their ability to “mobilize” and “home” in the distant site make their peripheral transplantation quite a feasible option for therapeutics especially at less accessible sites. Presently studies are going on to understand the mechanisms involved in the process. MSCs are assumed to follow similar steps as those of the leukocytes. For a cell to migrate, its adhesiveness to the local milieu has to be waived off followed by its directional movement to the particular site or location. The steps involved in homing include endothelial contact of the cells by tethering and rolling, resulting in deceleration of the cells into the bloodstream. Subsequently, activation of G-protein-coupled receptors and simultaneous activation-dependent arrest occurs. Finally, the cells transmigrate through the endothelium and underlying basement membrane (Butcher and Picker 1996). For resident MSCs migration and homing, mobilization occurs by downregulation of their adhesion molecules initiated by cytokines and/or chemokines such as platelet-derived growth factor (PDGF) AB and BB, and HGF (Son et al. 2006; Ponte et al. 2007; Liu et al. 2009; Baek et al. 2011). This is followed by general principles applicable to resident and transplanted cells. In the initial step of mobilization, endothelial selectins and expression of CD44 on MSCs have an important role to play in the initial step (Sackstein et al. 2008). Activation of MSCs to mobilize occurs through G-protein-coupled receptors that are typically chemokine receptors. For BM-MSCs homing, CXCR4-stromal-derived factor-1 (SDF-1) axis is critical (Moll and Ransohoff 2010; Angelone et al. 2017). Integrins play an important role in stable activation-dependent arrest of MSCs. The integrins form dimers that bind with endothelial cell adhesion molecules. Very late antigen-4

(VLA-4), formed by combination of integrin $\alpha 4$ and $\beta 1$, interacts with the VCAM-1 and is demonstrated to be involved in MSCs homing (Rüster et al. 2006; Segers et al. 2006). Diapedesis or transmigration of MSCs through the endothelial cell layer and basement membrane is brought about by lytic enzymes such as matrix metalloproteinases (MMPs). Among various MMPs, MMP-2 and MMP-9 are shown to have an important role as these enzymes preferentially degrade basement membrane components (collagen and gelatin) (Nagase and Woessner 1999; Steingen et al. 2008). Finally, MSCs recruitment/homing into the inflamed and damaged tissues may be brought by adhesion molecules such as galectin-1 and galectin-3 (Reesink et al. 2017).

1.2.4 Anti-inflammatory and Immunomodulatory Actions

MSCs are immune-privileged/immuno-evasive and bring in the immunomodulatory and/or anti-inflammatory actions as dictated by the available microenvironment (Gugjoo et al. 2019a, b; Gugjoo et al. 2020a; Gugjoo and Pal 2020). Their immuno-evasive nature, ability to avoid immune rejection, arises out of a lack of expression of the major histocompatibility complex (MHC)-II and minimal expression of the MHC-I and co-stimulatory molecules. Immunomodulatory actions may occur either through cell-cell contact, by their secretome or constitutively. Cell-cell contact-mediated actions arise through various MSCs receptors such as toll-like receptors (TLRs), intracellular adhesion molecules (ICAMs), VCAM, and Fas ligand-dependent interactions (Tomchuck et al. 2008; DelaRosa and Lombardo 2010; Akiyama et al. 2012). Transwell assays in canines show MSCs ability to secrete soluble factors that inhibit the immune response of the lymphocytes (Kang et al. 2008; Lee et al. 2011). Secretome-based immunomodulation/inhibition seems more potent as compared to the cell-cell contact interactions (Di Nicola et al. 2002). These cells modulate $CD8^+ CD4^+ T$ and dendritic cells and disrupt natural killer cells to prevent T-cell responses (Di Nicola et al. 2002; Wang et al. 2009). MSCs interaction with the CD4 and CD8 lymphocytes is activated in the inflammatory environment that bring in the higher anti-inflammatory actions. MSCs co-cultured with peripheral blood-derived mononuclear cells (PB-MNCs) and macrophages or under pro-inflammatory mediators (PGE₂, lipopolysaccharide, interferon- γ , tumor necrosis factor- α , and IL-2) reduce mononuclear cells (MNCs) production of inflammatory mediators such as TNF- α , inducible nitric oxide synthase (iNOS), and interleukin (IL)-1 β (Singer and Caplan 2011; Russell et al. 2016; Chae et al. 2017; Lara et al. 2017; de Moraes et al. 2016).

1.2.5 Antimicrobial Actions

It is now a well-accepted concept that MSCs may prevent microbial infections as has also been reported in numerous studies including the dogs (Johnson

et al. 2017). Such an action occurs either indirectly (modulate local environment and/ or amplify immune cell function) or directly (eradicate microbes through antimicrobial peptides, AMPs) (Cortés-Araya et al. 2018; Taghavi-Farahabadi et al. 2021; Silva-Carvalho et al. 2022). MSCs and/or secretomes may also potentiate antibiotic actions and could be useful against the biofilms (Johnson et al. 2017). MSCs exhibit pattern recognition receptors (PRRs) such as TLRs that may recognize pathogen-associated molecular patterns (PAMPs). Presently, two PAMPs including lipopolysaccharide (LPS) and polyinosinic: polycytidylic acid [Poly I : C)] are studied in detail in humans while such literature on animals such as dogs is limited. One of the studies shows that stimulation of canine BM-MSCs TLR 3 activates the IDO/kynurenine pathway thereby promoting their immunomodulatory activities (Deng et al. 2022).

Priming of MSCs with PAMPs such as LPS or pro-inflammatory mediators such as TNF- α stimulates them to secrete AMPs (Harman et al. 2017; Cortés-Araya et al. 2018). Direct antimicrobial action of MSCs occurs through AMPs such as cathelicidins, defensins, lipocalcins, and elafins. In dogs, BM-MSCs-secreted AMPs are apolipoproteins B and D, amyloid- β peptide, cathepsin B, protein S100-A4, galectin 3, CLEC3A, granulins, and transferrin (Humenik et al. 2019).

1.3 Sources of Dog MSCs for Proliferation

MSCs in dogs are harvested from almost all adult tissues (adipose, bone marrow, cornea, dental pulp, endometrium, limbus, muscle, olfactory epithelium, ovary, periodontal ligament periosteum, and peripheral blood) and fetal membranes (placenta, omentum, umbilical cord blood, umbilicus, and Wharton's jelly) as reviewed by Gugjoo and others in their book (Gugjoo et al. 2020b) (Fig. 1.2). In dogs, fetal membrane-based MSCs are limited in pets. This arises as owners prefer elective castration of their pets in the juvenile phase (Seo et al. 2009; Sultana et al. 2018; Borghesia et al. 2019). However, reproductive structures such as ovaries and/or uterus may be utilized for the purpose (Sultana et al. 2018; Phyo et al. 2023). To have sufficient numbers of the MSCs, the tissue samples are harvested to isolate and culture expand them. From solid tissue such as fat (adipose tissue), stromal vascular fraction (SVF) is harvested after tissue digestion with enzymes such as collagenases while from liquid samples such as bone marrow, MNCs fraction is isolated by the density gradient method. These cells are thereby transplanted in the specific culture media with the addition of fetal bovine serum or serum-free media and other factors for expansion. There are other techniques such as the explant method for solid tissues or the whole bone marrow method for liquid bone marrow samples (Gugjoo et al. 2020b; Gugjoo et al. 2020c). Clinical applications of stem cells require good manufacturing

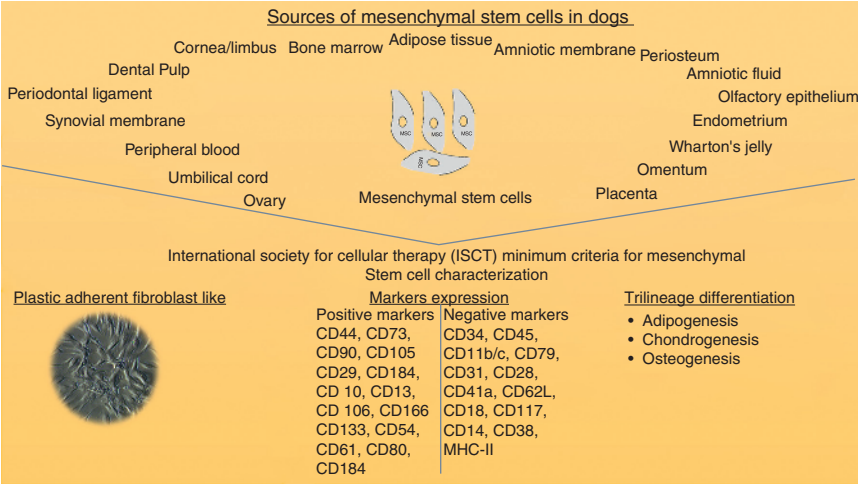


Fig. 1.2 Sources of mesenchymal stem cells and their characterization on the basis of the International Society for Cellular Therapy.

practices (GMPs) and good clinical practices (GCPs). These practices are to be followed during the entire process of tissue sample harvesting, cell isolation and culturing, and, finally, application (Gugjoo et al. 2019b). Fortunately, there is no dearth of GMP-compliant media and chemicals in the market (Bieback et al. 2008).

1.4 Why Do We Need to Culture Mesenchymal Stem Cells?

Natural donor tissues harbor MSCs in very minute and variable concentrations. This makes their cultural expansion a necessity. There is very limited concentration of MSCs to the extent of around 1 cell per 30K nucleated cells isolated from the iliac crest bone marrow aspirate (Muschler et al. 1997; Gugjoo et al. 2019a). To enhance cell concentration, one of the dog studies shows that 1.45 million, 2.33 million, 3.37 million, and 19.4 million MSCs are cultivated from each gram of the bone marrow, adipose tissue, muscle, and periosteum at 16 days culture period (Kisiel et al. 2012). Therefore, depending on the donor tissue, an approximate estimate of the cells available may be made. It is the key to remember that extended passaging/subculturing of the MSCs should be avoided due to their limited self-renewal potential. Therefore, the cells of low passage/subculture (preferably passage 3) are being used for therapeutics. It is also worth mentioning that the

cells from a particular source may show a predisposition toward a specific lineage and their secretome may also be variable (Gugjoo 2022). This may be dictated by the physiological status of the donor tissue (Dar et al. 2021).

1.5 Why to Characterize MSCs?

Donor tissue harbors variable cell populations and as such these cells are collected in toto when tissue sample is processed. Harvesting techniques utilized to collect the tissue sample from donor tissue/animals may also affect the cell population and their surface markers (DePompeo et al. 2019). After processing the liquid and solid tissue samples, MNCs fraction and SVF, respectively, are isolated and represent diverse cell types including a small fraction of MSCs. Canine MSCs tend to show variable expression of surface markers and their differentiation potential may also vary. This variation occurs across donors, tissue sources, location, and physiological status (Laura et al. 2008; Volk et al. 2012; Malagola et al. 2016; Bahamondes et al. 2017; Bearden et al. 2017; Bugueño et al. 2017; De Cesaris et al. 2017; Sasaki et al. 2018; Yaneselli et al. 2018; Rashid et al. 2021; Humenik et al. 2022). Not only the tissue source and age (Volk et al. 2012; Lee et al. 2017; Taguchi et al. 2019) but also the breed of the dogs matters (Bertolo et al. 2015). MSCs from younger dogs are more proliferative and express surface markers and have higher differentiation potential as compared to the older dogs (Volk et al. 2012; Bearden et al. 2017; Lee et al. 2017; Taguchi et al. 2019). Variable expression of surface markers might directly affect MSCs therapeutic potential. In dogs, hematopoietic markers such as CD34 and CD45 markers are uniformly not expressed while the expression of positive markers such as CD73 (ecto-5-nucleotidase), CD90 (THY1), and CD105 (endoglin) shows variability. CD73 plays a role in ensuring an anti-inflammatory environment; CD90 regulates cell–cell and cell–matrix interactions, cell adhesion, cell migration, cell apoptosis, T-cell activation, and fibrosis; CD105 is involved in cytoskeletal organization affecting cell morphology and migration (Teshima 2024).

MSCs from some of the dog breeds may have higher chondrogenic potential while others may show higher osteogenic differentiation potential (Bertolo et al. 2015). Further, under the *ex vivo* system, MSCs tend to undergo changes in their phenotype, chromosomes (genetic changes), and secretomes. This may be determined by the culture technique and the drugs or chemicals used in the culture process (Marycz et al. 2014; Guo et al. 2015; Jo et al. 2017; Kim et al. 2019; Scattini et al. 2023). As animal source supplements such as fetal bovine serum (FBS) have chances of infection besides, immune-related issues, alternatives such as serum-free media or autologous platelet lysate are being evaluated. One of the studies shows altered immune regulatory properties of dog BM-MSCs with the

serum-free media and therefore may be cautiously used (Clark et al. 2016). Serum-free medium incorporating specific growth factors may support the growth and proliferation of MSCs and their differentiation potential comparable to FBS (Devireddy et al. 2019). Autologous platelet lysate may be able to maintain cellular proliferation and viability and enhance their genetic potential for multi-lineage differentiation. However, the immunomodulatory potential of the cells is not demonstrated in these cells (Rashid et al. 2023). Extended culturing as mentioned above leads to altered cellular features and predisposes MSCs toward senescence (Martinello et al. 2011). It is worth mentioning that now-a-days, focus on specific markers is being given depending on the role of desired MSCs. For example, CD200 is considered to be a suitable marker for bone healing that MSCs must express when utilized for bone tissue engineering; CD44 is considered to be suitable for mobilization of MSCs and may be expressed in cases where MSCs are transplanted peripherally. There is a consensus from the Mesenchymal and Tissue Stem Cell Committee of the ISCT on some of the basic features of the MSCs that must be expressed. The guidelines put forth by the committee for MSCs characterization are that the cell should be plastic adherent and express surface markers such as CD90, CD73, CD105, CD106, and CD166 while simultaneously lacking hematopoietic markers such as CD45 and CD34. Besides, the cells must differentiate at least into adipogenic, chondrogenic, and osteogenic lineages (Dominici et al. 2006) (Fig. 1.2). It is pertinent to mention that the stored pool must harbor a higher percentage of the cells that are positive for these surface markers.

Apart from the specific MSCs features, it is very important to maintain sterility to prevent any untoward clinical reaction. As such endotoxin levels and infection-free cell cultures are to be maintained (Luo et al. 2021). Dogs must be screened for their common infections in order to consider them safe for transplantation. GMPs and GCPs are a must for their aseptic culture and transplantation, respectively (Rojewski et al. 2019). As MSCs undergo senescence with the extended culture, these cells are cryopreserved to maintain their viability and prevent any changes in their characteristic properties (Shahid et al. 2019). Specific formulations that include dimethyl sulfoxide (DMSO) and proline-based solutions are demonstrated to support MSCs viability and differentiation potential after cold storage (Horcharoensuk et al. 2022).

1.6 Factors Affecting MSCs Therapeutic Outcome

MSCs properties are affected by numerous factors such as donor, tissue source and its site, physiological phase, cell isolation technique and culture protocol, cryopreservation-thawing protocol, and clonal subpopulation (Dar et al. 2021; Dunn et al. 2021; Zhou et al. 2021; Ivanovska et al. 2022). General criteria set by

ISCT for human MSCs are commonly followed for dog MSCs as well. However, surface markers such as CD90, CD73, and CD105 are not strongly positive in all the cases (Gugjoo et al. 2019a). Considering the diverse actions these surface markers play such as cell–cell adhesion, migration, apoptosis, T-cell activation, cytoskeleton organization, and immune regulation, the variability in expression of these markers might determine clinical outcomes as well. Furthermore, MSCs may have variable properties depending on their site and source, besides the age of the donor as has been demonstrated in dogs. MSCs from different tissues or from the same tissue type at different locations and in different physiological phases of an individual may have different properties (Malagola et al. 2016; Bearden et al. 2017; De Cesaris et al. 2017; Sasaki et al. 2018). MSCs from younger donors often have better cell proliferation rates than the older ones (Volk et al. 2012; Guercio et al. 2013; Lee et al. 2017; Taguchi et al. 2019). These cellular properties are also affected by drugs such as anesthetics, antibiotics, and steroids and should therefore be considered while instituting the therapy (Marycz et al. 2014; Guo et al. 2015; Jo et al. 2017).

It is worth mentioning that there may be preponderance towards tumor formation as well. In addition to MSCs ability to suppress lymphocytes, these cells also support tumor-associated macrophages and promote angiogenesis. These cells tend to fuse with epithelial cells leading to epithelial-to-mesenchymal transition (EMT) (Gu et al. 2020; Liang et al. 2021). Add-on MSCs secretome harbors MMPs that tend to promote cancer cell growth and distant metastasis (Lee et al. 2013; Yang et al. 2015). Therefore, while instituting MSCs therapy, it is imperative to be cautious and continuously keep watching the underlying phenomenon. The mimicking of the intestinal stem cell organoid cultures may offer a useful option to understand MSCs mechanism of action in case of anal furunculosis (AF) and inflammatory bowel disease (IBD) (Kopper et al. 2021).

1.7 Stem Cell Regulations in Research and Therapeutics

Growing animal ethical considerations, human-companion animal bonds, pet insurance companies, animal-dependent companion support for aged and childless owners, expanding pharmacy for animals, and utilization of pets as experimental model animals for studies on animal drug testing make it extremely important to develop advanced therapeutic techniques and simultaneously devise ethical regulations for research and therapeutics. Furthermore, the one-health concept has increased the importance of the veterinary sector to provide healthcare-for-all aspects. Stem cell and/or stem cell-based products (SCPs), unlike small-molecule drugs, currently lack any standard safety test methods.

This arises as SCPs show substantial diversity based on their source, processing techniques, target species and ailments, and application sites. In relation to *ex vivo* production systems, manufacturers utilize variable sample sources and harvesting procedures, processing methods, assays, and equipment to develop a particular SCP. There is a tangible need to evaluate the SCPs through a more generalized means such as selecting donors and tissue samples, processing methods, validation assays, storage techniques, transportation, and delivery. Besides, an urgent need to understand the SCPs posology involving dose, route, and frequency is a must-have for developing standard practices (Petricciani et al. 2017).

Stem cells/SCPs including MSCs are included in the advanced therapy medicinal products (ATMPs) and are regulated by pharmaceutical rules (Oliver 2011; Gugjoo and Amarpal 2018; Gugjoo et al. 2018a, 2018b, 2019a). The stem cell-based therapeutics is started at a step when little of their physiology is understood. It is essential to have a regulatory framework established locally or globally as it has an important impact on the development and adoption of the drugs/devices/instruments. Because the therapeutic use of autologous MSCs with minimal manipulation comes under “regulatory discretion” of the Centre for Veterinary Medicine (CVM) of the Food and Drug Administration (FDA), the initial focus was put on these cells for commercialization. However, allogeneic MSCs or autologous differentiated or primed cells would require full drug approval (Harman 2015). In addition to the FDA, other standard organizations govern approval for SCPs and include the Australian Pesticides & Veterinary Medicines Authority, the European Medicines Agency for Veterinary Medicinal Products, and the Korea Food Drug Administration (KFDA). There is a growing market for animal stem cell therapy as a compound annual growth rate (CAGR) of 5.2% is projected for the next decade (2022–2032) as compared to the previous decade of 4.7%. Among various sources, the allogeneic source is most preferred and by the end of 2021, it accounts for around 66.9% of therapies. There is a major share of the animal stem cell market from North America, Europe, and East Asia. In South Asia, India has the maximum share. However, in India, there is no specific regulatory agency for animal stem cell therapy (<https://www.persistencemarketresearch.com/market-research/animal-stem-cell-therapy-market.asp>; <https://risingkashmir.com/a-new-era-in-veterinary-medicine-the-push-for-stem-cell-therapy-regulation-in-india/>). The guidelines are set but its implementation is currently under criticism.

Various companies offer kits to veterinarians to perform crude isolation and administration of stem cells at clinics. These kits remain to be approved by the FDA or any other agency as the claims are not supported by peer-reviewed clinical studies. These kits additionally make way for the sterility issues as clinical staff including practitioners and technicians are not trained in the cell biology of stem cell processing. Add-on some claim to provide activated cells which straightaway

violate current CVM policy (Oliver 2011; Boxer 2013; Norbert and Harman 2013; Harman 2015). Presently, stem cell-based therapy is under the research phase and is not a definitive therapy for any specific disease. However, stem cells, especially MSCs, are being used for varied clinical conditions as part of research for evidence-based stem cell medicine.

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