Chapter 2

Stromal Cell Ultrastructure

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Additional information is available at the end of the chapter

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Abstract

Regenerative medicine and tissue engineering therapies present an attractive treatment alternative to the current traditional clinical treatments. Stem cells are capable of self-renewal and multilineage differentiation. They also have the ability to create immunomodulatory microenvironment, and thus help to minimize organ damage caused by the inflammation and cells activated by the immune system. Human bone marrow mesenchymal stem/stromal cells (MSCs) have great potential for cellular therapy, as they possess the abilities to proliferate as well as to differentiate. MSCs are present in all tissues interacting with tissue cells and easy to isolate and expand in culture. Indeed, histological examination of MSCs is one of the main goals for studying their morphology. Both the light and the electron microscopes are essential tools where the histologist can identify the structure as well as the detailed ultrastructure of these cells. This will guide users to clearly understand their behavior, both *in vivo* and *in vitro.* Thus, the aim of this chapter is to give a spot of light on these cells and their histology.

Keywords: stem cell, stromal cell, biology, histology, ultrastructure

1. Introduction

Stem cells can be defined as undifferentiated cells that have the ability to self-renewal; proliferate into undifferentiated cells, and to differentiate into various mature specialized cells [1]. There are different types of stem cells that have been classified according to their potency. Cells are described as pluripotent that is, embryonic cells from the blastocyst (4–14 days after oocyte fertilization), they can differentiate into all cell types of the adult organism. If, in addition, they can form the extraembryonic tissues of the embryo, they are described as totipotent (1–3 days from oocyte fertilization) which can give rise to all the embryonic tissues and placenta. Multipotent stem cells that is, embryonic cells from the 14th day onward, have the ability to form all the differentiated cell types of a given tissue. The stem cells that maintain only one

lineage are described as unipotent [2]. In the trilaminar embryo, a middle mesodermal layer is formed between the ectodermal and endodermal cell layer. This mesodermal cell layer contains mesenchymal stem cells (MSCs), which develop into connective tissue (mesenchyme) and it maintains the progenitor stem cells that persist after birth [3].

2. Sources of stem cell

2.1. Embryonic stem cells

Embryonic stem cells (ESCs) have the greatest potential to differentiate into all cell types. ESCs are derived from the inner cell mass of the blastocysts. However, the use of ESC is associated with several ethical issues [4]. Also, safety concerns were raised with a high incidence of teratoma formation [5].

2.2. Induced pluripotent stem cells

Induced pluripotent stem cells (iPS) were first achieved by inducing a forced expression of specific genes that can reprogram human and mouse adult somatic cells into the undifferentiated cell [6, 7]. iPS have the same characteristics of ESCs, such as expression of pluripotency markers and differentiation capability [6].

2.3. Fetal stem cells

Fetal stem cells (FSCs) are derived either from a fetus or from extraembryonic structures. Various subtypes of FSCs were described according to their origin (i.e., amniotic fluid, umbilical cord, Wharton's jelly, amniotic membrane, and placenta). FSCs are ideal sources of cells for use in regenerative medicine. They are easily accessible, having a high proliferation rate. In addition, FSCs do not form teratomas [8] and overcome the ethical problem associated with ESCs [9].

2.4. Adult stem cells

In principle, adult stem cells are unspecialized (undifferentiated) cells. They are found in differentiated tissues and considered to be quiescent, but still capable of self-renewal and differentiation. These cells remain in their undifferentiated state until stimulated [10]. Adult MSCs have been isolated from different sites: bone marrow, adult peripheral blood, tooth pulp and liver [11].

3. Mesenchymal stem/stromal cells (MSCs)

3.1. History

The concept of mesenchymal stem/stromal cells (MSCs) was first introduced about half a century ago. In the 1970s, [12] Alexander Friedenstein described a population of bone marrow-derived cells of mesodermal origin. These MSCs were shown to have the ability to self-renew and to

differentiate into a multitude of mesodermal cell types [13–15]. Bone marrow MSCs represent a heterogeneous population derived from the nonblood-forming fraction of bone marrow, but have the ability to regulate hematopoietic cell development. *In vitro*, adult mesenchymal stem cells resident in this bone marrow fraction differentiate into bone, cartilage and fat [16]. Recently, a standardized nomenclature for MSCs has been proposed and the term "multipotent mesenchymal stromal cells" has been introduced [15] to refer to this population of fibroblast-like, plastic-adherent cells [17]. Their asymmetric division produces one identical daughter stem cell and a second progenitor cell that becomes committed to a lineage-specific differentiation program [18].

3.2. Importance and uses

MSCs produce many growth factors and essential cytokines needed for cell proliferation and differentiation [19]. They also support hematopoiesis in bone marrow and play an indirect role in supporting other cell types during tissue repair [20]. Adult stem cells could overcome many of the ethical and technical debate associated with ESC as they are isolated from adult tissues, including bone marrow stromal cells, adipose-derived stem cells and adult skin stromal cells [21]. However, because of their limited differentiation potential (multipotent), they are less likely to form tumors, although some are thought to be related to certain tumors [22].

3.3. Location

The exact location of these cells in vivo is not known, but recent work suggests that MSCs are located in the perivascular spaces as sub-endothelial cells surrounding the vascular sinusoids in the bone marrow [23]. Bone marrow contains three main cell types: endothelial cells, hematopoietic stem cells, and stromal cells. Bone marrow connective tissue network is called the stroma. The stroma consists of a heterogeneous population of cells that provide structural and physiological microenvironment to support hematopoietic cells and forms a complex extracellular matrix, which supports the hematopoietic process [23]. However, the frequency of MSCs in human BM has been estimated to be in the range of 0.001–0.01% of the total nucleated cells. Furthermore, the frequency of MSCs declines with age, from $1/10^4$ nucleated marrow cells in a newborn to about $1/10^6$ nucleated marrow cells in an 80-year-old person [24].

4. Biology of stromal cells/MSCs

4.1. Tissue distribution

Interestingly, MSCs reside in diverse tissues throughout the adult organism [25]. Nowadays, MSC populations have been obtained from many tissues other than the bone marrow, [26] including the adipose tissue [27] and placenta [28].

4.2. Properties

Mesenchymal/stromal cells (MSCs) have the ability to differentiate into a variety of different cells/tissue lineages; osteoblasts, chondroblasts, adipoblasts and reticular stromal cells [29].

MSCs possess potent immunomodulatory and anti-inflammatory effects and have been used as agents in autoimmune diseases [30]. They interfere with pathways of the immune response by means of direct cell-to-cell interactions and soluble factor secretion. In vitro, MSCs inhibit proliferation of T cells, B-cells, natural killer cells and dendritic cells [31].

4.3. Immunobiology

MSCs are believed to have critical roles in repairing damaged tissues. Tissue injury is associated with the activation of immune/inflammatory cells. In addition, inflammatory mediators, chemokines and leukotrienes, are often produced in the microenvironment by phagocytes in response to damaged cells [32]. Nevertheless, the function of the endothelial cells as a barrier is often broken down in damaged tissues. Thus, these inflammatory molecules and immune cells, together with endothelial cells and fibroblasts, result in the mobilization and differentiation of MSCs and replace the damaged tissue cells. The study of endogenous MSC migration is complex. Once MSCs have entered the microenvironment of injured tissues, MSCs start releasing many growth factors, including epidermal growth factor (tissue regeneration), fibroblast growth factor (cell survival and regeneration), platelet-derived growth factor (tissue repair), vascular endothelial growth factor (angiogenesis and wound healing), hepatocyte growth factor (intrinsic neural cell regeneration), angiopoietin-1 (angiogenesis) and stromal cell-derived factor-1 (neuroprotective effect). These growth factors, in turn, promote the development of fibroblasts, endothelial cells and tissue progenitor cells, which carry out tissue regeneration and repair [33].

4.4. Homing

Studies have shown that MSCs have the ability to migrate and to home to a variety of tissues. The migration process is represented by several distinctive steps and starts with the resistance and adhesive interactions between cells flowing through the bloodstream and vascular endothelium. The mechanisms used are assumed to follow the same steps that were described for leukocyte homing.

In the first step, the cells come into contact with the endothelium by tethering and rolling. Different molecules are involved in such process. The selectins on the endothelium are primarily involved and the expression of hematopoietic cell E−/L-selectin ligand which is a specialized form of cluster of differentiations (CD), CD44. This step is mediated by the homing receptors expressed on circulating cells which interact with their corresponding receptors expressed on the layer of endothelial cells. [34]. As regards the second step, the cells are activated by G-protein-coupled receptors, followed by integrin-mediated activation. MSCs express various integrins on their surface, among which integrin $\alpha 4/\beta 1$, which mediates cell–cell and cell- extracellular matrix interactions by binding to vascular cell adhesion molecule −1 and to the V-region of fibronectin, respectively. In damaged tissues, fibronectin is deposed together with fibrin at the injured site to stop the bleeding. The provisional matrix is then remodeled by macrophages and fibroblasts, determining an increase in V region-exposing fibronectin, which, in turn, allows MSCs to adhere and transmigrate into the extracellular matrix. In the last step, diapedesis or transmigration occur through the endothelium as well as through the underlying basement membrane. In this step one of the

matrix metalloproteinases (MMP) - which are lytic enzymes required to cleave the components of the basement membrane - the gelatinases MMP-2 and MMP-9 are the most important because they specifically degrade collagen and gelatin components of the basement membrane [35].

4.5. Characterization

MSCs isolated directly from bone marrow are positive for CD44. They are also positive for CD29, CD73, CD90, CD105 and CD166. On the other hand, they are negative for the hematopoietic surface markers such as CD11b, CD45, CD31, CD106, CD117 and CD135 [36]. As progress in phenotyping the MSCs and its progeny continues, the use of selective markers has resulted in the enhanced propagation and enrichment of the MSC population, while maintaining them in an undifferentiated state without diminishing the differentiation potential [37].

A part of a work [38] was carried out at Department of Trauma, Hand and Reconstructive Surgery, Johann-Wolfgang-Goethe University Hospital, Frankfurt, Germany. They demonstrated that MSCs expressed typical MSCs specific antigens CD73, CD90 and CD105 (hematopoietic surface marker) and were negative for the hematopoietic marker and lymphocytic markers CD34, CD45, respectively. According to the International Society of Cell Therapy, CD73, CD90 alongside CD105 are positively expressed on MSCs and remain the primary molecules used to identify MSCs [39]. The phenotypic characterization of MSCs from bone marrow has been further realized through the identification of the cytokine expression profile of undifferentiated cells. Constitutive expression of cytokines, such as granulocyte-colony stimulating factor, stem cell factor, leukemia inhibitory factor, macrophage-colony stimulating factor, and IL-6 and IL-11 is consistent with the ability of MSCs to support hematopoiesis [40].

5. Culturing

In order to avoid patient morbidity, the amount of MSCs that could be isolated from BM aspirate should be too small [12]. Therefore, they should be cultured in vitro to enable the expansion of MSCs to generate millions of cells which can be used for further therapeutic applications [39]. It was stated that MSCs retain more potential to differentiate after the third passage (P) [41]. In addition, over 70% of clinical trials used MSCs from 1 to 5 passages [42]. Moreover, a study reported that MSCs from 7 to 9 passages underwent osteogenic differentiation more than cells of later passages. Moreover, recent data indicated that reactive oxygen species-handling mechanisms (i.e., antioxidative activity/reduction potential) become disrupted in later passages, a condition, which was not observed in the lower passage [43].

Although several researchers [41] showed that with the long-term expansion of MSCs and with several sub-culturing, the cells lose their differentiating ability, a study performed [44] reported that no change at the level of genetic expression or differentiation capability of long-term cultured MSCs. Furthermore, MSCs have a stable phenotype over many generations

in vitro [45]. Another study [46] reported that MSCs retained their multilineage differentiation potential till passage 10 (P10) and maintain high levels of telomerase activity and long telomere length up to P10, but steady decline in the efficiency of proliferation in all cell populations after P10. Furthermore, MSCs showed a marked increase in the time required for cell doubling and showed an enlarged, flattened cellular morphology at P15, after which they ceased to undergo cell division but remained viable in culture. Thus, cells from passage 9 were used for differentiation as it was needed to obtain sufficient cell numbers for use through extensive cell quantity amplification and later passages were avoided [47].

6. Histology

Studying the behavior of MSCs in vitro has become an urgent need to give more insights on their behavior in vivo and their mechanisms in initiating osteogenesis. Indeed, histological examination of MSCs is one of the main goals for studying their morphology in vitro by light microscope. Although it is a primary step, yet, it is not sufficient to rely on it alone, to detect their behavior during their differentiation process, and as such it has to be accompanied by ultrastructure examination to correlate between their morphology and behavior.

6.1. Light microscope

MSCs are characterized by being star-shaped cell with thin long processes [48]. Using hematoxylin and eosin stains, MSCs are characterized by pale cytoplasm, large vesicular nucleus and multiple thin processes (**Figure 1**).

6.2. Phase contrast microscope

Regardless of the issue of origin, all MSCs share characteristics by consensus definition: they are spindle-shaped and plastic-adherent. In our study, [38] isolated human bone barrow MSCs

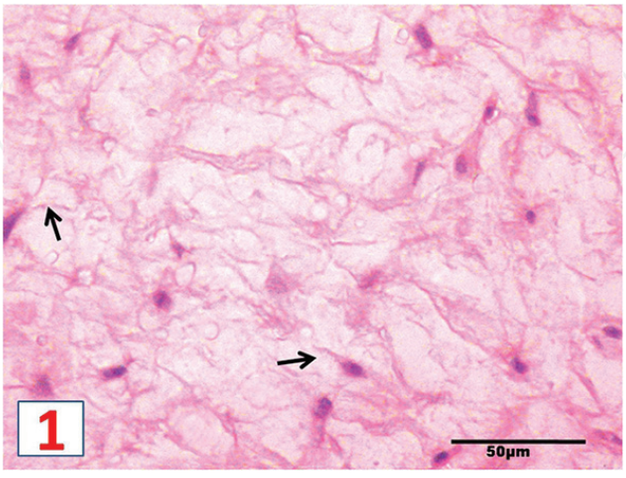

Figure 1. Light microscopic picture of the umbilical cord showing MSCs with many thin processes (arrow). Each cell exhibits a vesicular nucleus. Scale bar 50 µm.

revealed that the cells were adherent to the surface of tissue culture plastic flask. Furthermore, the cells were spindle in shape; which is considered as a second important characteristic of mesenchymal cell morphology. Researchers [38] described a population of adherent cells in culture till P5 (**Figure 2**). Most of the cells exhibited fibroblast-like spindle shape and showed vesicular nuclei with prominent nucleoli. Moreover, in P9, the adherent cells remained attached to the surface with their characteristic spindle shape (**Figure 3**). The cells exhibited vesicular nucleus, prominent nucleolus and multiple processes [38].

6.3. Electron microscope

6.3.1. Scanning electron microscope (SEM)

The two-dimensional morphology of MSCs demonstrated by scanning electron microscope (SEM) [38] showed the spindle-shaped cells with eccentric nuclei and several thin cytoplasmic processes extending from the edge of the cell surface in P5 and P9. In addition, cells in P 9 maintained their spindle shape (**Figure 4**). These SEM results were also reported [49].

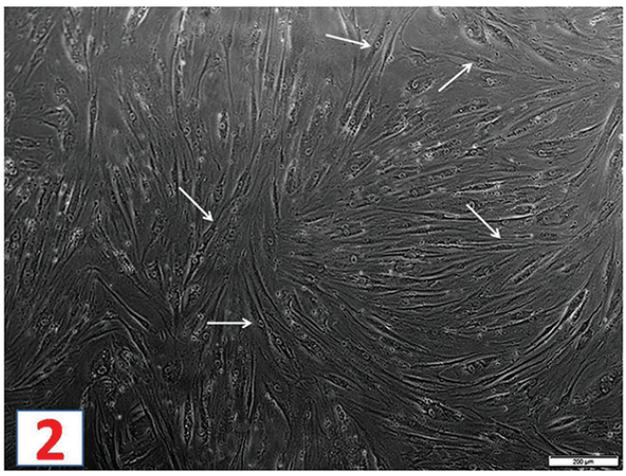

Figure 2. Cultured human bone marrow derived stromal cell from passage 5, showing adherent cells with their characteristic spindle shape (arrow) [38]. Scale bar 200 μm.

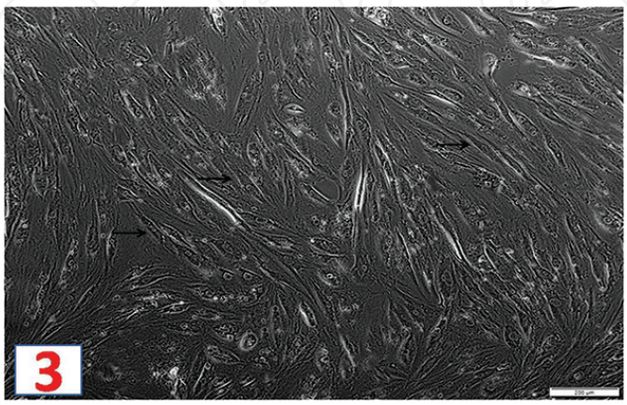

Figure 3. Cultured human bone marrow derived stromal cell from passage 9, showing adherent cells with their characteristic spindle shape (arrow) [38]. Scale bar 200 μm.

6.3.2. Transmission electron microscope (TEM)

Electron microscopic examination of MSCs in culture revealed the presence of euchromatic nucleus associated with abundant cell organelles which are considered as an indicator of an active cell (**Figure 5**). The spindle-shaped cells showed large irregular, euchromatic nucleus and the peripheral heterochromatin was slightly condensed along the inner surface of the nuclear membrane and nuclear pores (**Figure 6**). The cytoplasm showed many elongated profiles of rough endoplasmic reticulum and multiple mitochondria (**Figure 7**). Cytoskeletal structures were seen as fine filaments running parallel to the long axis of the cell near the nuclear membrane as well as beneath the cell membrane (**Figure 6**).

The same features of active MSCs were noticed after 14 days in culture. The cells exhibited a large euchromatic nucleus with numerous profiles of rough endoplasmic reticulum and multiple rounded mitochondria. In addition, the cell surface showed thin pseudopodia (**Figure 7**). Cytoskeletal filaments were irregularly dispersed in the cytoplasm as well as around the nucleus (**Figure 7**). Such observation was explained by the fact that the intracellular organelles architecture is organized by the cytoskeleton [36, 50, 51].

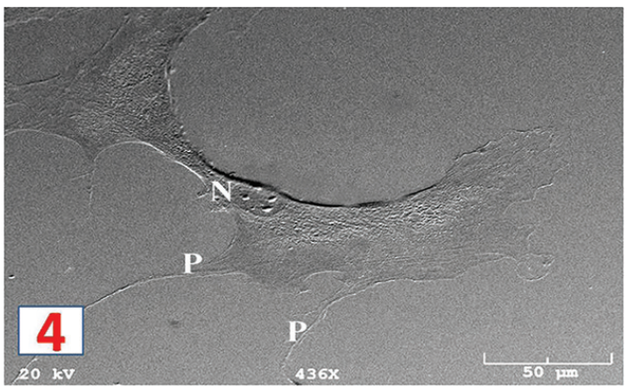

Figure 4. Cultured human bone marrow derived stromal cell, showing spindle shape cell with an eccentric nucleus (N) and multiple processes (P) [38]. Scale bar 50 µm.

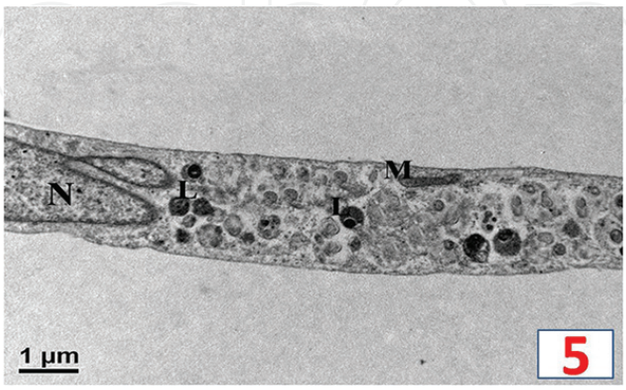

Figure 5. Transmission electron micrograph of cultured human bone marrow derived stromal cell on day 7. The cell is spindle in shape with an euchromatic nucleus (N). The cytoplasm shows mitochondria (M) and multiple lysosomes (L) [38]. Scale bar 1 µm.

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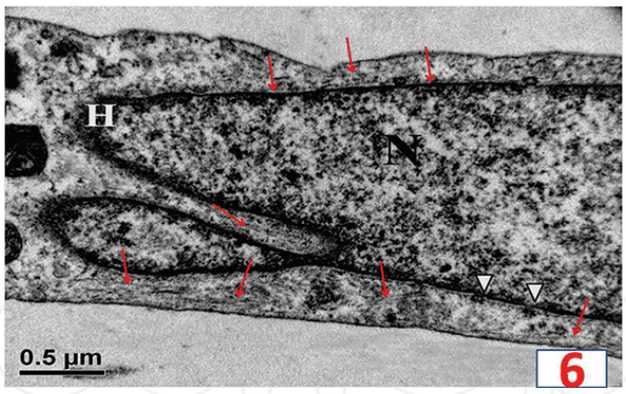

Figure 6. Transmission electron micrograph of cultured human bone marrow derived stromal cell showing part of the same cell exhibiting an euchromatic nucleus (N) with nuclear pores (arrow heads). The peripheral heterochromatin (H) is seen along the inner aspect of the nuclear membrane. Fine cytoskeletal filaments are noticed parallel to the long axis of the cell near the nuclear and cell membranes (arrows) [38]. Scale bar 0.5 µm.

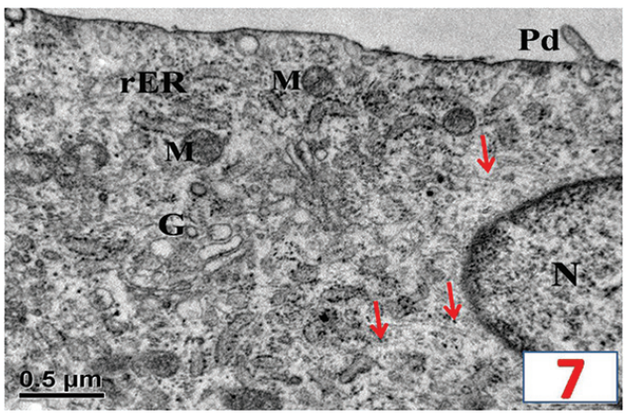

Figure 7. Transmission electron micrograph of cultured human bone marrow derived stromal cell on day 14. The cytoplasm exhibits numerous profiles of rER, mitochondria (M), and well-developed Golgi complex (G). The cell membrane exhibits a pseudopodium (Pd). Cytoskeletal filaments are irregularly dispersed in the cytoplasm (arrows). Part of an euchromatic nucleus is also seen (N) [38]. Scale bar 0.5 µm.

Moreover, after 21 days in culture, the cells showed clearly demarcated nucleolus (**Figure 8**). In addition, numerous large macro vesicles associated with the mature face of the Golgi complex were clearly depicted (**Figure 9**). These cells are now ready for differentiation once in the appropriate media. The structure of these cells would differ during the process of differentiation accordingly.

Another ultrastructure feature of MSCs is the presence of vesicles in the cytoplasm. Intercellular communication can be mediated through direct cell–cell contact or transfer of secreted molecules. Recently, a third mechanism has emerged that involves intercellular transfer of extracellular vesicles. Cells release into the extracellular environment membrane vesicles either of endosomal origin or of plasma membrane origin. They are named exosomes and microvesicles, respectively [52]. In the study [38] carried out on isolated MSCs, showed vesicular trafficking. (**Figure 10**) These vesicles were prominent after the cells were cultured in a

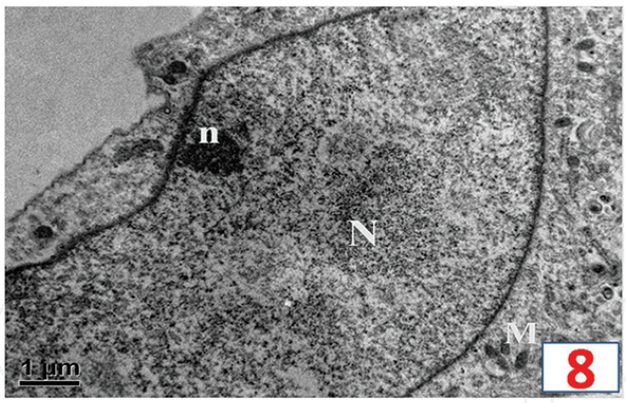

Figure 8. Transmission electron micrograph of cultured human bone marrow derived stromal cells on day 21, showing large euchromatic nucleus (N) with clearly demarcated nucleolus (n). The cytoplasm shows mitochondria (M) [38]. Scale bar 1 µm.

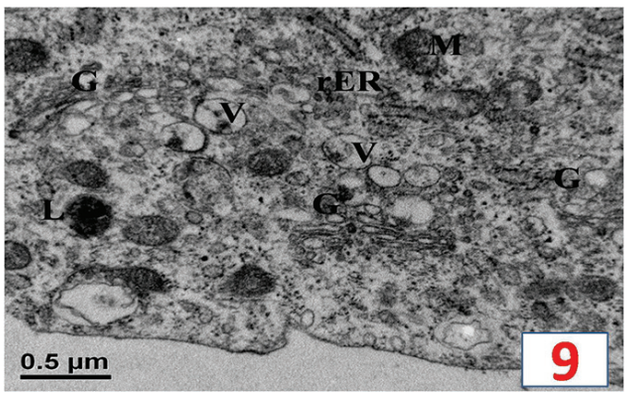

Figure 9. Transmission electron micrograph of cultured human bone marrow derived stromal cells on day 21, showing part of its cytoplasm with multiple well-developed Golgi complexes (G) associated with large secretory vesicles (V), numerous mitochondria (M), and lysosomes (L). The cytoplasm shows profiles of rough endoplasmic reticulum (rER) [38]. Scale bar 0.5 µm.

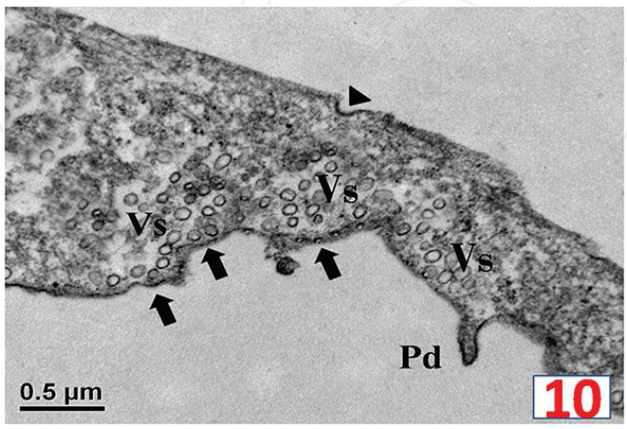

Figure 10. Transmission electron micrograph of cultured human bone marrow derived stromal cells. The cytoplasm shows several cytoplasmic vesicles (Vs) of variable sizes. A coated pit (arrowhead) and numerous subplasmalemmal vesicles are also seen (thick arrows). A surface pseudopodium (Pd) is seen [38]. Scale bar 0.5 µm.

media that stimulated its osteogenic differentiation. Microvesicles vary in size and are formed by the budding of the plasma membrane. Most cell types are known to produce microvesicles either constitutively or when stimulated during apoptosis or activation. The mechanisms involved in the mobilization of secretory microvesicles to the cell periphery, their docking, and fusion with the cell surface require the cytoskeleton (actin and microtubules), associated molecular motor proteins (kinesins and myosins) as well as other factors [53, 54]. The other clearly defined class of secreted membrane vesicles that originate from the endosomes are the exosomes. Exosomes were first discovered by Pan and Johnstone in 1983 [55]. They are formed by the invagination of endolysosomal vesicles to form multi-vesicular bodies. Exosomes are released by exocytosis. First, the cell membrane is internalized to produce endosomes. Subsequently, many small vesicles are formed inside the endosome by invaginating parts of the endosome membranes. Such endosomes are called MVBs. Finally, the MVBs fuse with the cell membrane and release the intraluminal endosomal vesicles into the extracellular space to become exosomes [56].

Exosomes directly interact with the signaling receptors of target cells [57]. After that, the exosomes fuse with the plasma membrane of recipient cells and deliver their content into the cytoplasm [58]. Finally, the exosomes are internalized into the recipient cells. Once in the recipient cell, some of these engulfed exosomes may merge into endosomes and move across the recipient cells to be released into the neighboring cells. In the other case, endosomes fused from engulfed exosomes will mature into lysosomes and undergo degradation [57, 59].

Lipids and proteins are the main components. The protein content of exosomes from different cell types contains different endosome-associated proteins (e.g., RabGTPase, SNAREs, Annexins and flotillin). They are also enriched in proteins that associate with lipid rafts, including glycosylphosphatidylinositol-anchored proteins and flotillin [60]. The other main component of exosomes is the lipid. In comparison to the plasma membrane, exosomes are highly enriched in cholesterol, sphingomyelin and ceramides at the expense of phosphatidylcholine and phosphatidylethanolamine [52]. In addition to the proteins and lipids, various nucleic acids have recently been identified in the exosomal lumen, including mRNAs, microRNAs and other noncoding RNAs [61].

The main functions of exosomes are their capacity to act as antigen-presenting vesicles, to stimulate immune responses [62]. Another main important feature of exosomes is being an ideal drug delivery vehicle. Meanwhile, research has been carried out encapsulating anticancer drugs into exosomes [63].

The function of MSC-derived exosomes has not been well defined. They act as an intercellular communication vehicle for modulating cellular processes. It was recently revealed that exosomes derived from MSCs play important roles in mediating the biological functions of MSCs [64].

A study demonstrated the electron microscopy of exosomes. They were cup-shaped and measured 40–100 nm in diameter. Exosomes are naturally secreted and well tolerated by the body. They are also safely stored and provide many therapeutic applications with avoiding the

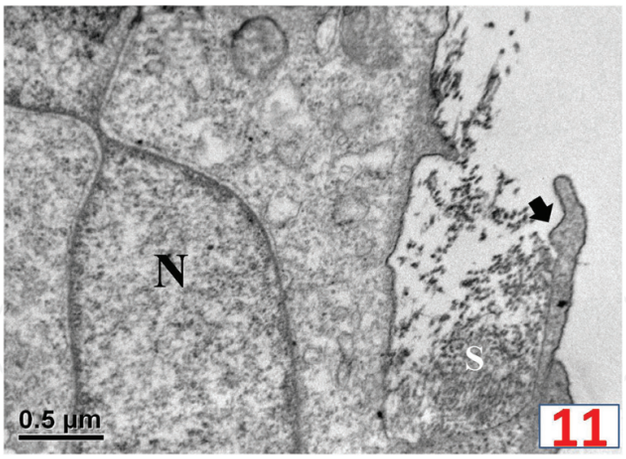

Figure 11. Transmission electron micrograph of cultured human bone marrow derived stromal cells. The cell surface of shows an open fibripositors (short arrow) with large amounts of secretory product (S) is observed. Note the euchromatic nucleus (N) [38]. Scale bar 0.5 µm.

risk of immunological rejection and malignant transformation [65]. Therefore, the use of MSCs to produce exosomes for drug delivery is the subject of the day [66]. Recently, liposomes are preferred drug delivery systems. It is a synthetic vesicle with a phospholipid membrane that has the ability to self-assemble into various sizes and shapes in an aqueous environment [67].

Another morphological feature detected is pseudopodia-like structures extending from the cell membrane (**Figures 7** and **10**). This might explain the capacity of the cells for migration within the receiving tissue. The main role of these structures is to transmit the produced material from one cell into another by extending the pseudopodia and communicating cells with each other as well as in cell signaling [68]. Interestingly, one of the most striking features during differentiation is the observation of finger-like extensions of the plasma membrane known as fibripositors (**Figure 11**). These fibripositors were located at the side of the cell and protrude into the spaces between cells. These fibripositors are the site where collagen fibrils were located. It was reported that the initial stage of extracellular matrix deposition results in arrays of short collagen fibrils completely enclosed within these fibripositors. These fibrils are then subsequently deposited extracellularly [69, 70].

It was reported that fibrils leaving the fibripositors were seen to run along the external surface of the cell. Tracking of fibrils revealed that the collagen fibrils in fibripositors were shorter than those extracellularly. Thus, these data suggested that fibripositors might be a place of fibril assembly. They determined that short fibrils become longer inside closed fibripositors, then protruding fibripositors (open), often project into the matrix, releasing fibrils extracellularly where individual fibrils then coalesce into bundles. Thus, fibripositors are specialized sites not only of fibril assembly, but also share in fibril transport extracellularly [71].

Another study declared that the fibripositors are dynamic structures and their formation and stabilization depend on the actin cytoskeleton [72]. This might explain the existence of the cytoskeletal filaments in the differentiating cells [38]. Accordingly, these cytoskeletal structures might be actin filaments. It is possible that fibripositors have been involved in the alignment of extracellular collagen fibrils in a parallel arrangement [73].

7. Conclusion

The MSCs maintained their undifferentiated histological structure till passage 9 for further tissue engineering. A detailed histological examination using the light and the electron microscopes is essential to understand the function of MSCs. In addition, exosomes represent a promising candidate for drug delivery vehicle.

Abbreviations

ESCs	embryonic stem cells
iPS	induced pluripotent stem cells
FSCs	fetal stem cells
MSCs	mesenchymal/stromal cells
CD	cluster of differentiation
MMP	metalloproteinases
P	passage
SEM	scanning electron microscope
TEM	transmission electron microscope
SNARE	soluble *N*-ethylmaleimide-sensitive factor receptor
Rab	Ras-related proteins in brain
GTP	guanosine triphosphate

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