Stem Cell Biology and Regenerative Medicine

Kursad Turksen *Editor*

Stem Cells: Current Challenges and New Directions

Humana Press

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ISSN 2196-8985 ISSN 2196-8993 (electronic)
ISBN 978-1-4614-8065-5 ISBN 978-1-4614-8066-2 (eBook)
DOI 10.1007/978-1-4614-8066-2
Springer New York Heidelberg Dordrecht London

Library of Congress Control Number: 2013948666

Printed on acid-free paper

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Springer is part of Springer Science+Business Media (www.springer.com)

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Chapter 1
Myogenic Potential of Stem Cells: In Vivo Assessment

Maximilien Bencze, Ingo Riederer, Gillian S. Butler-Browne, Wilson Savino, and Vincent Mouly

Abstract Stem cell therapy has been envisaged for treating disorders affecting skeletal muscle tissue. Several cell types have been identified for exerting a myogenic potential in certain conditions. In order to efficiently regenerate injured muscles while remaining safe for patients, these myogenic progenitors should present characteristics such as their availability to be isolated from patients, their growth and commitment performances and, if necessary, their capacity to be genetically corrected. Here, we present an overview of the main myogenic cell candidates that have been identified and tested in vivo, classifying them by their route of delivery (intra-muscular and systemic delivery), and focusing our attention on their regenerative capacity in animal models of Duchenne muscular dystrophy.

Keywords Stem cell therapy • Myogenic progenitors • Regenerative capacity • Duchenne muscular dystrophy • Delivery route

1.1 Introduction

Striated skeletal muscle is constituted by muscular fibres, which perform their characteristic contractile function. Muscle fibres (myofibres) are multinucleated post-mitotic structures that are supported in mechanic stress occurring during contraction by the surrounding basal lamina. Cytoplasm content, named sarcoplasm,

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K. Turksen (ed.), *Stem Cells: Current Challenges and New Directions*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4614-8066-2_1,

is characterised by contractile units called sarcomeres, and an abundance of mitochondria and masses of glycogen, which provide the ATP required for mechanic energy production. Muscle tissue is characterised by an important vascularisation, allowing a strong and rapid supply in nutriments and oxygen required by exercise.

1.1.1 Satellite Cells, the Functional Progenitor of Striated Muscle

Satellite cells have been described for the first time in 1961 by Mauro by morphological characteristics in electron microscopy [1]. They exhibit a small cytoplasm and are located beneath the fibres and their basal lamina. Their distribution within muscle tissue is preferentially near nuclei of myofibres (myonuclei), neuromuscular junctions or capillaries [2]. Satellite cells are the only myogenic precursors present at birth in humans [3] and are responsible for postnatal fibre growth. In adult healthy muscles, satellite cells are normally mitotically quiescent [4], but following a trauma or a pathologic lesion, satellite cells exit quiescence and get activated, up regulating transcriptional activity, and increasing cytoplasm volume in order to participate in muscle regeneration [5]. Whereas satellite cells are considered as the local myogenic precursors of skeletal muscle, other cell types that do not belong to the muscle lineage have been identified as capable of exerting a myogenic potential in vivo [6, 7]. However, most of these cells have not been assessed to significantly participate in physiological muscle regeneration.

As satellite cell type has been initially defined by morphologic and not phenotypic or genetic criteria, a significant disparity in satellite cell marker expression has been described, reflecting a phenotypic heterogeneity. However, it is well admitted in mouse and human that quiescent satellite cells strongly express the transcription factors Pax7, and in a lower proportion Myf5 [8]. Moreover, the adhesion proteins CD56 (N-CAM) and M-cadherin are also expressed [9].

Although satellite cells expressing MyoD, Myf5 and Pax7 are able to commit and differentiate in defined culture conditions into myogenic, osteogenic and adipogenic fate, suggesting that this cell type has a mesenchymal lineage plasticity [10], there is no definite evidence that they do so in vivo. Stem cell definition as a myogenic precursor implies the ability of an undifferentiated cell to both self-renew in order to maintain a stem cell pool and in parallel give rise to differentiated fibres. This dual fate is regulated through an asymmetric division allowing to keep a constant pool of precursors while the majority of the population can commit into differentiation and participate in muscle growth or regeneration [11]. However, in humans, satellite cells, like most somatic cells, are able to proceed to a limited number of divisions. Satellite cells isolated from newborn biopsies can make up to 60 divisions in vitro before replicative senescence occurs, partly due to telomeric erosion and the absence of telomerase expression in human satellite cells [12]. Thus, even if satellite cells are characterised by a lineage plasticity and a capacity of self-renewing, the existence of such a limit in their proliferative capacity prevents them to be called *bona fide* "stem cells", but only functional "progenitors" of muscle tissue.

1.1.2 Muscle Regeneration Process

Muscle regeneration is a well-orchestrated process, implicating the activation of satellite cells into myoblasts, their proliferation, their migration, and finally their differentiation and fusion to generate new myofibres [13].

A lesion occurring in muscle fibres activates quiescent satellite cells located on the injured fibres, which will proliferate and migrate preferentially to the lesion site [14]. This step is characterised by the activation of satellite cells, and the expression of the helix-loop-helix transcription factors myogenic regulatory factors (MRFs) Myf5 and MyoD [15–17]. Since quiescent cells do not express MyoD, its transcription will reflect their activation state. At that phase, satellite cells in proliferation phase are no longer called satellite cells but "myoblasts".

Activation and proliferation of satellite cells are regulated by exogenous factors such as growth factors and cytokines that can be delivered by surrounding cells and/or myoblasts themselves. Myoblasts can have two kinds of fate: a majority will proliferate and differentiate while a minority will go back to quiescence and restore the original pool of satellite cells. Then, they stop expressing MyoD but keep being positive for Pax7 satellite cell marker. The other and main part of myoblasts irreversibly commit into myogenic fate, differentiate into myocytes and fuse with other myocytes or injured myofibres to participate in muscle regeneration. Up regulation of transcription factors myogenin and then Mrf4 represent the first markers of differentiation [13, 16], concomitant with a down regulation of Pax7. Numerous studies suggest that MyoD is required for the transition from proliferation to differentiation. Cell models missing the gene coding for MyoD are characterised by a default of differentiation, suggesting that MyoD does play a role not only for activation but also for differentiation process [18, 19]. Myocyte enhancer factor-2 (MEF2) and myogenin are transitory expressed in early differentiation process and are required for terminal differentiation [20, 21], and trigger the expression of many muscle-specific genes, thus triggering the general process of coordinated differentiation. Myogenin-deficient mice express developmental abnormities and accumulate myoblasts that cannot fuse into mature myofibres [22, 23]. Once the differentiation program has been triggered, the expression of myogenic specific proteins progressively occurs such as myosin, dystrophin and spectrin.

1.1.3 Pathologies of Skeletal Muscles

Muscular dystrophies are a heterogeneous group of pathologies with or without genetic cause. Depending on the mutated gene and on the type of mutation, repartition and severity of clinical signs and kinetics of symptom appearance can largely differ. In some dystrophies such as oculopharyngeal muscular dystrophy (OPMD) and facioscapulohumeral muscular dystrophy (FSHD), a restricted number of muscles are affected, whereas in others such as Duchenne muscular dystrophy (DMD) or Becker muscular dystrophy (BMD), most skeletal muscles of the body are concerned.

This diversity in muscular dystrophies may obviously generate distinct approaches in stem cell therapy considering each disease's specificity.

DMD is a juvenile and progressive X-linked disease affecting approximately 1/3,500 male birth worldwide. It is caused by mutations in the dystrophin gene, which leads to the absence of functional protein on fibres, their mechanical fragility and their death by necrosis. Inflammatory infiltrate elicited by primary myonecrosis becomes rapidly cytotoxic and represents a dramatic issue in DMD pathogenesis, especially by favouring fibrosis [24, 25]. Due to repeated rounds of muscle degeneration and regeneration, satellite cells will finally reach senescence and will be no longer able to proliferate, differentiate and finally participate in muscle regeneration. This leads to abortive regeneration due to the exhaustion of the progenitors, and finally to progressive and irreversible muscular weakness, including of respiratory muscles which is, together with the cardiac defects, the most frequent cause of patient's death. Current treatments for DMD patients include the limitation of inflammatory processes [26] but there is yet no cure for this disease.

Ever since the identification of the first mutations in the dystrophin gene, gene and stem cell therapies have been envisaged to cure DMD by restoring dystrophin expression on myofibres. Cell therapy can be set up using two different approaches: patients can provide their own muscle progenitors for gene correction before by their re-engraftment (autologous graft). The progenitors can then participate in muscle regeneration and provide to the dystrophic tissue the missing gene without any immune response apart that potentially against dystrophin. However, the often limited proliferative capacity of the patient's cells, due to the cycles of degeneration–regeneration that already occurred, represents a real bottleneck for autologous cell therapy for DMD. The progenitors can also be provided by a non-dystrophic donor (heterologous graft), implicating an immunosuppression for the patient. Heterologous grafts present the advantage to avoid issues of genic correction of transplanted cells and of their proliferative capacity, but require a constant immunosuppression. For all these reasons, although DMD was the first muscle dystrophy to be targeted by cell therapy, it might be not the best clinical situation for this approach, as compared to other dystrophies where the muscles to be targeted by therapy are limited both in numbers and in volume, such as OPMD.

1.2 Stem Cell Therapy for Neuromuscular Disorders

1.2.1 What is Expected from a Good Cell Candidate?

The choice of good cell candidates relies on precise characteristics in order to ensure sufficient efficiency:

– The cell candidate has to be easily available in humans and in a sufficient quantity. For patients missing a large part of functional musculature, enduring a surgery removing a large biopsy that even reduces his or her mobility is obviously

inappropriate. For instance, isolating cells from blood would limit donor's impairment, as long as the cells present a myogenic potential.
- The cell candidate should be amplifiable in clinical conditions in order to obtain a sufficient amount of progenitors for regenerating the whole muscles.
- The myogenic potential has to be high and stable.
- The cell also needs to be genetically modifiable in order to integrate the missing gene to host tissue, in case of autologous cell therapy, which seems to be the best-adapted strategy.

The final fate of delivered cells has to be precisely determined in order to avoid that a significant part of the cells do not differentiate into an inappropriate cell type, which may finally impair muscle function. For instance, matrix deposition, in an uncontrolled inflammatory environment like in DMD muscles, becomes pathogenic by formation of permanent fibrotic tissue which hampers any therapeutic intervention and ultimately replaces dead myofibres (for review, see [25]). Any cell type, or its progeny, that may increase matrix deposition may thus increase also fibrosis, which represents a major issue in DMD pathogenesis.

A candidate should also be able to restore the satellite cell pool which can be activated and participate in the next rounds of regeneration, thus amplifying during the degenerative process the therapeutic benefit: round after round, this would improve the percentage of fibres expressing the missing protein.

1.2.2 Candidate Cells

Several types of myogenic precursors have been envisaged for cell therapy and their myogenic potential has been assessed both in vitro and in vivo. Concerning muscular dystrophies, most efforts of stem cell therapy have been focused on DMD since it is the most frequent dystrophy, although, as already stated, it may not be the best case for a proof of concept. Some candidates have been (and are currently) used in clinical trials. The main cell candidates available, their principal advantages and disadvantages, and recent advances in cell therapy are discussed, including their preferential delivery route: intramuscular or systemic.

1.2.2.1 Candidates for Intramuscular Delivery

Myoblasts

Since satellite cells, from which myoblasts are derived, are the postnatal precursors of skeletal muscle, responsible of both muscle fibre growth and regeneration, myoblasts were the first candidates considered for stem cell therapy. The first article assessing myoblast transfer therapy has been published by the group of T. Partridge in 1989, and used cultivated myoblasts in order to restore dystrophin expression in *mdx* mice (mice mutated for dystrophin, used as a model for DMD).

Control syngenic myoblasts with a wt dystrophin gene implanted into *mdx* muscles were incorporated into newly formed myofibres which then expressed dystrophin [27]. However, in clinical trials following this success some dystrophin-positive fibres detected were in fact revertant fibres, expressing a truncated form of the protein, which is common in *mdx* model and can be observed in DMD patients [28]. Furthermore, these clinical trials based on intra-muscular transplantation of allogenic myoblasts did not result in any clinical benefit [29, 30]; no muscle force improvement has been assessed and dystrophin expression in treated muscles was weak, even when associated with immunosuppression by cyclosporine A [31] or cyclophosphamide [32].

The confirmation of the poor efficiency of myoblast transfer therapy in clinical condition has incited further investigations in order to determine the reasons which could explain this lack of efficacy. Three main limiting factors of myoblast cell therapy have been identified in murine models of transplantation: a massive and precocious cell death, a lack of proliferation and a lack of migration of engrafted myoblasts.

Myoblast Death

A massive death occurs the first few hours after myoblast transplantation before any immune reject may occur [33, 34]. Beauchamp et al. described in their graft model of immortal myoblasts into dystrophic mice that by 24 h following MTT, over 90 % of the injected myoblasts are dead. The mechanisms implicated in this precocious cell death still remain unclear. It has been suggested that neutrophil infiltration on transplantation site could induce apoptosis by an lymphocyte function-associated antigen-1 (LFA-1)-dependant pathway [35]. More recent studies exclude a potential role of neutrophils, NK cells and macrophages [36]. The analysis of cell death using specific death markers showed that part of the myoblasts die by apoptosis and another part by necrosis [37]. Cell precocious death occurring by different pathways makes sense that causes may be multiple. Low survival of transplanted cells seems to be negatively correlated to the number of grafted cells suggesting that a large amount of cells may increase cellular stress, limit free oxygen and nutriments to injected myoblast and decrease the metabolite clearance [9].

Low Proliferation In Vivo

Conceptually, the massive cell death can be compensated by their proliferation in situ in order to efficiently participate in muscle regeneration. Whereas human myoblasts have a limited proliferative capacity in vitro due to the phenomenon of replicative senescence after a determined number of division [38, 39], this is not involved in the limited regenerative capacity in situ during MTT since myoblasts isolated from a young donor and capable of many divisions can rapidly reach millions in host muscles and would be sufficient to regenerate it entirely. However, the number of transplanted myoblasts does not seem to vary significantly during the days

following transplantation, suggesting that the proliferation of myoblasts post transplantation only compensates the precocious loss. Moreover, we have shown that the proliferation of human myoblasts occurs only during 3–5 days post transplantation in an immunodeficient host [40]. Several publications indicate that freshly isolated satellite cells or isolated myofibres associated to few quiescent satellite cells can very efficiently differentiate into muscle host after transplantation [41–43]. Amazingly, the regenerative capacity of freshly isolated murine myoblasts is dramatically higher than amplified myoblasts. For instance, 7–8 satellite cells associated with a single freshly isolated fibre can trigger dystrophin expression by more than hundred originally dystrophic myofibres. Furthermore, a part of myoblasts derived from these quiescent satellite cells dystrophin-competent can participate in the new pool of quiescent satellite cells further available for activation, proliferation and differentiation after another round of regeneration, thus further amplifying dystrophin expression [42]. In addition, $Pax7^{+}/myf5^{-}$ subpopulation of murine satellite cells have been described to be particularly efficient to colonise satellite cell niche and participate in consecutive rounds of regeneration [41]. Whether these observations can be confirmed in human cells still needs to be investigated.

Poor Migration of Myoblasts

A lack of effective migration by grafted myoblasts within the host muscle has been assessed in mouse [44, 45] and monkey models [46, 47]. This can be improved in the mouse when host tissue is previously irradiated [48], but this is obviously not compatible with clinical conditions. The improvement of migratory parameters in these cases seems to be linked to extracellular matrix (ECM) remodelling in muscle, which plays a determinant role in vivo. Myoblast dispersion is improved if the composition of ECM in laminins is increased [49] or if the secretion of matrix metalloproteases such as MMP-2 or MMP-9 is up-regulated [50, 51]. Repeating local injections in restricted area has been assessed to compensate for poor migration, but this approach makes the treatment of the whole musculature of DMD patients very complex [52, 53]. Furthermore, this cannot be carried out for vital muscles such as heart or diaphragm. Unfortunately, the mechanisms and molecules implicated in myoblast limited migratory in vivo remain unknown and would deserve to be better investigated.

An answer to this lack of migration of muscle progenitors would be to deliver them by a systemic route. The advantage of systemic route is that grafted cells can infiltrate the regenerative muscle through vessels and significantly improve their dispersion. This way of transplantation is not possible for myoblasts since they are not able to cross the endothelial blood vessel barrier [54].

Skeletal Muscle Aldehyde Dehydrogenase-Positive Cells

Aldehyde dehydrogenase 1A1 (ALDH1A1) is a cytosolic and ubiquitously distributed detoxifying enzyme. Cells positive for ALDH1A1 can be found in human bone

marrow, umbilical cord blood and peripheral blood. The cells characterised by a high expression of this protein do not exceed 3–4 % of the mononucleated cell fraction, but represent a stem cell subpopulation capable of differentiation into different cell types, including mesenchymal lineage [55]. In freshly isolated human adipose tissue-derived stromal vascular fraction, this proportion rises to 14 % [56].

Two distinct subpopulation of aldehyde dehydrogenase-positive cells can be isolated from skeletal muscle, based on the expression of CD34. These cells, called skeletal muscle aldehyde dehydrogenase (SMALD), present different phenotypic and functional characteristics. Whereas SMALD/34+ cells are likely associated with a mesenchymal profile, SMALD/34– cells do not basically express CD56 but can commit into a myogenic fate and effectively participate in muscle regeneration in vivo [57]. However, their characterisation in vitro still needs to be improved since they rapidly loose their initial ALDH+/34–/56– phenotype.

1.2.2.2 Candidates for Systemic Delivery

BMSCs and SP Cells

Initially promising experiments have been performed on bone marrow stem cells (BMSC) suggesting that some of these cells, whose precise phenotype remained unclear at that time, could participate in muscle regeneration in the mouse [58]. This participation in formation of muscle cells does not require Pax7 nor MyoD transcription factors as myoblasts do [59]. However, it appeared that the frequency of these fusion events is extremely low when injected intravenously in *mdx* mice and does not represent an effective stem cell therapy by BMSC: Dystrophin-positive fibres have been quantified and whereas clusters of dystrophin-positive fibres were apparent in muscle sections of grafted *mdx* mice, the proportion of these dystrophin-positive fibres did not statistically differ from the proportion measured in control animals, showing that these fusion events were very rare and potentially passive [60, 61].

Considering these results, cells derived from BMSC have been further investigated in order to isolate subpopulations exerting a better myogenic potential in vivo. Among them, bone marrow-derived side population cells (SP cells) have been identified by flow cytometry using their ability to efficiently exclude the vital DNA dye Hoechst 33342 [62]. This detoxification activity is mediated by the ABC transporter bcrp1 (ABCG2). SP cells can be isolated from mouse skeletal muscle: they are positive for stem cell antigen-1 (Sca-1), negative for hematopoietic markers such as CD45, CD43 and c-kit [63] and represent approximately 1 % of the mononuclear cells in adult mouse skeletal muscle. Intravenous injections of SP cells into irradiated mice suggested that these cells can participate in muscle regeneration. However, the percentage of donor-derived cells that had fused into myofibres from systemic delivery reached only 1–2 %. Alternative delivery method has been tested in order to improve donor cell engraftment. Grafted cells can migrate from the circulation into all hindlimb muscles of treated mice with an increased efficiency after an external muscle damage or in dystrophic muscles following exercise [64, 65]. However,

an intra-arterial injection of cultured cells derived from the SP leads to only 5–8 % of muscle fibres expressing the donor cells transgene [66]. More recently, the myogenic potential of SP cells was reinvestigated, and was found to be extremely low, although these SP cells could favour the engraftment of myoblasts by secreting factors enhancing myoblast proliferation and dispersion in vivo [67].

Pericytes/Mesoangioblasts

Mesoangioblasts are vessel-associated mesodermal progenitors that have been initially isolated from the mouse dorsal aorta but also exist in avian and mammalian species [68–70]. The human counterpart of mouse mesoangioblasts, which are suspected to correspond to cells previously defined as pericytes [30], express markers such as nerve/glial antigen 2 (NG2) proteoglycan and also alkaline phosphatase (ALP). In vitro, human mesoangioblasts can easily proliferate and spontaneously differentiate into myotubes, although they do not express initially myogenic markers. They are also easily transduced with lentiviral vectors. Very promising results have been collected concerning the myogenic capacity of these cells: in 2003, the team of G. Cossu has transplanted mouse mesoangioblasts by intra-arterial route into dystrophic mice and observed an amelioration in muscle structure and function [71]. Later, similar results have been observed by the same group on dystrophic (GRMD) dogs: They observed that allogenic canine mesoangioblasts transduced with mini-dystrophin significantly improved the deficient mobility of immunosuppressed GRMD dogs [72], suggesting that human mesoangioblasts may be a promising cell candidate for stem cell therapy in DMD patients. A phase I clinical trial with allo-transplantation of mesoangioblasts in DMD patients has been launched and preliminary results are not yet available.

CD133+ Cells

CD133 marker is expressed by a subpopulation of hematopoietic stem cells. These cells represent a very small fraction of the mononucleated cells present in the adult peripheral blood (also called AC133 cells) and cells purified from skeletal muscles based on the expression of this CD133 marker are also positive for some myogenic markers such as desmin.

Human blood-derived CD133-positive cells can undergo myogenesis when cocultured with myogenic cells, and their engraftment into immunodeficient *mdx* mice by systemic route contributes to the generation of myofibres and to the replenishment of satellite cell pool [73]. Functional tests showed that treated muscles recovered force after the transplantation. Furthermore, a comparison of their muscle regenerative potential to that of *bona fide* human satellite cells in immunodeficient mice showed that they are more effective than myoblasts to participate in host's regeneration and to produce satellite cells [74]. Genetic correction of blood-derived and muscle-derived CD133-positive cells isolated from DMD patients is also

feasible. The engraftment of these cells results in a significant recovery of muscle morphology, function and dystrophin expression in dystrophic mice, suggesting that an autologous stem cell therapy for DMD patients is possible using CD133-positive cells [75]. The possibility of extracting a muscular progenitor, which is capable of participating in a quantitative muscle regeneration, from blood patients is a trump card since it avoids for patients a muscle biopsy in already devastated muscles, in which myogenic cell quality is likely to be already altered. However, the myogenic fate of these blood-derived AC133 cells remains to be tightly controlled, since they can give rise to multiple progeny: It has been assessed that intra-muscular injections of human CD133+ cells accelerated muscle regeneration in a rat muscle injury model, likely due to an up regulation of VEGF secretion and due to their ability to differentiate into both endothelial and skeletal myogenic lineages [76]. However, the conditions to amplify these cells in vitro need to be further defined prior to envisaging clinical trials. An autologous transplantation of muscle-derived CD133+ cells has been performed in a DMD patient [77]. Stem cell safety has been investigated and no side effects have been observed. In this work, an increase of capillary proportion has been noticed in treated muscles.

1.3 Which Clues for an Effective Stem Cell Therapy?

The cell candidate for cell therapy of muscular dystrophies should be adapted to the type of dystrophy. For dystrophies concerning the majority of the body's musculature, heart and diaphragm, such as DMD, cell therapy would require a progenitor that can be administered through systemic delivery: it should therefore respond to chemoattraction to degenerative sites, and be capable of crossing vessels and colonising injured muscle tissue. Such a candidate should also present a strong and stable myogenic potential, but only once it is in a muscle environment in order to be safe for patients: The transplantation of high number of inadequate cells in circulation can generate vascular complications for patients such as thrombosis. In view of these requirements, mesoangioblasts and CD133+ cells could represent serious options for DMD or BMD, but further experiments or toxicity tests have to be performed, e.g. concerning the pluripotentiality of CD133+ cells isolated from the blood, prior to passing from bench to bedside. They should also be amplifiable in clinical conditions, including taking into account economic parameters. Autologous progenitors should be preferred to avoid immune suppression of the patients, since even immune-privileged cells may not keep this property once they differentiate into the myogenic lineage.

Amplified myoblasts (or other candidates for intramuscular delivery), despite their remaining limitations, still make sense for focused treatments of localised forms of muscular dystrophy such as OPMD, or eventually FSH. These diseases are characterised by the cohabitation in the same patients of injured and clinically spared muscles, these last ones potentially providing a source of autologous myoblasts. For late-onset dystrophies, autologous myoblasts may be used as long as the

effect of the mutation, present in all cells, is not rapidly occurring after transplantation. OPMD is such a late-onset disease, mainly characterised by a progressive weakness of eyelid and swallowing muscles with very late involvement of some proximal limb muscles. While myoblasts isolated from cricopharyngeal muscle present defects in proliferation, those isolated from spared muscles of OPMD patients do not present this defect [78]. A phase I clinical trial of myoblast transfer therapy has been launched, using autologous myoblasts isolated from non-clinically affected muscles (sterno-cleido-mastoidius, or vastus lateralis), expanded in vitro and transplanted into crico-pharyngeal muscle of patients. The first outcome of this trial is to assess feasibility and eventual toxicity, but the final aim will be to increase the motility of the pharyngeal zone. Preliminary data show that the treatment is safe and their function of swallowing is improved. For other diseases with an earlier onset, the requirement for a gene therapy of the candidate cells will have to be assessed.

Further investigations aiming at the improvement of the graft efficiency are still in process. Very recent data performed using human myoblasts transplanted into cryo-injured muscle of immmunodeficient mice gave more insights concerning the behaviour of human myoblasts in an in vivo context after transplantation. The peak of myoblast death occurs between 12 and 24 h following implantation [40]. Increasing human myoblast resistance to cellular stress prior to transplantation by up regulating the heat-shock protein expression inhibits the cell loss and generates a better participation of human progenitors to muscle regeneration [79]. Better understanding of the process leading to myoblast death would help designing better injection protocols and limit the requirement for extensive in vitro amplification, which may be detrimental to their in vivo efficiency [38]. We also observed that only 6 h post injection, only 10 % of myoblasts proliferates, and at 72 h, almost all myoblasts have started to differentiate, limiting their dispersion potential: At 5 days post transplantation, cell migration has ended. By conditioning myoblasts with an environment enriched in growth factors, the proliferation and dispersion of myoblasts are increased and extended, resulting in a higher number of fibres expressing human proteins and/or containing human nuclei 1 month post transplantation [40]. These results suggest that a modulation of the environment favouring proliferation (without preventing at long term the differentiation) and migration can significantly increase the regenerating capacity.

This is envisaged by two main ways:

- Co-injecting progenitors with other cell types: For instance, the immunosuppressive and trophic properties of mesenchymal stem cells may be interesting to exploit, even if they do not exert in vivo a sufficient myogenic potential [80, 81]. Muscle-derived CD31(−)/CD45(−) subpopulation of SP cells have been shown to increase in vivo the mitotic and migratory activity of myoblasts [67]; however they represent a very small fraction of muscular mononucleated cells. We have assessed recently co-injection in vivo of human myoblasts with human pro-inflammatory macrophages derived from blood monocytes, using an immunodeficient mouse model. We observed that this procedure extends the proliferative phase of myoblasts, delays the differentiation and significantly increases the

migration of myoblasts. Grafted pro-inflammatory macrophages do not participate themselves in regeneration by a passive fusion with myofibres but exert a trophic effect on myoblasts in vivo. Moreover, after several days, at least a part of pro-inflammatory macrophages switch their phenotype into anti-inflammatory phenotype, which favour myogenic differentiation [82, 83]. This phenomenon is explained in vitro by the fact that pro-inflammatory macrophages can change their secretion profile by phagocytizing apoptotic debris, delivering no longer inflammatory cytokines such as TNFα and IL1β, but mostly anti-inflammatory cytokines such as TGFβ and IL10 [82].

- Packaging progenitors with a customised scaffold: These last years emerged the idea of controlling the progenitor microenvironment by implanting them within a synthetic matrix, limiting cell stress and thus cell death upon implantation and favouring cell growth and dispersion from this artificial scaffold. This strategy has been tested using different types of matrix. Recently, promising results concerning myoblast cell death and regeneration kinetics have been obtained using photopolymerisable hydrogel [84] and/or scaffolds delivering insulin-like growth factor (IGF1; implicated in muscle growth and myoblast differentiation) and vascular endothelial growth factor (VEGF; favouring angiogenesis) [85]. However, how this strategy can be applied in a clinical context still needs to be determined.

Research concerning stem cells with a myogenic potential is still ongoing, in particular concerning embryonic (ES) and induced pluripotent stem (iPS) cells. These future potential candidate cells might be promising sources of progenitors for cell therapies. hES cells are derived from the inner cell mass of the embryonic blastocyst and can retain the potential to differentiate into cells belonging to ectoderm, mesoderm and endoderm lineages. However, therapeutic applications are still very far since hES cells, although poorly immunogenic, may be rejected once differentiated into the myogenic lineage, and can be tumorigenic, leading to formation of teratoma [86]. Multipotent mesenchymal precursors hES-derived have been transplanted and the formation of few myofibres was observed [87], but these results will require further confirmation. iPS cells can be generated from adult human dermal fibroblasts by transduction of four defined transcription factors: Oct3/4, Sox2, Klf4 and c-Myc [88]. As hES cells, however, a recent publication assessed that a conditional expression of Pax7 in human ES/iPS cells can generate myogenic precursors [89]. Their engraftment into dystrophin-deficient muscles produced human-derived dystrophin-positive myofibres and improved strength in a dystrophic mouse model. iPS cells represent a major improvement over hES cells since they can be isolated from the patient and thus be administered autologously. However, they still require multiple transduction, thus increasing the risk of insertional mutagenesis, and further progresses are required before any clinical application can be considered.

Acknowledgements The research conducted by the authors in the field of cell transplantation is supported by Association Française contre les Myopathies (AFM) and Association Institut de Myologie (AIM), Université Pierre et Marie Curie (UPMC), INSERM, CNRS, PHRC, ANR Genopath In-A-Fib, DPP-Nl and EC contract MYOAGE (contract HEALTH-F2-2009-223576) from the seventh FP.

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Chapter 2
Urine-Derived Stem Cells: Biological Characterization and Potential Clinical Applications

Guihua Liu, Chunhua Deng, and Yuanyuan Zhang

Abstract A subpopulation of urine-derived cells, termed urine-derived stem cells (USCs), possess stem cell capabilities, such as self-renewal and multipotential differentiation. These cells can differentiate into mesodermal cell lineages, such as osteocytes, chondrocytes, adipocytes, endothelial cells, and myocytes, including smooth muscle cell differentiation and endodermal lineages (e.g., urothelial cells). These cells maintain high telomerase activity and possess long telomeres; further, they retain a normal karyotype in vitro even after several passages. Importantly, these cells do not form teratomas in vivo. USCs express cell surface markers associated with pericytes and mesenchymal stem cells. These cells can be isolated from regular voided urine from each individual via a noninvasive, simple, and low-cost approach. The USCs isolated from one single urine specimen can generate up to 100 million cells at early passage, sufficient numbers to use for cell-based therapy for tissue repair.

Keywords Stem cells • Urine • Cell differentiation • Urinary tract system • Tissue regeneration

Abbreviations

3-D	Three-dimension
ECs	Endothelial cells
EFM	Embryonic fibroblast medium

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K. Turksen (ed.), *Stem Cells: Current Challenges and New Directions*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4614-8066-2_2,

EGF	Epidermal growth factor
FDA	Food and Drug Administration
HUVECs	Human umbilical venous endothelial cells
KSFM	Keratinocyte serum-free medium
MSC	Mesenchymal stem cells
PD	Population doublings
PDGF-rβ	Platelet-derived growth factor-B and -receptor
RPM	Revolutions per minute
SIS	Small intestinal submucosa
SMCs	Smooth muscle cells
UCs	Urothelial cells
UPCs	Urine-derived progenitor cells
USCs	Urine-derived stem cells
uUSCs	Stem cells collected from upper urinary tract
VEGF	Vascular endothelial growth factor
vUSCs	Stem cells collected from voided urine samples
vWF	Von Willebrand factor
α-SM actin	Alpha-smooth muscle actin

2.1 Introduction

Each tissue and organ in the body has a population of stem/progenitor cells which are involved in tissue repair and regeneration after injury. When these cells are harvested and expanded in culture, their potential to differentiate into various cell lineages allows them to be used for cell-based therapies and tissue engineering. Currently, cells for use in tissue repair are usually obtained from biopsies of specific tissues. The utility of expanded cell populations from such biopsies has been demonstrated in the production of tissue-engineered bladders and urethral tissue. However, potential complications of the biopsy procedure include bladder or urethral trauma, local tissue bleeding, infection and patient discomfort. To eliminate these complications and decrease medical costs, a noninvasive procedure to obtain cells would be highly desirable.

We recently demonstrated that it is possible to isolate and expand stem/progenitor cells from human-voided urine (voided USCs) [1–3] and urine obtained from the upper urinary tract (uUSCs) [4]. Approximately 0.2 % of cells collected from voided urine express markers characteristic of mesenchymal stem cells (MSCs), and they can expand extensively in culture. USCs have self-renewal capability consistent with stem cells. These cells can grow up from a single cell clone to large amounts of cells with an average doubling time of 20–31 h, depending on the passage number, and these cultures can be maintained for up to 57 population doublings [4, 5]. Importantly, USC can differentiate toward multiple bladder cell lineages as identified by the expression of urothelial, smooth muscle, endothelial and interstitial cell markers. In recent experiments, our study indicated that

urine-derived cells can give rise to additional specialized types, including osteocytes, chondrocytes, and adipocytes. The benefits of employing USCs are that these cells can be obtained noninvasively, using a simple, low-cost technology to harvest cells with good quality and quantity [1]. Here, we review the biological characterization and the potential clinical applications of urine-derived stem cells based on our previous data.

2.2 Isolation of USCs

In our previous description of the isolation of USCs [1], mid- and last stream urine was collected, and these urine samples were centrifuged. The supernatant was removed. The cell pellet was gently resuspended in mixed media composed of embryonic fibroblast medium (EFM) and keratinocyte serum-free medium (KSFM) (1:1 ratio) and the cells were plated in 24-well plates (*p0*). Three types of living cells exist in urine: differentiated, differentiating, and urine-derived stem cells. About 99 % of the living cells in urine did not attach to culture plates and were removed when the culture medium was changed. Morphologically, these cells were large and flat epithelial cells, suggesting that they were terminally differentiated. About 0.1 % of cells in urine are differentiating cells. These cells attached to culture dishes, expanded to about 10^3 cells within 2–3 weeks and consisted of at least four cell types based on morphology and phenotype. Some cells had a cobblestone appearance under phase contrast microscopy and they expressed uroplakin on immunofluorescence staining, indicating that they were of urothelial origin, while other cells were spindle shaped and expressed desmin, suggesting that they were of muscle origin. A third cell type had a circular appearance and expressed Von Willebrand factor (vWF), indicating an endothelial origin. Finally, cells with an elongated appearance were found to express c-kit, and these were considered to be interstitial cells. However, the number of all four differentiating cell types in primary culture gradually decreased after 3–4 weeks and did not grow after subculture.

About 0.2 % of the cells in urine have a phenotype consistent with multipotent stem cells and we designated them as urine-derived stem cells. These cells are easily cultured, appear genetically stable after a number of passages, and maintain the ability to give rise to more differentiated progeny. USCs comprised an average of 5–10 cells per 100 ml urine. USC clones were obtained from almost all of the urine samples we tested. Fresh urine showed the highest rate of colony formation (67 %) and urine stored at 4 °C showed the lowest rate (30 %). Urine from 13 to 40-year-old volunteers provided the highest rate of clone recovery. Catheterization significantly enhanced the number of USCs in urine compared to spontaneously voided urine, possibly because more cells were scraped off the inner bladder wall by the catheterization procedure. Collecting triple urine samples also increased the rate of clone formation compared to using single urine samples. A few days after being placed in a well, a single cell formed a cluster of cells that appeared small, compact and uniform (Fig. 2.1). A consistently high yield of cells was achieved from each clonal line.

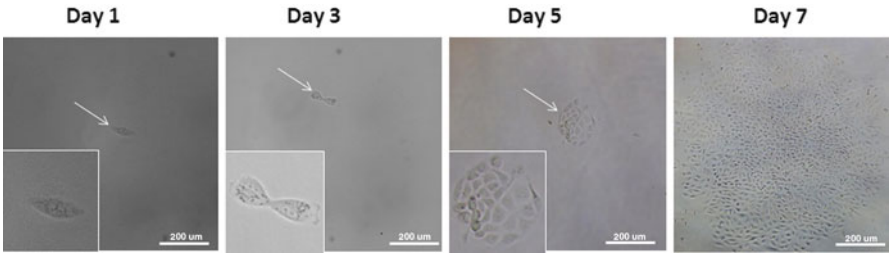

Fig. 2.1 One single USC (p0) that propagated into a clone. One single cell was founded at day 1 and it splited to two cells at day 3. A minor USC clone formed at day 5 and small, compact, and uniform "grain rice"-like clone was formed at day 7. Scale bar = 200 μm

About 2 weeks were required for cells to attain confluence at passage 1 in a 3 cm diameter well, 3½ weeks to expand to approximately one million cells in a 10 cm culture dish at passage 2, and 6–7 weeks to expand to approximately 100 million cells at passage 4. These cells showed normal exponential cell growth patterns with a steady increase in number during a 10-day culture period. These urine-derived cells also showed the ability to differentiate into various cell lineages as described below, and were capable of growing for up to 19 passages in vitro.

2.3 Bio-characterization of USCs

Cells from human urine specimens can be consistently cultured long term using a medium that we originally developed for culture of rat urothelial cells (USc) [6]. However, we found that the phenotype of the cultured human urine-derived cells was not that of primary urothelial cells. The primary cultures from urine did not show expression of UC-specific markers such as uroplakin I/IIIa, and cytokeratins (CK7, CK13, and CK19/20). Instead, the cells that we have designated as USCs displayed a surface marker phenotype consistent with MSCs and pericytes, namely CD44, CD73, CD90 (Thy-1), CD105 (endoglin), CD133, CD146, NG2, and PDGF-rβ. However, the percent of cells expressing these markers decreased with increasing passage number [7–11]. These cells were negative for the general hematopoietic cell marker CD45, hematopoietic stem cell markers, and other hematopoietic and endothelial lineage markers, including CD31 and CD34, indicating that these cells were not endothelial or hematopoietic progenitor cells.

USCs initially derived from a single cell are able to give rise to both UCs and SMCs [4]. After growth in medium containing epidermal growth factor (EGF, 30 ng/ml), the cells were efficiently induced to express the uroplakin and cytokeratin gene and protein markers [4]. After culture in a medium specialized for myogenic differentiation, the differentiated USCs expressed markers consistent with smooth muscle, including alpha-smooth muscle actin (α-SM actin), desmin, calponin, and myosin [1, 4]. We concluded that the urine-derived progenitors were at

least bipotential for the major bladder cell types. This result was surprising, because it was generally believed that muscle and uro-epithelial cells in bladder represent separate cell lineages derived from mesoderm and endoderm, respectively. We initially designated these cells as urine-derived progenitor cells, or UPCs, but we have recently observed that USCs can also differentiate to yield the characteristic cell lineages obtained from MSCs, such as osteocytes, adipocytes, and chondrocytes [12]. This led us to conclude that the urine-derived cells were stem cells capable of giving rise to both mesoderm and endoderm lineages.

The quality of cells obtained from urine is similar to that of the biopsy-derived cells described above. When differentiated, USCs express all proteins characteristic of the various bladder cell lineages. Karyotype analysis has demonstrated that these cells are genetically stable. Importantly, there is a major cost advantage to using USCs—it costs about US$50 to obtain cells from urine, versus about US$5,000 to isolate cells from a biopsy procedure. About 1.4×10^9 urothelial and SMCs are required for bladder tissue regeneration[1]. We estimate that three to four urine samples (about 25–40 USCs/800 ml urine) expanded for 4–5 weeks would yield a sufficient quantity of low passage, healthy cells for clinical tissue engineering applications. This time frame is comparable to that required for expansion from a tissue biopsy (7–8 weeks) [13]. USCs and the cells obtained through urological tissue biopsies come from the same urinary tract systems and have similar biological features. Therefore, collecting cells from urine could be an attractive alternative to the standard urological tissue biopsies currently used in cell therapy and tissue engineering.

2.4 Characterization of uUSCs

More recently, we found that some urine-derived cells from the upper urinary tract possessed characteristics similar to vUSCs, i.e., expansion capacity and bipotent differentiation to urothelium-like and SMC-like cells. The uUSCs can generate a large cell population from a single cell, like voided USCs. We observed that the average expansion capacity of uUSCs is 46.5 ± 8.6 population doublings (PD) (range 35–57 PD, $n = 4$). This implies that a single stem cell from the upper urinary tract, on average, can generate 1.0×10^{14} cells ($2^{46.5}$), within about 8 weeks. To retain good bipotent differentiation capacity, we typically use USCs below expansion passage 5 (p5). Under our optimized culture conditions, one single cell of uUSC can generate $2^{28.6} = 4.0 \times 10^8$ cells within about 4 weeks [4], at p5. It is known that 1.4×10^9 cells are required for both SMCs and UCs to create a tissue-engineered bladder[2]. Our recent data showed about 150 ml of urine obtained from the upper urinary tract via nephrostomy tube contains ten uUSC clones. Expansion of the stem cells from this volume of urine potentially can yield about 4×10^9 cells. Thus, assuming efficient differentiation, uUSCs can provide an adequate number of cells to engineer a neobladder. Importantly, uUSCs are a reliable cell source, as cell clones can be obtained from almost every urine sample [4]. It appears that uUSCs become voided USCs when urine drains from the kidney to the bladder for storage.

In chronic bladder diseases or muscle-invasive bladder cancer, uUSCs might be a good cell source for bladder tissue regeneration because the cells from the upper urinary tract are usually normal. In addition, the risk of finding ureter, renal pelvic, or kidney cancer in bladder cancer patients could be eliminated with careful scanning by a series of examinations. These scanning examinations include urine cytology, imaging tests (such as intravenous pyelogram, bone scan, computed tomography scan, magnetic resonance imaging, and lung X-ray), and cystoscopy/nephroureteroscopy and tissue biopsy from upper urinary tract. In treatment of end-stage bladder diseases or bladder cancer, using engineered bladder tissue with uUSCs as the cell source would be superior to current surgical procedures, i.e., bladder reconstruction using intestinal segments. Risks of use of bowel segments include: (1) tumorigenicity, as intestinal segments appear to be at an increased risk for malignancy, particularly adenocarcinoma, because of histological changes in the intestinal mucosa after long-term exposure to urine; and (2) complications such as stone formation and excess mucous secretion. Harvesting uUSC from patients who already have a nephrostomy tube in place would be a simple and low-cost approach to obtaining cells for engineering bladder tissue. Therefore, cells derived from upper urinary tract urine might be a good source for bladder tissue engineering in patients with bladder cancer [4].

2.5 Interaction of USC and Biomaterials for Tissue Engineering

Combining autologous stem cells with natural or synthetic biomaterial scaffolds provides a promising strategy for cellular delivery and engineering tissues. When combined with appropriate scaffold materials, USCs could be effectively used in urological tissue engineering. We seeded USCs or urothelial and smooth muscle cells differentiated from USCs onto a porous bacterial cellulose scaffold or modified three dimension (3D) porous small intestinal submucosa (SIS) scaffold under dynamic culture conditions to generate a cell-based tissue-engineered urinary conduit or urethra [2, 14]. Porous bacterial cellulose and SIS provided a 3D cell growth environment in vitro. As a nondegradable material, bacterial cellulose is an attractive candidate for creating a tissue-engineered conduit because this Food and Drug Administration (FDA)-approved biomaterial [15] is highly hydrophilic and causes little fibrosis when implanted [16]. This polymer is biosynthesized as a network of nanofibrils. The fibril entanglement and hydrogen bonding within the cellulose network provides high mechanical strength and a large surface area [17]. When implanted subcutaneously in rats, bacterial cellulose does not elicit fibrosis or induce proliferation of giant cells [16]. Bacterial cellulose has been shown to remain intact for 90 days when implanted subcutaneously [16]. We chose to use the 300–500 mm pore size range for the USCs seeding experiment because this range would allow adequate space for cell growth and extracellular matrix secretion and

remodeling, as well as for ingrowth of blood vessels from the native tissue after implantation. Therefore, it would be an alternative for bladder replacement when USCs are seeded within the porous bacterial cellulose scaffold.

SIS, another commonly used natural collagen scaffold, possesses a unique property, in which its permeability is "sided," or direction dependent. The mucosal to serosal direction is less permeable than the serosal to mucosal direction. When non-seeded SIS is used in urological applications, this "sidedness" property should be considered because it can assist in preventing urine leakage from the lumen of the urethra or bladder into surrounding tissues. However, in cell-based tissue engineering, this direction-dependent permeability appears less important in preventing urine leakage, because heavy cellular infiltration "fills up" the pores within the matrix to prevent leakage [18–24]. Therefore, USCs are able to form multilayered tissue structures and grow into the matrix as well when seeded on the more porous serosal side in vitro under dynamic culture conditions. The speed of dynamic culture also affects cell proliferation and multilayer formation on scaffold matrices. For example, it has been shown that when bladder cells are seeded on a collagen matrix such as decellularized bladder submucosa and cultured in dynamic conditions at 40 rpm (RPM), cell layer formation is enhanced compared to both 10 rpm and static culture conditions [25].

Additionally, epithelial–stromal cell interactions in cocultures play an important role in cell growth and are an efficient means of promoting cell growth, cell-matrix infiltration, and cell differentiation. The cell–cell communication present in coculture conditions facilitates cell signaling and thus promotes epithelialization [21]. Layered cocultures of urothelial and smooth muscle-differentiated USCs showed better cell growth and cell-matrix penetration and epithelialization compared to monoculture conditions [25]. The multilayered structure covered the entire surface of the polymer scaffold, with smooth muscle cells infiltrating the scaffold to a large extent.

2.6 Implantation of USCs In Vivo

To monitor the fate of differentiated USCs in vivo, cell-scaffolds were subcutaneously implanted into athymic mice and then tracked using immunohistochemical staining for human nuclear antigen. After USCs were induced to differentiate into urothelial and smooth muscle cells (SMCs), induced USCs (10^6 cells/cm^2) were seeded onto scaffolds such as bacterial cellulose or SIS in a layered coculture fashion under static and 3D dynamic (10 or 40 rpm) conditions for 2 weeks. Following the in vitro culture, the cell-scaffold constructs were then implanted in vivo for 4 weeks. This revealed that the porous scaffolds allowed three-dimensional growth of the cells, leading to formation of a multilayered urothelium and SMC-l matrix infiltration [2]. USCs that were induced to differentiate also expressed UC markers (Uroplakin-III and AE1/AE3) or SMC markers (α-SM actin, desmin, and myosin) after implantation into athymic mice for 1 month, and the resulting tissues were similar to those formed when UCs and SMCs derived from native ureter were used [14].

We also evaluated the effects of vascular endothelial growth factor (VEGF) overexpression on urine-derived stem cell survival and myogenic differentiation to determine whether these cells could be used as a novel cell source for genitourinary reconstruction. USCs were infected with an adenoviral vector containing the mouse VEGF gene (USCs/Ad-VEGF). USCs/Ad-VEGF was mixed with human endothelial cells (ECs) (total, 5×10^6 cells) in a collagen-I gel. These cell containing gels were subcutaneously implanted in an athymic mouse model. USCs expressed SMC markers after implantation in vivo, indicating that VEGF expression enhanced myogenic differentiation of USCs and muscle regeneration in vivo. This result might be due to a direct effect of angiogenesis, an indirect effect mediated by an autocrine factor that promotes muscle cell differentiation or both [26–31]. Our recent study demonstrated that VEGF expression by VEGF-expressing USCs, along with concurrent endothelial cell implantation, promoted angiogenesis, significantly improved in vivo cell survival and myogenic differentiation of USCs, and enhanced nerve regeneration within the graft, which maintained its size [30]. The safety of using cells that gene overexpress VEGF remains a concern due to the fact that overexpression of VEGF has been associated with urothelial cancer. The optimal dosing of VEGF and long-term follow-up after implantation of cells expressing VEGF requires further investigation. Autologous VEGF-expressing USCs combined with human umbilical venous endothelial cells (HUVECs) as an alternative cell source for urological cell therapy appears feasible and may be useful in genitourinary reconstruction, such as treating vesico-ureteral reflux and stress urinary incontinence with cell therapy or even in repairing urethral stricture and neuropathic bladder with tissue engineering technology.

2.7 Conclusion

There are several potential advantages to using USCs as a cell source for urological tissue engineering, including the following: (1) the cells can be easily harvested by a noninvasive method and grown in culture, as USCs do not require enzyme digestion or culture on a layer of feeder cells to support cell growth; (2) cells can be harvested from urine via noninvasive procedures rather than biopsies, and thus patient morbidity and potential complications such as urethral or bladder trauma and urinary tract infections are avoided; and (3) as USCs are autologous somatic cells, no ethical issues are involved in their use for tissue reconstruction, and no immune reaction to engineered implants should occur. Therefore, obtaining and using cells from urine could be an attractive alternative to the standard urological tissue biopsies currently used in cell therapy and tissue engineering.

Acknowledgments The authors would like to thank Dr. Jenifer Olson for her editorial assistance.

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Chapter 3
Immunoregulatory Properties of Mesenchymal Stem Cells: In Vitro and In Vivo

Pinar Çetinalp Demircan, Ayla Eker Sariboyaci, and Erdal Karaoz

Abstract Over the last few years, because of their self-renewal capacity and multilineage differentiation potency, mesenchymal stem cells (MSCs) have been thought to have important therapeutic potential. MSCs are considered to be effective in immune system by suppressing maturation of DC and the functions of T cells, B cells, and natural killer (NK) cells, by inducing regulatory T (Treg) cells. Although target cell–MSC interactions may play important role, the MSC-mediated immunosuppression also mainly acts through the secretion of soluble molecules and cytokines that are induced or upregulated following interactions with immune cells. The majority of data on the immunomodulation of MSCs are in vitro, although several studies have been in vivo. Various animal models such as mouse, baboon, and rat have been used to evaluate in vivo MSC immunoregulatory properties related to alloreactive immunity in SC and organ transplantations, autoimmunity, or tumor immunity. Clinical studies with MSC have aimed to demonstrate promising results in treating patients with cancer, reducing the incidence of GVHD after BM transplantation, improving and treating amyotrophic lateral sclerosis, Crohn's disease, metachromatic leukodystrophy, Hurler syndrome, rheumatoid arthritis, type 1 diabetes mellitus, lupus nephritis, and liver cirrhosis.

Keywords Mesenchymal stem cells • Immunoregulation • Immune cells • In vitro • In vivo

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K. Turksen (ed.), *Stem Cells: Current Challenges and New Directions*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4614-8066-2_3,

3.1 Immunoregulatory Properties of Mesenchymal Stem Cells: In Vitro and In Vivo

3.1.1 Mesenchymal Stem Cells

Multipotent mesenchymal stem cells (MSCs) are adult multipotent non-hematopoietic stem cells (non-HSCs) capable of self-renewal and generation of different cell lines. MSCs were first identified as an adherent and fibroblast-like population by Friedenstein and his colleagues who first isolated and characterized them from adult BM [1]. MSCs have also been identified from various postnatal tissues including cord blood [2], dental pulp [3, 4], natal teeth [5], adipose [6, 7], placenta [8], amnion [9], PB [10], pancreatic islets [11–13], and endometrium [14].

3.1.1.1 Isolation, Expansion, and Differentiation Capacity of MSCs

MSCs have been shown to be able to differentiate in vitro and in vivo into various mesodermal cell lineages including osteocytes, adipocytes, chondrocytes, muscle, and myelo-supportive stroma (Fig. 3.1) [15, 16]. In addition, some studies have

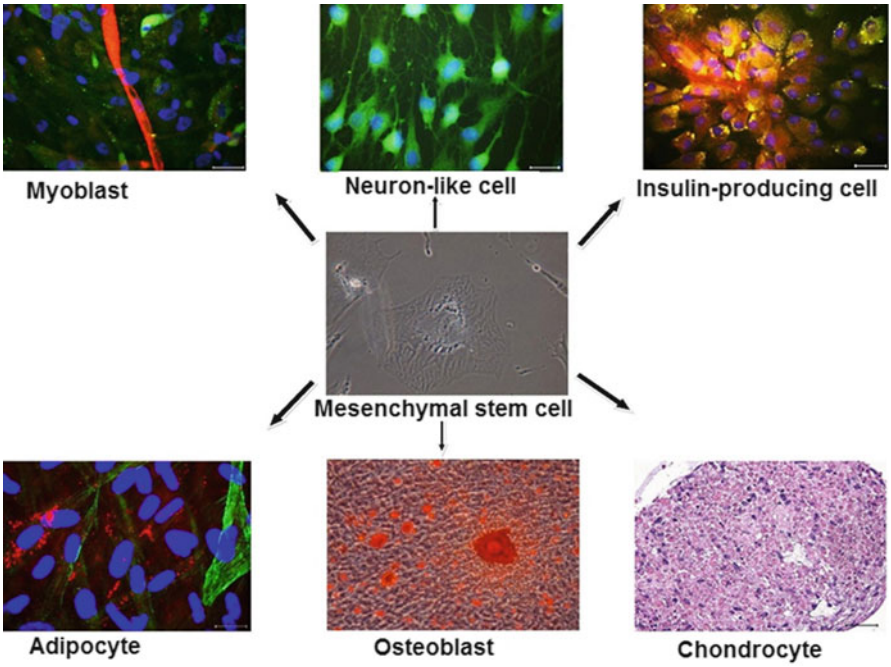

Fig. 3.1 MSCs have been shown to be able to differentiate in vitro and in vivo into various mesodermal, endodermal, and ectodermal cell lineages including osteocytes, adipocytes, chondrocytes, neuron-like and insulin-producing-like cells, muscle, and myelo-supportive stroma

reported the ability of MSCs to differentiate in vitro into tissues from other germ layers such as ectoderm (neurons) and endoderm (hepatocytes) [17]. In vitro, MSCs can be expanded as adherent cells, can clonally regenerate, and can give rise to differentiated progeny but generally have a limited in vitro life span due to a lack of activity of immortalizing enzyme telomerase, a phenomenon called replicative senescence [18, 19]. MSCs are isolated by gradient centrifugation of BM aspirates to isolate mononuclear cells that are then seeded in tissue culture plates in medium containing fetal bovine serum. Then, MSCs adhere to plastic surfaces and can be expanded in culture plates while non-adherent cells are removed in the culture medium [20]. Taking advantage of their plastic adherence characteristic and in some cases associated with enzymatic tissue digestion and density gradient centrifugation methods, these cells may also be isolated from various tissues as mentioned above. Because sometimes they are a heterogeneous population, evidenced by the different morphology and functional potentials observed, and they do not meet the criteria of a stem cell, the International Society for Cellular Therapy (ISCT) recently reclassified these cells as multipotent mesenchymal stromal cells [21]. In order to create a consensus and more uniformly characterize these cells, later the ISCT also published a position statement to propose a standard set of criteria to define the identity of a MSC [22].

3.1.1.2 Characterization of MSCs

Human MSCs are cells with fibroblast-like (fusiform) shape, and in their early growth in vitro have the ability to form fibroblastic colony-forming units (CFUs). They are negative for hematopoietic surface markers CD14, CD33, CD34, CD45, CD117, and CD133 and positive for CD13, CD29, CD44, CD54, CD55, CD73, CD90, CD105, CD166, and Stro-1 [23–25]. But, as no single antigen is exclusively expressed by human MSCs, three criteria have been proposed by the ISCT for their characterization [22, 26]:

- Adherence to plastic surfaces: MSCs must be plastic-adherent when maintained in standard culture conditions using tissue culture flasks.
- Potential to differentiate into osteocytes, adipocytes, and chondrocytes: ≥95 % of the MSC population must express CD105, CD73, and CD90, as measured by flow cytometry. Additionally, these cells must lack expression (≤2 % positive) of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA class II.
- Expression of stem cell (SC) surface antigens: The cells must be able to differentiate to osteoblasts, adipocytes, and chondroblasts under standard in vitro differentiating conditions.

Recently, a subset of MSCs were identified in vivo and prospectively isolated from adult mouse BM by phenotypical, morphological, and functional criteria as PDGFRα+ Sca-1+ CD45− TER119− cells, providing a useful method to identify MSCs [27].

3.1.2 Immunoregulatory Mechanisms of MSCs

Over the last few years, because of their self-renewal capacity and multilineage differentiation potency, MSCs have been thought to have important therapeutic potential.

MSCs are considered to be effective in immune system by suppressing maturation of DC and the functions of T cells, B cells, and natural killer (NK) cells, as well as by inducing regulatory T (Treg) cells, which enhance their regulatory effects [28, 29]. Recent studies in both animal and human systems have shown that although the mechanisms underlying the immunoregulatory effects of MSCs are still unclear, they are probably thought to be effective through cell-to-cell contact and a variety of cytokines and soluble factors via paracrine manner.

3.1.2.1 Initially Stimulation Is Necessary for Immunoregulatory Functions of MSCs

MSCs have suppressive and modulatory properties on immune system cells and they are excellent source of regenerative medicine. The majority of data on the immunomodulation of MSCs are in vitro, although several studies have been in vivo. The immunoregulatory effects of MSCs have been shown in alloimmune and autoimmune diseases such as graft-versus-host disease (GVHD) [30–32], osteogenesis imperfecta [33], arthiritis [34], and encephalomyelitis [35–37]. Initially MSC-mediated immunoregulation requires preliminary activation of the MSCs in these diseases. T cells and NK cells are activated by dendritic cells (DCs) in endothelial cells and then interferon-gamma (IFN-γ) cytokine released by them. IFN-γ stimulation could play a dual role in enhancing the efficacy of T-cell accumulation and secretion of some chemokines (CXCR and CCR) by parenchymal cells. MSC migration and activation are induced through the receptors of these chemokines located in the MSCs and IFN-γ in the damaged tissue [38, 39] (Fig. 3.2).

3.1.2.2 In Vitro Immunoregulation by MSCs

MSC Immunosuppression Is Mediated by Soluble Factors and Cytokines

Although target cell–MSC interactions may play important role, the MSC-mediated immunosuppression also mainly acts through the secretion of soluble molecules and cytokines that are induced or upregulated following interactions with immune cells (Fig. 3.3).

Effects of MSCs on T Cells

The earliest studies that investigated the immunosuppressive nature of MSCs were performed with human, baboon, and murine models, and demonstrated that MSCs

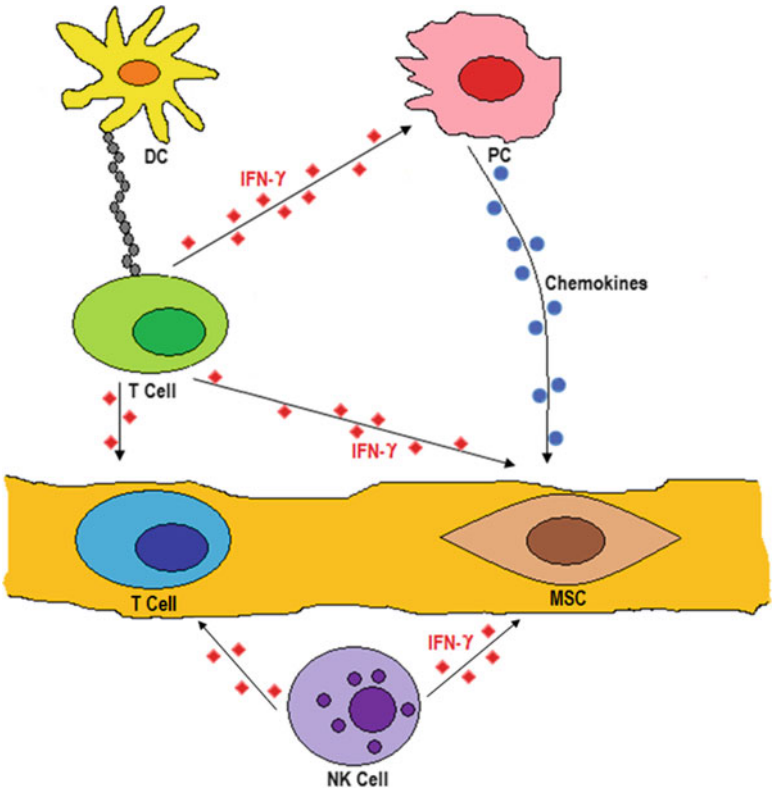

Fig. 3.2 Activation of T cells and NK cells by DCs and stimulation of MSCs through the chemokines and IFN-**γ** secreted by these cells in endothelial cells of damaged tissue

are able to suppress T-cell activation and proliferation in vitro and in vivo [28, 40]. However, the molecular mechanism underlying these effects is still unclear and needs to be explored in much greater detail; they probably require both cell-to-cell contact and a variety of cytokines and soluble factors in a paracrine manner. Several factors and mechanisms have been proposed as playing a major part in the immunosuppressive role of MSCs. Clearly, a major mechanism leading to inhibition of immune-cell effector functions is the arrest of the cell cycle in G0/G1, which results in the inhibition of cell proliferation [41–43].

Our and other's studies have confirmed the immunomodulatory effects of MSC-derived BM [28, 56, 57], adipose tissue (AT) [56, 58], Wharton's jelly (WJ) [58], peripheral blood (PB) [11], cordon blood (CB) [58–60], placenta [61], amniotic fluid (AF) [62], and dental pulp (DP) [64, 65] on immune cells when they are cocultured in transwell systems (cytokines and soluble factors with a paracrine mechanism) and mixed lymphocyte reactions (MLR; cell-to-cell contact and paracrine effects) [53, 55, 57].

On the basis of the data available, the indication is that following paracrine soluble factors have been reported to be involved in MSC-mediated T-cell suppression such

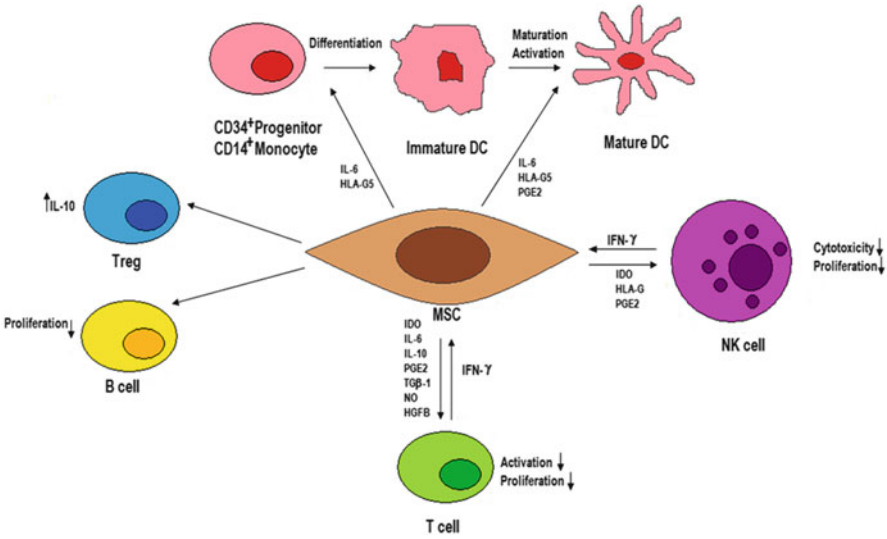

Fig. 3.3 Activated MSCs have modulatory effects on immune cells as T cell, B cell, DCs, and Tregs by secretion of some soluble mediators and cytokines such as human leukocyte antigen (HLA)-G, hepatocyte growth factor-**β**, indoleamine 2,3-dioxygenase (IDO), interleukin-6 (IL-6), interleukin-10 (IL-10), transforming growth factor-**β**, nitric oxide (NO), and prostaglandin (PGE2)

as hepatocyte growth factor (HGF)-β [1, 15, 42], transforming growth factor (TGF)-β [28, 44, 45, 65], indoleamine 2,3-dioxygenase (IDO) [46], prostaglandin E2 (PGE2) [47], nitric oxide (NO) [25], interleukin (IL)-6 [46, 65], human leukocyte antigen (HLA)-G [48, 49, 65], and vascular endothelial growth factor (VEGF) [50, 65]. Recently, it has been reported that intracellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 are essential for MSC–lymphocyte adhesion, and thus responsible for the immunosuppressive activity of MSCs [51, 65]. Additionally, some chemokines (stromal cell-derived factor, SDF-1; chemokine C-X3-C motif ligand 1, CX3CL1; chemokine C-C motif ligand 5, CCL5) and their receptors (chemokine C-C motif receptor 2, CCR2; CCR3; CCR4; C-X-C chemokine receptor type 4, CXCR4; and CX3CR1) have been shown to play a role in MSC migration and engraftment in injured tissues [52]. After this migration and engraftment, MSCs express some paracrine soluble factors and cytokines to inhibit T-cell activation and proliferation by suppressing the up-regulation of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, IFN-γ [53, 65], and IL-2 and IL-12 [54, 55] and by inducing the up-regulation of the anti-inflammatory cytokine IL-10 and regulatory T-cell (Treg; CD4, CD25, and Foxp3, forkhead box P3) markers [54, 65]. Furthermore it has been suggested that MSCs suppress cell division and induce the apoptosis of activated T cells [53, 65].

Only one study has indicated such a suppressive effect of hDP-MSCs, and the results of that study showed that the inhibitory effect of hDP-MSCs was greater than that of human BM-MSCs on the proliferation of phytohemagglutinin (PHA)-activated T cells [64].

In several studies, MSCs have been demonstrated to inhibit the expression of Th1 pro-inflammatory cytokines such as IFN-γ, IL-2, IL-12, and TNF-α, enhance T helper 2 (Th2) cytokine expressions, and induce Treg marker expressions [63]. Suppression of T-cell proliferation by MSCs has been reported to cause decreased IFN-γ production in in vitro cocultures [66]. Also in our laboratory we focused on the effects of pro-inflammatory cytokines by analyzing the decreased gene expression of IL-2, IL-6, IL-12, IFN-γ, and TNF-α, inhibited protein secretion levels of IL-2, IL-12, IFN-γ, and TNF-α, and inhibited expression levels of IL-2, IL-6, IL-12, IFN-γ, and TNF-α in MLR and transwell coculture systems [65].

Cytokines such as TGF-β1 and HGF-β1 are reported to be the primary mediators of T-cell suppression by MSCs [28]. They have been thought to carry out their suppressive effects in a synergistic manner, because when blocking antibodies against both cytokines were added to purify T cells, T-cell proliferation was completely restored [73, 74]. TGF-β1 and VEGF have been identified as tumor-associated factors that inhibit immune cell functions, and infusion of recombinant VEGF decreases the number of T cells [75, 76]. Furthermore, it has been shown that the addition of TGF beta receptor (rTGF-β) reduces T-cell proliferation in T cell/hBM-MSC MLR cocultures [28].

HLA-G, a nonclassical major histocompatibility complex (MHC) class I molecule, was firstly identified in cytotrophoblast and assumed a key role in tolerance of the fetus against the maternal. HLA-G is secreted by MSCs and has been demonstrated to exert immunosuppressive effects in MSC/T-cell cocultures [48, 65, 67]. It has also been shown to mediate MSC-induced Treg differentiation, inhibit the proliferation of T cells, and suppress cytokine secretion by T cells [48–50, 65].

IL-10 is also an important immunoregulatory cytokine produced by several cell types that promotes the development of Th2 cytokines and inhibits the production of Th1 pro-inflammatory cytokines, including IFN-γ, IL-2, IL-12, and TNF-α [65, 68, 69]. In addition to their linked expression, it has been demonstrated that both IL-10 and HLA-G5 are essential for full MSC-mediated immunosuppression and that they act in synergy [48].

IL-6 acts as both a pro-inflammatory and an anti-inflammatory cytokine and was identified as a mediator of MSCs; an inhibitor of T-cell proliferation, cytokine secretion, and cytotoxicity; and a stimulator of T-cell apoptosis [45, 46, 70]. However, it has been reported that IL-6 was able to stimulate T-cell proliferation [65, 71]. Furthermore, IL-6 is a potent pleiotropic cytokine that regulates cell growth and differentiation and plays an important role in the immune response. Dysregulated production of IL-6 and its receptor are implicated in the pathogenesis of many diseases, such as multiple myeloma and prostate cancer [72].

Plumas et al. [77] demonstrated that MSCs inhibit PHA-induced T-cell proliferation by inducing early apoptosis but have no effect on resting T cells. They confirmed that activated T cells died through an apoptotic mechanism in the presence of MSCs in MLR. In our study, it was found that hDP-MSCs significantly induced the apoptosis of PHA–CD3+ T cells in MLR and the transwell experiments on days 1 and 4 by using caspase IF labelling [65]. Similar to findings from other studies using time-lapse cameras, we observed apoptosis of PHA–CD3+ T cells in MLR

coculture in a video recording made with a time-lapse camera, with images collected every 4 min for the first 24 h of the experiment. We also determined that hDP-MSCs induced the apoptosis of PHA–CD3+ T cells in a video recording in the first 24 h [56, 65, 78].

CX3CR1 is a chemokine receptor that is reported to play a key role in the regulation of the immune response by Th1 cells and is important in some autoimmune diseases, such as multiple sclerosis (MS) [79, 80]. NCF-1 is an important gene in oxidative burst formation and regulates the severity of other autoimmune diseases, as well as indirectly modulating the degree of T-cell-dependent autoimmune responses [81]. During the late 1990s, several Th cytokines were identified; the IL-17A pleiotropic pro-inflammatory cytokine produced by Th17 cells has important functions in inflammatory responses [82]. Recently, it was shown that MSCs prevented the in vivo differentiation of naive CD4+ T cells into Th17 cells and inhibited the expression of IL-17, IFN-γ, and TNF-α by fully differentiated Th17 cells, as well as the production of Foxp3 and IL-10 [45, 83]. IP-10 (CXCL10) is a CXC chemokine that selectively chemoattracts activated T cells, NK cells, and monocytes through binding to its receptor, CXCR3, thus inhibiting cytokine-stimulated hematopoietic progenitor cell proliferation [84].

In our study, we have recently demonstrated any other immunoregulatory capabilities of hDP-MSCs on PHA–CD3− T cells in two different coculture systems (direct and indirect). Our results have indicated that hDP-MSCs could regulate T-cell responses by inhibiting the production of pro-inflammatory cytokines (IL-2, IL-6, IL-12, IL-17A, IFN-γ, and TNF-α by PHA–CD3+ T cells), inducing apoptosis in PHA–CD3+ T cells, and promoting the differentiation of Treg and the expression of IP-10 in MLR and transwell systems. We suggested that they exert all of these regulatory effects through the increased secretion of paracrine soluble factors and cytokines, such as HGF-β1, HLA-G, ICAM-1, IL-6, IL-10, TGF-β1, VCAM-1, and VEGF, when cocultured with PHA–CD3+ T cells [65]. We also observed that hDP-MSCs suppressed cytokines such as CX3CR1, IFN-γ, IL-6R, IL-17A, NCF-1, and TNF-α, and induced gene expression of CD4, CD25, Foxp3, and IP-10, by PHA–CD3+ T cells using real-time PCR in transwell experiments at day 4.

PGE2 is an important product of arachidonic acid methabolism, is synthesized by MSCs and stimulates IL-6 secretion. PGE2 inhibits T-cell mitogenesis and IL-2 production. It also plays a role as a cofactor in the induction of Th-2 and suppresses T lymphocyte proliferation [85, 86].

NO is another important mediator in suppression of lymphocyte cells. It has been reported that NO inhibits the proliferation of T-cell activation by suppressing STAT5 phosphorylation in G0/G1 phase of the cell cycle [86, 87]. NO has been shown to induce the apoptosis of immune cells not only by JAK/STAT pathway but also by MAPK and NF-kB pathway. It is also downregulates gene expression of many cytokines as well [88].

IDO is a tryptophan-catalyzing enzyme and has been reported to release from activated MSCs. Kynuerine is a degradation product of IDO and has been reported to inhibit T-cell response [89, 90]. Although IDO has been demonstrated to be synthesized by MSCs in several studies [28, 85, 90, 91], there are also studies indicating

that it is not [46]. T-cell proliferation was not determined when tryptophan and IDO inhibitor are added in MSC/T cell cocultures [92, 93].

Effects of MSCs on B Cells

MSCs may also regulate the immune response through interaction with B lymphocyte. MSCs may affect B cell proliferation, apoptosis, immunoglobulin (Ig) production, and chemotaxis [70]. When BM-MSCs and B lymphocytes from PB of healthy donors were cocultured with stimuli to B cell activation, the proliferation of B lymphocytes and immunoglobulin production (IgM, IgG, and IgA) were inhibited by the secretion of soluble factors by MSCs [20, 42, 94-97]. In addition, It has been reported that the proliferation and antibody production of B cells are suppressed by MSC soluble factors as PGE2, TGF-β, IDO, HLA-G, NO, and HGF-β [98]. In contrast, culture supernatant from MSCs had no effect, suggesting that the release of inhibitory factors requires paracrine signals from B cells. Mouse B cell proliferation is induced by either anti-CD40 monoclonal antibody and IL-4 or pokeweed mitogen and this proliferation is inhibited by MSC coculture [99]. The effects of MSCs on B cells are dose dependent, but the MSC/B cell ratios at which these effects have been observed may vary according to culture conditions. Most results have been observed at a 1:1 ratio [41], but recent studies suggest that lower ratios, such as 1:10 [100, 101] and 1:30 [102], are still effective [70].

It has been also shown that MSCs efficiently inhibit B cell proliferation through an arrest in G0/G1 phase of the cell cycle and not through induction of apoptosis [41, 97]. MSCs also modulated the chemotactic properties of B cells by reducing their expression of the chemokine receptors CXCR4, CXCR5, and CCR7 and their chemotaxis to CXCL12 and CXCL13 [41]. Rafei et al. have been indicated that the secretome of MSCs suppressed plasma cell immunoglobulin production as a result of MSC-derived CC chemokine ligands CCL2 and CCL7 processed by the activity of matrix metalloproteinases (MMPs). The neutralization of CCL2 or inhibition of MMP enzymatic activity abolished their suppressive effect and the MMP-processed CCL2 suppressed the STAT3 activation in plasma cells [103]. Furthermore, MSCs could decrease antihuman factor VIII (hFVIII)-IgG levels in hemophilic B6 mice [99]. In contrast, additional studies revealed that MSCs are able to promote the proliferation of B cells and their capacity to differentiate into Ig-secreting cells [100, 101, 104].

Effects of MSCs on DCs

DCs are the cells mostly specialized in uptake, transport, and presentation of antigens. DCs play a key role in the induction of immunity and tolerance, depending on the activation and maturation stage and, as recently suggested, the cytokine milieu at sites of inflammation [105]. Depending on their activation and maturation stage, DCs may act in the primary immune responses as either inducers of T-cell immunity

or mediators of T-cell tolerance [106]. The interactions between DCs and MSCs have been investigated in different studies to assess whether MSCs may alter DC maturation, differentiation, and functions, and also contribute to the generation of tolerogenic antigen-presenting cells (APCs). The presentation of alloantigens by APCs to T cells leads to T-cell activation and proliferation which are both suppressed by the presence of MSCs [28, 31, 107]; this result raises the question whether the immunoregulation by MSCs on T-cell functions becomes, directly or indirectly, also the role of DCs. MSCs have been demonstrated to interfere differentiation, maturation, and functions of DCs, generating immature DCs [20]. MSC roles in suppression of differentiation effect both monocytes and CD34+ progenitors into CD1a+ DCs, skewing their differentiation toward cells with features of macrophages. Molecules related to antigen presentation such as CD1a, CD40, CD83, CD80 (B7-1), CD86 (B7-2), and HLA-DR could be inhibited during the maturation of DCs in the presence of MSCs [108]. MSCs have been reported to alter the cytokine profile of DCs by inducing the secretion of the anti-inflammatory cytokine IL-10 and by inhibiting the secretion of the pro-inflammatory cytokines such as IFN-γ, IL-12, and TNF-α [54, 55, 85, 109] in cocultures. Thus Th1is inhibited, while IL-10 secretion is induced by endotoxin-stimulated DCs, and regulatory T cells are also increased [70]. Taken together, these results propose that MSCs inhibit the differentiation and activation of dendritic cells, resulting in the formation of immature DCs that present a suppressor or an inhibitory phenotype. Transwell experiments have indicated that the suppressive effect of MSCs on DC differentiation is mediated by soluble factors as well as cell–cell contact may mediate MSC modulation of DC maturation [109, 110]. The production of IL-6 and HLA-G by MSCs may contribute to the inhibitory effect of MSCs on DC differentiation [46, 111, 112]. HLA-G and IL-6 induce the development of tolerogenic DC by suppression maturation/activation of myeloid DC [111, 113]. Alternatively, PGE2 may be an interesting candidate factor. It is reported that inhibition of PGE2 synthesis has been generated by the secretion of TNF-α and IFN-γ by DCs cocultured with MSCs [85]. In addition to direct suppression of T-cell proliferation, the induction of regulatory APCs may be a key mechanism by which MSCs indirectly suppress proliferation of T cells [112].

Effects of MSCs on NK Cells

NK cells present innate cytolytic activity that mainly targets cells that lack expression of HLA class I molecules. Killing by NK cells is modulated by a balance of signals transmitted by activating and inhibitory receptors interacting with HLA molecules on target cells. However, NK cells play a role by lysing autologous tumor cells regulated by their stimulating receptors [113]. The mechanisms underlying MSC-mediated NK cell regulation have been partially unstitched [20, 42, 73, 74, 94–96]. Soluble factors or cell-to-cell contact mediate different effects depending on the experimental settings [114]. MSCs have been indicated to inhibit both IL-2- and IL-15-induced NK proliferation and IFN-γ production [29, 66, 73, 85, 115]. In vitro

studies on the interactions between NK cells and MSCs are also of potential interest for cancer immunotherapy involving NK cells, as well as for GVHD treatment and prevention [116, 117]. Actually short-time coculture of IL-15-stimulated NK cells and MSCs leads to the suppression of NK cytolytic activity against both the HLA class I-negative and -positive cells. This regulation is associated with the reduction of IL-15-induced cytokines, such as IFN-γ, IL-10, and TNF-α, and it needs cell-to-cell contact [118]. Similar results have been obtained with long-time coculture of IL-2-activated NK cells with MSCs, leading to the decrease of killing against the HLA class I-negative K562 cell line [117]. In this study, MSCs have been reported not to inhibit the lysis of freshly isolated NK cells, whereas NK cells cultured for 4–5 days with IL-2 in the presence of MSCs have reduced cytotoxic potential against K562 target cells (HLA class I-negative targets) [117]. Taken together, these data show that MSCs may inhibit NK functions against HLA class I-negative and -positive targets. MSCs have been demonstrated to inhibit NK proliferation and cytotoxicity and change cytokine secretion through their factors as IDO, PGE2, HLA-G, TGF-β, HGF-β, and IL-10 [48, 119]. On the other hand, MSC-dependent inhibition of IL-15-activated NK cells requires both cell–cell contact and soluble factors, such as TGF-β1 and PGE2 that are produced during MSC/NK coculture [29]. Immunosuppressive effect of MSCs has been studied and a 1:1 ratio of MSC/NK coculture was found to be more effective than a ratio of 1:10 [119].

Taken together, persuasively demonstrate that MSCs are capable of regulating the function of different immune cells in vitro, particularly involving the suppression of T-cell proliferation, suppression of cytokine secretion by T cells, stimulation of T-cell apoptosis, induction of Treg cells, suppression of the proliferation and antibody production of B cells inhibiting proliferation, cytotoxicity of NK cells, changing cytokine secretion of NK cells, and suppression of maturation/activation of DC (Table 3.1).

The mechanisms underlying the immunoregulative effects of MSCs are still uncertain and several different results have been suggested. Furthermore, the in vivo biological studies of these in vitro observations have been shown and similar results were obtained.

As a result of all these in vitro and in vivo studies and results, we may suggest that allogeneic MSCs also have the important potential of being used in regenerative medicine and improving clinical applications, including cellular therapy in autoimmune diseases, tissue/organ engineering, BM engraftment, and inhibition of GVHD.

3.1.2.3 In Vivo Immunoregulation by MSCs

Animal Models of MSC Immunoregulation

Various animal models have been used to evaluate in vivo MSC immunoregulatory properties related to alloreactive immunity in SC and organ transplantations, autoimmunity, or tumor immunity (Table 3.2). Preliminary data in a mouse model revealed that when allogeneic or xenogeneic rat HSCs were transplanted with their

Table 3.1 In vitro studies of MSCs in molecular interactions of immunoregulation

Cytokines and soluble factors	Proposed mechanisms	References
IDO	Inhibits T-cell response	[66, 67]
	Suppresses the proliferation and antibody production of B cells	[76]
	Inhibits proliferation, cytotoxicity of NK, changes cytokine secretion of NK	[24, 97]
HGF-β	Inhibits T-cell proliferation	[1, 50]
	Suppresses the proliferation and antibody production of B cells	[76]
	Inhibits proliferation, cytotoxicity of NK	[24, 97]
	Changes cytokine secretion of NK	
HLA-G	Mediates Treg differentiation	[24–26, 44]
	Inhibits the proliferation of T cells, suppress cytokine secretion by T cells	[76]
	Suppresses the proliferation and antibody production of B cells	[89, 91]
	Suppresses maturation/activation of DC	[24, 97]
	Inhibits proliferation, cytotoxicity of NK	
	Changes cytokine secretion of NK	
ICAM-1	Upregulates the adhesive capability of T cells	[27]
IL-6	Inhibits T-cell proliferation	[18, 20, 47]
	Inhibits cytokine secretion and cytotoxicity	[89, 91]
	Stimulates T-cell apoptosis	
	Suppresses maturation/activation of DC	
IL-10	Promotes the development of Th2 cytokines and inhibits the production of Th1 pro-inflammatory cytokines	[24, 45, 46]
	Inhibits proliferation, cytotoxicity of NK	[24, 97]
	Changes cytokine secretion of NK	
NO	Inhibits the proliferation of T-cell activation by suppressing STAT5 phosphorylation in G0/G1 phase of the cell cycle	[63, 64]
	Suppresses the proliferation and antibody production of B cells	[76]
PGE2	Suppresses T lymphocyte proliferation, inhibits T-cell mitogenesis, inhibits IL-2 production, induces Th-2 proliferation	[62, 63]
	Suppresses the proliferation and antibody production of B cells	[76]
	Suppresses secretion of TNF-α and IFN-γ by DCs	[62]
	Inhibits proliferation, cytotoxicity of NK, changes cytokine secretion of NK	[24, 97]
TGF-β	Inhibits T-cell proliferation	[1, 50]
	Suppresses the proliferation and antibody production of B cells	[76]
	Inhibits proliferation, cytotoxicity of NK, changes cytokine secretion of NK	[24, 97]
VCAM-1	Upregulates the adhesive capability of T cells	[27]
VEGF	Decreases the number of T cells	[52, 53]

Abbreviations: *IDO* indoleamine 2,3-deoxygenase, *HLA-G* histocompatibility antigen, class I–G, *ICAM-1* intercellular adhesion molecule-1, *IL-6* interleukin-6, *IL-10* interleukin-10, *HGF-β* hepatocyte growth factor-β, *NO* nitric oxide, *PGE2* prostaglandin E2, *TGF-β* transforming growth factor-β, *VCAM-1* vascular cell adhesion molecule-1, *VEGF* vascular endothelial growth factor

marrow microenvironment under the kidney capsule, the rat MSCs induced immunological tolerance [120]. In the same way, in utero sheep co-transplanted with sheep HSCs and human MSCs show increased levels of engraftment and shorter periods of hematopoietic reconstitution [121]. One of the first in vivo studies

Table 3.2 In vivo immunoregulation by MSCs in various disease models in animals

Animal model	MSCs	Outcome	References
Mouse			
Murine melanoma tumor model	Allogeneic mouse; IV and SC infusion	Promotion of tumor growth	[122]
STZ-induced diabetes	Xenogeneic mouse; intracardiac infusion	MSC grafted kidney and pancreas in STZ NOD SCID mice and may be useful in enhancing insulin secretion and perhaps improving the renal lesions	[126]
GVHD	Allogeneic mouse; multiple IV infusions	MSCs controlled the lethal GVHD	[32]
GVHD	Allogeneic mouse; single IV infusion	MSCs did not improve GVHD	[124]
Allogeneic BMT	Syngeneic and allogenic mouse; single IV infusion	MSCs were capable of increasing the engraftment rate in recipient mice	[123]
MS model (EAE)	Syngeneic mouse; multiple IV infusion	Prevention of EAE development; MSCs are home to inflamed lymphoid tissues reducing disease progression	[36, 37]
CIA	Allogeneic mouse; multiple IV infusion	MSCs did not confer any benefit	[71]
Endotoxin-induced ALI	Syngeneic mouse; injected into the trachea; intrapulmonary	MSCs markedly decrease the severity of endotoxin-induced acute lung injury and improve survival in mice	[129]
Bleomycin (BLM)-induced chronic lung injury	Syngeneic mouse; single into the jugular vein	MSCs protect lung tissue from bleomycin-induced injury with anti-inflammatory effect by blocking TNF-α and IL-1, two fundamental pro-inflammatory cytokines in lung	[130]
CIA (RA)	Allogeneic mouse; single intraperitoneal injection	A single injection of MSCs prevented the occurrence of severe, irreversible damage to bone and cartilage. MSCs reduce joint inflammation and increase Treg generation	[131]
Kidney ischemia reperfusion injury	Syngeneic mouse; multiple IV infusion and injected intracortically in three separate locations in the kidney sagittal midline	MSCs are helpful in the restoration of tubular epithelial cells with an anti-inflammatory effect and do not make a significant contribution to the restoration of epithelial integrity after an ischemic insult	[132]
Experimental autoimmune hearing loss (EAHL)	Syngeneic mouse; multiple IP infusions	MSCs are one of the important regulators of immune tolerance with the capacity to suppress effector T cells and to induce the generation of antigen-specific Treg cells	[136]

(continued)

Table 3.2 (continued)

Animal model	MSCs	Outcome	References
Baboon			
Skin graft transplantation	Allogeneic baboons; multiple IV infusion and subcutaneous injection	MSCs led to prolonged skin graft survival when compared to control animals	[31]
Rat			
Ischemic acute renal failure (ARF)	Allogeneic rat; multiple IV infusion	Protection against renal ischemia/reperfusion injury. Highly significant renoprotection obtained with MSCs in early infusion	[125]
Allogeneic heart transplantation	Allogeneic rat; single immediately and later IV infusions	MSCs injected intravenously migrated to the heart during chronic rejection	[127]
Allogeneic heart transplantation	Allogeneic rat; multiple IV infusions	MSCs co-injected with cyclosporine accelerate rejection. MSC injections were not only ineffective at prolonging allograft survival but also tended to promote rejection	[56]
Myocardial infarction (induced by occlusion of the left coronary artery)	Syngeneic rat; multiple intramyocardial injections	MSCs showed an anti-inflammation role and MSC transplantation might partly account for the cardiac protective effect in ischemic heart disease	[128]
Myocardial infarction	Allogeneic and syngeneic rat; single intramyocardial injection	MSCs significantly improved ventricular function for at least 3 months after implantation. Allogeneic (but not syngeneic) cells were eliminated from the heart by 5 weeks after implantation, and their functional benefits were lost within 5 months	[135]
GVHD	Allogeneic rat; single IBM cavity	MSCs prevent lethal GVHD	[134]
Fulminant hepatic failure (FHF)	Allogeneic rat; multiple IV and extracorporeal perfusion	Human MSCs protect against hepatocyte death and increase survival in mice after the injections of the hepatotoxin D-galactosamine	[133]

RA reumatoid arthritis, *STZ* streptozotocin, *GVHD* graft-versus-host disease, *MS* multiple sclerosis, *EAE* experimental autoimmune encephalomyelitis, *ALI* acute lung injury, *CIA* collagen-induced arthritis, *BM* bone marrow, *IBM* intra-BM, *BMT* bone marrow transplantation, *MSCs* mesenchymal stem cells, *IV* intravenous, *SC* subcutaneous, and *n.a* not applicable

demonstrated that systemic infusion of allogeneic MSCs derived from baboon BM prolonged the survival of allogeneic skin grafts to 11 days compared with 7 days in animals not treated with MSCs [31].

In vivo effects of MSCs have been observed in other reports treated with GVHD after allogeneic SC transplantation. Systemic infusion of in vitro-expanded MSCs derived from AT could control lethal GVHD in mice transplanted with haploidentical HSC grafts [32]. Only infusions of MSCs early after transplantation in this study were effective in controlling GVHD. Additionally, it was recommended repeated infusions of MSCs are required to improve GVHD and this might be explained by a recent observation that the infusion of a single dose of MSCs at the time of allogeneic BM transplantation (BMT) did not affect the incidence and severity of GVHD in mice. This might explain why the infusion of a single MSC dose in allogeneic BMT does not affect the incidence and severity of GVHD in mice [70, 124]. MSC infusion is not always followed by their stable engraftment and function. In vivo studies have shown that the administration of allogeneic MSCs into an MHC-mismatched host may result in their rejection [123]. In vivo effects of MSCs have demonstrated that MSCs prevented the rejection of allogeneic tumor cells in immunocompetent mice. MSCs infused systemically or adjacent to subcutaneously implanted B16 melanoma cells resulted in enhanced tumor formation, whereas melanoma cells injected alone were eliminated by the host immune system [122].

BMT is becoming a powerful strategy for the treatment of hematologic disorders (leukemia, aplastic anemia, etc.), congenital immunodeficiencies, metabolic disorders, and also autoimmune diseases. Using various animal models for autoimmune diseases, it has recently been shown that BMT can be used to treat autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), immune thrombocytic purpura, insulin-dependent diabetes mellitus, chronic glomerulonephritis, and also a certain type of non-insulin-dependent diabetes mellitus. A study using long bones of cynomolgus monkeys developed a novel strategy for allogeneic SC transplantation using perfusion method plus intra-BM (IBM) injection of stem cells. They have found that “IBM-BMT” (injection of whole BMCs [containing HSCs and MSCs] into the BM cavity) is the best strategy for allogeneic BMT. They have recently developed a new “Perfusion Method (PM)” for BMCs while minimizing the contamination of BMCs with T cells from the PB. The donor-derived hemopoietic cells quickly recover even when the radiation doses used as the conditioning regimen are reduced. Recipient mice, rats, and even monkeys show neither GVHD nor graft failure. IBM-BMT will become a valuable strategy for the treatment of various intractable diseases, including autoimmune diseases evaluated in other studies in this issue [134].

MSC-based immune modulation is considered a potential novel strategy for autoimmunity. The infusion of MSCs was only effective at disease onset and at the peak of the disease, but not after disease stabilization. In contrast, infusion of MSCs had no beneficial effects on collagen-induced arthritis (CIA) as tested in a murine model of RA [71]. In a mouse model of RA (DBA/1 mice immunized with type II collagen in Freund’s adjuvant), a single injection of MSCs prevents the occurrence of severe, irreversible bone and cartilage damages, by inducing T-cell

hyporesponsiveness and modulation of inflammatory cytokines, such as TNF-α [131]. The immunoregulatory ability of murine MSCs has been studied to treat myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis (EAE), a model of human MS in mice. In that effort, MSCs were injected intravenously before disease onset (preventative) as well as at different time points after disease occurrence (therapeutic). MSC administration before disease onset strikingly ameliorated EAE, and overall, the therapeutic scheme was effective when MSCs were administered at disease onset as well as at the peak of disease, but not during the chronic phase. It has been shown that mouse MSCs may improve EAE through the induction of peripheral T-cell tolerance against the pathogenic antigen; however, MSCs seem to be effective only at the disease onset and peak, but not after disease stabilization [36, 37].

The therapeutic efficacy of human adipose tissue-derived (hAT)-MSCs has been studied in experimental autoimmune hearing loss (EAHL) in mice. Systemic infusion of hAT-MSCs significantly improved hearing function and protected hair cells in established EAHL. The hAT-MSCs decreased the proliferation of antigen-specific Th1/Th17 cells and induced the production of anti-inflammatory cytokine interleukin-10 in splenocytes. They also induced the generation of antigen-specific CD4+ CD25+ Foxp3+ Treg cells with the capacity to suppress autoantigen-specific T-cell responses. Their experiment demonstrated that hAT-MSCs are one of the important regulators of immune tolerance with the capacity to suppress effector T cells and to induce the generation of antigen-specific Treg cells [136].

MSCs may also provide protective effects in rat models of kidney and myocardial injury, based on ischemia/reperfusion processes, by secreting soluble immune-modulating factors [125]. To study the effect of human MSCs in the development of diabetes immunodeficient recipient mice (NOD SCID) were used, chemically rendered diabetic by streptozotocin injections. Infusion of hMSCs increased peripheral insulin levels and reduced glycemic levels. As well as in the kidney, human DNA infused as hMSCs was detected in the pancreas. Islets appeared larger in pancreata from hMSC-treated diabetic mice compared with islets from untreated diabetic mice. Additionally islets had an increase in mouse insulin immunoreactivity [126, 137]. Heart transplant models in rats have shown that MSC injection not only failed to prolong allograft survival but also when MSCs were co-administered with low-dose cyclosporine, it accelerated allograft rejection. However MSCs injected intravenously migrated to the heart during chronic rejection [56, 127]. The effects of MSC differentiation on the immune characteristics of cells evaluated and monitored cardiac function for 6 months after post-myocardial infarction MSC therapy. MSCs significantly improved ventricular function for at least 3 months after implantation. The long-term ability of allogeneic MSCs to preserve function in the infarcted heart is limited by a biphasic immune response whereby they transition from an immunoprivileged to an immunogenic state after differentiation, which is associated with an alteration in MHC-immune antigen profile [128, 135]. In a study, functional and survival effects related to the immunomodulatory properties of MSCs were tested in an endotoxin model of acute lung injury (ALI). MSCs increased survival at 48 h. MSC administration mediated a down-regulation of pro-inflammatory

responses to endotoxin while increasing the anti-inflammatory cytokine IL-10. They concluded that treatment with intrapulmonary MSCs markedly decreases the severity of ALI and improves survival in mice [129]. A study used bleomycin (BLM)-induced lung injury mouse model was reported that IL-1 receptor antagonist (IL1RN) mediates the anti-inflammatory and antifibrotic effect of MSCs during lung injury. They identified that subpopulations of murine and human MSCs secreted high levels of IL1RN. In vitro and in vivo data provided that production of IL1RN by MSCs protects lung tissue from BLM-induced injury with anti-inflammatory effect by blocking TNF-α and IL-1α, two fundamental pro-inflammatory cytokines in lung [130].

Another group observed that there is no effect of BM-derived stem cells on restoration of tubular epithelial cells during repair of the postischemic kidney. Upon i.v. injection of BM-MSCs, postischemic functional renal impairment was reduced, but there was no evidence of differentiation of these cells into tubular cells of the kidney [132]. A study using rats undergoing fulminant hepatic failure model reported that the administration of MSC-derived molecules in two clinically relevant forms can provide a significant survival benefit. In conclusion, they describe the first use of the secreted and metabolic functions of MSCs to derive a new class of immunotherapeutic [133].

Clinical Experience of MSC Immunoregulation

There is no unique and hierarchically prevalent mechanism responsible for MSC immunoregulation, but there is a redundant panel of mechanisms that suggests the in vivo relevance of immunoregulation by the stromal cell compartment. Some contradictory results have been produced by different groups, probably due to different experimental factors related to MSC origin, culture conditions, and lymphocyte subset and activation state. In a general manner, these data suggest that both soluble factors and cell-to-cell contact are involved. MSC regulatory effects are operational in vivo, as MSC infusion can significantly prolong the survival of MHC-mismatched skin grafts in baboons [31], lower the incidence and cure the refractoriness to treatment of GVHD after allogeneic HSC transplantation in humans [116], and improve EAE in mice [37]. Thus, it is important to know the kinetics, mechanisms, and administration modalities of MSC-based immune therapies to achieve clinical benefit with no or only a small number of potential side effects [70].

Because of their hypoimmunogenic properties, MSCs are considered a potential strategy to prevent graft rejection and GVHD. First clinical study was performed in 2004 and MSCs were shown to accelerate the recovery in a patient with a severe case of GVHD. Haploidentical MSCs were used to treat severe, refractory, grade IV acute GVHD of the gut and liver in a patient. No toxicity after MSC infusion, rapid disappearance of symptoms, and strong immunosuppression in vivo were observed [116]. Growing number of follow-up studies involving MSCs have been reported since. Clinical studies have aimed to demonstrate promising results in treating patients with cancer, reducing the incidence of GVHD after BMT, improving the recovery

of patients after amyotrophic lateral sclerosis, and treating of fistulas in patients with refractory perianal Crohn's disease, metachromatic leukodystrophy, Hurler syndrome, rheumatoid arthritis, type 1 diabetes mellitus, lupus nephritis, and liver cirrhosis. Selected clinical trials to date potentially seeking to exploit the immunomodulatory properties of MSCs to achieve their desired therapeutic goal and involving administration of MSCs in http://www.ClinicalTrials.gov are listed in Table 3.3 [137].

There are different phase II clinical trials running to study the optimal MSC dose and administration schedules that have the best efficiency in preventing or treating GVHD following allogeneic HSCs. Seven patients of different ages, diagnosis, and disease status were treated with allogeneic (three cases) or haploidentical (four cases) MSCs together with HSCs. A neutrophil count of $>0.5 \times 10^9$/l and a platelet count of $>30 \times 10^9$/l were both achieved after a median of 12 days. Acute GVHD grade 0–I occurred in five patients, and grade II acute GVHD in the two other patients, evolving into chronic GVHD in one patient [141]. Comparable results have been obtained in a European phase I–II study: 14 children received haploidentical HSC grafts in combination with expanded MSCs derived from donor BM. Faster leukocyte recovery was observed, with no immediate adverse effects [142]. A phase II study has conducted enrolling 55 patients with severe steroid-resistant grade II to IV acute GVHD. Thirty patients displayed a complete response and nine patients a partial response. Sixteen patients had stable or progressive disease. Survival of patients with complete response was significantly higher than the patients with partial or no response. No side effects were observed [138]. Another report on patients with leukemia, however, showed effective prevention of acute GVHD but a higher incidence of relapses in patients who were co-transplanted with MSCs and MHC-identical allogeneic HSCs [139]. Co-transplantation of third-party donor HSCs with cord blood transplants has been shown to overcome the limitation posed by low cellularity of cord blood units for unrelated transplants in adults. The co-infusion of MSCs from the same HSC donors was therapeutically effective for severe acute GVHD but no significant differences in cord blood engraftment and incidence of GVHD were observed [140]. In another clinical trial, 13 patients with steroid-refractory acute GVHD were treated with BM-MSCs. Two patients showed clinical responses [144]. A clinical study enrolling 32 patients with acute GVHD was performed. Patients with grade II to IV GVHD were randomized to receive two treatments of MSCs in combination with corticosteroids. 66 % complete responses and 16 % partial responses were reported. No MSC infusion-related toxicities or ectopic tissue formation were found. Comparing the low and high MSC dose, there was no difference between safety and efficacy results [145].

Four treatment-refractory patients were treated with allogeneic MSCs. The patients presented a stable 12- to 18-month disease remission, showing improvement in serologic markers and renal function [143]. In terms of type 1 diabetes, the Juvenile Diabetes Research Foundation announced its intent to fund the commercial entity Osiris to evaluate the immunomodulatory effects of prochymal®, a formulation of immunomodulatory adult BM-MSCs, for the purpose of improving disease management in individuals with type 1 diabetes [32, 145].

Table 3.3 Selected clinical trials potentially seeking to exploit the immunomodulatory properties of MSCs to achieve their desired therapeutic goal and involving administration of MSCs

Title	Condition/therapy	Responsible party/sponsor	Recruitment status
Safety and efficacy study of umbilical cord-derived mesenchymal stem cells for rheumatoid arthritis	RA Human umbilical cord-derived allogenic MSCs	Alliancells Bioscience Corporation Limited, China	Active, not recruiting
Umbilical mesenchymal stem cell and mononuclear cell infusion in type 1 diabetes mellitus	Type 1 diabetes mellitus Human umbilical cord-derived allogenic MSCs	Fuzhou General Hospital, China	Active, not recruiting
Co-transplantation of islet and mesenchymal stem cell in type 1 diabetic patients	Type 1 diabetic patients BM-derived autologous MSCs	Fuzhou General Hospital, China	Recruiting
Phase 2 study of human umbilical cord-derived mesenchymal stem cell for the treatment of lupus nephritis	Lupus nephritis Human umbilical cord-derived allogenic MSCs	CytoMed & Beike, China	Recruiting
Human menstrual blood-derived mesenchymal stem cells for patients with liver cirrhosis	Liver cirrhosis Human menstrual blood-derived MSCs	S-Evans Biosciences Co., Ltd., China	Recruiting
OTI-010 for graft-versus-host disease prophylaxis in treating patients who are undergoing donor peripheral stem cell transplantation for hematological malignancies	Hematological malignancies BM-derived autologous MSCs	Johnson Comprehensive Cancer Center, USA National Cancer Institute (NCI)	Active, not recruiting
Mesenchymal stem cell infusion as prevention for graft rejection and GVHD	Hematological malignancies BM-derived donor MSCs	University Hospital of Liege, Belgium	Completed
Safety and efficacy study of umbilical cord blood-derived mesenchymal stem cells to promote engraftment of unrelated hematopoietic stem cell transplantation	Acute leukemia Human umbilical cord-derived allogenic MSCs	Medipost Co., Ltd.	Active, not recruiting
Mesenchymal stem cell transplantation to patients with relapsed/refractory aplastic anemia	Acute leukemia BM-derived allogeneic MSCs	Guangzhou General Hospital of Guangzhou Military Command, China	Recruiting
A pediatric study of a plerixafor containing regimen in second allogeneic stem cell transplantation	ALL, AML, MDS, CML, JMML, NHL BM-derived allogeneic MSCs	St. Jude Children's Research Hospital, US	Recruiting
Mesenchymal stem cells in multiple sclerosis (MSCIMS)	MS BM-derived autologous MSCs	University of Cambridge, UK	Completed

(continued)

Table 3.3 (continued)

Title	Condition/therapy	Responsible party/sponsor	Recruitment status
Mesenchymal stem cells for the treatment of multiple sclerosis (MS)	MS BM-derived autologous MSCs	Hadassah Medical Organization, Israel: Ministry of Health	Active, not recruiting
Evaluation of autologous mesenchymal stem cell transplantation (effects and side effects) in MS	MS BM-derived autologous MSCs	Royan Institute, Iran	Recruiting
Autologous mesenchymal stem cell (MSC) transplantation in MS	MS BM-derived autologous MSCs	University Hospital Case Medical Center, USA Cleveland Clinic Foundation Food and Drug Administration	Recruiting
Safety and efficacy of umbilical cord mesenchymal stem cell therapy for patients with progressive MS and neuromyelitis optica	MS NMO Human umbilical cord-derived allogenic MSCs	Shenzhen Beike Bio-Technology Co., Ltd., China	Recruiting
Prochymal™ adult mesenchymal stem cell for treatment of moderate-to-severe Crohn's disease	Crohn's disease BM-derived allogeneic MSCs (prochymal)	Osiris Therapeutics, USA	Completed
Dose-escalating therapeutic study of allogeneic bone marrow-derived mesenchymal stem cells for the treatment of fistulas in patients with refractory perianal Crohn's disease	Crohn's disease BM-derived allogeneic MSCs (prochymal)	Leiden University Medical Center (LUMC), The Netherlands	Recruiting
Efficacy and safety study of allogenic mesenchymal stem cells for patients with chronic graft-versus-host disease (MSCsTc GVHD)	GVHD BM-derived allogeneic MSCs	Chinese Academy of Medical Sciences, Zhejiang University, China	Active, not recruiting
Umbilical cord blood-derived mesenchymal stem cells for the treatment of steroid-refractory acute or chronic graft-versus-host disease (GVHD-MSC)	GVHD Human umbilical cord-derived allogenic MSCs	Samsung Medical Center, Korea	Recruiting
Mesenchymal stem cell infusion as treatment for steroid-resistant acute GVHD or poor graft function	GVHD BM-derived autologous MSCs	University Hospital of Liege, Katholieke University Leuven, Belgium	Recruiting
Evaluation of the role of mesenchymal stem cells in the treatment of GVHD	GVHD BM-derived donor MSCs	Chiristian Medical Collage, India	Recruiting

Treatment of refractory GVHD by the infusion of expanded in vitro allogeneic mesenchymal stem cell	GVHD BM-derived autologous MSCs	University of Salamanca, Spain	Recruiting
Multicenter clinical trial for the evaluation of mesenchymal stem cells from adipose tissue in patients with chronic graft-versus-host disease (CMM/EICH/2008)	GvHD AT-derived allogenic MSCs	Fundación Progreso y Salud, Spain	Recruiting
Allo-HCT MUD for nonmalignant red blood cell (RBC) disorders: sickle cell, thal, and DBA: reduced intensity conditioning, Co-tx MSCs	GVHD BM-derived allogeneic MSCs	Stanford University, USA	Recruiting
Treatment of steroid-resistant GVHD by infusion MSC (MSC for GVHD)	GVHD BM-derived allogeneic MSCs	UMC Utrecht, The Netherlands	Recruiting
Safety and efficacy of prochymal for the salvage of treatment-refractory acute GVHD patients	GVHD BM-derived allogeneic MSCs (prochymal)	Osiris Therapeutics, USA	Completed
Safety and efficacy study of adult human mesenchymal stem cells to treat acute gastrointestinal graft-versus-host disease	GVHD BM-derived allogeneic MSCs (prochymal)	Osiris Therapeutics, USA	Completed
Efficacy and safety of prochymal infusion in combination with corticosteroids for the treatment of newly diagnosed acute GVHD	GVHD BM-derived allogeneic MSCs (prochymal)	Osiris Therapeutics, USA	Recruiting
Prochymal infusion for the treatment of steroid-refractory acute GVHD	GVHD BM-derived allogeneic MSC s (prochymal)	Osiris Therapeutics, USA	Competed
Efficacy and safety of adult human mesenchymal stem cells to treat patients who have failed to respond to TP steroid treatment for acute GVHD	GVHD BM-derived allogeneic MSCs (prochymal)	Osiris Therapeutics, USA	Recruiting
Follow-up study to evaluate the safety of prochymal for the treatment of GVHD patients	GVHD BM-derived allogeneic MSCs	Osiris Therapeutics, USA	Active, not recruiting
Donor mesenchymal stem cell infusion in treating patients with acute or chronic GVHD after undergoing a donor stem cell transplant	GVHD BM-derived donor MSCs	Case Comprehensive Cancer Center, USA National Cancer Institue (NCI)	Recruiting

BM bone marrow, *AT* adipose tissue, *RA* rheumatoid arthritis, *Prochymal* allogenic human MSCs produced by Osiris Therapeutics, *ALL* acute lymphoblastic leukemia, *AML* acute myeloid leukemia, *MDS* myelodysplastic syndrome, *CML* chronic myeloid leukemia, *JMML* juvenile myelomonocytic leukemia, *NHL* non-Hodgkin lymphoma, *NMO* neuromyelitis optica; Date: 29.03.2012

In conclusion, the earliest studies that investigated the immunosuppressive nature of MSC demonstrated that MSCs are able to suppress immune system cells especially T-cell activation and proliferation in vitro and in vivo. The outcome of ongoing clinical results, as well as of studies in patients and animal models, demonstrates that the anti-proliferative and immunomodulatory properties of MSCs combined with their immunological favor may offer a new strategy in the treatment of autoimmune diseases such as Crohn's disease, metachromatic leukodystrophy, Hurler syndrome, rheumatoid arthritis, type 1 diabetes mellitus, lupus nephritis, and liver cirrhosis. While extensive research documents the role of hematopoietic stem cells in autoimmune diseases, few studies have addressed if and how MSCs contribute to their etiopathology. Stem cell transplantation in such diseases aims to destroy the self-reacting immune cells and produce a new functional immune system, as well as substitute cells for tissue damaged in the course of the disease by controlling and protecting vital organs from inflammatory. It is possible that they exert all of these regulatory effects through the increased secretion of paracrine soluble factors and cytokines such as HGF-β1, HLA-G, ICAM-1, IL-6, IL-10, TGF-β1, VCAM-1, and VEGF. However, the current knowledge of the immunobiology and clinical application of MSC needs to be reinforced by carrying out systematic studies in murines, large animal models, and humans.

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Chapter 4
Current Research on Stem Cells in Parkinson's Disease: Progress and Challenges

Ria Thomas and Jan Pruszak

Abstract Parkinson's disease is thought to be one of the most promising candidates for future cell therapeutic avenues using stem cells. It is characterized by focal degeneration of dopamine neurons in the midbrain, is amenable to neural transplantation approaches as verified by successful engraftment of fetal-derived tissue, and the cell type of interest can be generated from pluripotent sources. In the following chapter, we give an overview of fundamental principles of stem cell research in the context of Parkinson's disease. We summarize common approaches of dopaminergic in vitro differentiation, discuss current efforts for cell identification and isolation, and touch upon novel developments in direct phenotype conversion through epigenetic reprogramming.

Keywords Parkinson's disease • Pluripotent stem cells • Dopaminergic neurons • Surface markers • Reprogramming

Abbreviations

AA	Ascorbic acid
AADC	Aromatic L-amino acid decarboxylase

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K. Turksen (ed.), *Stem Cells: Current Challenges and New Directions*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4614-8066-2_4,

BDNF	Brain-derived neurotrophic factor
BMP	Bone morphogenetic protein
DA	Dopaminergic
DAT	Dopamine transporter
db-cAMP	Dibutyryl cyclic adenosine monophosphate
EGFR	Epidermal growth factor receptor
ES cells	Embryonic stem cells
FGF	Fibroblast growth factor
GDNF	Glial cell line-derived neurotrophic factor
hPSC	Human pluripotent stem cell
iDA	Induced dopaminergic cells
IGF-1R	Insulin-like growth factor-1 receptor
iN cells	Induced neuronal cells
iPS cells	Induced pluripotent stem cells
miRNA	microRNA
PD	Parkinson's disease
RA	Retinoic acid
SDIA	Stromal cell-derived inducing activity
TGFβ	Transforming growth factor beta

4.1 Introduction

4.1.1 The Promises of Stem Cell Research

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the progressive loss of dopaminergic (DA) neurons in the ventral midbrain (mesencephalon). DA neurons are present in both the human forebrain and midbrain, and are organized into distinct groups named A8 to A17. The mesencephalic DA groups in the ventral tegmental area (A10), the substantia nigra pars compacta (A9), and the retrorubral field together account for 90 % of the total DA neuronal population in the brain [1, 2]. Of these, A9 DA neurons are of greatest biomedical interest due to their selective loss being a characteristic pathological feature of PD. The adjacent A10 group is only slightly affected. Research is ongoing with respect to elucidating the underlying mechanisms of this selective vulnerability, and a few candidate factors involved in this DA neuronal protection have been identified (e.g., G-substrate [3], Rab3b [4], orthodonticle homeobox-2 (Otx2) [5]). In addition to the DA neurons, other neuronal populations such as the norepinephrine neurons in the locus coeruleus region are clearly affected, and are known to contribute to the diverse pathological and clinical factors of this disease entity. Nevertheless, the degeneration of mesencephalic DA neurons is accepted to be the major cause of the devastating classical triad of cardinal motor symptoms (bradykinesia, tremor, rigor) [6] and as the main aspect of suffering by the patients [7]. In most cases it remains unclear

why nigral DA neurons are lost over the course of years to decades, i.e., the major fraction of PD patients suffers from the idiopathic form. In parallel, we have gained increasing insights into a number of largely genetic, familial subtypes of this disease [8] that may also aid in elucidating the underlying pathophysiological mechanisms. As of now, while pharmacological approaches (e.g., L-DOPA substitution [9, 10]) as well as neurosurgical intervention (e.g., deep brain stimulation [11]) can be efficacious in a number of patients, there is unfortunately no curative treatment available. The rather circumscribed nature of the lesions with focal loss of a specific neural subtype has been exploited experimentally and also clinically by transplanting DA neurons and/or midbrain DA precursor cells. As the A9 midbrain DA neurons contribute to the complex basal ganglia circuitry via projections extending to the dorsolateral striatum as the target area, neurosurgical cell replacement approaches have implanted extrinsic cell sources directly into the striatal target region [12–14]. Importantly, ample animal experimental data and clinical trials have provided sufficient and indisputable proof of principle that cell therapy can work in this paradigm. Some patients show clear clinical motor improvements for more than a decade after receiving the graft [13–16]. In these and a number of other patients, involuntary dystonic and hyperkinetic movements (dyskinesias) have been observed as adverse events. Moreover, it is being discussed whether the grafts might be susceptible to the ongoing neurodegenerative process, illustrating the cell therapeutic opportunities as well as the remaining challenges of PD cell therapy [17]. In these past and present trials, fetal tissue material was used. For a single unilateral transplantation, typically three to six fetal mesencephali obtained from ca. 6 to 11 weeks of gestation have to be obtained, anonymously and after informed consent, from maternal donors undergoing elective abortions. First and foremost, this raises considerable ethical concern. It also requires substantial logistical efforts in the procurement and preparation of the tissue. Finally, tissue quality and thereby overall quality of the therapeutic agent, i.e., the cell suspension, have been largely dependent upon the technical standards of both the obstetrician as well as the skilled dissector ultimately isolating the ventral midbrain region by microdissection from the fetal brain. This has made comparison of results from different trials occasionally difficult [18–20]. Recent multi-institutional efforts [21] aim at equilibrating such considerable procedural variability by establishing and adhering to common technical, neurosurgical, and follow-up standards. In summary, however, while showing convincing results, it remains somewhat unlikely and undesirable that fetal tissue would match our standards for a widely applicable, reliable, and ethically suitable cell source in the treatment of neurological disease including PD long term.

Recent progress in stem cell biology has greatly expanded the options for potential future biomedical benefit for patients suffering from PD and other progressive neurodegenerative disorders. The hope is that experimental strategies using cell replacement as well as the development of PD-related in vitro screens can be applied. Among the stem cell sources available (adult neural stem cells, fetal neural stem cells, mesenchymal stem cells, etc.) human pluripotent stem cells (hPSCs), i.e., embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, exhibit the vastest expansion and differentiation potential (Fig. 4.1a–c). ES cells are derived

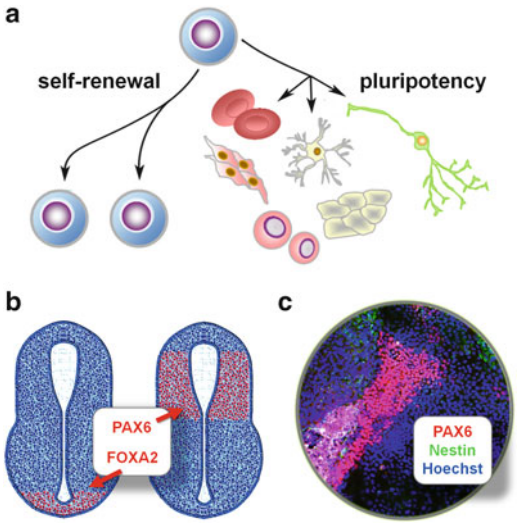

Fig. 4.1 Self-renewal and differentiation in the embryo and in the culture dish. (**a**) Pluripotent stem cells are characterized by their potential for extended self-renewal and their ability to spontaneously differentiate into cell types derived from all three germ layers including neuroectodermal derivatives. (**b**) Schematic of expression domains in the developing embryological neural tube. Reliable patterns occur, shaped by tissue gradients of patterning factors, morphogens and interactions of the cells with one another, and the extracellular matrix in a three-dimensional environment. For example, *FOXA2* is expressed in the floor plate, while a *PAX6* expression domain is found in the latero-dorsal region at this stage. (**c**) In a dish of neurally induced hPSCs, random patterns do arise (illustrated here by PAX6). However, we do not fully control or understand the microenvironmental inputs sufficiently, to predict these, to yield a synchronized onset of the desired gene expression, or to generate wholly homogenous neural culture systems

from the inner cell mass isolated from human blastocysts after in vitro fertilization procedures. iPS cells are reprogrammed from somatic cells such as human dermal fibroblast samples obtained from patient donors by transient expression of transcription factors associated with pluripotency (such as *OCT4*, *C-MYC*, *KLF4*, *SOX2*, *LIN28*) [22]. A range of novel biomedical opportunities have, thus, materialized, including potential applications in cell therapeutic paradigms (transplantation) as well as in in vitro screens where patient-derived iPS/PSC derivatives could be studied to elucidate the underlying pathophysiological mechanisms and to identify new drug targets. For the latter, PD patient-derived iPS cell-derived neurons can be used to assess disease-related effects of the underlying genetic aberrations. Epigenetically, truly pluripotent sources are the least restricted, resulting in the opportunity to differentiate into the broadest range of cellular phenotypes. Consequently, biomedically relevant cell subpopulations such as DA neurons can be faithfully differentiated from such pluripotent sources. Importantly, in principle, DA differentiation can occur spontaneously, which is exemplified by the DA differentiation observed within teratoma formation of non-primed PSCs and after

transplantation of PSCs to the kidney capsule [23]. Apart from fetal neural stem cells obtained from fetal midbrain tissue, such spontaneous DA differentiation does not occur with any of the other cell sources. In addition, hPSC differentiation represents an unprecedented, modifiable model of early human development. However, both pluripotent cell types share the necessity of controlling and appropriately directing their vast growth and differentiation potential in order to exploit them for biomedical applications. Upon withdrawal of pluripotency-promoting conditions (fibroblast growth factors (FGFs), feeder cell layers) hPSCs tend to differentiate spontaneously in vitro, however, in a somewhat unpredictable manner toward derivatives of all germ layers. Over the past few years it has been well established that human ES as well as iPS cells can be patterned toward neural and DA neuronal phenotype through a variety of directed differentiation protocols [2, 24–27]. Moreover, the established proof of principle that mouse as well as human ES and iPS cell-derived DA neurons can yield functional recovery in rodent models of PD can be considered highly promising for future cell therapeutic and other biomedical avenues [28–32]. However, are these expectations warranted? Can basic and translational stem cell research fulfill its promises and eventually deliver? Which basic biological studies and biotechnological accomplishments are essential before clinical translation of these sources can be realized?

4.1.2 Remaining Major Challenges

Undoubtedly, the issue of tumor formation remains a critical factor, when aiming for exploiting pluripotent sources. The vast expansion potential of PSCs makes them the most attractive source for long-lasting, reliable, and plentiful cell production. However, this also requires that this broad expansion and differentiation potential can be well controlled. Upon transplantation, undifferentiated, immature ES and iPS cells develop into tumors containing derivatives of all three germ layers which are used to assess pluripotency in the teratoma assay [33, 34]. Therefore, one needs to be able to rigorously exclude that any contaminating PSCs remain "hidden" within the differentiated neural cell population before clinical application. In addition to teratoma formation, insufficiently patterned neural stem or progenitor cells also need to be excluded from cell preparations before application. Otherwise, insufficiently patterned cells may generate unwanted progeny of other phenotypes in vivo, and may proliferate sufficiently to yield neuroepithelial tumors, for instance [35]. While these tumors may not exhibit oncogenic transformation or malignancy, their inherent potential for extended self-renewal must be controlled. What has sometimes been noticed to a lesser extent is that even for in vitro applications (e.g., screens), overgrowth of the population of interest by other nonneural or proliferative neurally derived cellular contaminants has to be avoided as this may decrease the sensitivity of the assays and mask any potentially detectable effects [36–39]. Any pharmacologically relevant effect of interest on, for instance, a fraction of 10 % of

DA neurons in an otherwise heterogeneous dish of cells may be masked by the remaining larger fraction of unwanted cells.

In current differentiation protocols, attempting to mimic the sequence of nervous system development in the embryo, a neural induction phase is followed by DA patterning and ultimately terminal DA differentiation. Upon a closer look, however, it is clear that the resulting cultures remain quite heterogeneous, and that, for instance, transcription factor expression patterns that would be clearly distinct and spatially appropriately confined within certain domains in the developing embryo are arbitrarily distributed in the dish (Fig. 4.1b, c). For instance, while neuroepithelial *PAX6* expression may be precisely confined to the dorsolateral domain of the developing neural tube at a certain stage in the embryo, expression in the dish may be quite patchy, regardless of the fact that overall media composition and thereby macroenvironmental exposure to growth factors are identical for all cells in the dish. Consequently, not only the remaining pluripotent cells and unpatterned cells can result in tumor formation, but also the phenotypic accuracy of the generated cells remains somewhat unclear [28, 29, 35].

4.1.3 Steps Toward Realizing the Promises of Stem Cell Research

Therefore, the declared aim of continued research efforts in this field is to optimize neuronal differentiation from PSCs in order to generate pure and properly patterned functional DA neurons in vitro. Current stem cell research with this particular biomedical scope addresses these challenges from two different angles:

4.1.3.1 Understanding Development to Further Enhance DA In Vitro Differentiation

This challenging, not necessarily readily attainable, goal addresses the classic embryological problem of how the multitude of signaling cues guide differentiation and morphogenesis within a given living system, i.e., the embryo or in this case the Petri dish culture (Fig. 4.2a). In this specific context, how do transcriptional programs, supracellular organization, and microenvironmental influences result in temporally and spatially appropriately patterned DA neurons?

4.1.3.2 Isolating the Population of Interest from a Heterogeneous Pool of Cells

This second approach is of rather pragmatic nature. While we may currently be unable to fully control and precisely direct hPSC-derived neural cells exclusively to the DA population of interest, we are able to exploit increasingly specific marker

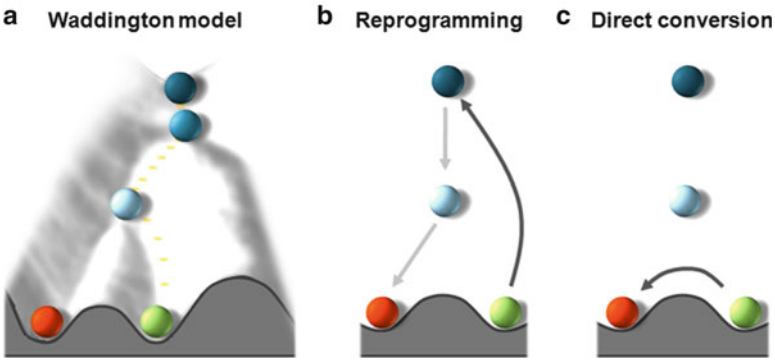

Fig. 4.2 Waddington model, stem cell differentiation, and (re)programming. (**a**) In this schematic, a pluripotent cell's potential to differentiate is guided by the hills and valleys of Waddington's epigenetic landscape down to its destined cell fate, becoming increasingly restricted in its intrinsic differentiation potency (adapted and redrawn from Waddington 1957 [137]). (**b**) Reprogramming, e.g., by the application of Yamanaka factors or by nuclear transfer, reverts somatic cells back to the early pluripotent stage, an epigenetically blank slate that is open for de novo differentiation. (**c**) Induced neuronal (iN) cells are generated by epigenetically converting a nonneural somatic cell directly to a neural/neuronal phenotype through transient expression of key determinants of neuronal phenotype

sets to enrich this neuronal subset from a heterogeneous source using cell sorting approaches.

4.2 Understanding Development to Further Enhance DA In Vitro Differentiation

4.2.1 How to Recognize a Midbrain DA Neuron?

Midbrain DA phenotype is characterized by its functionally relevant set of specific markers that can be analyzed by gene expression profiling or detected by immunofluorescence analysis using a wide range of well-established antibodies. Dopamine is produced from the amino acid tyrosine in a two-step reaction. The first step catalyzed by tyrosine hydroxylase (TH) converts tyrosine to L-DOPA, which is then converted to dopamine by the enzyme aromatic L-amino acid decarboxylase (AADC; DOPA decarboxylase). Two transporters required for DA neurotransmission include the plasma membrane dopamine transporter (DAT), which helps in removing dopamine from the synapse, thus helping to terminate its action, and the vesicular monoamine transporter (VMAT2), which helps in the recycling of dopamine from the synaptic terminal. While DAT is exclusively expressed by DA neurons, TH and

AADC are expressed by all catecholaminergic neurons. A protein that is widely present in the A9 group of DA neurons is the G-protein-gated inwardly rectifying potassium channel subunit (Girk2) which thereby serves as a valid marker to identify the specific subgroup of DA neurons affected in PD [13, 40]. Conversely, calbindin is a marker expressed by the dopamine neurons projecting to the limbic nucleus accumbens region and is not expressed in cells projecting to the dorsolateral motor putamen [13]. Additional DA neuronal markers include the glial cell line-derived neurotrophic factor (GDNF) signal transducing receptor c-Ret [41] and aldehyde dehydrogenase 2 (ADH2, Raldh1). Transcriptionally, LIM homeobox transcription factors 1a/b (LMX1A/ LMX1B) are two of the specifiers involved in early stages of midbrain DA development. LMX1A activates neurogenin2 (NGN2) by the intermediate activation of MSX1 (Msh homeobox 1). NGN2, in turn, blocks the action of the homeobox protein Nkx6.1 to allow the neuronal generation before they enter the differentiation pathway [42]. The *Wnt1* gene contributes to midbrain DA development by activating *Otx2*, which in turn inhibits Nkx2.2, thereby overcoming its negative effect on DA development [43]. Finally, the DA neurotransmitter phenotype is determined by the Cys4 zinc finger nuclear receptor-type family member *Nurr-1* (nuclear receptor-related protein 1; in humans: *NOT1*) and the paired-like homeodomain transcription factor-3 (*PITX3*; pituitary homeobox-3) [44].

Apart from immunocytochemical and expression analyses to verify DA phenotype, there are a few functional assays to assess DA differentiation. To evaluate neurotransmitter release and reuptake, high-performance liquid chromatography (HPLC) is used to measure dopamine release evoked by potassium chloride stimulation, as well as its metabolites homovanillic acid and DOPAC [45]. Detecting the presence of other neurotransmitters such as epinephrine, norepinephrine, or serotonin can help determine the homogeneity or the purity of the induced population [46]. Electrophysiological characteristics of DA neurons that help in distinguishing them from other neuronal populations include pacemaker activity, i.e., the presence of spontaneous action potentials at a rate of 1–9 Hz [47, 48], as well as the characteristic fluctuations in intracellular calcium ion concentration associated with the spontaneous firing of action potentials [49]. To yield meaningful results from in vitro screens or to physiologically execute their function after neural transplantation, our SC-derived DA populations will need to match the above physiological phenotypic profiles as closely as possible.

4.2.2 In Vivo DA Development as a Template for Stem Cell In Vitro Differentiation

When aiming for replicating physiological embryologic midbrain development in the dish, one profits from a decade-long history of fundamental biological studies aimed at elucidating fate specification of this important neuronal subpopulation. As gastrulation leads to the formation of the three germ layers in the embryo, pluripotent cells can give rise to ecto-, meso-, and endodermal derivatives. In vivo, the

mesoderm-derived notochord induces the overlying neuroectoderm to give rise to the neural plate, and, following its invagination at around 3–4 weeks after conception, eventually the neural tube. Subsequently, a range of rostro-caudal patterning cues results in the formation of three bulges that give rise to the forebrain, midbrain, and hindbrain vesicles. Neurons are initially produced in the ventricular zone in proximity to the lumen of the respective neural tube regions and subsequently migrate to reach their destination in the outer or the marginal zone. The underlying changes in cell number, shape, polarity, adhesion, migration, and eventually process extension and synapse formation heavily depend on the spatially and temporally fine-tuned action of signaling molecules secreted by the notochord, floor plate, and the isthmic organizer, for example. Early on, BMPs are negative regulators of neural development, and endogenous antagonists (noggin, chordin, follistatin) inhibit BMPs and lead to induction of neural tissue [50, 51]. The midbrain–hindbrain boundary or the isthmic organizer is established by the homeobox transcription factors engrailed-1 and engrailed-2 [52], and by the dose-dependent action of gastrulation brain homeobox-2 (*GBX2*) and *OTX2* [53, 54]. It serves as an important signaling center with its secretion of FGF-8 and Wnt-1, inducing the development of the midbrain toward its anterior and the hindbrain toward its posterior part [55]. FGF8 from the isthmic organizer and sonic hedgehog (SHH) from the notochord together determine the location where midbrain DA neurons are born. SHH from the notochord acts mainly via the forkhead box protein-A2 (*FOXA2*) to regulate midbrain neural development by inducing neurogenesis, regulating DA phenotype, and regulating the survival and maintenance of midbrain DA neurons [56–58]. Further regional specification of the brain is determined by the interplay of transcription factor domains and secreted factors such as FGFs, Wnts, and RA that jointly promote regionally appropriate phenotype development. For example, for RA a rostro-caudal gradient has been well established [59], and high concentration of RA results in the development of spinal cord-level motor neurons, while intermediate concentrations aid in midbrain-type DA development in vitro [60]. In summary, while we continue to gain insight into the precise transcriptional networks involved in specifying and maintaining appropriate DA phenotype [57, 61], the above proteins are also well established to serve as faithful markers of bona fide midbrain DA neurons differentiated from human stem cells.

4.2.3 Overexpression and Gene Engineering in Stem Cell Differentiation Systems

An admittedly crude, yet powerful, strategy to generate the desired cell type from a pluripotent source is to forcefully change its transcriptional program by genetic overexpression of phenotype-defining transcription factors. Through application of powerful DA transcriptional regulators such as *Nurr1*/*NOT1* [62], *PITX3* [63], or *Lmx1a* [61], downstream genetic programs become expressed and features of DA neurons can be enhanced. Such studies may contribute to further elucidating the sequence of

gene regulatory circuits controlling DA phenotype. However, they do not necessarily result in cell sources that would be equivalent to a midbrain DA neuron or superior to a spontaneously differentiated DA neuron derived from PSCs. Recreating the appropriate physiological gene dosage relationships within the transcriptional networks characteristic of DA phenotype remains difficult. Consequently, cells are present that can be largely non-physiological. For example, *Nurr1* overexpression in murine ES cells led to TH expression in non-neuronal cells [64]. Moreover, the utility of such genetically modified cell phenotypes for transplantation or biomedically relevant in vitro assays is highly questionable. In addition to transcriptional control, it has become increasingly clear to which profound extent posttranslational modifications and expression regulation play a role for phenotype establishment and maintenance [65, 66]. For instance, a range of non-coding RNAs have emerged as being able to control neural differentiation [67]. Given their frequent promiscuity for a range of targets it is sensible that microRNAs (miRNAs) may be involved in fine-tuning DA phenotype establishment and maintenance. Genome-wide screens could be applied to identify miRNAs that control the critical transitions from proliferative neural stem cell to differentiated DA neuron. The miRNA most abundant in the brain, miRNA-124, is known as a powerful regulator of neural stem cell differentiation including in ES cell paradigms [68]. The miRNA-133b has been implied in mesencephalic development and appears to be able to modulate Pitx3 in retinal as well as midbrain DA neuronal cells. Furthermore, it has been reported to be upregulated in a subset of PD patient samples [69–71]. Other links have been drawn between miRNA-7 and α-synuclein (associated with PD neuropathology), for example [72], or the miRNA-433 and the DA survival-promoting FGF20 [73]. While more detailed analysis on such putative associations and into the potential underlying mechanisms is certainly warranted, a growing number of miRNA candidates appear to be involved in DA phenotype development and maintenance or the neurodegenerative processes underlying PD [74, 75]. A lot remains to be learned before exploiting these insights into fine-tuning transcriptional and posttranslational control of DA phenotype generation and maintenance to improve stem cell in vitro differentiation or to potentially enable future in vivo neuroprotective strategies.

4.2.4 Modulating Gene Expression 2.0: Epigenetic Programming and Direct Conversion

Yet, along those lines and inspired by iPS cell technology, recent studies have spurred basic biological curiosity to switch phenotypes more readily (Fig. 4.2b, c). This resulted in a new experimental playing field termed “direct conversion”. A non-neuronal, differentiated somatic cell can now be epigenetically reprogrammed into a neuronal cell without passing through the intermediate pluripotent state by expression of master regulators of the respective neuronal phenotype, a process referred to as transdifferentiation. It is thought that this could, in principle, circumvent some of the remaining issues inherent to pluripotent cells such as

tumorigenicity and low yield of the target cell population. Several studies have demonstrated the possibility of deriving induced neuronal (iN) cells and induced DA neurons (iDA) from mouse and human fibroblasts. The first study used the POU domain transcription factor Brn2 (POU3F2), the BHLH family member Ascl1 (Mash1), and the pan-neural transcription factor Myt1l (NZF-1) (BAM factors) to generate iN cells from mouse fibroblasts at about 2–7 % efficiency [76]. The functional status of cells generated in this manner was confirmed by staining for mature neuron-specific markers and by the analysis of electrophysiological properties of the cells. In another study, the BAM factors along with *NeuroD1* were able to convert human fibroblasts into mature functional neurons at an efficiency of 2–4 % out of which 10 % stained positive for the facultative DA marker TH [77]. Interestingly, transdifferentiation of fibroblasts to neurons through inclusion of miRNAs involved in neuronal lineage development has also been explored. The known neurogenic miR9/9* and miR124, along with *NeuroD2, Asc1l,* and *Mscl1,* produced iN cells at about 10 % efficiency [78]. In a separate study miR124 along with *Brn2* and *Msc1l* has been shown to produce functional iN cells, however, at a slightly lower efficiency of 4–8 % [79]. A few of the neurons thus produced showed properties of glutamatergic and GABAergic subsets but no other subsets were detected.

First proof toward the generation of DA neurons via direct conversion from somatic cells came from a study that used the BAM factors along with the DA lineage-promoting genes *Lmx1a* and *FoxA2* to generate iDA from human fibroblasts. About 10 % of the neural cells generated that way belonged to the DA lineage as confirmed by the presence of specific markers and electrophysiological analysis [80]. A different set of genes, *Lmx1a, Nurr1,* and *Ascl1/Mash1,* was used to generate functional iDA from both mouse and human fibroblast cells. This combination was able to successfully generate iDA from patient fibroblasts [81]. A related study successfully generated iDA from human fibroblasts using the factors *MASH1*, *NGN2*, *SOX2*, *Nurr1*/*NOT1*, and *PITX3* and used these cells for in vivo studies on a rat model for PD. The transplantation led to a symptomatic relief as assessed by behavioral studies and immunohistochemistry [82]. Also, a polycistronic vector coding for Ascl1, Lmx1b, and Nurr1 was able to produce midbrain-like DA neurons (A9) from astroglia (a plentiful source innate to the CNS) at an efficiency of 18–20 % [46]. While conceptually most exciting, at the time of writing, additional limitations are inherent to these "direct conversion" approaches, ranging from low efficiency of transdifferentiation and the cytotoxicity associated with some protocols to issues of genomic integration and use of viral vectors. Moreover, since the cells being produced do not pass through an intermediate proliferative stem cell stage, the quantity of the final cells produced is dependent on the initial number of cells used, thus putting a constraint on the quantity of material available.

Transdifferentiation of somatic cells into induced neural precursor cells (iNPCs) rather than terminally differentiated neurons could help overcome a few of the aforesaid problems to a significant extent. Transient expression of the four reprogramming factors *Sox2, Klf4, c-Myc,* and *Oct4* paired with neural reprogramming culture conditions could convert mouse fibroblasts to iNPCs [83]. However the use of *Oct4* and *c-Myc* still puts this protocol at a disadvantage due to the tumor

Table 4.1 Soluble factors used for neural induction of hPSCs

Factor	Concentration	Example protocols
Noggin	250–500 ng/ml	Chiba et al. (2008) [92] Chambers et al. (2009) [93] Cooper et al. (2010) [125] Nasonkin et al. (2009) [126] Soldner et al. (2009) [26] Sonntag et al. (2007) [28]
SB431542	10–20 μM	Chambers et al. (2009) [93] Mak et al. (2012) [94] Morizane et al. (2011) [95] Smith et al. (2008) [127] Patani et al. (2009) [128]
LDN-193189	100 nM	Kriks et al. (2011) [45]
Dorsomorphin	1–5 μM	Mak et al. (2012) [94] Morizane et al. (2011) [95] Zhou et al. (2010) [129]

The factors most commonly used to neuralize hPSCs in current differentiation protocols are listed, providing the range of concentrations and selected example protocols

formation potential of the iNPCs produced. An improved experimental avenue is represented by the use of *FoxG1* and *Sox2*, which resulted in the conversion of mouse fibroblasts to a neural stem cell population capable of differentiating into neurons or astroglia. The addition of *Brn2* as an extra factor produced cells with a trilineage differentiation potential, which apart from the aforementioned two lineages could also differentiate into oligodendrocytes [84]. An alternative method of generating mouse and human tripotent neural stem cells with merely a single factor, *Sox2*, has also been reported [85]. When combined with the respective neuronal subset-specific key regulators, such approaches could lead to the production of well-defined, expandable subsets such as DA precursors at higher efficiencies.

4.2.5 Generating DA Neurons from PSCs by Modulating Culture Conditions

In addition to direct modulation of transcriptional cascades by genetic engineering or by epigenetic approaches (see above), conventional approaches in stem cell biology comprise the modulation of in vitro culture conditions, guiding the pluripotent source toward the differentiated mature progeny of biomedical relevance. In addition to providing extracellular matrix substrates (such as collagen, fibronectin, laminin, poly-ornithine), the major parameters utilized to direct hPSC toward DA differentiation are soluble factors added to the culture media. Profiting from insights into physiological neural and midbrain development, these include the application of recombinant factors in a sequence mimicking neural induction, midbrain patterning, and DA differentiation. As a recent development, small molecules with equivalent effects have become available to substitute for some of the recombinant proteins

Table 4.2 Soluble factors used for DA patterning

Factor	Concentration	Example protocols
Sonic hedgehog	100–500 ng/ml	Mak et al. (2012) [94] Chambers et al. (2009) [93] Perrier et al. (2004) [2] Shintani et al. (2008) [91] Soldner et al. (2009) [26] Sonntag et al. (2007) [28] Yang et al. (2008) [130] Zhou et al. (2010) [129]
Purmorphamine	10 μM	Li et al. (2008) [131] Stacpoole et al. (2011) [132]
FGF8a	100 ng/ml	Cooper et al. (2010) [125]
FGF8/8b	50–100 ng/ml	Cooper et al. (2010) [125] Perrier et al. (2004) [2] Shintani et al. (2008) [91] Soldner et al. (2009) [26] Yang et al. (2008) [130]
Retinoic acid	100 nM	Cooper et al. (2010) [125] Okada et al. (2004) [60]

DA patterning factors most commonly applied in hPSC differentiation paradigms, respective concentrations, and example references are shown

which may enable future scale-up and make protocols more economical. Growth and patterning factors are being applied over the course of weeks to months in vitro. The range of concentration and duration of application may orient itself after in vivo time frames but is otherwise largely empiric. An overview is given in the following paragraphs and Tables 4.1, 4.2, and 4.3.

4.2.5.1 Neural Induction: Generation of Neural Stem Cells from PSCs

Pluripotent cells can be differentiated either in suspension culture or as adherent culture. In suspension culture, they spontaneously form aggregated spherical embryoid bodies capable of giving rise to all the three germ layers [86]. In adherent culture, the cells can be grown either on a feeder layer or under feeder-free conditions. Different types of feeder cells being used for neural induction include (1) stromal derived PA6 and MS5 cell lines that possess stromal cell-derived inducing activity (SDIA) capable of directing ES cells to differentiate toward neural lineage [87–89]; (2) telomerase-immortalized human fetal midbrain astrocytes capable of inducing DA differentiation [29]; (3) human amniotic membrane matrix capable of inducing differentiation into neural precursors [90]; and (4) bone marrow-derived stromal cells from mice with neuralizing activity [91]. However, these feeder cell layers secrete poorly defined factors that aid in neural differentiation.

Table 4.3 Soluble factors promoting DA differentiation and survival

Factor	Concentration	Example protocols
BDNF	10–20 ng/ml	Ko et al. (2009) [133] Mak et al. (2012) [94] Chambers et al. (2009) [93] Perrier et al. (2004) [2] Soldner et al. (2009) [26] Sonntag et al. (2007) [28] Yang et al. (2008) [130] Zhou et al. (2010) [129]
GDNF	10–20 ng/ml	Ko et al. (2009) [133] Mak et al. (2012) [94] Chambers et al. (2009) [93] Perrier et al. (2004) [2] Soldner et al. (2009) [26] Sonntag et al. (2007) [28] Yang et al. (2008) [130] Zhou et al. (2010) [129]
TGFβ3	1 ng/ml	Perrier et al. (2004) [2] Soldner et al. (2009) [26] Sonntag et al. (2007) [28] Yang et al. (2008) [130]
FGF20	1–10 ng/ml	Correia et al. (2007) [134] Shimada et al. (2009) [135]
AA	200 μM	Hong et al. (2008) [136] Ko et al. (2009) [133] Perrier et al. (2004) [2] Soldner et al. (2009) [26] Sonntag et al. (2007) [28] Yang et al. (2008) [130] Zhou et al. (2010) [129]
Db-cAMP	1–500 μM	Ko et al. (2009) [133] Mak et al. (2012) [94] Perrier et al. (2004) [2] Soldner et al. (2009) [26] Sonntag et al. (2007) [28] Yang et al. (2008) [130]

Commonly applied DA differentiation and survival factors, concentrations, and example references are shown

More commonly, as a better-defined alternative, soluble factors are being used to direct pluripotent cells toward the neuroectodermal lineage. BMP antagonists such as noggin have become widely applied [28, 92]. Alternatively, small-molecule inhibitors (LDN193189, dorsomorphin, SB431542) of the underlying Smad/ALK signaling pathways can be used [45, 93–95]. After in vitro neuralization of the original cells, neural stem cells and neural progenitors derived from PSCs can be further expanded or patterned toward the phenotype of interest (see Table 4.1).

4.2.5.2 Midbrain DA Patterning

Even though many different factors are being utilized in different protocols, basic components that play a major role in midbrain DA patterning include SHH and FGF8. SHH represses dorsal characteristics and promotes the differentiation of DA neurons with forebrain phenotype by the expression of *Nkx2.1* [96]. Together SHH and the rostro-caudal patterning factor FGF8 can induce a forebrain DA neuronal phenotype. However, an early addition of FGF8 during neuroepithelial formation can promote midbrain DA neuronal formation [2, 97] (see Table 4.2).

4.2.5.3 DA Neuronal Differentiation

The various main factors used in the terminal stages of our DA neuronal culture protocols in order to promote DA differentiation, maintenance, and survival include transforming growth factor (TGF3β), brain-derived neurotrophic factor (BDNF), GDNF, dibutyryl-cAMP (db-cAMP), and ascorbic acid (AA) [2, 28, 32, 93]. TGFβ signaling plays an important role in midbrain DA neuronal development. By itself, TGFβ is not sufficient to induce the full midbrain DA neuronal system and requires SHH and FGF8 for its proper action [98]. TGFβ acts via GDNF and together they exert a neuroprotective function and help in their survival [99, 100]. The growth factors BDNF, GDNF, and db-cAMP are thought to help in neural differentiation and the formation, elongation, and survival of DA neurite outgrowth [101]. Empirically, treatment with AA has been known to increase DA neuronal differentiation. However, the exact pathway by which it acts remains unknown [102]. Besides the aforesaid basic factors, FGF20 has also been used in some protocols due to its inductive effect on DA neuronal differentiation and its ability to decrease apoptosis among the differentiated neurons [103, 104]. While important pillars of DA differentiation protocols have been identified (see Table 4.3), the fraction of cells in vitro that differentiate toward the desired phenotype of interest remains low, and further methodological groundwork and empirical protocol development are required.

4.2.6 The Next Steps?

Regardless of the sophistication applied in genetic and epigenetic modulation of the transcriptional program, of ECM composure, and overall growth factor exposure (see preceding sections), it remains difficult to control the microenvironmental conditions that influence a cell's development in direct interaction with its immediate neighbors. By applying more complete concepts of intercellular interdependence during development (cell–cell signaling), eventually more efficacious and homogeneous in vitro differentiation paradigms of neuronal subtypes including DA neurons could be devised. In addition to circumscribed signaling via growth and other surface receptor signaling pathways, other signals could include density-dependent

mechanisms (contact inhibition, crowd-control) [39, 105, 106], sensors of mechanical parameters (cytoskeletal, tensile forces [107]), and electrochemical coupling (membrane potential changes, electrical gradients, gap junctional signaling [108]). Taken together, we continuously gain a better understanding of the complex network of cell–cell and cell–environment interactions, mechanical and biochemical signals that govern neural tissue development in vivo. Such insights may be very well applied to most recent developments in the area of optimizing three-dimensional (3D) differentiation protocols.

Standard 2D culture on a plastic or a glass substrate, while being an excellent source of cells for a wide variety of applications, is at a serious disadvantage in terms of its poor morphological and physiological organization. It mimics the cellular atmosphere in a rather poor manner and is not a true representative of the cellular environment found in organisms. 3D differentiation cultures to the neural lineage add an extra dimension to the conventional monolayer culture and promote cell organization, morphogenesis, and differentiation to a larger degree and hence may be capable of generating better in vitro models of development and disease. Three-dimensional stratified cortical epithelium [109, 110] and retinal cells [111] have been successfully generated from floating cultures of ESC grown in minimal medium and supplemented with specific factors. By varying the 3D culture conditions, and keeping most other factors unchanged, the formation of the above tissues occurred in a mutually exclusive manner which could be attributed to cell–cell interactions that would not have been possible in a conventional monolayer culture [112]. However, this technology has many challenges that need to be addressed before one can employ it for the generation of in vitro models and tissues. Even the well-established 3D cultures are affected by the lack of intermembrane interactions and spatiotemporal gradients of humoral factors and oxygen which play a critical role for the development of tissues. Microfluidic platforms which can recreate these gradients and cell surface-mediated interactions between different cell types could be a solution [113]. Coculture of neurons and glial cells on a microfluidic platform was able to successfully establish tissue-like neuronal-glial interactions [114]. Hence, one can speculate whether fundamental biological insights into supracellular signaling and technological developments in microfluidics and 3D culture systems could lead to the generation of better functional models of the complex ventral midbrain DA system in the future.

4.3 Isolating the Population of Interest from a Heterogeneous Pool of Cells

4.3.1 DA Neuronal Cell Sorting

For now, given the vast developmental and tumorigenic potential of pluripotent cells, cell purification and enrichment strategies will have to be exploited. Our

earliest studies used a simple green fluorescent protein (GFP) marker-based, positive selection strategy in which selection for Sox1-GFP (neural precursors) eliminated tumor-generating cells from murine neuronal differentiation culture [115]. However, mere enrichment of neural precursors including long-term self-renewing neural stem cells may be insufficient to avoid neural tumors in hPSC paradigms [29, 38]. In addition, the ratio of the actual DA cells had been quite low, and further enrichment for this biomedically most relevant fraction was deemed necessary. However, process-bearing neuronal cells are generally perceived as being notoriously difficult to sort via FACS in terms of cell survival. Nevertheless, within a certain fetal developmental time window (ca. E11 to E18 in the mouse; ca. 6–12 weeks of gestation in humans) post-mitotic DA neurons can be dissociated from fetal midbrain tissue into a single-cell suspension with good survival. In our experience, by appropriately adjusting sorting parameters (nozzle size, sort pressure, and speed) and the post-sort culture conditions, if a viable neuronal cell suspension can be generated from the tissue of origin, it can also be FACS-purified without further detrimental loss of cells. For instance, we previously used synapsin-GFP as a mature neuronal reporter to enrich hPSC-derived neurons for in vitro study and for transplantation [116]. Work from our group and others has underlined the feasibility of achieving functional engraftment of DA neurons into rodent brain after flow cytometric purification [30, 31, 117]. Moreover, Pitx3-GFP was applied as an A9 midbrain DA-specific marker, and high-purity ES cell-derived cultures were established post sorting [117]. The resulting functional recovery of PD rodent models receiving cell transplants of these purified cell suspensions underlines the feasibility of subjecting the rather fragile DA neuronal subset to cell sorting [30]. Lately, we and others have made considerable progress to substitute for the fluorescent genetic reporters (GFP) by developing an increasingly specific neural surface antigen code based on cluster of differentiation (CD) surface molecule markers [38, 118]. Such methodological developments have the potential to yield medically applicable protocols in the future. Sorting the CD15-/CD24high/CD29low [116] or the CD184-/CD44-/CD15low/CD24+38 subsets, respectively, enabled the establishment of pure neuronal cultures in vitro and of tumor-free grafts. To further resolve the surface molecular signature of specific neural sub-lineages derived from pluripotent stem cells, more detailed analysis is ongoing. Regarding DA phenotype, the surface marker Corin has been exploited to identify and isolate ventral mesencephalic midbrain floor plate progenitors as precursors of DA neurons [57, 119]. Furthermore, Ganat et al. utilized FACS-based strategies to identify surface marker candidates expressed by murine midbrain DA neurons, such as the acetylcholine receptor subunit beta-3 (CHRNB3) and the guanylate cyclase-C receptor (GUCY2C) [30]. As these are not exclusively expressed on midbrain DA neurons, it will be interesting and fruitful to investigate with which other surface marker combinations these candidate markers are coexpressed on this particular cell type.

In addition to their utility for cell isolation by FACS, or also by immunomagnetic cell separation or immunopanning, surface molecules identified to correlate with specific developmental stages or sub-lineages of neural development are worth studying in their own right due to their functional role for context-appropriate cell

development. A given cell senses its environment through its combinatorial expression of surface molecules such as growth factor receptors and adhesion molecules, and thereby interacts with and responds to soluble factor gradients, the extracellular matrix, and neighboring cells comprising its developmental niche [120]. Microenvironmental cues and membrane-mediated downstream signaling pathways enable a cell's context-appropriate development. A wide range of surface molecule families are known to be present during neural development, and provide ample opportunity for further functional study. The integrin family of surface receptors is one particularly salient example. We found β1-integrin (CD29) to be tightly regulated in a stage-specific manner during hPSC neural differentiation [118]. High levels were present on Pax6-positive neural stem cells and neuroepithelial rosettes. In contrast, β-III-tubulin-positive cells and doublecortin-positive cells, i.e., neurons and neuroblasts, expressed low to no levels of β1-integrin. Correspondingly, Hall et al. found high β1-integrin levels on human fetal neural stem cells [121]. Given β1-integrin's role at the center of the integrin family and its ability to form heterodimers not only with other integrin subunits but also with other growth factor receptors (e.g., IGF-1R or EGFR [122]) and other surface molecules (e.g., cadherins, nectins [123, 124]), current research aims at characterizing the heterodimers present during hPSC neural differentiation and at defining their functional role. We postulate that elucidating surface molecule-mediated signaling pathways will be critical to enhance our understanding of how microenvironmental conditions control neurodevelopmental programs including DA specification.

4.4 Conclusion

In summary, current hPSC research offers a number of future clinical options for pathophysiological investigation, diagnosis, and cell therapy in the context of PD. PSC-derived neuronal cultures yield DA neuronal cells that exhibit a number of features of midbrain DA phenotype. Precise equivalency and phenotype stability remain to be established in direct comparison to fetal midbrain DA neurons. Current research strategies aiming for clinical translation put their efforts into, on the one hand, identifying marker sets that allow for isolation of DA neuronal cells while eliminating unwanted proliferative cell types. On the other hand, fundamental biological studies into the regulatory networks that govern midbrain DA development are being continued. Exciting developments in this latter regard include the notion that relatively small sets of transcriptional key regulators may suffice to program somatic cells toward DA phenotype. All this is likely to synergize with the ongoing parallel efforts on the clinical level using fetal mesencephalic tissue aimed at identifying clinical parameters such as the most appropriate PD patient collective, optimal surgical strategies, cell preparation, and postsurgical follow-up, and eventually aid in the clinical translation of stem cell-based applications for PD.

Acknowledgments RT is funded through the excellence cluster "Spemann Graduate School of Biology and Medicine" of the German Research Foundation (DFG). Our research program on hPSC neuronal differentiation is funded by DFG grant PR1132/3-1 (to JP). The authors thank Christopher J. Hindley for critically reading the manuscript.

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Chapter 5
Stem Cell Applications in Retinal Diseases

Güngör Sobacı

Abstract In contrast to limbal-conjunctival autograft transplantation (LCAT) which has been mainstay of treatment for some ocular surface diseases for decades, stem cell therapy (SCT) is still in its infancy stage in the retina clinic today. Currently, there is an unmet need for treatment of most of the retinal disease with blinding outcome. Recent advances in stem cell (SC) technology provide the basis for optimism for the development of SC-based curative strategies to treat retinal blindness. Beginning in mid-2011, FDA-approved clinical protocols using human embryonic stem cell (hESC)-derived RPE for both dry age-related macular degeneration (AMD) and Stargardt macular dystrophy (SMD) are underway. There are, however, several important challenges even from basic molecular mechanisms which need to be overcome in order for SCT to be a viable option for physicians. This article constitutes an up-to-date summary of therapeutic use of SCs, and aims to contribute understanding of the current and potential use of SCT in the retina.

Keywords Retina • Macula • Degeneration • Dystrophy • Experimental • Clinical • Stem cell • Therapy

5.1 Introduction

For over 30 years, bone marrow stem cells (BMSC), and more recently, umbilical cord blood stem cells (UCBSC), and mesenchymal stem cells (MSC) have been used as therapeutic agents in the treatment of human diseases successfully.

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K. Turksen (ed.), *Stem Cells: Current Challenges and New Directions*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4614-8066-2_5,

Owing to its accessibility, Limbal-Conjunctival Stem Cell Therapy (LCAT) has been the only stem cell therapy (SCT), namely cell-based therapy already in use in ocular diseases.

The retina, as part of the central nervous system (CNS), is made up of neurons which degenerate progressively throughout life. Neurons in the human retina, like those in other mammalian species, have been considered to have little ability to regenerate or repair after injury well over a century. In recent decades, however, it has become apparent that the mammalian visual pathway still exhibits considerable plasticity.

There are many retinal neurodegenerative diseases destined to blindness. Socioeconomic burden of these neurodegenerative diseases cannot be underestimated even in developed economies. Stargardt macular dystrophy (SMD) as an hereditary disease in the young, and age-related macular degeneration (AMD) as an acquired one in the elderly are among the most common of them. When only advanced AMD is considered, nearly 30 million people in the United States and Europe suffer from AMD, which represents a US$25–30 billion worldwide market that has yet to be effectively addressed. After endless efforts in preclinical studies, clinical trials on the use of human embryonic stem cell (hESC) on SMD and AMD were realized in mid-2012.

Stem cell therapy/SCT) may affect the eye, the retina in particular, in two mechanisms of action: (1) by cell replacement therapy: either by transplanting SC/progenitor cell (PC), or, by recruiting endogenous progenitor-like populations residing within the retina, (2) Enhancement of the neurotrophic factors within the recipient milieu. One or both mechanism(s) may be effective, depending on the spatial and/or temporal changes within the microenvironment of the degenerating retina, and/or the type of SC used.

There is still unmet need for therapy of almost all types of hereditary diseases and many of acquired retinal diseases. With progress in molecular therapeutic approaches in SCT, a hope for curative therapy of these diseases otherwise destined to blindness has appeared in recent years.

5.2 Stem Cell Therapies for Replacement of the Photoreceptors

Photoreceptors are principal cell types that detect light, transform into visual signal, and transfer visual signals through other retinal neurons to the brain. Photoreceptors become compromised in retinal diseases such as hereditary macular degeneration (HMD), retinitis pigmentosa (RP), retinal detachment (RD), and AMD.

Currently, there is no proven therapy available to rescue the blindness caused by photoreceptor diseases. Photoreceptor replacement has been shown to be feasible in animal models of these diseases. Almost all stem cell sources have been tested for their ability to replace photoreceptors. There has been ongoing effort to replace photoreceptors from embryonic or fetal tissues for decades [43]. Functional photoreceptor replacement could be obtained in MacLaren et al.'s study, in which freshly

dissociated, postmitotic rod photoreceptors were transplanted to the subretinal space; however, the number of cells could not be increased in vitro due to their postmitotic state [44]. One important obstacle to the clinical use of photoreceptor cell transplantation for human retinal disease is that an appropriate source of the precursor cells is required. Postmitotic photoreceptor precursor cells can easily be derived from tissue of the early postnatal mouse retina (P1–P5). However, equivalent human retinal cells could be derived from second-trimester fetuses, which aside from ethical considerations [60]. Recent studies have shown progress in a somewhat different strategy to cell-based therapy in the retina. New rod and cone photoreceptors have been successfully generated from ESCs of mouse, monkey, and human or from induced pluripotent stem cells (iPSC) [28, 36]

The generation of photoreceptor cells from ESC was first described by the Takahashi group through a series of elaborate steps [60]. ESC protocols have recently been replicated in iPSC, generating both human photoreceptor and RPE phenotypes [28]. Proof of principle has already been shown in the retina that photoreceptors may be replaced by transplantation of neural progenitor cell (nPC) [27]. Success in refinement of the selection of donor photoreceptor precursor cells increased the number of integrated photoreceptor cells, which is a prerequisite for the restoration of sight. Most importantly, several groups have demonstrated successful transplantation of photoreceptors to the degenerating mouse retina, showing synaptic connections between grafted cells and host retina and functional improvements in vision in these models. Lamba et al. showed that retinal PCs derived from hESC may migrate into mouse retinas following intra-ocular injection, settle into the appropriate layers and express markers for differentiated cells, including both rod and cone photoreceptor cells [27, 35]. Currently, well-studied SCs, MSCs have also been shown to transdifferentiate into neuron-like cells even into photoreceptors in presence of norrin in medium [10, 34]. Wang et al. showed that hNPCs rescue photoreceptor degeneration in Royal College of Surgeons (RCS) rats in the long-term [71, 72]. Lu et al. reported that subretinally implanted human adult BM-derived somatic cells rescue vision in a rodent model of retinal degeneration [38]. This study showed that immunosuppression and persistence of SCs are not required for functional success. West et al. however, showed that integration to the neuroretina and long-term survival are possible if the host is immunosuppressed [73]. Simon et al. also showed that Muller glial cells induce SC properties in retinal progenitors in vitro, and promote their further differentiation into photoreceptors [63]. Francis et al., in a non human primate study showed that subretinal transplantation of forebrain neural PCs is feasible and effective [16]. Intraocular delivery of neurotrophic factors via stem cells (SC) slowed down the photoreceptor cells and retinal ganglion cell (RGC) loss experimentally [61]. Neural stem cells (NSCs), via brain-derived neurotrophic factor (BDNF), have been shown to improve cognition in Alzheimer's disease (AD) model [31]. Retinitis pigmentosa (RP) refers to a subset of inherited retinal degenerations, for which over 180 disease associated loci have been mapped and of these over 130 genes have been identified that mutated genotype may result in severe vision impairment. Cell replacement by recruitment of SC in the diseased retina and/or cell-based neurotrophic factor delivery seems to be operative in RP in general.

5.3 Stem Cell Therapies for Replacement of the Retinal Pigment Epithelium

The structural and functional changes in the Retinal Pigment Epithelium (RPE) have always been interest of focus in development of outer retinal diseases/disorders. Long-term safety and function of RPE from hESC in preclinical models of AMD has been shown. Li et al. showed that endogenous BM derived SCs express RPE cell markers and migration into the focal areas of RPE damage [38]. Fan et al. showed that MSC may recruit in the laser-induced choroidal neovascular membrane (CNM), and this may be modulated by engineered MSCs to produce the antiangiogenic pigment epithelial-derived factor (PEDF) at the CNV sites, thereby inhibiting the growth of CNVs and stimulating regressive features [15]. In their well designed study, Lu et al. showed that subretinal transplantation of hESC-derived RPE in both the RCS rat and Elov14 mouse, which are well-known animal models of retinal degeneration and Stargardt, respectively gave functional RPE structures in the long-term [42]. Vaajasaari et al. have developed a progressive differentiation protocol for production of functional RPE-like cells from hESCs and hiPCs [69]. Furthermore, their results showed that RPE-like cells could be differentiated in xeno-free and defined culture conditions, which is mandatory for Good Manufacturing Practice (GMP)-production of these cells for clinical use.

In an acute retinal degeneration model developed using intravenous sodium iodate (NalO3) in toxic dose, we showed that intravitreally applied GFP(+) MSC which can be tracked by in vivo (intravitreally) using autofluorescence technique may restore the RPE/photoreceptor functions in rabbit (Fig. 5.1) [52].

Our study results were confirmed by Gong et al. [19]. They showed that BM-MSCs transplanted into the subretinal space of sodium iodate-injected rats have the ability to differentiate into RPE, photoreceptor, and glial lineage cells. Literature review shows that dose, time, and the location of SC transplantation may affect the course of photoreceptor degenerations differently. For now, RPE cells and photoreceptors derived from patient-specific iPSC can serve as a valuable tool in

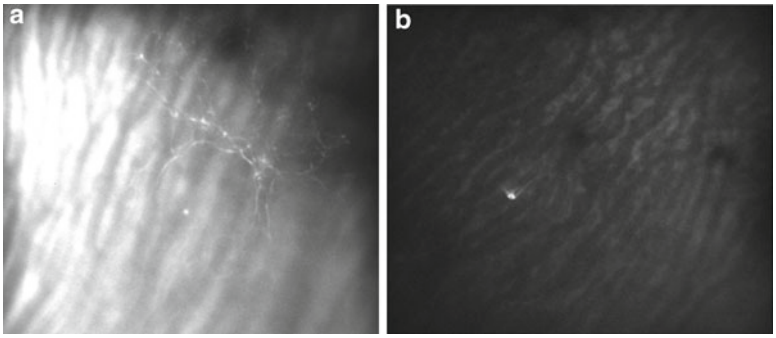

Fig. 5.1 In vivo autofluorescence trace of intravitreally implanted GFP(+)-MSC (5th day) (**a**) and settled into the degenerated retina (20th day) in the rabbit (**b**)

elucidating the mechanism of pathogenesis and drug discovery for geographic atrophy. In iodoacetic acid-induced retinal injury model, Zhou et al. showed that iPSCs of swine can differentiate into photoreceptors in culture, and these cells can integrate into the damaged swine neural retina, thus, laying a foundation for future studies of retinal stem cell transplantation [77].

5.4 Stem Cell Therapies for Replacement of the Retinal Neurons

Compared with photoreceptor replacement, the replacement of RGC is much more complex. Neurons in the central nervous system including the eye fail to regenerate their axons after injury. NSCs have significant role in retinal histogenesis; so that they affect final cell number of all the retinal cells including photoreceptors. The modulation of pro- and anti-apoptotic related genes including Bax, Bcl-2, Bcl-X, and Xiap have been shown to be effective in protecting RGCs from injury, at least transiently and to promote neuritic outgrowth [33, 62].

Fetal or embryonic retinal progenitors could be grown in vitro and used for transplantation [32]. Neurospheres grown from the adult pigmented ciliary epithelium (CE) could also be transplanted to the retina [9]. NSCs derived from the hippocampus show a remarkable ability to integrate into the retinal layers and form morphologically normal appearing retinal neurons (Takahashi et al. [66]). This neurogenic potential has been confirmed by the comparative study on human ciliary epithelium (CE) and Muller cells [4]. Both MSC and nonpigmented CE expressed markers of neural progenitors, including SOX2, PAX6, CHX10, and NOTCH. Nestin, a neural stem cell marker was only expressed by MSC. Non-pigmented CE displayed epithelial morphology, limited photoreceptor gene expression and stained strongly for pigmented epithelial markers upon culture with neural differentiation factors [46]. Bahia et al. also suggested that human pigmented CE does not proliferate or exhibit neurogenic properties in vitro, non-pigmented CE does not differentiate into retinal neurons in vitro, Müller glia that express neural progenitor markers differentiate into retinal neurons in vitro [4].

Evidence of neural integration following transplantation has been presented [46, 71] however, as typified by these studies, accurate discrimination of donor and host is a challenge because graft–host synapses are difficult to distinguish from intra-graft synapses. Goldenberg-Cohen showed that primitive stem cells derived from BM express glial and neuronal markers and support revascularization in injured retina exposed to ischemic and mechanical damage [18]. There are many examples of generating human neuronal cells from iPS and a preliminary report of generating ganglion cells from ESC [23]. In RCS rat model, Tian et al. showed that subretinal transplantation of retinal SC was associated with increased Muller cell de-differentiation, and slow of the progression of retinal morphological degeneration and prevention of the functional disruption [67]. Harper et al. showed that transplantation of BDNF-secreting MSC provides neuroprotection in chronically hypertensive rat eyes [21].

Oligodendrocyte precursor cells and Muller cell-derived stem cells have been shown to slow optic nerve degeneration by integrating into the degenerating nerve fiber layer in the rat glaucoma model [5, 6]. In addition, in rat eyes with optic nerve transection, neuroprotective effect of intravitreal injections of MSC has been shown [37].

5.5 Stem Cell Therapies for Restoration of Retinal Immunity

Recent studies have showed that cultured MSCs secrete various bioactive molecules which have anti-apoptotic, immunomodulatory, angiogenic, antiscarring, and chemoattractant properties [24, 51, 65]. These properties in the light of available data from our clinic and other disciplines suggest that intravitreally applied MSC therapy would be of benefit in uveitis treatment.

Recent preclinical studies are summarized in Table 5.1 [7, 25, 30, 31, 37, 49–51, 53, 57–59, 68, 75, 76].

5.6 Clinical Stem Cell Therapy Trials for Retinal Diseases

There have been early attempts to restore/replace macular function through RPE replacement in AMD with subfoveal CNM. Among the most noticeable of clinical studies, Van Meurs and Van Den Biesen performed autologous retinal pigment epithelium-choroid translocation after the removal of a subfoveal CNM in six patients with exudative AMD. In a short term follow-up they showed that this method was feasible and may result in a surviving and functioning graft for more than 1 year [70]. MacLaren et al., in 10-patient clinical interventional study, showed that autologous human RPE-choroid sheet replacement for denuded areas after excision of CNM can restore vision in neovascular AMD, and ex vivo gene transfer to this RPE-Choroid sheet was feasible; however, complication rate was high [45]. These procedures using autologous adult RPE cells failed to replace or restore naive RPE function due mostly to postmitotic nature of the donor cells.

Currently, there is no SCT protocol used in the retina clinic. However, search in the "www.clinicaltrials.gov" yields eighteen ongoing studies on retinal diseases. These are:

1. Autologus Bone-Marrow Derived Mononuclear Stem Cell Transplantation in Ocular Lesions of Behcet's Disease (Study NCT00550498). This phase I, interventional study on safety of MSC therapy conveyed by Iranian doctors has been terminated in July 2011, because of the lack of improvement obtained in three cases and development of retinal detachment in two of ten cases.
2. Hematopoietic Stem Cell Transplantation in Autoimmune-Related Retinopathy (ARRON) (Study NCT00278486). This phase I toxicity/efficacy/survival study, recruiting participants, is expected to be finalized in the mid-2012 now terminated due to insufficient patient follow-ups. This study has been terminated due to in compliance of the patients with follow-up.

Table 5.1 Recent preclinical studies on stem cell therapy in retinal diseases

Retinal diseases	Donor cell type (species)	Target (species)	Outcomes	Reference
Retinal degeneration	h/mBM-SCs	Retinal cells (m)	Retinal degeneration	[7]
	hBM-somatic cells	Photoreceptors®	Cells were differentiated into photoreceptors r BM-MSCs retinal cells (r) expressed a rod photoreceptor Bipolar and Amacrine cell	[53]
	Primate ESCs	Primate retinal cells	Coculturing is efficient for inducing ESC	[53]
	mESCs	Retinal cells (m)	Differentiates to various retinal cells	[75]
Neural retina repair	mPostmitotic rod photoreceptor precursor cells	Rod photoreceptors (m)	Presence of photoreceptors upto 12months	[49]
Retinitis pigmentosa	mBM-MSCs	Microglia (m)	Microglia may be protective in RP	
	hESC	Subretinal (m)	Produce photoreceptors	
	hnPC	Subretinal (r)	Long-term functional	[58]
	hUTC, hPTC,hADF and hMSC	Photoreceptors (r)	Umbilical tissue-derived cells gave large areas of photoreceptor rescue; MSCs produced only localized	[25, 68]
	hiPSCs	hRPE (culture)	iPSCs differentiate into functional RPEs	[57]
	hESC-derived RPE	Subretinal (m/r)	hESCs could serve a potentially safe and inexhaustible source of RPE	[42]
Glaucoma	hMSCs	Retinal ganglion cells (r)	BM-MSCs deliver neurotrophic factors	[30, 50]
	hRPE	Photoreceptors, rods and electroretinogram	Partial preservation of rod and cone	
	Photoreceptors (r) cones (r)		hRPE and hSC grafts can survive and rescue	[51, 59]
TON	mBMli(−)SC	Optic nerve	Neural and Glial markers	[37]
	hUCBSCs	Optic nerve	Optic nerve protected, with secretion of BDNF and	[76]
ION	mBMlin(−)SC	Optic nerve	Neural and Glial markers	[31, 37]

h human, *m* mouse, *r* rat

Key:

AMD age-related macular degeneration, *BM-SC* bone marrow stem cells, *iPSC* induced pluripotent stem cells, *ESC* embryonic stem cell, *MSC* mesenchymal stem cell, *RP* retinitis pigmentosa, *RPE* retinal pigment epithelium, *UTC* umbilical cord tissue-derived cells, *PTC* placenta tissue-derived cells, *ADF* adult dermal fibroblasts, *MSC* mesenchymal stem cells, *BM* bone marrow, *SC* stem cells, *TON* traumatic optic neuropathy, *ION* ischemic optic neuropathy

3. Safety and Tolerability of Sub-retinal Transplantation of hESC-derived RPE (MA09-hRPE) Cells in Patients with Advanced Dry Age Related Macular Degeneration (Dry AMD) (Study NCT01344993). This phase I/II, open-label, multi-center, prospective study, aiming to determine the safety and tolerability of sub-retinal transplantation of hESC derived RPE (MA09-hRPE) cells in patients with advanced dry AMD is the second to the HESC treatment in the spinal cord injury as a FDA-approved HESC treatment. This study which began to enroll first patient in July 2011 by Dr Schwartz from Jules Stein, UCLA, CA, USA plans to involve 12 patients (for dose escalation in cohorts of three patients in each). The initial results of clinical trials of subretinal transplantation of human hESC-RPE cells in patients with SMD and dry AMD are expected to be finalized in the mid-2013.
4. Sub-retinal Transplantation of hESC-Derived RPE(MA09-hRPE) Cells in Patients With Stargardt's Macular Dystrophy (Study NCT01345006). As was in hESC-RPE for dry AMD by Dr Schwartz, this phase I/II, open-label, multi-center, prospective study aims to determine the safety and tolerability of sub-retinal transplantation of hESC-derived RPE (MA09-hRPE) cells in patients with Stargardt's Macular Dystrophy (SMD), and expected to be finalized in the mid-2013. This study will involve 12 patients (for dose escalation of 50,000–200,000 cells/enrolled into several centers across the United States. The initial results of clinical trials of subretinal transplantation of hESC-RPE cells in patients with Stargardt's macular dystrophy and dry AMD showed preliminary safety and possible visual acuity benefits, especially in SMD.
5. Autologous Bone Marrow-Derived Stem Cells Transplantation for Retinitis Pigmentosa (Study NCT01068561). This phase I nonrandomized open-label short-term safety study executed by Brazilian doctors completed in the last year showed that a single intravitreal injection of autologous BM MSC was safe in patients with RP. A phase I trial of intravitreally injected autologous BM-derived mononuclear cells for hereditary retinal dystrophy demonstrated no evidence of toxicity with possible visual acuity benefits but no structural or functional changes. Long-term results are awaited.
6. Cell Collection to Study Retinal Diseases (Study NCT01432847). This study aims to collect skin fibroblasts, hair keratinocytes, and CD34+ blood cells from patients with Best Disease, L-ORD, and AMD and from age-, gender-, and ethnicity-matched healthy participants for generation of the iPS cells of patient and healthy volunteers, and to analyze molecular mechanisms involved in disease initiation and progression. In addition, the iPS cell-derived RPE cells will be used to perform high-throughput (HTP) drug screens aimed at suppressing the molecular phenotypes of the disease to identify potential therapeutic agents for these diseases. In addition, two clinical trials on the extraocular/metastatic disease of retinal origin (retinoblastoma) are included in this site.
7. High-Dose Thiotepa Plus Peripheral Stem Cell Transplantation in Treating Patients With Refractory Solid Tumors (Study NCT00003173). This is phase II trial to study the effectiveness of high-dose thiotepa plus peripheral stem cell transplantation in treating patients with refractory solid tumors including

retinoblastoma. This study has been completed. Authors report that intensive multimodality therapy including high-dose chemotherapy with autologous hematopoietic stem cell rescue was curative for the majority of patients with stage 4a metastatic retinoblastoma (disseminated metastatic disease not involving the CNS, including extradural/dural disease without parenchymal or leptomeningeal disease) treated; this therapy may be beneficial for some patients with stage 4b retinoblastoma (CNS disease, including trilateral retinoblastoma). Longer follow-up has been suggested to determine whether it is curative or not [12, 13].

8. Combination Chemotherapy, Autologous Stem Cell Transplant, and/or Radiation Therapy in Treating Young Patients with Extraocular Retinoblastoma (Study NCT00554788). This multinational, multicenter, phase III trial studying the side effects and treatment effect of intensive multimodality therapy together with autologous SC transplant in young patients with extraocular retinoblastoma (stage 2 or 3 disease: orbital and/or regional involvement) is recruiting patients.
9. Clinical Trial of Autologous Intravitreal Bone-marrow CD34+ Stem Cells for Retinopathy (NCT01736059). Sponsored by California Davis, this phase-1 study focuses on treatment of various retinopathies (retinal vein occlusions, retinitis pigmontosa) now recruits patients.
10. Safety and Tolerability of Sub-retinal Transplantation of Human Embryonic Stem Cell Derived Retinal Pigmented Epithelial (hESC-RPE) Cells in Patients With Stargardt's Macular Dystrophy (SMD) (NCT01469832). This study aims to evaluate the safety and tolerability of RPE cellular therapy in patients with SMD, and to evaluate potential efficacy endpoints to be used in future studies RPE cellular therapy is currently recruiting patients.
11. A Phase I/IIa, Open-Label, Single-Center, Prospective Study to Determine the Safety and Tolerability of Sub-retinal Transplantation of Human Embryonic Stem Cell Derived Retinal Pigmented Epithelial(MA09-hRPE) Cells in Patients With Advanced Dry Age-related Macular Degeneration(AMD) (NCT01674829) This study aiming to evaluate the safety of the surgical procedures when used to implant MA09-hRPE cells and to assess the number of hRPE cells to be transplanted in future studies is currently recruiting patients.
12. Safety and Tolerability of Sub-retinal Transplantation of hESC Derived RPE (MA09-hRPE) Cells in Patients With Advanced Dry Age Related Macular Degeneration (Dry AMD) (NCT01344993). This study aiming to safety and tolerability trial to evaluate the effect of subretinal injection of human embryonic stem cell derived retinal pigment epithelium cells in patients with dry Age Related Macular Degeneration (AMD) is currently recruiting patients.
13. Safety and Tolerability of MA09-hRPE Cells in Patients With Stargardt's Macular Dystrophy (SMD) (NCT01625559). This study aiming to evaluate the safety and tolerability of RPE cellular therapy in patients with SMD Group is currently recruiting patients.
14. Study Of Implantation Of Human Embryonic Stem Cell Derived Retinal Pigment Epithelium In Subjects With Acute Wet Age Related Macular Degeneration And Recent Rapid Vision Decline (NCT01691261). This study

aiming to evaluate the safety and feasibility/efficacy of treating subjects with wet age-related macular degeneration (AMD) in whom progressing vision loss is not yet open.

15. Intravitreal Bone Marrow-Derived Stem Cells in Patients With Advanced Age-related Macular Degeneration (AMDCELL) (NCT01518127). The Brazilian group in this study aims to to evaluate the behavior of intravitreal injection of of autologous bone marrow stem cells in patients with age related macular degeneration, now reciting patients.
16. Autologous Bone Marrow-Derived Stem Cells Transplantation For Retinitis Pigmentosa (RETICELL) (NCT01560715). The Brazilian group from Sao Paulo passing phase-2 stage in this study aims to evaluate the short-term safety and efficacy of a single intravitreal injection of autologous bone marrow stem cells in patients with retinitis pigmentosa.
17. Effect of Intravitreal Bone Marrow Stem Cells on Ischemic Retinopathy (RetinaCell) (NCT01518842). Study from Sao Paulo group aiming to evaluate the behavior of the intravitreal use of bone marrow derived stem cells in patients with ischemic retinopathy is recruiting patients.
18. Study of Human Central Nervous System Stem Cells (HuCNS-SC) in Age-Related Macular Degeneration (AMD) (NCT01632527). This study as a open-label dose-escalation investigation of the safety and preliminary efficacy of unilateral subretinal transplantation of HuCNS-SC cells in subjects with Geographic Atrophy secondary to Age-Related Macular Degeneration (AMD) is expected to be finalized mid-2014.

Today SCT is experimental in nature. However, several anecdotal reports of the success with SCT for neurodegenerative disease including retinal ones from India and China have appeared in peer-reviewed journals recently (www.scientificamerican.com).

5.7 The Challenges in Application of Stem Cell Therapy in Retinal Disease

Early attempts to treat animal models of neurological disease, including retinal degenerations, with human ESC failed to show a significant functional contribution for the transplanted neurons, and in some of those implanted intravitreally in slow degenerating mouse model resulted in teratoma formation [41]. Currently, no evidences of tumorigenesis have been reported when ESC-derived retinal PCs were used in preclinical studies. However, in Arnhold's experimental study, tumor formation has been shown when neurally selected mouse ESC was transplanted into rodent retina [2].

Cumulative evidences from the literature suggest that stem cell therapy in the eye, even in the local applications, would not be free from the side effects/complications. These are:

1. Currently available clinical studies is still far from expectancy for SC replacement therapy in neurodegenerative disease, like glaucoma, benefit from SCT. Several studies have shown that, although NSCs are able to migrate into the host retina, they do not express retinal phenotypes. On the other hand, retinal stem cells undergo differentiation but are unable to migrate and integrate with the host retina [1, 56].
2. Maintenance of epigenetic mechanisms including DNA methylation, chromatin remodeling, and the noncoding RNA-mediated process, and epigenetic regulators which are key players in stem cell biology and their dysfunction are not fully understood [40].
3. There have been evidences for similarities and differences between hESC lines in self-renewal, and spontaneous and directed differentiation. This is probably due to inherited variation in the sex, stage, genetic background of embryos used for hESC line derivation, and/or changes acquired during passaging in culture [30]. Similarly, MSCs from the two different mouse strains, namely, C57BL/10 and mdx, exhibit differences in proliferative and myogenic abilities. Change in mouse MSC behavior in mdx mouse study has been indicated to the lack of dystrophin protein [39].
4. There is insufficient safety data on the ideal developmental stage for optimum integration of donor cells. For successful integration, the grafted photoreceptors should assume the correct orientation, with an inner synapse and an outer photoreceptive segment positioned against host inner retina and RPE, respectively. It may be that for effective integration, SCs need to be differentiated some way along the photoreceptor lineage before transplantation. A lack of integration is likely to show a tendency for rosette formation. Previous experiments with RPE sheets showed that long-term rescue of photoreceptor cells in the RCS rat, could be accomplished using young, healthy RPE cells at early stages of the disease process [71].
5. Cliary epithelium (CE) has been identified to be a source of SC; however, the true stem cell nature of the CE and its possible application in cell therapies has now been questioned [8].
6. Currently, the percentage of cells able to integrate and make connections is low (approximately 0.1 %) [55]. Given that millions of photoreceptors will probably be needed to restore meaningful vision, the challenges for the cell suspension approach for generation of sufficient numbers of cells and full integration are not overlooked.
7. There is still ongoing controversy on the best route for application of retinal SCT. Unusually, Singh et al. showed that the intravenous route resulted in optimal localization of donor cells at the site of injury. These cells incorporated into injured retina in a dose-dependent manner [64]. The data presented in this study reflect the importance of dose and route for stem cell-based treatment designed to result in retinal regeneration.
8. There is insufficient long-term safety data on the ideal developmental stage for minimum immunogenicity and teratogeneity of donor cells. Transplantation into the retina may be hampered by gliosis, although this may be less marked

than other CNS sites. Clinically, it is recognized that intra-retinal RPE migration is a feature of RP, which may imply that gliosis could still allow for cell integration to some extent. On the other hand, as in first clinical trial of MSC therapy for Behcet disease, intraocular gliosis might result in termination of the clinical trial (Study NCT00550498).

9. iPS clones also vary in pluripotency and differentiate less efficiently than ESCs, which show robust neuronal differentiation [26]. Interestingly, this variability is independent of the type of vector used in and probability of tumorogeneity.
10. Neural transdifferentation of MSCs are still on debate. Hill et al. showed that human umbilical cord blood-derived MSCs do not differentiate into neural cell types or integrate into the retina after intravitreal grafting in neonatal rats [22], which is contrary to report on optic nerve repair by Zhou [76].
11. Although the eye is often considered an immune privileged site, recent evidence suggests that immune suppression, particularly of T-cell mediated pathways, is needed for the long-term survival of transplanted photoreceptors [20] Presence or maintenance of the immunoprivilages of the vitreous and the subretinal space which are probable sites for SC applications are still questioned, since the blood-retinal barrier might be disrupted in SCT.
12. There are interrelation(s) between the retinal cells for both anatomical and functional in nature. It is widely accepted that photoreceptors will not survive without a functional underlying RPE layer; thus, any iPSC transplantation strategy to treat diseases that affect both layers, AMD, should aim to replace the RPE cells prior to photoreceptor transplantation. It is also well known that the cone photoreceptors cannot survive without the rods (rod-derived cone viability factor).
13. Candidates for retinal cell replacement surgery must have some retinal cone and rod photoreceptors intact. The retinal cell replacement surgery primarily serves to repair the nerve synapses in the retina, the macula lutea, and the fovea. The surgery cannot generate new photoreceptors.
14. It is not known whether vision can be restored in a severely degenerate retina with prolonged photoreceptor loss.
15. There is still need for better understanding of basic SC science. Patient-specific iPS cells may lead to customized cell therapy. However, regeneration of retinal function will require a detailed understanding of eye development, visual system circuitry, and pathology in retinal degeneration [50].
16. Another challenge for stem cell therapy is the ongoing disease in the host microenvironment including the extensive remodeling, changes in neuronal migration, and rewiring of synaptic connections that is likely to differ between retinal degenerations [17].
17. The role of iPSC-derived cells to subvert the need for immunosuppression remains to be determined.
18. Postmitotic photoreceptor precursors can functionally integrate into the adult retina; however, the number of photoreceptor precursors present in the differentiated cell population (0.2 % Nrl(+) cells) is still far from functionally successful grafting. Stem cells might need to be directed toward a postmitotic "progenitor phenotype" in vitro before transplantation in order to achieve optimal integration and functionality [74].

19. Several studies have shown that, although NSCs are able to migrate into the host retina, they do not express retinal phenotypes. On the other hand, retinal stem cells undergo differentiation but are unable to migrate and integrate with the host retina [40, 56].
20. Much like the rest of the mature CNS, the retina is implastic and inhibitory to cellular migration. This could be attributable to the adult retinal environment being unable to provide all signals needed for retinal neuronal development and even being inhibitory. Due mostly to blood-retinal barrier effect, about 1 % of intraocularly transplanted cells commonly migrate into the retina [29, 52]. However, following injury, strong activation of glia, i.e. astrocytes and microglia, occurs that releases several molecules to restore retinal homeostasis.
21. There is still no scientifically proven model for inducement of IPC from CB cells.
22. It is not easy task to find out causative gene mutation in locus with countable and predicted genes; they cannot be identified even though all molecular screening tests including next-generation sequencing approach.

5.8 Breakthroughs in Stem Cell Therapy for Retinal Diseases

These are:

1. Substantial benefit could be obtained in retinal SCT due to SC-delivered growth factors from newer sources of SC. This may serve as a source of neurotrophic factors which slows down photoreceptor cells and RGC loss in experimental models. For example, placental mesenchymal stromal cells induced into neurotrophic factor-producing cells have been shown to protect neuronal cells from hypoxia and oxidative stress [39].
2. Success in refinement of the selection of donor photoreceptor precursor cells increased the number of integrated photoreceptor cells, which is a prerequisite for the restoration of sight.
3. It is generally agreed that when the human ES cells are subjected to an extensive differentiation program prior to transplantation, they do not form teratomas when transplanted to either the vitreous or the sub-retinal space.
4. Newer surgical techniques (for retinal and subretinal delivery of SC) made delivery of SCs easier and safer. A prototype instrument for subretinal transplantation of iPS-RPE sheets subretinally has been produced.
5. Plastic compressed collagen constructs suitable vehicles for drug and cell delivery into the eye. This may be used to deliver Muller cell-derived Retinal Ganglion Cells in to the damaged retina. Newer ultrathin parylene film seeded with hESC-RPE has been developed for stem cell application.
6. Newer transduction and genetic modification techniques such as transcription and growth factors are available. This can be promoted by treatment with small molecule inhibitors of the Notch pathway and retinal lasering [10, 64].

7. A progressive differentiation protocol for the production of functional RPE-like cells from hESCs and hiPSCs in xeno-free and defined culture conditions, which is mandatory for Good Manufacturing Practice (GMP)—production of these cells for clinical use defined by Vaajasaari et al. [69]. These novelties enabled more straightforward GMP guidelines today.
8. Storing biological material from SC source including milk teeth stem cells is more convenient and cheap. GMP guidelines are also more straightforward today.
9. The recent development of human iPS cells, specifically their induced differentiation into cells with human photoreceptor phenotype, has now provided us with the opportunity for embryo-free autologous transplantation [50]. This avoids the use of ESC-related concerns on ethical and biological basis. These iPS cells, being autologous, may obviate the need for chronic immune suppression. Technical advances in cloning have enabled the generation of iPS cells from human fibroblasts [50] and without the c-Myc gene, which significantly reduces the risk of tumor formation [48]. Recently this method has been optimized to avoid retroviruses by using episomal vectors, which do not become incorporated into the genomic DNA enabling free of viral and transgene sequences [17, 50, 74]. Human iPS cells have now been cloned from patients with neurological diseases such as Parkinson's disease, muscular dystrophy, and Huntington's disease [3, 11, 54].
10. The iPS cell is innovative technology that turns somatic cells into ESC-like cells via the transduction of several key genes. It can be also used to elucidate the disease mechanism. iPS clones derived from patients could be used for treatment via ex vivo correction of the gene defect before reintroduction into the host.
11. The recent success in culturing a whole optic cup in vitro has shown how large numbers of photoreceptors might be available [14]. They have demonstrated that optic-cup morphogenesis in that simple cell culture depends on an intrinsic self-organizing program involving stepwise and domain-specific regulation of local epithelial properties. The in vitro generation of a three-dimensional retina offers the potential of modeling retinal disease and testing pharmacological therapies. This development also opens new possibilities of growing artificial retinal tissue sheets rather than simple cell grafting.
12. Controlled delivery system such as functionalized magnetic nanoparticles may serve as treatment delivery system, especially for previously inaccessible part of eye (the optic nerve).

5.9 Conclusion and Future Remarks

Recent studies in molecular biology and molecular surgery have provided a springboard for application of SCT in the retina today. Early results of the clinical studies seem promising. Since integration efficacy of SCs is both disease and stage-dependant, a customized approach for each retinal disease will be needed to ensure

the optimal conditions for successful therapy. As experimental evidence strongly suggests, combinations of gene- and cell-based therapies are required to overcome morphological and functional impairment attributable to neurodegenerative disease of the retina.

Recent approval of SCT in patients with Stargardt and AMD opened a novel treatment avenue not only in ophthalmology, but also in medicine. There are, in fact, valid controversies; therefore, cautious optimism for these new SCTs suggested for retinal diseases is still valid. With the aid of recent developments in SCT, there will be growing numbers of clinical protocols addressing SCTs for retinal diseases in near future.

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Chapter 6
Potential Use of Dental Stem Cells for Craniofacial Tissue Regeneration

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Abstract Mesenchymal stem cells such as bone marrow stromal cells and Adipose-derived stem cells are widely being used for clinical applications in regenerative medicine. Dental stem cell sources such as dental pulp stem cells, stem cells from human exfoliated deciduous teeth, periodontal ligament stem cells, stem cells from apical papilla, dental follicle progenitor cells, and tooth germ stem cells have also been started to be used for the same purposes. Since most dental-derived stem cells are of cranial neural crest origin, their use in the engineering of craniofacial structures holds promise in the near future. This chapter will discuss the potential applications of adult stem cells in craniofacial tissue engineering. Current knowledge about adult stem cells of dental and non-dental origin will be reviewed with respect to their regenerative capabilities and therapeutic potentials

Keywords Dental stem cells • Craniofacial tissue engineering • Differentiation

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K. Turksen (ed.), *Stem Cells: Current Challenges and New Directions*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4614-8066-2_6,

Abbreviations

MSC	Mesenchymal stem cell
BMSC	Bone marrow stromal cell
ASC	Adipose-derived stem cell
DPSC	Dental pulp stem cell
SHED	Human exfoliated deciduous teeth
PDLSC	Periodontal ligament stem cell
SCAP	Stem cell from apical papilla
DFPC	Dental follicle precursor cell
TGSC	Tooth germ stem cell
hTGSC	Human tooth germ stem cell
TGF-β	Transforming growth factor-β
BMP	Bone morphogenetic proteins
PEGDA	Poly (ethylene glycol) diacrylate
PD	Population doubling rate
ALP	Alkaline phosphatase
BSP	Bone sialoprotein
DSP	Dentin sialoprotein
NeuN	Neuronal nuclear antigen
GAD	Glutamic acid decarboxylase
NFM	Neurofilament M
GFAP	Glial fibrillary acidic protein
CNPase	2,3-Cyclic nucleotide-3-phosphodiesterase
DMP 1	Dentin matrix protein-1
EMD	Enamel matrix derivatives
CAP	Cementum attachment protein
CP-23	Cementum protein-23

6.1 Introduction

In humans, the healing of craniofacial tissues frequently results in limited regeneration due to size and character of the defect. Functional replacement of such lost or damaged craniofacial tissues is one of the specific goals of tissue engineering [1, 2]. Recent developments in tissue engineering initiated new alternatives by utilizing biomaterials [3], gene therapy [4], signaling molecules [5] and stem cells [6] to regenerate craniofacial structures, aiming at the ideal of restitutio ad integrum. Until now, much has been learned about the single use of various biomaterials in the craniofacial region [7]. Various materials, such as natural or synthetic polymers [8, 9], ceramics, and composites [10], were used as tissue engineering scaffolds to promote cell migration and differentiation, extracellular matrix synthesis, and vascularization. Also, bioactive molecules were added to these scaffolds to enhance cell attachment,

new tissue formation, and angiogenesis [11]. However, none of these cell-free approaches were able to establish optimal tissue regeneration. Since mesenchymal stem cells (MSCs) play a pivotal role in the development of craniofacial structures, tissue engineering approaches using MSCs hold promise of providing a treatment for people suffering from craniofacial tissue and organ deficiencies [12, 13].

The craniofacial region involves various components, such as bone, nerves, connective tissue, glands, fat, teeth, and muscle. From this perspective, the reconstruction of these structures using stem cell-based approaches is a complex issue, but not impossible. Various attempts to date have been made to engineer the periodontium [14], cementum [15], temporomandibular joint [16], bone, [6] and fat tissue [17] using stem cells. Especially, MSCs derived from the bone marrow stroma (BMSCs) have been used extensively in craniofacial tissue engineering [18, 19]. Bone marrow-derived MSCs have the potential to differentiate into various lineages, and have therefore, been also clinically applied for treating different tissue disorders [20, 21]. Studies have shown that these multipotent adult stem cells are present in various tissues and organs, such as the nerve, skin, adipose, tendon, synovial membrane, and liver [22–26]. However, due to some reasons, such as diseases of bone marrow or surgical trauma during bone marrow isolation procedures, researchers are looking for alternative stem cell sources that require minimally invasive collection procedures.

Recent studies have revealed the presence of adult stem cells in tissues of dental origin as well [27]. Dental stem cells have the capability to undergo osteogenic, odontogenic, adipogenic, and neurogenic differentiation [28]. Since MSCs from dental tissue are obtained during regular orthodontic procedures, usage of that type of stem cell is easy, cost-effective, and does not raise additional safety and ethical concerns. Six different types of stem cells were isolated from dental tissues, such as dental pulp stem cells (DPSCs) [27], stem cells from exfoliated deciduous teeth (SHED) [29], periodontal ligament stem cells (PDLSCs) [30], stem cells from apical papilla (SCAP) [31], dental follicle precursor cells (DFPCs) [32] and tooth germ stem cells (TGSCs) [33]. Indeed, one important feature of these dental-derived cells is their ectomesenchymal origin, which makes them a good candidate for tooth regeneration studies [28].

6.2 Adult Stem Cells of Non-Dental Origin

Mesenchymal stem cells (MSCs) are populations of adult cells that reside in various tissues and organs, especially in the bone marrow, and maintain their regenerative potential through asymmetric mitotic cell division [18]. In other words, they have the ability to renew themselves, while differentiating into several specialized cell types of mesenchymal origin, termed as multipotency [34]. Upon need, tissue-specific MSCs have the genetic potential to repair or regenerate tissues from which they derive [12].

6.2.1 Bone Marrow-Derived Mesenchymal Stem Cells

Among various cell sources, Bone Marrow-Derived Mesenchymal Stem Cells (BMSCs) have been extensively studied for regenerating different types of tissues. These cells are frequently isolated from bone marrow aspirates from the iliac crest and live in close contact with the hematopoietic stem cells that have been successfully used in the treatment of leukemia for several decades. Under established culture conditions, BMSC is a heterogeneous cell population [35]. However, these mixed populations of BMSCs can be purified and homogenous groups can be immune selected using various surface markers [36].

Although no single marker to date has been shown to identify the MSCs, several markers have been reported to be typical for BMSCs. These markers include CD29, CD44, CD73, CD90, CD105, CD146, CD166, and STRO-1 as positive, CD11b, CD14, CD34, CD45, and HLA-DR as negative [35, 37–39]. According to the minimal criteria proposed by International Society for Cellular Therapy, human MSCs must at least express CD73, CD90, and CD105, and lack expression of CD14 or CD11b, CD79 alpha or CD19, CD34, CD45, and HLA-DR surface molecules [40].

BMSCs are plastic adherent and have the ability to produce colonies when seeded at very low cell densities, termed as clonogenicity [35]. Moreover, it has been shown that BMSCs are capable of differentiating, at least, into mesodermal cell lineages, such as bone, cartilage, tendon, adipose, and muscle [18]. Besides, several studies reported the transdifferentiation potential of BMSCs into cells of different germ layers, including neurons [41], hepatocytes [42], retinal cells [43] and myofibroblasts [44]. The plasticity of BMSCs is still controversial since it is not clear whether the expression of tissue-specific markers is caused by transdifferentiation or cell fusion of other bone marrow cells [45].

The use of BMSCs for promoting the biologic potential of scaffolds in craniofacial tissue engineering, especially the hard tissue regeneration, has gained interest within last 10 years. Stem cell delivery may be a particularly effective treatment alternative for craniofacial bone defects with an impaired healing. However, there is a need for optimal carrier materials that enable the delivery and maintenance of stem cells at the defect site. Various scaffold materials have been used in combination with BMSCs, including ceramics [46], calcium phosphates [47], synthetic polymers [48], composites [49] and titanium meshes [50] in vitro. Besides, animal studies (including rat, dog, pig, sheep species) mostly provided the evidence that the application of BMSCs in bony defects increased osteogenesis compared to untreated defects without MSCs [6, 51–54]. Recently, it has been shown that anatomically shaped human bone grafts can be engineered using BMSCs in controlled perfusion bioreactor systems [55].

However, translational research, involving human subjects, is more important for the establishment of a human craniofacial cell therapy protocol. The first pioneering study came from Warnke et al. 2004 [56]. They showed the repair of an extended mandibular discontinuity defect by growth of a custom bone transplant with bone marrow precursor cells inside the latissimus dorsi muscle of an adult male patient. Instead of

culture expanded cells, freshly isolated cells were used in this study and the patient related outcome was satisfying. In further studies, researchers also tried autologous stem cell transplantation for the treatment of maxillofacial defects in human subjects (Table 6.1). For a detailed understanding of bone regeneration using autologous stem cells, there are recent reviews on craniofacial bone tissue engineering [57–59].

Craniofacial structures also contain cartilage tissues in various regions, such as ear, nose, and temporomandibular joint. Since one direction of differentiation for BMSCs is the chondrogenic lineage, various attempts, mostly using 3D culture systems, have been made to establish cartilage regeneration in vitro [60–62]. The differentiation potential of BMSCs towards chondrocytes depends on supplementation with growth factors, mainly transforming growth factor-β (TGF-β) and bone morphogenetic proteins (BMPs) [63]. The in vitro regeneration of cartilage using BMSCs have been shown by utilizing different scaffold systems, growth factors and gene therapy [9, 64, 65]. There are also several reports on human subjects about the transplantation of BMSCs for cartilage repair [66, 67]. Besides, the clinical outcomes of BMSC implantation versus autologous chondrocyte implantation have recently been evaluated in a cohort study of 72 patients [68].

In recent years, it has been reported that mandibular condyle can be also engineered using BMSCs due to their osteogenic and chondrogenic differentiation ability [16]. BMSCs isolated from adult rats were induced in osteogenic and then chondrogenic culture medium, separately. Differentiated cells were photoencapsulated in a poly (ethylene glycol) diacrylate (PEGDA) hydrogel in two separate layers resembling the natural form of human mandibular condyle and then transplanted into immunocompromised mice. Histological results showed that the two stratified separate osteogenic and chondrogenic layers maintained their phenotypes after transplantation [16, 69]. Especially, the intercellular matrix of the chondrogenic layer exhibited a strong staining with cartilage related markers, such as safranin O and transplanted cells displayed characteristics of native chondrocytes.

6.2.2 Adipose-Derived Stem Cells

In recent years, Adipose-Derived Stem Cells (ASCs) have become an alternative multipotent cell source for use in craniofacial tissue engineering [13]. ASCs share some similarities with BMSCs by means of immunophenotype, differentiation potential, and clonogenicity [70, 71]. In vitro differentiation of ASCs into osteogenic, chondrogenic, adipogenic, and myogenic lineages have been confirmed in various studies [72, 73]. Especially, the osteogenic potential of ASCs has been intensively studied through the combination of various grafting materials both in vitro and in vivo [73–76]. Also, animal [74, 75] and human [77] studies utilizing ASCs have demonstrated the bone regenerative potential of these cells in different conditions. In a recent clinical study, Thesleff et al. 2011 [77] have successfully repaired large calvarial defects with the combination of beta-tricalcium phosphate graft material and autologous culture expanded ASCs in four patients.

Table 6.1 Human studies of craniofacial bone regeneration using MSCs

[ref.]	Patient numbers	Procedure	Cell source	Serum	Scaffold	Osteogenic medium	Growth factor	Cell number (cells/ml)
[78]	6	Sinus lifting/onlay plasty	hBMSCs	Xenogenic	β-TCP	+	PRP	1×10^7
[79]	14	Sinus lifting/onlay plasty	hBMSCs	Xenogenic	Thrombin/calcium chloride	+	PRP	1×10^7
[80]	6	Sinus lifting	hBMSCs	Autologous	β-TCP/HA	–	–	n/a
[81]	2	Alveolar cleft	hBMSCs	Autologous	DBM	–	–	2.5×10^6
[82]	1	Postextraction	hBMSCs -BRCs	Xenogenic	Gelatin sponge	–	–	1.5×10^7
[83]	23	Sinus lifting (osteotome)	hBMSCs	Autologous	Thrombin/calcium chloride	+	PRP	n/a
[77]	4	Cranioplasty	hASCs	Autologous	β-TCP	–	–	1.5×10^7
[84]	3	Alveolar cleft	hBMSCs	Autologous	β-TCP/HA	–	PDGF+PRF	$2{,}5\times10^6$

BRC bone repair cells, *TCP* tricalcium phosphate, *HA* hydroxyapatite, *DBM* demineralized bone matrix, *PRP* platelet rich plasma, *PDGF* platelet-derived growth factor, *PRF* platelet rich fibrin, *n/a* not available

Another potential application of ASCs is the reconstruction of soft tissues for facial cosmetic purposes due to their adipogenic properties. Although the number of published articles on this area is very few, ASC enriched fat grafts hold promise for the repair of mastectomy defects [85] and facial defects due to abnormalities, such as the progressive hemifacial atrophy [86]. Recently, several animal studies have suggested that ASCs could also be used for the repair of the facial nerve [87, 88]. Decellularized allogenic artery conduits seeded with ASCs were used for the reconstruction of transected facial nerves of rats and these tissue engineered constructs provided beneficial effects on functional facial nerve regeneration, but the findings were inferior to the nerve autografts [87].

In vitro differentiation of stem cells towards different lineages is usually performed with the use of various supplementations and growth factors. It is well established that both these exogenous factors [88] and the tissue environment [89] play a crucial role in the differentiation potential and extracellular matrix production of these cells. Recent knowledge also suggests that MSCs, either cultured in conditioned media [90] or co-cultured with other cell types [91], improve their differentiation ability towards the desired lineage. Although this evidence favors the use of non-cranial-derived MSCs (BMSCs, ASCs, etc.) in craniofacial tissue engineering [92], important differences exist between the characteristics and therapeutic potential of MSCs from different sources. BMSCs from iliac bone and alveolar bone have been shown to have different characteristics in terms of cellular activities. For example, iliac BMSCs formed more compact bone in vivo and were more responsive to osteogenic and adipogenic differentiation in vitro and in vivo, whereas alveolar BMSCs proliferated faster, expressed increased levels of ALP and deposited more calcium in vitro [93].

These data provide the evidence that the origin of MSCs must be taken into account when planning a differentiation route of MSCs for treating craniofacial discrepancies. Since the neural crest cells are thought to contribute to the development of most craniofacial tissues and organs, a regeneration protocol that utilizes stem cells of cranial neural crest origin might be more beneficial to achieve this goal.

6.3 Adult Stem Cells of Dental Origin

6.3.1 Stem Cells from Mature Dental Tissues

Although quite limited, human dental pulp has the ability to repair itself when either caries or trauma does not involve the pulp cavity [94]. This means that ectomesenchymal progenitor cells remain in the pulp tissue after the eruption of human teeth and are also responsible for the formation of new dentin. Previous studies reported that these progenitors can be induced to differentiate into odontoblast-like cells and are capable of producing dentin-like mineralized nodules [95, 96]. Using a human wisdom teeth model, the characterization of these heterogeneous populations of dental pulp stem cells (DPSCs) was first performed by Gronthos et al. 2000 [27].

Table 6.2 Immuno phenotyping of adult stem cells from different sources

	BMSCs	ASCs	DPSCs	SHED	PDLSCs	SCAP	DFPCs	TGSCs
CD3	–	–	–	–	–	n/a	–	n/a
CD9	+	+	+	n/a	+	n/a	+	n/a
CD10	+	+	+	+	+	n/a	+	n/a
CD13	+	+	+	+	+	+	+	n/a
CD14	–	–		–	–	–	–	–
CD29	+	+	+	+	+	+	+	+
CD31	–	–	–	–	–	n/a	–	n/a
CD33	–	–	–	–	–	n/a	–	n/a
CD34	–	–	–	–	–	–	–	–
CD44	+	+	+	+	+	+	+	+
CD45	–	–	–	–	–	–	–	–
CD56	–	–		+	n/a	n/a	+	n/a
CD59	+	+	+	n/a	+	n/a	+	n/a
CD73	+	+	+	+	+	+	+	+
CD90	+	+	+	+	+	+	+	+
CD105	+	+	+	+	+	+	+	+
CD106	+/–	+/–	+	+	+	+/–	+/–	n/a
CD117	–	–	–	–	–	–	–	n/a
CD133	–	–	–	n/a	n/a	n/a	+	–
CD146	+	+	+	+	+	+	+	n/a
CD166	+	+	+	+	+	+	+	+
STRO-1	+	+	+	+	+	+	+	+
SSEA-4	+	+	+	+	+	+	+	+
HLA-DR	–	–	–	–	–	–	–	–
OCT4	+	+	+	+	+	+	+	+
NANOG	+	+	+	+	+	+	+	+
Nestin	+	+	+	+	+	+	+	+
Sox2	+/–	+	+	+	+	+	+	+
Rex-1	+	+	+	+	+	+	+	n/a
ALP	+	+	+	+	+	+	+	+

+/– contradictory results in the literature, *n/a* not available

DPSCs have some similar characteristics with BMSCs such as high proliferation rate, colony-forming ability, differentiation potential under normal culture conditions [37] and also express several important mesenchymal markers, such as CD44, CD90, and CD105 (Table 6.2) [28]. Besides their dentinogenic potential, DPSCs have been reported to differentiate into osteogenic, chondrogenic, adipogenic, and myogenic lineages [97–99]. Recently, CD117 positive DPSCs have been reported to differentiate into high-purity hepatocyte-like cells [100].

Additionally, ecto-mesenchymal stem cells can also be isolated from the pulp of resorbing milk teeth, termed as stem cells from exfoliated deciduous teeth (SHED) [29]. When compared with DPSCs and BMSCs (Table 6.2), SHEDs are highly proliferative with an increased population doubling (PD) rate [101]. These cells have been shown to express STRO-1 and Oct-4, two important cell surface markers of

multipotent stem cells (Table 6.2) [102]. As seen in DPSC cultures, SHEDs express osteo/odontogenic cell markers, including alkaline phosphatase (ALP), bone sialoprotein (BSP), Cbfa1, and dentin sialoprotein (DSP) [29, 103]. SHEDs also express several neural markers, such as β III-tubulin, neuronal nuclear antigen (NeuN), glutamic acid decarboxylase (GAD), nestin, neurofilament M (NFM), glial fibrillary acidic protein (GFAP) and 2,3-cyclic nucleotide-3-phosphodiesterase (CNPase) [29]. In a previous study, SHED-derived neural-like spheres were transplanted into the striatum of parkinsonian rats and an improvement in the behavioral impairment was achieved [104]. Also, it has been recently reported that tooth-derived stem cells, SHEDs [105] and DPSCs [106], could be a useful tool for functional recovery after spinal cord injury. Adipogenic, myogenic, and chondrogenic differentiation have also been reported from SHED [107].

One treatment strategy in the craniofacial region using dental pulp-derived stem cells (DPSC and SHED) might be the regeneration of tooth structures, including pulp and dentin. When transplanted into immunocompromised mice, DPSCs displayed an ability to form dentin pulp-like complexes [108]. However, transplanted SHEDs were capable of establishing dentin pulp-like tissue [29]. Additionally, it has been shown that SHEDs have a higher capacity of osteogenic and adipogenic differentiation compared to DPSCs [101, 109]. Two recent studies demonstrated the osteogenic potential of SHED in critical size bone defects in pig mandibular [110] and mouse calvaria [111] in vivo. Using DPSCs, endodontic perforations were successfully repaired with a tissue engineering approach, involving dentin matrix protein 1 (DMP1) signaling molecule and a collagen scaffold, in immunocompromised mice [112]. Especially, the transplantation of $CD31^{-}/CD146^{-}$ side populations of DPSCs into an amputated in vivo pulp model resulted in complete pulp regeneration with vascular and neuronal compartments [113].

The periodontal ligament (PDL) is an interfacial connective tissue between alveolar bone and cementum, and contains progenitor cell populations that are responsible for the maintenance of the tooth in the alveolar socket against mastication forces. These progenitor cells have long been known to differentiate into cementoblasts and osteoblasts [114]. A previous study reported that these periodontal-derived stem cells display characteristics (osteogenic, adipogenic, and chondrogenic) similar to mesenchymal and other dental stem cells (Table 6.2), and termed them as periodontal ligament stem cells (PDLSCs) [30]. Especially, the expression of chondrogenic genes, early osteoblastic and adipogenic markers were enhanced in $STRO\text{-}1^{+}/CD146^{+}$ immunoselected PDLSC cultures [115]. Besides their osteogenic potential, PDLSCs express important markers for tendo/ligamentogenesis, including scleraxis and tenomodulin [116]. Moreover, a periodontium-like structure, including cementum and PDL, can be regenerated following transplantation of PDLSCs into immunocompromised mice [30, 117]. Several animal studies [118, 119] reported that autologous PDLSCs transplanted into surgically created periodontal defects were able to regenerate periodontal tissues and differentiate into functional osteoblasts and fibroblasts, thereby providing a treatment alternative for periodontitis.

Another treatment strategy using PDLSCs is the formation of a periodontal-like tissue around dental implants, in order to challenge the concept of osseointegration with biointegration. An organized periodontal tissue was found around titanium

implants seeded with PDLSCs and placed into maxillary molar sites of rats [120]. A similar approach involving human subject also revealed that new tissue with PDL characteristics, such as lamina dura and motility similar to teeth, was established at the bone implant interface [121]. Recently, it has been shown that heterogenous cultures of PDLSCs contain stem cells of neural crest origin, thus making them a useful tool in neuroregenerative and/or neurotrophic medicine [122].

6.3.2 Stem Cells from Immature Dental Tissues

During tooth development, ectomesenchyme-derived dental papilla cells are known to be responsible for root formation. While the root is being formed, dental papilla is entrapped by dentin that is produced by odontoblasts of dental lamina origin [123]. So, the dental pulp takes its final form and dental papilla protrudes more apically forming a cell rich zone at the apex. Previous studies have indicated that stem cells are also present in this apical part of dental papilla of the developing permanent teeth [31]. Therefore, these stem cells derived from the apical papilla (SCAP) can only be isolated from the apex of immature teeth at a certain development stage [124].

SCAP expresses several mesenchymal markers and lack hematopoietic markers similar to DPSCs and SHED (Table 6.2) [125]. Interestingly, SCAP expresses CD24 that is normally not present in DSPC and SHED cultures [28, 126]. Besides, when stimulated, these cells can undergo osteogenic and odontogenic differentiation in vitro [125]. Although the expression levels of osteo/dentinogenic markers in SCAP are lower than in DSPCs, SCAP have been reported to exhibit an increased proliferation rate, higher PD, better tissue regeneration capability, higher telomerase activity, and migration capacity in a scratch assay [127]. Additionally, ex vivo expanded SCAP was also found to differentiate into adipogenic and neurogenic lineages, as seen in DPSC and SHED [31]. A recent data suggested that canonical Wnt/β-catenin signaling favored the proliferation and odonto/osteogenic differentiation of SCAP [128]. Additionally, it has been reported that both SCAP and PDLSC could be used together in the regeneration of a root/periodontal complex capable of supporting a porcelain crown [127].

Dental follicle is a loose connective tissue and it surrounds the developing tooth (including enamel organ and dental papilla) before eruption. It is believed that DF is responsible for the establishment of periodontium, cementum, and alveolar bone until the tooth takes its final place [129]. This ectomesenchyme-derived sac-like tissue can be easily isolated during the extraction of impacted teeth. Recent evidence suggested that progenitor cells in the dental follicle (DFPCs) are plastic adherent and form clonogenic colonies similar to other dental stem cells when cultured in vitro [32]. DFPCs display fibroblastic morphology and express putative stem cell markers Notch-1 and Nestin [130]. Under specific culture conditions, DFPCs differentiated into osteogenic, neurogenic, and adipogenic lineages [32, 131]. When stimulated with enamel matrix derivatives (EMD) or BMP-2/-7, DFPCs expressed cementoblast markers, such as cementum attachment protein (CAP) and cementum protein 23 (CP-23) [132].

When supplemented with dexamethasone and/or insulin, human DFPSCs have been found to produce mineralized nodules in vitro. During osteogenic differentiation, the expression of some related genes (Osx, DLX-5, runx2, and MSX-2) remained unaffected [133]. However, the upregulation of DLX-3 as a response to osteogenic induction was found to influence the cell viability and osteogenic differentiation in DFPSC cultures [134]. Besides, bovine-derived DFPCs formed fibrous tissue surrounded by a mesothelium-like structure, but not cementum or bone, when transplanted into immunodeficient mice [32]. DFPCs are also capable of differentiating towards neurogenic lineage. After cultivation in serum replacement medium, containing culture supplement for glial cells, neurosphere-like cell clusters were established from DFPCs, and these cells were further differentiated into neuron-like cells by subculturing them on laminin and poly-L-ornithine substrates [135]. On the other hand, TGF-β was demonstrated to improve glial-like differentiation of DFPCs, but not neural like [136]. Recently, DFPC cell sheets were shown to have a better regeneration potential for periodontal tissues than PDLSC sheets, when subcutaneously transplanted into nude mice [137].

6.3.3 Tooth Germ Stem Cells

Until now, most studies cultured stem cells derived from immature tooth tissues in two portions by dissecting the dental follicle and apical papilla, separately. So, either DFPC or SCAP cultures were established. However, adult stem cells, that are responsible for tooth development, are derived from both ectoderm and the underlying mesenchyme. Therefore, reciprocal signaling pathways between these cell groups should be considered in designing a culture system from third molars [138]. The hypothesis of our studies was that the whole tooth germ should be used for preserving the stemness of the culture when isolating stem cells from immature third molars. Besides, the perfect dissection of the tooth germ tissue into dental follicle and apical papilla portions is impossible at the stage of early crown formation (unpublished data), thereby leaving some remnants from the adjacent tissue. Thus, in our cultures we have decided to isolate stem cells from the whole developing tooth organ, as done in the literature [139], and termed them as tooth germ-derived stem cells (TGSCs) (Fig. 6.1).

Human tooth germ tissues are derived from third molars and they are quite unique since embryonic tissues remain quiescent and undifferentiated until around age 6. Thus, human TGSCs are considered to be an ectomesenchymal source for isolating primitive pluripotent stem cells that could differentiate into multiple lineages. In our previous studies, we were able to isolate and characterize MSCs from human dental follicle (DFPCs) [140] and human tooth germ (hTGSCs) [33]. In the later study, we showed the differentiation of hTGSCs into osteogenic, adipogenic, and neurogenic cells, as well as tube-like structures in Matrigel assay [33]. Significant levels of sox2 and c-myc messenger RNA (mRNA) and a very high level of klf4 mRNA expressions were observed when compared with human embryonic stem cells. Recently, another group reported that stem cells derived from third molars of young donors

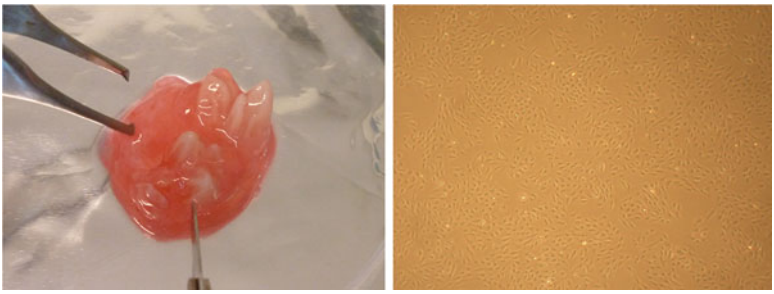

Fig. 6.1 Dissection of tooth germ tissue and morphology of TGSCs derived from pig (10× obj)

(10, 13, and 16 years old) could be reprogrammed to a pluripotent state (induced pluripotent stem (IPS) cells) by using retroviral vectors containing oct3/4, sox2, and Klf4 [141]. Expression of developmentally important transcription factors could render hTGSCs an attractive candidate for autologous transplantation since they can differentiate into various tissue types, such as osteoblasts, neurons, and vascular structures [33].

Interestingly, primary cultures of TGSCs readily express early neural stem cell markers, including nucleostemin, nestin, vimentin, and β-III tubulin [33]. Furthermore, the cryopreservation did not lead to a major change in the undifferentiated state of TGSCs [142]. According to the expression of neurogenic markers (β-III tubulin, nestin, and neuronal intermediate filament NFL), TGSCs also protect their neurogenic potential following long term cryopreservation [142], thereby making them a potential source for the treatment of neurodegenerative disorders. In a similar study [139], the neurogenic and hepatogenic characteristics of human tooth germ precursor cells (TGPCs) were evaluated. Especially, the transplantation of undifferentiated TGPCs into immunocompromised rats with experimentally established liver fibrosis led to improvement of liver function [139].

Although the number of published articles about TGSCs is extremely low, current findings provide important clues about the primitive characteristics of these cells. Thus, further studies, including transplantation protocols, needed to evaluate their regenerative potential in the craniofacial tissue engineering.

6.4 Conclusion

Stem cell sources have extensively been used for the treatment of craniofacial tissue defects since they have the capacity to originate a wide range of tissues. Generally, MSCs are preferred for such tissue regenerations. However, dental stem cells have also a self renewal and multilineage differentiation capacity. Besides, they are originated from cranial neural crest. Therefore, they have great potential to get used in craniofacial tissue engineering applications.

Acknowledgements Authors gratefully acknowledge the various grants from ITI (International Team for Implantology) Research Grant (750-2011), TUBITAK Joint Research and Development Project (111 M031) and TUBİTAK 1002 (110 M404).

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Chapter 7
Pediatric Diseases and Stem Cells: Recent Advances and Challenges

Duygu Uçkan

Abstract Stem cell treatments hold promise in the pediatric field. Children have increased regenerative potential when compared with adults. In addition, the number of stem cells needed for therapeutic efficacy is much lower in children based on their lower body weights. Therefore, sufficient number of cells may readily be collected from donors and/or may be expanded ex vivo in lesser number of passages. All of these factors are expected to enable more efficient, less expensive, and timely application of stem cells in clinical practice. In this review, we will cover the areas potentially suitable for stem cell therapies in children including inborn errors of metabolism, transplantation, and autoimmune/inflammatory conditions. Hematopoetic and mesenchymal stromal/stem cells will be emphasized as the most available stem cell sources for clinical application at present. In addition, the invaluable role of doing research with the use of induced pluripotent stem cell lines obtained from the cells/tissues of inherited rare diseases is highlighted and future application areas are included.

Keywords Cell therapy • Hematopoietic stem cell • Immunomodulation • Immunosuppressive • Inborn error • Induced pluripotent stem cells • Inherited disease • Mesenchymal stem cell • Mesenchymal stromal cell • Pediatrics • Stem cells • Transplantation

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K. Turksen (ed.), *Stem Cells: Current Challenges and New Directions*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4614-8066-2_7,

Abbreviations

ALD	Adrenoleukodystrophy
BMT	Bone marrow transplantation
BPD	Bronchopulmonary dysplasia
cGMP	Current good manufacturing practices
CD	Crohn's disease
EBMT	European Group for blood and marrow transplantation
ESC	Embryonic stem cell
GLD	Globoid cell leukodystrophy
GCSF	Granulocyte colony stimulating factor
GVHD	Graft versus host disease
HSCs	Hematopoetic stem cells
HSCT	Hematopoetic stem cell transplantation
HIV	Human immunodeficiency virus
i.v.	Intravenous
iPSC	Induced pluripotent stem cell
MIOP	Malignant infantile osteopetrosis
MLD	Metachromatic leukodystrophy
MPS	Mucopolysaccharidosis
MSC	Mesenchymal stem cell/mesenchymal stromal cell
NEC	Necrotizing enterocolitis
OI	Osteogenesis imperfecta
RDEB	Recessive dystrophic epidermolysis bullosa
SDF-1 alpha	Stromal-derived factor 1 alpha
UCB	Umbilical cord blood
UCBT	Umbilical cord blood transplantation

7.1 Hematopoetic Stem Cells in Pediatric Diseases

7.1.1 Hematopoetic Stem Cell Transplantation (HSCT) in Inborn Errors of Metabolism

In many of the childhood inborn errors of metabolism the disease course is associated with progressive and serious defects and there is no treatment alternative other than hematopoietic stem cell transplantation (HSCT) from HLA-matched donors. The need to use intensive conditioning regimens in HSCT is still an unresolved problem and is the main cause of increased morbidity/mortality associated with this treatment. Critical timing, detailed pre-transplant preparation of the patient and a multidisciplinary approach are important issues to achieve maximum benefit from the procedure. In most cases, appropriate timing of HSCT is considered as transplanting soon after the appearance of the first sign and symptoms of the disease.

The results of HSCT are promising in a number of such diseases and in selected cases [1–10]. Recently, umbilical cord blood transplantation (UCBT) from unrelated donors has become an attractive practice in patients with lysosomal storage diseases (i.e., Hurler's disease). The promising results with this approach have been attributed to availability of timely transplants by increased capacity of Cord Blood Banks and achievement of better enzyme levels through secretion of the missing enzyme by the cord blood cells [5, 8, 11].

Among inborn errors, lysosomal storage diseases and peroxisomal disorders are the leading ones that have been shown to benefit from HSCT Achievement of higher enzyme levels is important for effectiveness; therefore, the use of noncarrier donors is preferred to obtain optimal results in transplants from family members. The results of HSCT from HLA-matched family or unrelated donors are quite good in patients with Hurler disease or mucopolysaccharidosis type 1 (MPS-IH) when transplanted early in the course of the disease. These patients show acceleration in growth and development, and respiratory, cardiac, neurocognitive improvement or stabilization is achieved. Accumulation of glycoaminoglycans, GM2, GM3 gangliosides in the brain can be prevented by replacement of the missing enzyme [7, 12–14]. Long term survival rate of 85 % (despite 56 % sustained engraftment rates) has been reported in a multicenter study involving 146 patients with this disease [12].

HSCT data has gathered in other members of lysosomal diseases, i.e., metachromatic leukodystrophy (MLD) and globoid cell leukodystrophy (GLD) as well. In MLD, the results obtained are not as favorable as those with Hurler's disease whereas patients with GLD has responded well to HSCT in timely transplants and in selected cases, although the number of treated patients is limited [6]. Among peroxisomal diseases X-linked cerebral adrenoleukodystrophy (ALD) is the main indication that favorable results may be obtained by early transplant. In this disease, accumulation of long chain fatty acids renders the cellular membrane vulnerable to second hit by trauma, inflammation, or infection. HSCT from an HLA-identical donor at an early phase of the disease is a standard therapeutic option. As opposed to lysosomal diseases, the mechanism of action is not through cross correction of the missing enzyme but in fact by elimination of cellular infilltrate and inflammation [1, 2, 6]. The decision of transplant is made according to the degree of demyelination. The Loes neuroimaging score indicates the extent of demyelinated areas in parietooccipital regions of cerebral hemispheres in cerebral ALD and has been shown to be directly associated with posttransplant outcome. Five year survival rates of 92 % has been reported in patients with lower scores [15, 16].

The need for HSCT in rapidly progressive neurometabolic diseases including Tay Sachs, Sandhoff disease, Niemann Pick A, gangliosidosis, neuropathogenic Hunter and Gaucher diseases—with neurological involvement—is controversial. It has been suggested that patients transplanted in centers with experience may show stabilization of signs and symptoms of their disease following HSCT. Rare diseases such as Maroteaux-Lamy syndrome (mucopolysaccharidosis, MPS VI), Sly syndrome (MPS VII), Gaucher's disease, Fucosidosis, alpha-mannosidosis, aspartil glycoaminuria, mucolipidosis II (I-cell disease),Wolman disease are considered to be among diseases in which HSCT may be effective [1–3, 16]. In a recent

large scale review study comparative benefits and harms of HSCT versus standard therapies or disease natural history in children with inherited metabolic diseases, malignant solid tumors, or autoimmune diseases have been evaluated [17]. Among metabolic diseases, compared to the natural history of the disease, benefit in overall survival or neurological symptoms has been found, respectively, in Wolman's disease and in Farber's disease Type 2/3 (high strength). On the other hand, no benefit of single HSCT for overall survival compared to symptom management has been reported in Niemann-Pick Type A (low strength). Additionally, no benefit has been detected in GaucherType III disease in neurodevelopmental symptoms compared to enzyme replacement therapy (low strength), in juvenile form of GM1 and juvenile Tay-Sachs (both low strength) compared to symptom management or disease natural history, in Sanfilippo disease compared to symptom management, substrate reduction therapy, or disease natural history (low strength), and in Hunter's disease compared to symptom management or disease natural history (low strength). Based on the results obtained in that comparative review study, the authors state that "evidence demonstrating benefit or harm of HSCT versus standard therapies or disease natural history has been found insufficient for most pediatric indications including inborn errors of metabolism" [17].

In the recent years promising new areas for the use of hematopoetic stem cells (HSCs) in pediatric practice have been defined. Emerging uses for pediatric HSCs have been reported as the use of; HSCT for treatment of autoimmune disorders to induce tolerance in solid-organ transplant recipients, umbilical cord blood (UCB) in patients with inherited metabolic disorders, i.e., Hurler syndrome and the use of HSCs for delivery of gene therapy and selected stem cells in human immunodeficiency virus (HIV) infection [18].

Although HSCT is considered as a standard therapy for many pediatric indications, the beneficial effect of this treatment method is counterbalanced by the toxic effect of the conditioning regimens contributing to unacceptable morbidity/mortality and preventing its common use. Development of more effective and safer therapeutic strategies is necessary to achieve cure in inborn errors of metabolism in childhood. Cellular and/or gene therapy approaches may present an alternative in this area.

7.1.2 Regenerative Potential of HSCs

At present, the clinical application of stem cells as a means of standard therapy involves the field of HSCT, and stem cell use for regenerative purposes is applicable only under experimental protocols. HSCT practice has increased considerably since the initial successful bone marrow transplant (BMT) performed in the 1960s till the current worldwide activity of over 30,000 HSCTs annually [19–23]. The regenerative potential of donor-derived HSCs was first noted in the 1980s in patients who received BMT for inherited metabolic diseases in whom the favorable effects were

attributed to secretion of the missing enzymes by donor cells and to contribution of genetically healthy cells of donor monocyte-macrophage origin to the repair process [1–9]. The last decade has highlighted the potential regenerative role of HSCs in nonhematological diseases (including cardiovascular, neurological) and in nontransplant setting [24–31]. The increasing interest in the stem cell field in regenerative medicine has exploded since then.

The use of HSCs for organ repair has rapidly expanded after the observations made in experimental models suggesting the contribution of donor-derived HSCs in the repair process of damaged organs following HSCT. Several investigators have demonstrated migration of donor-derived HSCs to the sites of organ damage in recipients of HSCT [29, 30, 32, 33]. Donor origin of cells were identified in the recipient tissues by demonstration of donor sex in gender-mismatched transplants or with specific markers [34–37]. The modified microenvironment at the site of injury has been shown to play an important role in attracting the stem cells to the injured tissue mainly through the stromal-derived factor 1 alpha (SDF-1alpha)/CXCR4 axis and contribution into the healing process [38–40]. It is known that HSCT patients suffer from multisytem damage due to the effects of the toxic conditioning regimens and the donor-derived immunological reactions. The findings in the biopsies of the involved organs with acute graft versus host disease (GVHD) have indicated the contribution of donor bone marrow-derived cells to endothelial and epithelial cell renewal in HSCT recipients [35, 41]. In another study the investigation of the autopsied tissues of a patient with Hunter disease after UCBT has revealed the presence of donor-derived weak bands both in the liver and in the cerebrum. The immunoreactivity in the brain showed positivity for CD68-positive microglia/monocytes predominantly in perivascular spaces and some in the brain parenchyma [42]. Although HSCT is not a standard indication in Hunter disease [6] the detection of donor-derived cells in the brain parenchyma after UCBT has suggested the potential of HSCs for treatment of neurological symptoms in this neurodegenerative disease as well. Similar incidental findings have strengthened the perspective about the regenerative potential of stem cells and paved the way of cellular therapies.

7.1.3 Cellular Therapy Experience with HSCs and Other Cell Types (in Adults and Children)

In the last decade, increasing number of reports demonstrating donor-derived cells in injured organs of recipients have contributed to acceleration of the translational research studies involving the use of stem cells for regenerative purposes and initiation of cellular therapy practices in several disciplines of medicine. Since 2008 the European group for Blood and Marrow Transplantation (EBMT) and a joint committee of scientific organizations have added data collection on “novel cellular therapies” in the regular annual activity report of HSCT [23]. For the year 2010,

69 teams from 21 countries (from Europe and other) provided data on 1010 patients in whom the indications for cellular therapy included; graft versus host disease (GVHD), musculoskeletal disorders, cardiovascular disorders, epithelial disorders, autoimmune diseases, and neurological disorders. The reported use of autologous cells were mainly for musculoskeletal (39 %) and cardiovascular (32 %) disorders whereas allogeneic cells were used for GVHD (58 %) and epithelial disorders (23 %). The cell type used for regenerative purposes was mesenchymal stem/stromal cells (MSCs) in 49 %, HSCs in 28 % of grafts whereas more mature cells including chondrocytes (10 %), dermal fibroblasts (4 %) and keratinocytes (1 %) were also used for some occasions. The analyses revealed extensive manipulation of the grafts including ex vivo expansion of cells in 63 % and transduction in 10 %. The cell delivery route was intraorgan (45 %), intravenous (31 %), on a membrane or gel (20 %) or by the use of 3D scaffolds (4 %). The report has shown that the number of yearly cell therapy applications has increased significantly from the previous report of 2008 involving data from 656 patients [27, 31].

Beneficial effects of cell therapies in different disease/injury states have been suggested by several investigators [25, 27, 43, 44]. On the other hand, there are increasing reports focusing on adverse events with these novel therapies [45, 46]. Great majority of these applications involve adult patients. The application of novel cellular therapies in pediatric practice has been based primarily on the immunomodulatory/anti-inflammatory properties of MSCs in GVHD and in the HSCT setting [27, 31, 47, 48]. However, individual applications for regenerative purposes are lately being reported and the potential of those therapies in childhood diseases is being emphasized [49–56].

The source of stem cells for regenerative purposes may be of autologous or allogeneic origin. The use of HSCs for organ repair necessitates the use of autologous sources. HSCs provoke immune response in the host, thus HLA matching and pre-transplant conditioning of the patient with toxic preparative regimens is necessary for allogeneic use of HSCs as done in the HSCT setting [10]. Therefore, HSCs are generally not suitable cells for regenerative applications in the allogeneic setting. Instead, autologous use of these cells (obtained in sufficient numbers from the bone marrow or mobilized peripheral blood) has aroused a lot of interest among clinicians since it is a feasible approach without the need for ex vivo manipulation and free from the immunological reactions due to allogeneic cells [27, 31, 43].

On the other hand, some types of stem cells including embryonic stem cells (ESCs) and UCB cells are applicable only under allogeneic conditions except for the extremely rare occasions where autologous cryopreserved cord blood stem cells are available for that particular patient. The use of ESCs is highly restricted due to ethical concerns and the high risk of oncogenic potential [57, 58] as opposed to MSCs which are considered among the most suitable cells for cellular therapy. Mesenchymal stem/stromal cells can be isolated either from autologous tissues (bone marrow, adipose tissue, and many other tissues), or allogeneic sources including placenta/cord tissues or from designated or HLA-fully mismatched third party donors [47–49, 59–66].

7.2 Mesenchymal Stem/Stromal Cells (MSCs)

Mesenchymal stem/stromal cells have emerged as the main type of stem cells for use in regenerative medicine. These cells are of stromal origin, differentiate into all cells of connective tissue and can escape from immune rejection; therefore they are candidate cells to be used in allogeneic setting without the need for HLA-matching as opposed to HSCs. It has been shown that MSCs (particularly those from fetal/placental origin) have immunomodulatory effects and low immunogenicity. They inhibit T-cell alloreactivity in vitro and secrete soluble factors to induce immune suppression [47, 48, 56, 59–66]. Mesenchymal stem/stromal cells can be obtained from several tissues including bone marrow, adipose tissues, dental tissues, placental and cord tissues, skin, visceral organs, and body fluids. These cells necessitate in vitro expansion in culture in order to reach sufficient numbers for therapeutic applications or even for research purposes [67–77].

Mesenchymal stem/stromal cells contribute to the regeneration process: (1) by migration towards damaged/injured tissues in response to signals (chemokines, other soluble factors) released from the changed microenvironment at the site of injury, (2) by providing cell to cell contact and receptor-ligand interactions in the injured tissues to initiate regenerative processes, (3) by secretion of growth factors, enzymes, chemokines, angiogenic factors to help recovery of the injured tissue cells, (4) by secretion of anti-inflammatory, immune-suppressive, immune-modulating factors to prevent excess inflammation during healing process, (5) by differentiation into cells of connective tissue origin, (6) by rarely transdifferentiation into cells of injured tissue type, (7) by fusion with injured cells (in rare occasions) and inducing their functionality. In addition, their migratory capacity and resistant/stromal phenotype renders MSCs as promising vehicles for gene therapy [48, 56, 63, 67–82].

Based on the above characteristics, MSCs appear as candidate cells to be used in regenerative medicine, HSCT, organ transplantation, autoimmunity, inherited diseases, and even in cancer [49, 55, 56, 59, 69, 83–90]. Their migratory capacity and resistant character provide advantage in gene therapy by directing the gene-transferred cells towards cancerous tissues to target selective cancer cell kill. The results of experimental studies in animals have shown promising results in cancer models with the use of gene-modified MSCs [91–94].

7.2.1 Immunomodulatory and Hematopoetic Supportive Roles of MSCs for Clinical Translation

In the field of HSCT, acute GVHD is the main condition in which most clinical experience with MSCs is being accumulated in Europe and USA in clinical studies. It is believed that MSCs' immunosuppressive and anti-inflammatory properties contribute to achievement of a favorable clinical response in this inflammatory condition.

The results from a multicenter, phase II experimental study from Europe reporting data from 55 patients with steroid refractory acute GVHD have demonstrated that MSC infusions (1–5 infusions/patient) did not show side-effects during or immediately after infusions and suggested this treatment as an effective therapy for patients with steroid-resistant, acute GVHD regardless of the donor type [48]. Mesenchymal stem/stromal cells are poor Ag-presenting cells, do not express MHC class II or co-stimulatory molecules, can induce tolerant phenotype and induce immunosuppression through secretion of soluble factors. All of these factors are suggested to have a role in achievement of clinical response in this inflammatory condition. The initial promising results then led to initiation of further clinical trials in GVHD and other HSCT coomplications with variable results [48, 56, 59, 86, 95–98]. A recent clinical study has summarized the first experience of using a premanufactured universal donor formulation of human MSCs (Prochymal) in 12 children for therapy resistant (steroid + other immunosuppressives) grade III-IV acute GVHD involving gastrointestinal system (in all), skin, or liver . The MSCs were prepared from healthy donors with any HLA type. The intravenous (i.v.) infusion schedule was twice weekly for 4 weeks and the administered cell numbers ranged between 2 and 8×10^6 cells/kg/dose. No acute toxicities were reported and the administration was found safe. The clinical response was complete in 58 % of patients and the gastrointestinal symptoms resolved in 75 % of children. The authors conclude that MSC administration in children is safe with no acute toxicity and no ectopic tissue formation, and holds potential for treatment of acute GVHD even in a patient population with grave prognosis [59].

Mesenchymal stem/stromal cells have also been used for prevention of graft rejection in HSCT and/or for treatment of refractory cytopenias by providing microenvironmental support for donor hematopoesis [84, 98, 99]. It has been shown that bone marrow-derived MSCs remain host-derived despite successful hematopoietic engraftment after allogeneic HSCT including patients with lysosomal and peroxisomal storage diseases [99]. Co-administration of allogeneic MSCs with HSCs is believed to assist in hematopoietic engraftment particularly in mismatched situations. Another observation with MSCs in the HSCT setting has been the reversal of tissue toxicity (including hemorrhagic cystitis, colon perforation with peritonitis, and pneumomediastinum) and successful treatment of extensive hemorrhages with the use of MSCs. The authors point out to a less addressed issue with the use of MSCs in stimulation of clotting and vasoconstriction as contributary factors in wound healing [56].

In spite of the promising preliminary observations there is debate questioning the efficacy of MSCs when used as a single cellular therapy agent in regenerative applications. Still, in HSCT practice the beneficial effects of MSCs as a sole cellular therapeutic tool (in repeated doses) is generally acknowledged particularly in the management of acute GVHD. This preliminary experience prompted initiation of clinical studies in another inflammatory condition, Crohn's disease (CD).

Previous experience with autologous HSCT in CD has suggested that the results are encouraging but the toxicity associated with this therapy is a limiting factor. Mesenchymal stem/stromal cell therapy, being a less aggressive approach, appears

to be more acceptable among clinicians and has shown some clinical benefit in treatment refractory CD whether used systemically or by local route into the fistulas [89, 100]. However, the results from large scale studies are not available to make conclusion about the effectivity of MSCs in this condition.

Another promising field for use of MSCs has been considered as autoimmunity states. In an experimental study, umbilical cord derived MSCs have been found to be effective in lupus nephritis. A Th1 to Th2 shift, inhibition of lymphocyte proliferation, and suppression of pro-inflammatory cytokines were all found to be contributary factors for achievement of favorable response [90]. Although the initial observations with the use of MSCs in autoimmune and inflammatory conditions seems to be encouraging, the experience in this field is still limited and the final immunomodulatory response is greatly dependent on the microenvironmental condition of the patient and/or the injured tissue(s). Some researchers have reported that MSCs may inversely induce an immune reaction under defined conditions such as in the nonmyeloablative setting or at lower doses. Moreover, the source, the number of cells infused and the cell preparation steps may all contribute to the nature and the degree of immunomodulatory effect [63, 101]. Therefore delibarate use of MSCs may carry risks and every single patient needs to be evaluated for that special occasion before going further with treatment.

7.2.2 Basis of MSC Use in Pediatric Diseases

The clinical use of MSCs has aroused interest in the pediatric field and pioneering clinical applications of MSCs have been reported in inherited diseases of childhood [84, 85, 87]. These cells are attractive for pediatricians due to their regenerative potential, the ease of administration by i.v. infusion or directly by local implantation to the site of injury if suitable, availability of cells for both autologous and allogeneic use (including from HLA-mismatched donors) and, at least short-term safety profile. Their migratory properties towards the site of injury confers advantage for intravenous use [39, 40, 78]. The main difficulty in clinical use of MSCs is the need for ex vivo expansion of cells which requires extensive manipulation under "Current Good Manufacturing Practice (cGMP)" conditions [102, 103]. In spite of the conflicting reports, it is generally agreed that cells obtained especially from advanced passages in culture may carry the risk of cytogenetic transformation and oncogenic potential [104–106]. Therefore, long-term safety of MSC therapies remains unproven.

Clinical application of MSCs in children has advantages both technically and for achievement of a better biological response. The smaller size of children when compared to adults renders availability of higher number of cells for therapy. Based on the preliminary clinical experience with MSCs, the effective cell dose for clinical use has been suggested as $1–2 \times 10^6$ cells/kg of the patient [47, 48, 59, 85–88]. The cell numbers needed for efficiency may easily be reached even in short-term cultures when expanding cells for infants and small children. The availability of higher

number of cells and in a shorter time period may indicate increased effectivity. Additionally, it has been shown that younger individuals display an increased regenerative potential which may also contribute to improved effectiveness of cell-based therapies [107]. Thus, the efficacy of MSC application in children is expected to be higher when compared with adults.

In general, MSC therapies appear promising but the preliminary clinical experience has shown limited effectiveness. The optimal cell dose, site of application, stem cell source, schedule of administration are not yet determined and patient-specific, condition-specific individualized treatment protocols are not available. Repeated infusions of MSCs are often needed to achieve more efficient and sustained responses [48, 59]. Therefore many weeks of cell culture and multiple passaging may be required particularly for treatment of heavy patients to reach sufficient cell numbers/kg of the patient. Such an extensive in vitro manipulation increases the risk of cytogenetic and oncogenic evolution or cell senescence [105, 106]. In small children and infants the required cell dose is much less rendering early passage cells available for therapy. The in vitro cell manipulation steps are reduced which may suggest a hopefully safer product in terms of oncogenicity.

7.2.3 MSCs in Inherited Diseases of Childhood

Clinical experience in children with cell based and gene therapy with MSCs has been expanding since the initial use of these cells in the early 2000s in inherited diseases and in HSCT setting. Osteogenesis imperfecta (OI) is characterized by a defect in type I collagen production and the patients suffer from severe growth failure, multiple fractures, and progressive bony deformities. The initial clinical experience with HSCT in OI has been reported by Horwitz et al. [84] who had previously shown somewhat beneficial role of BMT in patients with severe OI carrying COLIA1 or COLIA2 mutations and bone formation and mineralization was observed after the procedure. This preliminary study showed the migration of donor MSCs (which were in the bone marrow inoculum) to defective bones and suggested a contributary role in the regenerative process [84]. The researchers then used MSC therapy in type III OI patients ($n=5$) with severe defect and reported accelerated growth and walking without support in all patients at 6 months evaluation. However, at longer term follow-up, the beneficial effects of MSC therapy was somewhat lost and the authors brought forward the issue of repeated infusions [84, 85]. A recent experimental study in mice with OI has supported the human data to show the therapeutic potential of cellular therapies in this disease. By the use of three dimentional microcomputerized tomography and histopathologic analyses, the researchers have shown significant improvements in bone structure of mice after HSCT revealing an increase in trabecular number, widening and bone volume, decrease in trabecular space and bone fractures, prevention of kyphosis, and achievement of weight gain [109]. These findings suggested a therapeutic role for cell therapy (MSCs and possibly HSCs) in regeneration of the skeletal disorders.

Childhood diseases that are candidates for cell and gene therapies are heterogenous including inherited diseases with systemic and severe defects and a progressive course. Some of these disorders (i.e., Hurler disease, adrenoleukodystrophy) have been shown to benefit from HSCT whereas there are many in which this treatment strategy is ineffective. MSC therapy may be used in co-transplant setting with HSCT to improve organ dysfunction and to increase engraftment and to suppress GVHD [48, 59, 87, 88, 98]. Alternatively, MSCs may be used to provide missing enzyme or other factors or in gene therapy context in lysosomal diseases. Experimental and preliminary studies have demonstrated functional recovery by MSCs transduced by retroviral/lentiviral vectors to secrete enzymes which are deficient in lysosomal storage diseases. Further studies have also been focusing on MSC gene therapy strategies in hematological diseases to provide missing factors, i.e., in hemophilia, or by lentiviral mediated genetic correction of HSC and MSCs in Fankoni aplastic anemia. However, these studies are either not at the clinical phase or have just started [79, 110–116].

As in adult studies, the majority of the reported clinical experience in children with the use of MSCs has involved the HSCT setting where the anti-inflammatory/immunomodulatory properties of MSCs are believed to play a role in management of GVHD (as stated above). Thus, the clinical use of MSCs has initially been limited to life-threatening, severe complications of HSCT or progressive, severe systemic diseases like inborn errors. However, the achievement of promising responses (although not optimal) and at least short term safety has led to expansion of the application areas to include non-HSCT setting and regenerative medicine.

7.2.4 MSCs in Pediatric Regenerative Medicine

In this section we will higlight potential areas in pediatrics to demonstate the utility of MSCs in the regeneration process of some tissues.

7.2.4.1 Implications in the Neonatal Period

Bronchopulmonary Dysplasia (BPD)

MSC therapy shows promise in management of pulmonary diseases including chronic obstructive lung disease, pulmonary hypertension [117]. In pediatric practice, neonatology is a potential field where several disorders may be candidates for cell and gene therapies. A beneficial effect of systemic treatment with bone marrow-derived MSCs and MSC-conditioned media has been shown to contribute to ameliorate lung parenchymal and vascular injury in vivo in the hyperoxia murine models of bronchopulmonary dysplasia (BPD) [50, 118, 119]. Several mechanisms have been involved including activation of endogenous lung epithelial stem cells. Bronchioalveolar stem cells are capable of self-renewal and differentiation in

culture, and proliferate in response to bronchiolar and alveolar lung injury in vivo. Exposure of these cells to MSC-condioned medium in culture has stimulated their growth efficiency, indicating that MSCs and MSC-derived factors may induce bronchoalveolar stem cells for repair of alveolar lung injury found in BPD [120]. In another study preconditioning of marrow MSCs with 95 % oxygen in culture and then use in in vivo studies showed production of higher levels of naturally occurring antioxidant stanniocalcin-1 in MSCs [119]. All of these studies suggest a favorable role in management of newborn infants suffering from BPD.

Congenital Malformations of the Respiratory System

Among respiratory pathologies in pediatrics, laryngotracheal agenesis is a disease of infancy that is considered as a candidate disorder for stem cell and tissue engineering approaches [51]. The favorable experience with clinical transplantation of a tissue-engineered airway in an adult patient with end stage bronchomalacia has led to consideration of this engineering approach in similar pathologies. The team obtained donor cadaver trachea, treated chemically to obtain cell-free scaffold on which patient's own bone marrow MSCs were seeded. The donor trachea was readily colonized by epithelial cells and MSC-derived chondrocytes and a tissue-engineered airway with mechanical properties that allow normal functioning was obtained [121]. This approach of combining cell therapy with decellularized scaffolds appears to be promising especially in the field of fetal tissue engineering and pediatric surgery for fetus/infants with laryngotracheal agenesis. In fetuses with airway obstruction the use of human amniotic stem cells together with decellularized scaffolds is considered as a promising approach. The implications of a similar tissue engineering approach in other congenital malformations is also discussed [51, 55].

Cerebral Palsy/Hypoxic Ischemic Encephalopaty/Stroke

Attenuation of severe brain injury by human UCB-derived MSCs have been shown in newborn rat models. Middle cerebral artery occlusion is a commonly used experimental model for severe perinatal injury, neonatal stroke [52]. Intraventricular administration of human UCB-MSCs 6 h after occlusion has been shown to induce improvement both clinically (weight gain, survival, rotarod, and cylinder test performance) and by imaging and histological studies in neonatal rats [122]. There is increasing evidence from in vitro and in vivo preclinical studies that stem/progenitor cells may have multiple beneficial effects on outcome after hypoxic-ischemic injury. The mechanism of action, the optimal type, dose, and the method of administration of stem cells is unclear. Except for a small number of studies showing no benefit of cellular therapy in such injury models, satisfactory responses are obtained in general [123]. Wang et al. demonstrated increased proliferation of neural stem cells in hyperbaric oxygen treated newborn rats with hypoxic-ischemic brain damage [124]. Recently, neuroprotection by VEGF-transfected neural stem cells was suggested in

neonatal cerebral palsy rats [125]. Furthermore, a hematopoetic growth factor, erythropoetin at low doses, has been shown to ameliorate brain damage in periventricular leukomalacia in rats by targeting late oligodendrocyte progenitors known to be vulnerable to hypoxia-ischemia [126].

A recent study describing the use of marrow stromal cells in cerebral hemorrhage may also have implications in the neonatal period. The researchers developed intracranial hemorrhage by stereotactic injection of 0.5 U collagenase type IV in the striatum of adult Wistar rats. Two hours later animals were subjected to intracerebral injection of 2×10^6 allogeneic marrow MSCs. Survival of donor cells expressing neuronal and astroglial markers was shown in the brain tissue indicating induction of endogenous neurogenesis and inhibition of apoptosis of newly forming neural cells [127].

In summary, the promising findings in these different injury models whether hypoxic, hemorrhage, or other causes, suggest that MSC treatment may have implications in improvement of cerebral disorders particularly in the neonatal period.

Gastrointestinal System: Necrotizing Enterocolitis (NEC)

Formula feeding, bacterial colonization of the gut, hypoxia, and hypoperfusion are involved in pathogenesis of NEC. Intestinal epithelial cells, and intestinal stem cells are damaged leading to impairment of gut barrier function. In our previous study human bone marrow derived MSCs were administered intraperitoneally to neonatal Sprague-Dawley rats with NEC and migration of Fe-loaded human cells to intestines was shown on histological studies by Prussian blue and beta-2 microglobulin immunostaining. Improvement in clinical and histological findings suggested a potential therapeutic role for MSCs in NEC in the neonatal period [128]. A further study in a rat model of NEC revealed the protective effect of heparin-binding EGF-like growth factor on intestinal epithelial cell lineages including intestinal stem cells. The protective effect of this growth factor was confirmed in hypoxic conditions in ex vivo crypt-villous organoid cultures and was found to be dependent on EGF receptor activation, and was mediated via the MEK1/2 and PI3K signaling pathways [129].

7.2.4.2 Sepsis

Several recents reports focus on MSC treatment in sepsis. MSCs show promise in the treatment of sepsis by their intrinsic ability to home to injured tissue,use paracrine mechanisms to change the local environment (by secreting soluble factors to limit systemic and local inflammation), decrease apoptosis, stimulate angiogenesis, activate resident stem cells, induce immune modulation and exhibit direct antimicrobial activity [130]. The murine model for sepsis is induced by cecal ligation puncture procedure. Administration of bone marrow derived MSCs to mice before or shortly after the injury has shown to reduce mortality and improve organ function

[131]. The beneficial effect of MSCs was at least partly attributed to IL-10/IL-10R crosslinking on macrophages suggesting reprogramming of macrophages by MSCs through prostaglandin E2 release. Further animal studies confirmed favorable effects of MSCs in reduction of mortality, bacteremia, acute lung injury, and improvement of myocardial function during endotoxinemia. Enhanced phagocytic activity of blood monocytes was found as a contributary factor [132–135]. MSCs have also been shown to play a role in the treatment of E. Coli induced acute lung injury in mice [136]. Based on the antiinflammatory properties of UCB-derived MSCs, human UCB-MSCs were used in Escherichia coli (E. coli)-induced acute lung injury in mice through intratracheal route and increased survival and attenuation of lung injury was demonstrated. The favorable effects were attributed to down-modulation of the inflammatory process and enhancement of bacterial clearance.

7.2.4.3 Bone

Considering the connective tissue origin of MSCs, one of the most promising fields for therapeutic use of MSCs is bone diseases, both acquired and inherited [137, 138]. Experimental studies in mice have shown dynamic migration of transplanted MSC to the fracture site, modulation of the injury-related inflammatory responses and their contribution to the initiation of the repair process. Using MSC expressing luciferase, time- and dose-dependent and CXCR4-dependent MSC migration at the fracture site has been shown by bioluminescence imaging. Fracture healing was improved and correlated with an increase in cartilage, bone content, and changes in callus morphology. Additionally MSCs were shown to engraft at the callus endosteal niche and initiate callus formation with contribution of BMP-2 expression [138]. Thus, MSC therapy may have important implications in management of skeletal problems in rare pediatric diseases including OI, malignant infantile osteopetrosis (MIOP) and neurofibromatosis by contributing to the repair process of multiple fractures, pseudoarthrosis, nonunions frequently observed in these disorders. In a previous study from our center, in patients with MIOP a defect in adipogenic differentiation of bone marrow-derived MSCs has been demonstrated and the possible beneficial role of MSC therapy is discussed [139]. In another study investigating MIOP mutations, the use of MSCs in patients with extrinsic defects (RANKL mutation) is addressed [140].

7.2.4.4 Skin

MSCs contribute to healing of skin defects and have aroused interest in the field of plastic surgery. The regenerative effect has been attributed to the stromal support characteristics of MSCs providing secretory, angiogenic, antiinflammatory, and antifibrotic effects enabling healing without scar formation [141, 142]. Differentiation of marrow derivated MSCs into vascular endothelial cells and dermal fibroblasts

and contribution of growth factors including VEGF, bFGF, EGF, and SDF to achievement of skin expansion have been demonstrated in a recent experimental model in pigs [143]. Implications in pediatrics include severe burn injury, a rather common accidental event in childhood, and inherited skin diseases including recessive dystrophic epidermolysis bullosa (RDEB),a skin fragility disorder associated with mutations in type VII collagen gene resulting in defective anchoring fibrils at the epidermal-dermal junction. Subepidermal blistering induced by recurrent trauma disrupts epidermal homeostasis. The quality of life is poor and stem cell therapies including HSCs, MSCs have been used in experimental studies and in a very limited number of clinical cases. These studies have shown that healthy donor cells from the hematopoietic graft migrate to the injured skin, contribute to an increase in the production of type VII collagen, maintain skin integrity, and reduce blister formation [144–146]. Still, a major impact on the disease course has not been reported by stem cell treatments alone and the toxicity risk of HSCT especially on the skin and mucous membranes remains as a concern. The supply of extracellular matrix proteins by stem cells, particularly of MSCs, carries a potential in the regeneration process of skin/appendages/connective tissue defects in RDEB and craniofacial and other congenital malformations in childhood. The use of gene modified cells appear to be a promising approach in management of skin disorders. A recent study has shown correction of RDEB in an animal model by the use of genetically modified epidermal grafts [147]. On the other hand there has been increasing interest in development of induced pluripotent stem cell (iPSC) lines from children with rare inherited diseases including RDEB to be used in disease models and to study genetic correction in expendable cells [148].

7.2.4.5 Cardiovascular

Cardiovascular diseases are among those in which clinical experience with stem cell treatments is increasing most rapidly particularly in adults. Many experimental and clinical reports suggest improvement in damaged cardiac function, vascular healing, regeneration in infacted myocardium, and favorable short- and long-term outcomes after HSC or MSC treatments [24, 25, 43, 44]. However, some studies also exist questioning the efficacy and reporting the adverse events of cellular therapies in cardiovascular diseases [45, 46]. Except for a few reports on pediatric cases almost all reports involve adult practice. Studies in pediatric field usually involve inherited storage diseases and cardiomyopathies [49, 53, 54]. In an experimental mice model of inherited cardiomyopathy with targeted mutation of delta-sarcoglycan gene, the use of granulocyte-colony stimulating factor (G-CSF) treatment with allogeneic BMT markedly increased donor-derived MSCs in the marrow and induced their mobilization into the peripheral blood after BMT. G-SCF also induced donor cell recruitment to the heart after induction of myocardial damage with isoproterenol in cardiomyopathic mice. However the improvement of left ventricular function was temporary in that study [149].

7.2.4.6 Renal

The immunoregulatory functions of MSCs have implications in the treatment of renal diseases as well. One example is lupus nephritis in which UCB-derived MSCs were effective in significantly delaying the development of proteinuria, decreasing anti-dsDNA, alleviating renal injury, and prolonging life span in an in vivo experimental model [90]. In another study, we investigated the effects of human marrow derived MSC therapy on acute kidney injury in a rat model. The combined regenerative effects of erythopoetin (darbepoetin) and MSCs were investigated in an ischemia/reperfusion model and a significant clinical, laboratory, and histological improvement was obtained. The results suggested concomitant application of darbepoetin and MSCs as a potential novel renoprotective therapy for patients after having sustained an ischemic renal insult [150].

7.2.4.7 Hepatic

The use of MSCs has revealed striking results in hepatic diseases, particularly in regression of liver cirrhosis by supporting the function, proliferation, and differentiation of endogenous hepatocytes under appropriate conditions. MSCs have been used in both experimental animal models and in humans with fulminant hepatic failure, end-stage liver diseases, and inherited metabolic disorders [151–153]. Intraportal transplantation of human MSCs has been found to prevent death in pigs from fulminant hepatic failure [153]. In a toxic hepatic fibrosis model induced by application of CCl4, regression of fibrosis and improvement in albumin expression has been reported after the use of bone marrow cells; in another study in a toxic insult model-acetaminophen induced acute liver injury-favorable response has been obtained by the use of marrow cell therapy [154, 155]. Such applications may have implications in pediatric practice in inherited diseases and in the common occurrence of intoxications in childhood. Okura et al. have demonstrated reduction of serum cholesterol levels in heritable hyperlipidemic rabbits by portal vein injection of human adipose-derived multipotent progenitor cells. After transplantation,injected cells were localized in the portal triad and integrated into the hepatic parenchyma. They expressed human albumin, human alpha-1-antitrypsin, human Factor IX, human LDL receptors, and human bile salt export pump and showed hepatocytic differentiation [156]. These favorable findings may suggest the use of a similar therapy approach in human version of hyperlipidemia (familial hypercholesterolemia) and in other inherited liver diseases of childhood.

7.2.5 Adverse Effects and Risks of MSC Therapies

The clinical experience with the use of MSCs in pediatric diseaes has shown that systemic infusion of cells is generally well tolerated and has not caused serious

immediate side effects such as thromboembolic events, severe anaphylaxis, or pulmonary symptoms, when used at standard recommended doses of $1–2 \times 10^6$/kg cells. Mild side effects or allergic reactions have been reported which were mainly attributed to the use of fetal bovine serum during in vitro culture expansion of MSCs [48, 85, 87, 98].

In spite of the reports suggesting short term safety of MSCs there is lack of knowledge about their fate and effects at long term. The possibility of cytogenic transformation, oncogenicity, replicative senescence has been described in long-term cultures and with extensive manipulations and repeated passaging [104–108]. Therefore generally earlier passage cells are preferable for clinical use.

Recently, the safety of the use of even earlier passage of cells is being questioned. As opposed to many reports, Jeong et al. have shown tumor formation at the site of cell implantation in experimental injury models [157]. Therefore careful monitoring of chromosomal status of in vitro expanded cells is recommended before administration.

MSCs possess highly secretory properties making them suitable for the repair processes. However, this property may cause undesired response in a tumor bearing host by stimulating the growth or by modifying other biological behaviors of the malignant cells by providing stromal support. It has been shown that malignant cells and MSCs interact with each other and tumor microenvironment may stimulate oncogenicity in MSCs. Liu et al. have shown malignant transformation of MSCs in co-culture experiments by using rat bone marrow MSCs and malignant rat glioma C6 cells. The culture system was without direct cell–cell contact and soluble factors were held responsible [158, 159].

In paralel to these investigations with MSCs the risk for oncogenic transformation has been regarded as a general feature of cellular and gene therapy applications and strategies are being developed to prevent this adverse effect [160]. Di Stasi et al. reported [161] development of a method to prevent oncogenicity of other cellular therapies by inducing apoptosis to eliminate the infused cells in case of adverse events. They devised an inducible T-cell safety switch that is based on the fusion of human caspase 9 to a modified human FK—binding protein, allowing conditional dimerization. When exposed to a synthetic dimerizing drug, the inducible caspase 9 becomes activated and leads to the rapid death of cells expressing this construct. Five children who had undergone HSCT for leukemia were treated with the genetically modified T cells. In patients who developed GVHD a single dose of dimerizing drug was given and more than 90 % of the modified T cells were eliminated within 30 min after administration and ended the GVHD without recurrence. The authors conclude that inducible Caspase 9 cell-suicide system may increase the safety of cellular therapies and expand their clinical applications [161].

Replicative senescence is another undesired event and a limiting factor for in vitro expansion and differentiation of MSCs. A new approach for prevention of replicative senescence is enhancement of the proliferative capacity of hMSCs by ectopic expression of telomerase which will then allow for long-term culture. However, hMSCs with constitutive telomerase expression demonstrate unregulated growth and even tumor formation. An attractive strategy is development of

inducible immortality in hTERT-MSCs by using inducible Tet-On gene expression system and hTERT expression may be modulated [108].

In spite of the reports highlighting a stimulatory role for MSCs on oncogenicity, lack of long-term engraftment potential of MSCs has been suggested as an advantage for the host to overcome the risk of malignant transformation. The majority of in vivo experimental studies with the use of MSCs have failed to track infused cells in recipient tissues at long term. It is hypothesized that MSCs may undergo apoptosis after displaying an active role in the healing process. This property suggests a possibly safer in vivo profile in terms of oncogenicity [90].

A recently addressed issue about MSCs is their fate and functionality after systemic infusion. Instant blood-mediated inflammatory reaction, has been shown to compromise the survival and function of systemically infused islet cells and hepatocytes. Investigators tested this effect for MSCs and reported that MSCs displayed high amounts of prothrombotic tissue/stromal factors on their surface to trigger this reaction after blood exposure particularly after long-term culture. The use of early passage cells only elicited minor reaction. The authors state that, this adverse event can potentially compromise the survival, engraftment, and function of the cells [162].

7.3 Induced Pluripotent Stem Cells (iPSCs) in Pediatric Diseases

Main focus of this reveiw article is on HSCs and MSCs, as the most suitable stem/progenitor cells for clinical use. Other cell types including ESCs, and other organ specific stem cells have not been addressed. İnduced pluripotent stem cells may be considered as a stem cell source for future applications and is too early to consider them as a therapy alternative [163–171]. Yet, iPSC section is included here based on the exciting research potential in pediatrics particularly in inherited and rare diseases.

Basic research with human materials in rare pediatric disorders is challenging due to the difficulty to obtain sufficient number of cells and tissue materials from patients and to develop cell lines with unlimited life span for advanced research. Using the iPSC technology forced expression of specific pluripotency genes are induced in somatic cells from the blood or tissues of patients. This way, somatic cells are reprogrammed to behave as ESCs [163]. These cells are suitable to be used in experimental studies for research and for testing of therapeutic agents, particularly in rare diseases with a limited tissue/cell source from patients including inborn errors of metabolism. In paralel with the expanding work with iPSCs libraries of patient-specific human iPS cell lines are being generated modeling inherited metabolic disorders [163–171].

An exciting area in the iPSC field is the potential of genetic correction of the gene mutated iPSCs as a therapy alternative in future. However, at present, these

cells do not appear to be suitable for use in humans due to the risk of oncogenicity that may be induced during reprogramming of the somatic cells to obtain iPSCs. A recent report about genetic correction of α1-antitrypsin deficiency in gene mutated-iPSCs has provided the first evidence for application of human iPSCs in treatment of inherited genetic disorders [164, 165]. In clinical practice it is challenging to collect/expand adequate number of cells for gene therapy applications. One major advantage of the use of iPSCs is the possibility of generating unlimited quantities of cells for autologous transplantation. The results of another study also provide evidence for therapeutic applications by the use of these cells. iPSCs were generated from patients with Hurler disease and were differentiated into both hematopoietic and nonhematopoietic cells. These findings showed that the missing enzyme of Hurler disease, α-L-iduronidase, was not required for stem cell renewal. Thus Hurler-iPSCs can be used to generate autologous hematopoietic grafts which will overcome the immunologic complications of allogeneic transplantation [167]. In addition, generation of nonhematopoetic cells can carry the potential to treat anatomical sites not fully corrected with HSCT. These findings involving either differentiation or gene correction of mutated iPSCs represent a future therapy alternative. Clinical translation needs resolution of safety issues.

Besides several inherited diseases this technology has been used in experimental studies involving muscular dystropy disorders to generate an unlimited source of myogenic cells [166]. Tedesco et al. recently developed iPSCs from fibroblasts and myoblasts of patients with limb-girdle muscular dystrophy 2D (carrying mutations in the gene encoding α-sarcoglycan). Then the iPSCs were used to generate mesoangioblast-like cells which were expanded in culture and genetically corrected in vitro with a lentiviral vector carrying the gene encoding human α-sarcoglycan and a promoter that would ensure expression only in striated muscle. Interestingly transplantation of the genetically corrected human iPSC-derived mesoangioblasts into α-sarcoglycan-null immunodeficient mice resulted in functional improvement of the dystrophic muscle and muscle fibers expressing α-sarcoglycan were generated [168].

Recent studies are focusing on functional aspects of cells derived from iPSCs as well. In Marfan disease fibrillin-1 gene coding for an extracellular matrix protein is defective. In this disease model skeletal cells derived from iPSCs were shown to display exactly similar phenotype (in osteogenesis and chondrogenesis) as those derived from ESCs carrying the mutation [169]. In another study patient-specific human iPS cell lines for inherited metabolic disorders of the liver were used to investigate functionality. Those cell lines showed typical disease specific pathologies (i.e., elevated lipid and glycogen accumulation in iPSCs from patients with glycogen storage disease type 1a). A 3-step differentiation protocol in chemically defined conditions was used to differentiate these cell lines into hepatocytes. Mature hepatocyte functions were obtained including albumin secretion, cytochrome P450 metabolism [170]. Further studies demonstrated liver engraftment potential of hepatic cells derived from patient specific iPSCs [171]. Thus, iPSC lines are suitable for modeling rare inherited diseases and to investigate therapeutic strategies.

7.4 Challenges in Stem Cell Use and Recent Advances: Highlighting Pediatric Issues

7.4.1 Limited Efficacy of Stem Cell Treatments in Humans and Combined Therapeutic Approaches

Stem cell therapies appear to induce promising responses in animal injury models. But, the preliminary experience in humans has shown limited efficacy in regenerative applications, including the pediatric field. The results obtained by MSCs, accepted as the most suitable cellular therapy agents for organ repair, has not reached an optimal stage, perhaps due to the relatively limited experience in this field till present. The beneficial role of repeated applications, combinatorial approaches including gene therapy and other cellular and/or pharmacological therapies are being addressed.

One of the most challenging fields in medicine is management of neurological disorders; thus stem cell therapies and combination approaches have aroused much attention. Neuroprotective features of MSCs and their potential use in regenerative medicine are addressed in detail by many research groups [28–30, 172, 173]. Recent studies indicate that targeting different pathogenic mechanisms may provide better response than cell therapy alone in systemic/complex diseases including neurodegenerative disorders. Lysosomal storage and peroxisomal disorders are candidate diseases for combination therapies with cell and gene therapies. Recent clinical applications of gene therapy have been described in ALD and MLD with the use of genetically transduced autologous HSCs and MSCs, respectively [113, 174, 175]. An experimental study has shown migration of HoxB4 transduced bone marrow cells of MLD mice to the brain, contribution into oligodendrocyte regeneration by carrying therapeutic proteins and induction of favorable clinical response [176].

Generally, cell and gene therapy can supply a persistent source of the deficient enzyme but clinical results are usually not as expected. Addition of a second therapeutic strategy including substrate reduction, antiinflammatory or other pharmacological agent is being investigated in order to achieve synergistic effects [177–179]. Organ specific stem cells, i.e., neural stem cells have been suggested to carry a therapeutic potential in experimental studies in inborn errors and/or neurodegenenerative diseases including leukodystrophies, Sandhoff disease, hypoxic-ischemic encephalopathy [180, 181]. Alternatively, combination of a central nervous system-directed adeno-associated virus (AAV)2/5-mediated gene therapy with BMT has shown synergistic effects and significantly improved motor function and life span in an experimental mouse model [181]. Somewhat promising results have come from studies in infantile neuronal ceroid lipofuscinosis caused by the loss of palmitoyl protein thioesterase-1 (PPT1) activity. Gene therapy, neuronal stem cells, or small molecule drugs were shown to produce some clinical benefit and partial histological improvement [182].

Stem cell and combination approaches present a therapy alternative for muscular dystrophies (Duchenne, Becker muscular dystrophy); another disappointing area

among neuromuscular diseases [183–185]. Transplantation of myoblasts (satellite cells or other myogenic cell populations) or stem cells has been performed to promote muscle regeneration. MSCs, being as the stem cells that all connective tissue cells including myoblasts are derived from, have not fullfilled the expectations in achievement of satisfactory clinical response. The use of mesoangioblasts has produced satisfactory response in animal models of muscular dystrophy and clinical testing in children with Duchenne muscular dystrophy has been initiated [168, 185]. Another strategy is the use of adeno-associated viral vectors to deliver synthetic dystrophin genes for DMD in experimental setting [186].

7.4.2 Challenges in Getting Access to Cells/Tissues from Children for Stem Cell Use and Research

Development of effective therapeutic strategies for inborn errors of childhood is a challenging area. One reason is the difficulty to obtain cells or tissue materials from young individuals and/or to expand in vitro to have unlimited cell source for clinical use or for research studies. Many of these diseases are rare and animal models either donot exist or human correlation may be poor. Therefore, there is paucity of functional basic research studies in these rare diseases, particularly in the stem cell field. Furthermore, cell and gene therapy applications necessitate quite a high number of cells and are generally based on administration of determined number of cells/kg of the patient. Thus, therapeutic options with stem cells are also limited. The iPSC technology has emerged as a critical step to overcome this problem and appears as a promising area for advancement of research in inherited rare diseases and for development of efficient therapeutic strategies for future use. This technology has been used in experimental studies in several inherited diseases of childhood to have infinite cell source for research [163, 170, 171]. Gene therapy technologies are under investigation [164, 168]. iPSCs for pediatric use and research are covered above in more detail.

Although the results are promising it is too early to consider iPSCs as a therapy alternative. Current use of this technology provides a precious tool for stem cell research and for drug testing studies.

7.4.3 Limitations and Regulations in Children in Clinical and Basic Research

Currently, application of stem cells in children for regenerative purposes, excluding HSCT for standard indications, is mostly restricted to rare disorders in orphan disease category and to progressive, life-threatening diseases. At present, stem cell treatments are applicable only under experimental protocols and not yet considered as means of standard therapy. This includes the use of MSCs, other types of adult

stem cells, ESCs (highly restricted), and even the use of HSCs for regenerative purposes. The ethical restrictions and regulations in children limit not only clinical use but also basic research with patient materials. The intensity of regulations differ between countries and among centers. Therefore, establishment of iPSC cell lines is a valuable technique to enable many scientists all over the world get access to stem cells obtained from rare inherited diseases, share their experiences and contribute to advancement of research not only in these rare disorders but also in common diseases as well, by contributing to understanding mechanisms of diseases.

The restrictions and regulations in children in the stem cell field is a necessity to prevent stem cell abuse. Extreme caution is advised for the use of stem cells for therapeutic purposes. On the other hand, rare inborn errors of metabolism in orphan disease category, that are associated with multisystem defects and are progressive and life-threatening are considered as candidate diseases for new therapeutic strategies including gene and cell therapies. Therefore, stem cell basic and clinical research performed under appropriate ethical rules is encouraged and may contribute to establishment of new therapeutic strategies in these severe disorders lacking an effective therapy alternative.

7.4.4 Limitations in Fetal and Prenatal Issues in the Stem Cell Field and Recent Advances

Fetal and prenatal field is a specific period of pediatric practice and carries strict regulations and limitations for research and therapy. From the stem cell point of view, the prenatal period is precious as a unique source of stem cells that are less differentiated and highly potent carrying a regenerative potential [61]. In addition, the immunotolerant state during the prenatal period increases the engraftment potential and the efficiency of cell therapies. It has been shown that intrauterine transplantation of allogeneic HLA-mismatched fetal MSCs to a human fetus with severe OI in the 32nd week of gestation reverted the phenotype into a mild one and the favorable response persisted during several years of follow-up [187].

Further advancements in cell and gene therapies have enabled the applicability of these approaches in the prenatal period for correction of a genetic defect before irrepairable tissue damage has occurred. Transduction of first trimestr MSCs from fetal blood with lentivirus for introduction of genes of interest and intrauterine transplantation to the fetus has been described [188]. In another article by David et al, the indications determined by "The NIH Recombinant DNA Advisory Committee for P*renatal/Fetal Gene Therapy Indications*" for life-threatening disorders or other nonlethal diseases have been reported. The advantages of prenatal application are addressed and defined as the availability to target genes to a large population of stem cells, achievement of a higher vector to target cell ratio due to the smaller size of the fetus and immune tolerance to the transgenic protein by early gestation delivery [189].

Another interesting field that has emerged with pre-implantation genetic diagnosis practices is the availability of the use of affected-spare blastocysts with specific

known inherited mutations in basic research studies. The mutated embryos are normally discarded. It is discussed that these affected blastocysts can be used for the derivation of disease-bearing human embryonic stem cells for studying the molecular and pathophysiological mechanisms underlying the genetic disease [190].

Fetal period offers a good opportunity for the field of hematology to study hematopoesis. Fetal hematopoesis has been studied well in experimental animal models. During mammalian embryonic development hematopoesis has been described as a migratory phenomenon, starting from the yolk sac blood island to the aorta-gonad-mesonephros region, fetal liver, and subsequently, to the fetal bone marrow. Recent studies focus on the supportive role of fetal stromal niches (primary hematopoetic niches), particularly from fetal liver in achievement of hematopoetic differentiation from embryonic stem cells and in globin switch [191]. These findings may be useful to overcome the problematic issue of ex vivo expansion of HSCs and may have implications in HSCT and HSC-gene therapy field.

7.4.5 Adverse Effects of Stem Cell Therapies and Long-Term Safety Issues

Considering the long life expectancy in the childhood period long-term safety issue becomes a critical factor when considering stem cell therapies in children. At present, the experience with the clinical use of stem cells is limited and long-term adverse effects are not known. A major risk is the potential of oncogenicity particularly with the use of ESCs or other stem cells that require extensive in vitro manipulation [57, 95, 105, 106, 108, 192]. In order to eliminate or at least to minimize this potential risk, the use of ESCs is strongly discouraged, and avoidance of extensive manipulation to the stem cell product is recommended. Careful monitorization of the chromosomal status of the in vitro expanded cells is another important preventive measure. In addition, administration of stem cell treatments (i.e., MSCs) to patients with malignancy may play a role in development of an aggressive biological behavior of the tumor cells by receiving stromal support from the MSCs or may carry a risk of MSCs achieving oncogenic potential in the tumor microenvironment [158, 159]. Recent research studies have focused on apoptotic and suicidal mechanisms to overcome this serious risk of cell and gene therapies [160, 161].

7.5 Conclusion

Stem cell field holds several advantages for clinical application in pediatrics. The regenerative capacity of children is higher than adults. Higher number of cells/kg can be achieved during culture expansion particularly in small children, and the duration of cell preparation is shorter. These factors may play a role in increasing the efficacy of these treatments. On the other hand, stem cell basic research in the

pediatric field is an exciting area and may contribute to development of new gene/cell therapy strategies and combinatorial approaches by understanding of mechanisms of disease. Inherited diseases are in fact knock out systems and research with patient stem cells or induced pluripotent stem cell (iPSC) lines obtained from their cells/tissues is invaluable. Therefore, the "stem cell" topic seems to be a fruitful area for pediatric research and development and presents a promising therapeutic alternative at longer term.

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Chapter 8
Stem Cell Therapy: From the Heart to the Periphery

Bernd Stratmann and Diethelm Tschoepe

Abstract Peripheral artery disease remains a clinical challenge—together with coronary artery disease, it accounts for increased morbidity and mortality in the concerned patients. Therapeutic concepts are often limited because of underlying co-morbidities and generalised atherosclerosis. The search for new forms of intervention follows several directions with stem cell therapy or therapeutic angiogenesis being one of the most promising approaches. The following chapter should provide an overview on the significance of the disease and the limitations of currently applied procedures. The biological concept which is the driving force of improvement in this special clinical situation is presented, and a brief overview on the history of stem cell therapy for vascular regeneration is given. So far, regarding peripheral artery disease, this story is a story of success, and future clinical approaches will take into account new sources of stem cells beside bone marrow to successfully treat patients with the disease, even in palliative situations.

Keywords Peripheral artery disease • Therapeutic angiogenesis • Revascularisation • Ischaemia • Perfusion

8.1 Introduction

Stem cells of variable sources have demonstrated significant potential for vascular regeneration in peripheral arterial disease. Preclinical studies proved beneficial effects in animal models of critical limb ischaemia in terms of revascularisation, and clinical trials showed clear advantage in stem cell-treated patients with critical limb ischaemia with regard to symptoms and wound healing. Because many of these

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K. Turksen (ed.), *Stem Cells: Current Challenges and New Directions*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4614-8066-2_8,

patients are not eligible for revascularisation or endovascular procedures, there is an urgent need for novel therapies to improve the clinical situation and the quality of life. These no-option patients are treated in clinical trials with stem cell preparation of various sources like bone marrow, peripheral blood, or adipose tissue.

8.2 The Burden of the Disease

Coronary (CAD) and peripheral (PAD) arterial diseases are major causes of morbidity and mortality around the world, and millions of patients with CAD and PAD are treated by different medications, bypass surgery, or angioplasty. Risk factors for atherosclerotic PAD are mainly, but not exclusively, smoking and diabetes and are, therefore, comparable with those for atherosclerosis in the cerebrovascular and coronary circulation. Atherosclerotic occlusive disease of the lower extremities is the most frequent form of atherosclerosis, and PAD is a strong marker for atherothrombotic disease in other vascular beds [1]. PAD is present in approximately one-half of all patients with foot ulcers accounting for chronic wounds due to insufficient perfusion [2]. Along with polyneuropathy, PAD causes foot ulceration which is the major cause of non-traumatic lower-limb amputations. As a rather mild form of PAD, intermittent claudication (IC) is characterised by pain upon walking, limiting the pain-free walking distance. Chronic ischaemic rest pain, ulcers, or gangrene attributable to objectively proven arterial occlusive disease characterise critical limb ischaemia (CLI) which is the most advanced and severe form of PAD. Only 20 % of patients with critical limb ischaemia describe previous symptoms of intermittent claudication [3]. PAD is estimated to develop in 500–1,000 individuals per million persons in the general population; the prevalence of all stages of PAD in the general population is estimated to be 4.2–35 % and varies by country [4, 5]. Progression of PAD towards CLI is expected to occur for 4.3–9.6 % of the PAD patients, eventually resulting in amputation of the affected limb [3, 5]. For Germany, data are available from the observational German Epidemiological Trial on Ankle Brachial Index (getABI study) from 2004, in which the ABI of consecutive, unselected patients aged 65 years or older with bilateral Doppler ultrasound measurements was determined. A total of 6,880 patients were included (42.0 % male, mean age 72.5 years). The prevalence of PAD for men/women as indicated by an ankle-brachial index (ABI) <0.9 was 19.8 %or 16.8 %, respectively [6]. In the US population, the prevalence of PAD was 4.5 % in the general population but increased to 9.5 % in persons with diabetes [7]. PAD develops at a younger age among patients with diabetes as compared to the general population [8]. Diabetes mellitus causes almost 50 % of all non-traumatic amputations of the lower extremities worldwide, and more than 80,000 procedures are performed annually [7]. The lifetime risk for amputation in diabetic patients is 10–15 %, which is 10–30 times higher in comparison to the general population [9–12]. Leg amputation due to atherosclerotic PAD corresponds to a mortality rate of around 30 % and a 5-year prognosis with survival rates of less

than 5 years [12–16], and even asymptomatic PAD by itself is a significant predictor of cardiovascular morbidity and death [17]. The presence of cardiovascular risk factors and co-morbidity importantly contributes to the reduced survival. CLI has important functional implications, and its impact on the quality of life, assessed as quality of life indexes, has been reported to be similar to those of terminal cancer patients [18, 19]. In addition, CLI is associated with higher numbers of surgical interventions and hospitalisation [20, 21].

8.3 Treatment Concepts and Why They Do Fail

Despite therapeutic and technical advances in endovascular surgery, CLI continues to be associated with a high risk of (cardio)vascular events, including major limb loss, myocardial infarction, acute coronary syndrome, stroke, and death, especially in patients with diabetes mellitus [22–24]. Due to the widespread nature or the distal location of the occlusions and due to the presence of co-morbidities, PAD patients are at highest risk for peri-procedural complications and death. Because of the high operative risk or unfavourable vascular involvement, up to 40 % of patients do not qualify for such surgical interventions or endovascular procedures [25–27]. Thirty percent of patients undergoing amputation previously underwent one or more revascularisation attempt [28]. This accounts for about 100,000 major leg amputations in the European Union, and 120,000 in the United States [13, 29]. There is an unmet need for new strategies to offer these patients an additional and viable therapeutic option. The prognosis of death is around 20 % within 6 months of CLI diagnosis and rises to more than 50 % at 5 years after diagnosis [13, 30]. These extraordinary high mortality rates exceed those seen in any other pattern of occlusive disease like symptomatic coronary artery disease [1, 31] and reflect the severity of systemic effects associated with a diagnosis of CLI.

Treatment decisions in CLI are individualised and should take into account life expectancy, functional status, anatomy of the arterial occlusive disease, as well as surgical risk and are often multidisciplinary. Open surgical bypass was regarded as the most effective treatment strategy for limb revascularisation in these patients for a long time. Endovascular procedures treatment options were improved and are part of clinical routine in the angiologic treatment of PAD. Multimorbidity of the patients with extensive co-morbidities (atherosclerosis or heart disease), the anatomic location of the lesion, or the extent of the disease limit surgical interventions, and in subgroups primary amputation remains the only treatment option. Perioperative mortality in 5–20 % of the patients accounts for the bad prognosis of amputation as well as the risk for a second amputation in 30 % of cases, with only 25–50 % of subjects achieving full mobility [26]. The median cost of successful limb salvage is half of the costs for the management of a patient after amputation [26, 32]. These patients with no option of either surgical or endovascular revascularisation might benefit from stem cell therapy and/or tissue engineering strategies that aimed at

accelerating the natural processes of vascularisation, angiogenesis, and tissue repair [33]. Several clinical studies reveal that the injection of bone marrow-derived mononuclear cells (BMC) results in improvement in symptoms and healing of ulcers in patients with CLI up to stage IV of Fontaine's classification [34].

8.4 Basics of Vessel Formation and Biology

The key steps in vessel formation comprising endothelial cell activation, migration, proliferation, and reorganisation are highly regulated in a complex balance of pro- and anti-angiogenic mechanisms. The de novo synthesis of blood vessels from endothelial progenitors which differentiate into endothelial cells and fuse into luminal structures is called vasculogenesis. During embryonic vascular development, pluripotent stem cells differentiate to endothelial cells, which upon development form a primitive vascular network by assembly, called the primary capillary plexus. Vascularisation of several organs, like the endocardium of the heart and the dorsal aorta, occurs by vasculogenesis. In adult neovascularisation, migration and differentiation of bone marrow-derived endothelial progenitor cells (EPCs) are involved [33, 35]. Hypoxia and the key transcriptional system hypoxia-inducible factor (HIF) are major inductors for both angiogenesis and vasculogenesis by enhancing the synthesis of pro-angiogenic factors like vascular endothelial growth factor (VEGF), angiopoietin, and inducible nitric oxide synthase (iNOS) [36–38].

Arteriogenesis refers to an increase in the diameter and calibre of pre-existing arteriolar collateral connections. Perivascular cells are recruited within this process, and expansion and remodelling of the extracellular matrix occurs. Arteriogenesis results in the increase of collateral vessel size and wall thickness with shear stress rather than hypoxia being the main stimulus of arteriogenesis [39]. Shear stress leads to an upregulation of cell adhesion molecules for circulating monocytes, which subsequently accumulate around the proliferating arteries and provide the required cytokines and growth factors [38, 40].

Angiogenesis is defined as the sprouting of new capillaries from an existing vascular structure, a process that is triggered by endothelial cell migration and proliferation. Remodelling of the extracellular matrix (ECM), tubule formation, and expansion of the surrounding vascular tissue as well as remodelling of newly formed vessels into 3-dimensional networks with regression of unnecessary microvessels are key elements of angiogenesis. Angiogenesis occurs as a sprouting of small endothelial tubes from pre-existing capillary beds in response to local hypoxia. It is mediated by hypoxia-induced release of cytokines like VEGF and related growth factors [41]. The resulting capillaries are rather small, with a diameter of about 10–20 μm, and cannot sufficiently compensate/substitute for a large occluded transport artery. Organs like the brain, the kidneys, and the developing limbs are vascularised by angiogenesis. Angiogenesis is likely to be the very first mechanism for most new blood vessel growth in the adult, regardless whether it is a result of physiologic or pathologic stimuli like cancer growth [42, 43].

8.5 Role of Stem Cells in Therapeutic Angiogenesis: Manufacturing and Biological Challenges

Stem cells have demonstrated significant potential for regeneration in peripheral arterial disease in both animal and human studies. While results of clinical trials have been variable with respect to myocardial infarction and dilated cardiomyopathy, they have clearly proven benefit for patients with critical limb ischaemia and peripheral arterial disease [34]. Preclinical trials have demonstrated the angiogenic and vasculogenic potential of autologous bone marrow-derived stem cells (BMCs) in the treatment of PAD [44–47]. BMC are preferred in the cellular therapy of vascular diseases since bone marrow can be easily accessed, is renewable, and is an autologous source for regenerative cells. The use of purified and selectively expanded cell populations may allow a more target organ-specific stem cell therapy in the future. For therapeutic purposes, 50–250 ml adult bone marrow blood is aspirated from the iliac crest under local anaesthesia [34]. Mononuclear cells are separated from the whole bone marrow aspirate by density gradient centrifugation [48]. To overcome open preparation procedures and the application of several washing steps, newer protocols apply closed-tube procedures which reduce contamination risk. In summary, good manufacturing practice processes to produce a quality-controlled and contamination-free cell product [49, 50]. During cell preparation, viability needs to be determined several times and finally must reach approximately 95 % to guarantee functionality. Cell product characterisation by fluorescence-activated cell sorting or a cell counter is needed for product release. This manufacturing process can be performed within one working day in an ambulatory setting.

BMCs comprise several cell populations having the capacity to proliferate, migrate, and also differentiate into various mature cell types, best analysed in the application after myocardial infarction. Among these cells are haematopoietic stem cells [51–54], mesenchymal stem cells [55, 56], endothelial progenitor cells [57, 58], and side population cells [59, 60]. The angiogenic properties of BMCs have been attributed to the differentiation of these pluripotent stem cells into endothelial cells, thereby generating new blood vessels [61]. In addition, BMC releases cytokines and growth factors that promote angiogenesis. BMC releases vascular endothelial growth factor (VEGF), and the chorioallantoic membrane is an ischaemic environment, stimulating vasculogenesis [62, 63]. The most important cell populations involved in angiogenesis are CD133+ cells [64], CD117+ cells [65], and CD34+ cells [66], in addition to the mesenchymal stem cells [67].

Endothelial progenitor population comprises a heterogenous population of cells such as CD34-/CD133+/VEGFR2+ and CD34+/CD133+/VEGFR2+, in addition to the mature endothelial cells. The CD34-/CD133+/VEGFR2+ fraction is the precursor of the CD34+/CD133+ population and shows more potent vasoregenerative capacities [68, 69]. Endothelial progenitors are reported to be mobilised by several agents such as chemotherapeutic agents [70], metronomic chemotherapy [71], and erythropoietin [72]. These agents can be used therapeutically either to enhance angiogenesis in ischaemic cases or to reduce angiogenesis in cases of malignancies.

8.6 Working Concepts

New vessel formation to improve tissue perfusion through the three mechanisms vasculogenesis and/or angiogenesis in the ischaemic tissue as well as collateral vessel formation via arteriogenesis is a main topic therapeutic neovascularisation relies on. Positive effects on classical perfusion markers like TcPO2 could be clearly demonstrated. Physiological effects like collateralisation or angiogenesis have scarcely been described and could not always be attributed to clinical success [73].

Whether these effects can be attributed to the incorporation of stem cells into the wall of the new vessel, or homing stimuli released by platelets, or to the cytokines released by chemo-attracted BMCs inducing proliferation of resident endothelial cells remains an open issue. Imaging techniques to follow up with the injected cells are improving and deliver first results in animal models but also prove a diminished survival of the cells [74, 75]. An interesting finding is that hypoxia induces progenitor cell mobilisation through HIF-1α induction of SDF-1 and controls subsequent differentiation into endothelial cells through HIF-1α-regulated VEGF expression [36, 37, 76]. Mesenchymal stem cells mobilise to sites of ischaemia and adopt a partial endothelial phenotype when exposed to similar vasculogenic stimuli such as hypoxia. Mesenchymal stem cell recruitment and subsequent endothelial differentiation within ischaemic tissue may indeed be driven through the HIF-1α/SDF-1/VEGF pathway. Kinnaird et al. were able to show that cultured human BM-derived stromal cells promote arteriogenesis through paracrine mechanisms [77]. This concept is supported by Heil et al., who suggest that in the adult organism, bone marrow cells (BMCs) do not promote vascular growth by incorporating into vessel walls but rather act as “cytokine factories or depots”, promoting vascular growth by paracrine effects [78]. Findings by Jin et al. also support this concept by which ischaemia induces plasma elevation of stem and progenitor cell-active cytokines, including sKitL (Soluble Kit-ligand) and thrombopoietin, and, to a lesser extent, progenitor-active cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and erythropoietin [79].

Based on the experience from the application of stem cells in heart diseases, it is known and estimated that the regenerative potential of bone marrow-derived stem cells may be explained by at least any of 4 mechanisms [80]: (1) transdifferentiation of BMCs to cardiac myocytes [81, 82]; (2) cytokine-induced myocyte growth [83, 84] induced by cytokines (like vascular endothelial growth factor, insulin-like growth factor, platelet-derived growth factor) and increase of residual viable myocytes (especially in the zone of the infarcted area); (3) stimulation of intrinsic myocardial stem cells (endogenous stem cells) [83]; and (4) induction of cell fusion between transplanted BMCs and resident myocytes [85, 86], which was taken as an explanation for transdifferentiation.

Precise mechanisms cannot be given; it seems to be a cocktail phenomenon that boosts the effect—in the heart as well as in the periphery.

8.7 Cellular-Based Therapeutic Concepts in PAD/CLI

Therapeutic angiogenesis using stem cells, autologous progenitor cells, growth factors such as basic fibroblast growth factor, and transcription factors such as hypoxia-inducible factor-α that induce synthesis of angiogenic cytokines have been used in critical limb ischaemia patients who lack options for endovascular or surgical revascularisation [34]. Single growth factor therapy proved to be insufficient in the treatment of CLI [87], whereas cellular-based therapies are reported to be successful at various study sites [34]. The fact that bone marrow cells are composed of extensive complex cell fractions containing many kinds of undifferentiated stem cells and differentiated cells obviously guarantees for successful application. Implantation of autologous bone marrow cells is proven to be an effective and feasible technique of inducing therapeutic angiogenesis in both clinical and experimental studies. However, the angiogenic potency might differ among cell fractions of bone marrow cells, and which of these play a key role is yet unclear.

Injection of unfractionated bone marrow mononuclear cells has been reported to promote neovascularisation of ischaemic tissues effectively. This angiogenic effect may be related to their ability to induce vascular and muscle regeneration by direct de novo vascular and muscle differentiation or paracrine mechanisms through vascular endothelial growth factor secretion as described before. The working concept of BMCs in humans cannot be answered finally. Some but not all studies report neovascularisation and angiogenesis during treatment which is supposed to trigger wound healing in chronic wound situations like diabetic foot [48, 73].

Our recently published results of the prospective clinical trial to evaluate the safety and efficacy of non-expanded and expanded bone marrow-derived mononuclear in the case of diabetic critical limb ischaemia prove the safety and functionality of stem cell treatment in this population [73]. The study enrolled critical limb ischaemia patients who were no-option cases. A typical example is given in Fig. 8.1, showing a patient who was successfully treated with bone marrow-derived mononuclear cells [48, 73].

The route of administration of stem cells was by intramuscular or intra-arterial injection. The cell product was injected in the calf muscle or infused in the arteria femoralis. We used 40–50 ml bone marrow as starting material which was expanded over a time period of 12 days leading to an accumulation of mesenchymal stem cells and compared this cellular product to bone marrow cells. Currently, three routes of stem cell administration in critical limb ischaemia are applied: intramuscular, intra-arterial, or a combination of both. With the intra-arterial administration into the common femoral artery of the ischaemic leg, mononuclear cells are supposed to reach the region of maximum ischaemia by blood flow [44, 88]. While travelling in the circulation, nutrient and oxygen supply are preserved and provide a favourable environment for survival and engraftment, but the uptake from the circulation may be limited and cells may be damaged and loose potential due to shear stress. In this type of delivery, homing requires migration of cells out of the vessels into the

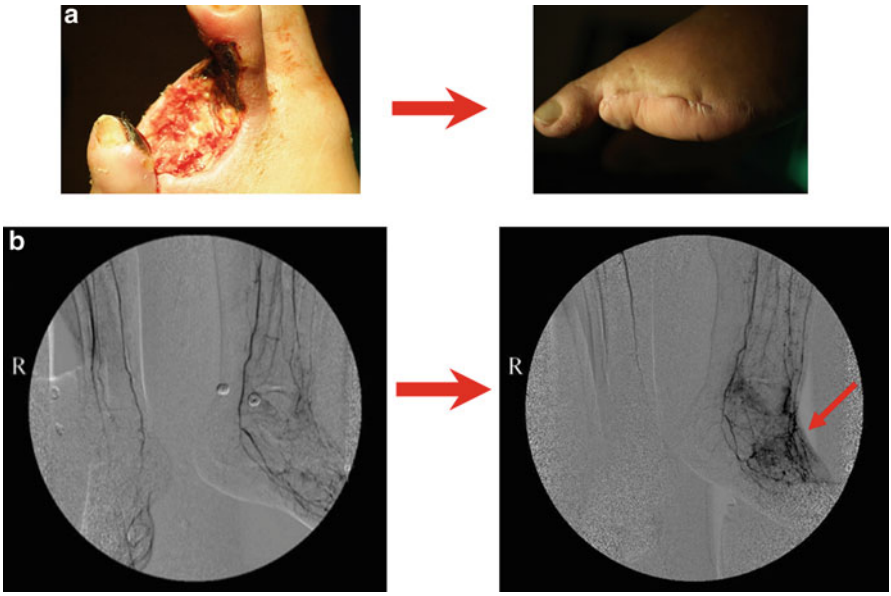

Fig. 8.1 Use of autologous bone marrow stem cell therapy leads to clinical improvement and 100 % wound healing in a patient with peripheral arterial occlusive disease. (**a**) *Left*: the situation at treatment beginning is shown, *right*: after 20 weeks. (**b**) Fine needle angiogram before autologous bone marrow stem cell therapy (*left*) and 20 weeks after the therapy (*right*), new collateralisation of capillary vessels of the forefoot is highlighted by the *arrow*

surrounding tissue, which makes ischaemic tissue targets less efficiently [89]. The intramuscular injection of stem cells with creation of a cell depot with paracrine activity in the ischaemic area overcomes this issue, but it is less clear what the fate of these muscle-deposited cells is [38]. The survival of the cells may be decreased, because of the injection site being nutrient and oxygen depleted [89, 90]. In our trial, outcome was not related to the application route, and there was no clear benefit of expanded cells, which were enriched in mesenchymal cells [73].

Most studies on cell therapy for critical limb ischaemia used the entire mononuclear cell fraction. Angiogenic effects of bone marrow mononuclear cells and peripheral blood mononuclear were shown to be equal in stimulation of neovascularisation [91]. The dose of infused cells plays a pivotal role in the effectiveness of therapy in terms of time to effect. Typically, the total cell number of mononuclear cells ranged from 0.1 to 101×109 cells [92–94]. The fraction of CD34+ cells in the isolated mononuclear cells population varies from 0.6 to 2.4 % in the therapeutic angiogenesis using cell therapy studies [95, 96]. Classical parameters to prove success is reduction in ischaemic pain, improved walking distance, improved ankle-brachial index and transcutaneous oxygen pressure, and signs of wound healing. Some studies also prove therapeutic effects by angiography [34, 73, 97, 98].

8.8 The Beginning: Preclinical and Clinical Applications

Preclinical studies showing that application of BMCs, including EPCs, into ischaemic limbs increases collateral vessel formation led to the evaluation of safety and feasibility of these cell-based therapies in patients with PAD. Clinical benefits can be noticed in form of a decrease in ischaemic symptoms and an increase in ankle-brachial index, transcutaneous oxygen pressure index, and exercise tolerance. The working hypothesis is that the observed clinical effects are associated with an improvement in angiogenesis, formation of new collaterals and/or augmentation of endothelium-dependent vasodilation. It is beyond the scope of this contribution to comment and review all trials done so far; there are excellent and actual reviews which the reader is referred to [34, 99, 100].

The Therapeutic Angiogenesis by Cell Transplantation (TACT) study by Tateishi-Yuyama et al. was the first larger study on the use of BMC in limb ischaemia [96] and delivered the concept for many other following studies. It was an open pilot study in which efficacy and safety of autologous implantation of BMC was established and a randomised controlled confirmatory part, comparing the efficacy of BMC vs. peripheral blood PBMC treatment. In the PBMC part, patients with bilateral leg ischaemia were randomly injected with BMC in one leg (active treatment) or with PBMC into the other as a control. At 4 weeks, ankle-brachial index (ABI) was significantly improved in legs injected with BMC compared with those injected with PBMC. Similar improvements were detected for transcutaneous oxygen pressure, rest pain, and pain-free walking time. Legs injected with PBMC cells showed much smaller increases of ABI and TcPO2. The improvements in the BMC-injected legs were sustained at 24 weeks [96]. The TACT study served as a basis for many other protocols of comparable studies which proved safety and feasibility of BMC treatment in CLI. The use of BMC instead of PBMC is more favourable due to the fact that bone marrow punction is more rapidly and less variable in terms of quality and number of cells. PBMC collection requires expensive G-CSF injections over 5 consecutive days and plasmapheresis for several hours, making this process costly and time-consuming. Lu et al. were able to prove in a double-blind placebo-controlled trial that bone marrow-derived mesenchymal stem cells are more potent than bone marrow mononuclear cells in terms of time to wound healing, painless walking time, ABI, TcPO2, and magnetic resonance angiography (MRA) analysis. No difference was detected regarding pain relief and amputation rate [101].

Since the TACT publication, there have been more than 30 reported therapeutic cell trials in patients with PAD. Recently, Fadini et al. did a review of the literature searching for effective autologous cell therapy studies for the treatment of PAD [34]. They found 108 reports, of those 42 were clinical trials and 37 fulfilled the criteria to be meta-analysed. In general cell therapy was effective in improving surrogate indexes of ischaemia, subjective symptoms, and hard end points (ulcer healing and amputation). In all trials with data on ankle-brachial index and transcutaneous oxygen pressure, ABI and TcPO2 improved significantly during therapy;

regarding only controlled trials, the effect on ABI was smaller, but still significant, whereas the effect on TcPO2 was not detectable with statistical significance. Increase in walking capacity and pain relief was demonstrated in all trials. Ulcer healing significantly improved in the active treatment group vs. the control group in controlled cell therapy trials. Amputation rate was documented in only two controlled trials of cell therapy indicating a significant benefit in terms of limb salvage as compared to control treatment. This meta-analysis demonstrates that cell therapy is able to significantly improve ABI, TcPO2, rest pain, pain-free walking distance, ulcer healing, and limb salvage but the physiological explanation remains obscure.

The National Institute of Health publishes clinical trials that have been approved by the Food and Drug Administration, European medicines agency, and other national regulatory bodies under an identification number and activation status in www.clinicaltrials.gov as shown as of September 2012. Currently, 34 studies are listed if "critical limb ischemia and stem cells" are entered as search terms and results are controlled for inclusion criteria. Of those, 9 studies are recruiting patients, 14 are completed, 5 are not recruiting, and the remaining 4 studies have an unknown status. These studies will deliver more knowledge on the effect of this therapy. Current studies prove that cellular therapy is well tolerated and offers rising hope for patients with peripheral arterial disease. Administration of autologous bone marrow mononuclear cells is easy to perform, inexpensive, and safe, with a definite ameliorating effect on limb ischaemia. However, specification of the target cell population, route of administration, and dose escalation needs to be evaluated in larger case-controlled studies.

8.9 Future Directions

Several sources of autologous stem cells have been tested in preclinical and clinical trials: adipose-derived stem cells (ASC), bone marrow-derived mesenchymal stem cells (BM-MSC), human umbilical vein endothelial cells (HUVEC) and embryonic stem cell-derived endothelial cells (ESC-EC), embryonic stem cell-derived mesenchymal stem cells (ESC-MSC), and induced pluripotent stem cell-derived endothelial cells (iPSC-ECs). Since iPSCs can be derived from a variety of tissues and have high replicative capacity, they are potentially an unlimited source of autologous therapeutic cells. Preclinical studies using iPSC-derived cells have shown promise for treatment of sickle-cell anaemia, Parkinson's disease, ß-thalassemia, and peripheral arterial disease (PAD) [102–105]. Genetic and epigenetic abnormalities are the main issues raised against iPSC-ECs but may be obviated with careful generation, culture, and selection of iPSCs [106]. In addition to safety concerns, there are manufacturing hurdles to overcome for therapeutic application. Current reprogramming methods are inefficient, although reprogramming methods continue to improve. Whether these cell types will replace currently available clinical routines or will remain further alternative remains to be seen.

Stem cells are a promising reagent for vascular as well as tissue repair, but many obstacles need to be overcome before they can be widely used in clinical routine. Imaging techniques have pointed to the problem of limited cell viability of transplanted cells in ischaemic tissues that remains a major concern. Although the studies suggest that the cells can exert a therapeutic effect even without prolonged survival, strategies to enhance viability by using survival factors (i.e. HIF1α, Akt, bcl2) are under development. The addition of soluble factors and three-dimensional extracellular matrices may further promote cell survival and/or angiogenesis and add a new quality to this up to now successful story.

8.10 Conclusion

Benefits were reported from clinical trials using different sources of stem cells in patients with PAD including improvement of ABI and TcPO2, reduction of pain, and lower major amputation rates. Nonetheless, large randomised, placebo-controlled, double-blind studies are necessary and are currently ongoing to provide stronger safety and efficacy data on cell therapy. Current literature is supportive of intramuscular (bone marrow) cell administration as a relatively safe, feasible, and possibly effective therapy for patients with PAD who have no option for conventional revascularisation.

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Chapter 9
Stem Cells in Wound Healing

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Abstract Cutaneous wound healing encompasses a well-organized process with related but distinct phases, namely, as inflammatory response, proliferative phase, and remodeling. These interrelated events are orchestrated by different types of cells, chemokines, and hormones to repair the injured area and support the integrity of the tissue. During the wound healing, the cellular responses against the injury are mainly coordinated by mesenchymal stem cells which generate paracrine signals and invoke hemopoietic stem cells, hair follicle stem cells, endothelial precursor cells, and epidermal stem cells to differentiate into resident tissue cells. These cell types have a particular role in each step of healing phases and accelerate the wound closure.

This chapter focuses on the involvement of stem cells in various phases of wound healing and recent therapeutic strategies utilizing stem cell therapy and technology for the treatment of tissue injury.

Keywords Wound healing • Stem cell • Stem cell therapy

Abbreviations

ASCs	Adipose-derived stem cells
BM	Bone marrow
BM-MSCs	Bone marrow derived MSCs
HSCs	Hematopoietic stem cells
MSCs	Mesenchymal stem cells
TA	Transit amplifying

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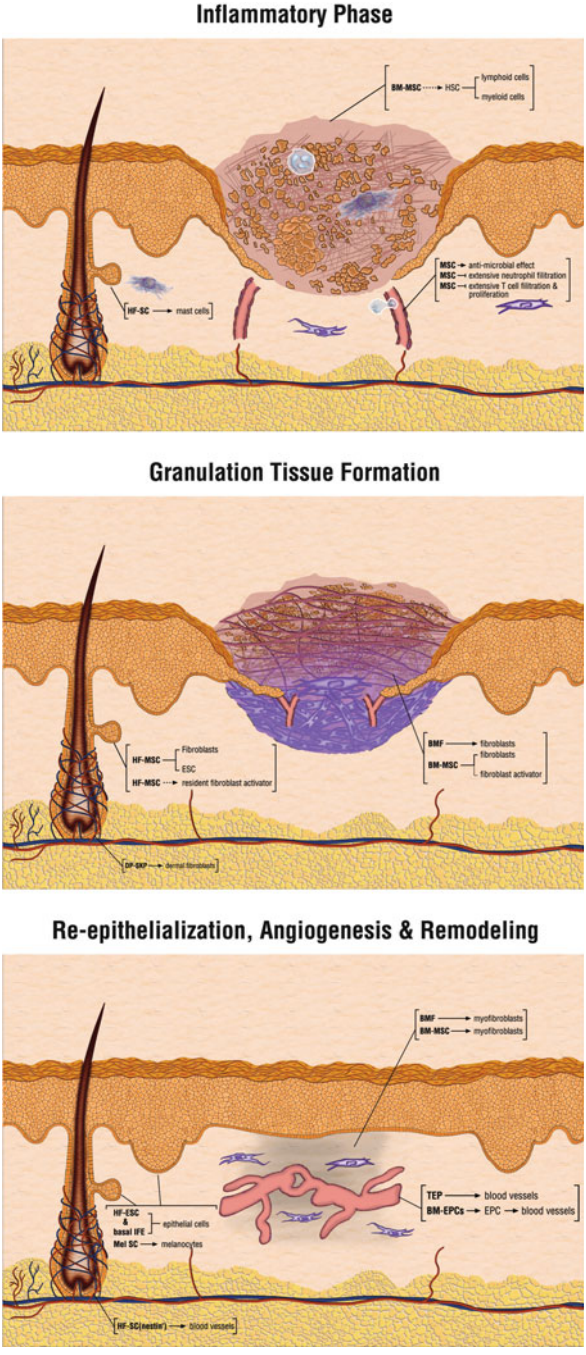
K. Turksen (ed.), *Stem Cells: Current Challenges and New Directions*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4614-8066-2_9,

9.1 Introduction

Adult skin is composed of two tissue layers: multilayered stratified squamous epidermis which serves as a barrier against environment and an underlying collagen-rich connective tissue layer which provides a mechanical strength [1]. During the cutaneous wound healing, maintenance and support of the skin are provided by proliferation, migration, differentiation, and apoptosis of different cell types [2]. Wound healing is achieved by consecutive yet overlapping phases such as hemostasis, inflammation, scar tissue formation, and subsequent remodeling [3] (Fig. 9.1). Following a normal cutaneous injury, platelets migrate into the wound area forming a temporary shield the homeostatic plug that prevents the leakage of blood constituents into the wound bed and blockades the invasion of microorganisms [4]. The homeostatic plug composes of a platelet-embedded fibrin and fibronectin-rich matrix that serves as a provisional matrix for the cell migration, attachment for the reservoir of cytokines, and growth factors [5]. A few hours later, the recruitment of macrophages and neutrophils kick-starts the process of inflammation in response to the changes on the endothelial cell surface proteins and due to release of chemoattractive signals such as TGF-ß (transforming growth factor-beta), platelet-derived growth factor (PDGF), and formylmethionyl peptides produced by bacteria [6]. Leukocytes are pulled out from the blood circulation by the help of selectin family members that weakly bound on endothelial cell surface. Later on the firmer adhesion of leukocyte is provided by ß2 class of integrins which results in diapedesis in the manner of transmigration from blood vessel through the dermis [7]. Neutrophil infiltration is necessary to clean the bacteria and other microorganisms from the injured area, which is either then phagocyted by macrophages or taken out by eschar after a few days. At the same time circulating blood monocytes help to increase the number of macrophages infiltrated into the wound area to clean the remaining pathogens [8, 9]. In addition to their role in phagocytosis of the debris and neutrophils, macrophages are necessary for secreting cytokines and growth factors to trigger the cell proliferation, migration, and matrix remodeling necessary for the formation of granulation tissue and new blood vessels [9]. After the inflammation phase, the next step is the scar tissue formation in which the granulation and epithelialization of the wound area take place [3]. During the overlapping phases of reepithelialization and granulation, keratinocytes proliferate and migrate over the newly laid granulation tissue

Fig. 9.1 Distinct phases of wound healing emphasizing the contribution of different stem and progenitor cell populations in the inflammatory (*top panel*), granulation tissue formation (*middle panel*), and reepithelialization, angiogenesis, and remodeling. *Stripped arrows* represent the paracrine signaling mediated by indicated stem cell population, while *open-ended arrows* describe the differentiation of designated stem cell population into a particular resident cell type. *MSC* mesenchymal stem cell, *BM-MSC* bone marrow-derived MSC, *HSC* hematopoietic stem cells, *HF-SC* hair follicle stem cells, *HF-MSC* hair follicle-derived MSC, *BMF* bone marrow-derived fibrocytes, *DP-SKP* dermal papilla skin progenitor cell, *ESC* epithelial stem cell, *HF-ESC* hair follicle-derived ESC, *basal IFE* basal interfollicular epidermis cells, *MelSC* melanocyte stem cell, *TEP* tissue endothelial progenitors, *BM-EPC* bone marrow-derived endothelial progenitor cells


Inflammatory Phase
BM-MSC HSC
lymphoid cells
myeloid cells
MSC anti-microbial effect
MSC extensive neutrophil filtration
MSC extensive T cell filtration & proliferation
HF-SC mast cells
Granulation Tissue Formation
HF-MSC Fibroblasts
ESC
HF-MSC resident fibroblast activator
BMF fibroblasts
BM-MSC fibroblasts
fibroblast activator
DP-SKP dermal fibroblasts
Re-epithelialization, Angiogenesis & Remodeling
BMF myofibroblasts
BM-MSC myofibroblasts
TEP blood vessels
BM-EPCs EPC blood vessels
HF-ESC & basal IFE epithelial cells
Mel SC melanocytes
HF-SC(nestin+) blood vessels

which is characterized by the invasion and proliferation of fibroblasts and the formation of new blood vessels at the dermis [5, 10, 11].

Granulation tissue is formed by subsequent recruitment of macrophages, fibroblasts, and endothelial cells to the wound area. Macrophages constantly provide the cytokines necessary for recruitment of fibroblasts and endothelial cells for the formation and development of fibrous tissue new blood vessels, respectively [6, 9]. Upon induction by cytokines and growth factors, fibroblasts from the edge of the wound proliferate and migrate into the clot by secreting extracellular matrix (ECM)—degrading matrix metalloproteinases (MMPs) to replace the provisional matrix with new ECM. During this proliferative phase fibroblasts synthesize the new connective tissue (granulation tissue) containing a matrix of elastin fibronectin, collagen fibers, proteoglycans, and other proteins [12]. As the wound edges approach to each other, myofibroblasts, a specialized population of fibroblasts with smooth muscle cell-like contractile property, arise from the wound-edge fibroblasts through a process regulated by PDGF, TGF-ßs, and fibroblast growth factor (FGF) [13]. Myofibroblast is responsible for wound closure by increasing the wound tensile strength through collagen I, III, VI, and XII matricellular protein deposition and concomitant wound contraction by contractile bundles of actin stress fibers pulling up the collagen fibrils, thus reducing the wound furrow [13, 14]. Following fibroblasts, angiogenic endothelial cells populate the newly laid granulation tissue and form new blood vessels to deliver the oxygen and crucial nutrients for cellular metabolism [15]. While the granulation tissue turns to a scar tissue, the immature collagen synthesized by fibroblasts is remodeled to larger collagen bundles to increase the intermolecular cross-links and tensile strength of the newly formed tissue [3, 13]. The remodeling phase is characterized by the increase in the collagen bundle diameter which becomes more orientated and cross-linked to each other during the maturation phase and the degradation of hyaluronic acid and fibronectin [5, 12]. Inflammatory cells underneath the migrating edge of the epithelium and myofibroblasts and vascular cells following the wound closure undergo apoptosis forming the acellular scar resulting in transformation of the granulation tissue to the scar tissue [3, 5].

9.2 Types of Stem Cells Implicated in Wound Healing

Numerous studies have provided significant evidence for the importance of stem cells from different origins during the wound healing starting from the earliest phase of the wound healing “inflammation” to the maturation phase “reepithelialization and remodeling” [2]. Superficial wounds may be able to heal without enrolling stem cells; however, repair of deep tissue injury or wounds requires the contribution of not only stem cells with mesenchymal and hematopoietic origin but also resident tissue-specific stem cells during different well-concerted repair cascades necessary for restitution of tissue integrity after injury [2, 16].

Mesenchymal stem cells (MSCs) are non-hematopoietic, adherent multipotent cells of stromal origin with differentiation potential into precursor cells for the bone, cartilage, muscle, tendon, stroma, and adipose tissues [16, 17]. According to recent studies MSCs can be found in all vascularized tissues as pericytes that are pleiotropic in character and bear the ability to respond to each event in the environment [17, 18]. MSCs can be isolated from the adipose tissue, from the amniotic fluid, and most commonly from the bone marrow and do not cause immune rejection [16, 17, 19]. Owing to their differentiation into mesenchymal lineages, MSCs express many different surface marker but do not express endothelial and hematopoietic markers such as CD11, CD14, CD31 (PECAM-1), CD33, CD34, CD45, and CD133 [20]. According to International Society for Cellular Therapy (ISCT) records, MSCs should have the expression of CD73, CD90, and CD105 (SH2 or endoglin) but must lack the expression of CD45, CD34, monocyte, macrophage, and B cell markers [21]. In addition, MSCs have also been reported to express CD73 (SH3 and SH4), CD106 (VCAM-1), CD44 (hyaluronic acid receptor), CD90 (Thy 1.1), CD29, STRO-1, CD54 (ICAM-1), CD13, CD47, CD146, CD49a, CD164, and CD166 surface markers [20]. Apart from these surface antigens, different surface receptors are expressed on MSCs such as growth factor receptors EGFR (epidermal growth factor receptor), bFGFR (basic fibroblast growth factor receptor), IGFR, PDGFR (platelet growth factor receptor), and TGF-ß1RI, TGF-ß1RII [20]. Expression of chemokine receptors including CCR1, CCR2, CCR4, CCR6, CCR7, CCR9, CCR10, CXCR1, CXCR2, CXCR4, CXCR5, CXCR6, and CX3CR1 and cytokine receptors (Docheva 23–24) such as IL-1R, IL-3R, IL-4R, IL-6R, IL-7R, IFN-γR (interferon-gamma receptor), and TNFI, TNFIIR [22] was also shown to be putative MSC markers. In addition, MSCs are characterized by surface molecules including ICAM-1, ICAM-2, VCAM, and ALCAM that are involved in cell-to-cell interactions [23]. The microenvironment milieu not only induces the MSC differentiation but also affects their paracrine signaling in response to various signals [24]. In the cutaneous wound healing, MSCs can control necessary cellular functions such as reduction of the inflammation, promotion of the angiogenesis, and induction of the cell migration and proliferation [2, 17, 24].

The key regulatory cells of wound healing such as the endothelial cells, fibroblasts, and keratinocytes are under the control of MSC paracrine signaling, especially involving the growth factors such as PDGF, VEGF (vascular endothelial growth factor), bFGF (basic fibroblast growth factor), keratinocytes growth factor (KGF), TGF-ß, and mitogens [16, 25]. It was reported that these paracrine signals endorse the proliferation of dermal fibroblasts, keratinocytes, and endothelial cells under in vitro conditions [24–26] which are necessary to accelerate the wound closure.

During the first step of the wound healing named as inflammation, oxidative stress condition generates a low-oxygen environment named as hypoxic condition that induces the bone marrow-derived MSCs (BM-MSCs) to increase their proliferation and migration capacity resulting in angiogenesis [27, 28]. Although bone marrow-derived cells are widely in use for wound healing experiments, it was reported that multipotent cells harvested from fat tissue called adipose-derived

stromal cells were able to give the same response as BM-MSCs under similar hypoxic conditions [29].

Hematopoietic stem cells (HSCs) are pluripotent cells which are able to give rise to all types of mature blood cells such as lymphoid (T, B, and natural killer cells) and myeloid cells (granulocytes, erythrocytes, monocytes, megakaryocytes) taking part in early stages of wound healing [30]. HSCs are able to differentiate into long-term repopulating cells (LT-HSCs), short-term repopulating cells (ST-HSCs), and restricted progenitor cells known as multipotent progenitors. Pluripotency of HSCs can be proved by producing long-term repopulation of multilineage clones in vivo [31]. The difference between mature (committed) and immature (not committed) cells can be discriminated by the expression of mature blood linage (lin)-specific markers. One of the well-known markers to identify the human HSCs is the cell surface antigen CD34. To obtain enriched population of HSCs, more surface receptor markers also need to be examined along with CD34. For example, $CD34^+$ $CD38^-$ cells are able to give long-term repopulating cells, whereas $CD34^+$ $CD133^+$, $CD34^+$ c-kit^+, and $CD34^+$ KDR^+ (vascular endothelial growth factor receptor-2) cell surface markers are used for the isolation of pure human HSCs population [32].

Although $CDCP1^+$ (CUB-domain-containing protein) marker is not expressed on mature blood cells, it can only be used to increase the purity of HSCs population along with $CD34^+$ and $CD133^+$ but not to distinguish the HSCs from other cell types [32]. Sca-1 in addition to c-kit and Thy-1 surface markers are restriction markers for the murine HSCs [33]. The expression of CD34 marker in murine is different from human HSCs [34]. Recent experiments showed that CD34 is expressed around 50 % in bone marrow of juvenile mice, whereas in the adult mice, 90 % of the HSCs do not express CD34. Therefore, for the pure HSCs isolation, $CD^{-/low}$ and c-kit^+ Sca-1^+ lin^- markers are used in HSCs purification from the adult mice bone marrow [35, 36]. In the bone marrow, HSCs are mingled together with endothelial cells which secrete SDF-1 (stromal-derived factor-1) that is recognized by CXCR receptor on HSPCs surface. SDF-1/CXCR4 association is important for the maintenance of HSPCs in the bone marrow under the normal conditions. In case of inflammation, SDF-1/CXCR4 complex is disrupted, and hematopoietic stem and progenitor cells (HSPCs) are released to the peripheral blood [37]. Another mechanism that increases the HSPCs number in the peripheral blood is the upregulation of neutrophil number in response to inflammation. Neutrophils secrete proteases such as matrix metalloproteinases-9 (MMP-9), neutrophil elastase, and cathepsin G, resulting in upregulation of SDF-1, CXCR4, SCF (stem cell factor), c-kit, and VCAM-1 (vascular endothelial factor-1) which are involved in the liberation of hematopoietic progenitor cells (HPCs) in blood circulation [38, 39].

Another type of stem cells called bone marrow-derived circulating endothelial precursor (CEP) cell takes role in the angiogenesis during the wound healing. Bone marrow-derived CEP cells can be isolated from adipose and granulation tissues [40]. CEP cells are similar to embryogenic angioblasts which are able to give rise to migratory endothelial cells. These migratory endothelial cells can proliferate, circulate in the blood and recruited to the site of inflammation, and differentiate into mature endothelial cells in response to hypoxia-inducible factor-1-induced stromal cell-derived factor-1 that is expressed under the hypoxic conditions [40, 41].

The observations that the skin deprived of hair follicles exhibit a perturbation in the harmony of wound healing phases with delayed wound closure suggested an important role for the hair follicles as the harborer of epithelial stem cells (ESCs) [42]. ESCs in three different sections of epidermis, namely, the interfollicular epidermis (IFE), the sebaceous gland, and the outer root sheath of hair follicle in the hair follicle structure, not only have the self-renewal capacity and regenerate their specialized progeny during phases of hair follicle growth (i.e., growth (anagen), cessation (catagen), and rest) but also have the capacity to construct all the cells of stratified epithelial in response to epidermal injury [10, 42, 43]. The IFE runs down to the outer root sheath (ORS) of hair follicle through the infundibulum (upper segment) and isthmus (middle segment), while the sebaceous glands are ensured just above the ORS [44]. In mice, transcriptional profiling identified α_6-integrin^{low} CD34$^-$ and Sca-1$^-$ expression in ESC from the upper infundibulum [45], while bulge cells revealed CD34$^+$ keratin15$^+$ and CD200$^+$ phenotype [11, 46]. Similarly, in human system hair follicle stem cell markers were identified with CD200^{high} and α_6-integrin^{high} profile for interfollicular and follicular ESC [46] and keratin15$^+$ CD34$^-$ and connexin43$^-$ expression pattern for bulge ESC [44, 47]. Connective tissue sheath (CTS) and papilla (CTP) surrounding the hair shaft found the fibroblasts, endothelial cells, mast cells, and macrophages in granulation tissue after wounding and show pluripotency in vitro by generating extramedullary HSCs [48] and cells with adipogenic, osteogenic [49], and neural differentiation potential [50].

9.3 Stem Cells in Inflammation

During the inflammation, infiltration of neutrophils and monocytes is the main event to trigger the sequential steps of wound healing process and clean the bacteria from the injured area (Fig. 9.1; top panel). The duty of the MSCs is to regulate the microbial activity by producing not only LL-37 which is an antimicrobial peptide originally identified to be stored in specific granules of neutrophils but also immune modulative factors that induce immune cells to increase their cell surface receptors that prompt phagocytosis for the duration of inflammation [51]. Moreover, in nonhealing chronic wounds, MSCs are responsible for the reduction of excessive T cell and in turn neutrophil infiltration [52]. Upon injury, inflammation induces the T cell activation and the production of inflammatory molecules such as TNF-α, IFN-γ, IL-α, and IL-1ß, resulting in increased neutrophil infiltration. Inflammatory milieu induces MSCs to release nitric oxide (NO) which represses T cell proliferation through the inhibition of transcription factor Stat-5 phosphorylation [53]. Moreover, NO suggested to have a negative effect on the nuclear factor kappa B and mitogen-activated protein kinase which are known to upregulate expression of proinflammatory cytokines playing an important role in neutrophil infiltration [17]. Given that neutrophils not only degrade the newly forming extracellular matrix by releasing a large amount of collagenase but also destroy the growth factors such as PDGF and TGF-ß that are necessary for healing by releasing elastase [2, 3, 9], the presence of

MSCs downgrading excessive T cell activation and neutrophil infiltration is critical in controlling the intensification of the inflammation process.

In addition to the function of MSCs during the inflammation, bone marrow-derived HSCs take part in the generation of the inflammatory cells. Bone marrow-derived HSCs are known as the main source of leukocytes [31]. BM-MSCs orient the HPCs to differentiate into dendritic cells which give rise to the formation of granulocytes such as eosinophil, basophile, and especially neutrophils in a process called myelopoiesis [54, 55]. Another source of the resident leukocyte population in the skin is the stem cells of hair follicle bulge which have the potential to differentiate along mast cell upon induction [56]. In a response to cutaneous wound, hair follicle cells secrete the stem cell factor known as mast cell proliferative factor to establish a signal for the differentiation of mast precursor cells to mature into mast cells [9, 57]. These intradermally deposited HPC and mast precursor cells could be the source of macrophages, dendritic cells, and mature mast cells during the wound healing [2].

9.4 Stem Cells in Granulation Tissue Formation

In the proliferative phase of wound healing, resident and stem cell-originated neoteric fibroblasts, endothelial and epithelial cells are recruited to the wound area and induced to proliferate in response to a cocktail of host factors secreted during the inflammatory phase (Fig. 9.1; middle panel). Fibroblasts are the quintessential contributor of granulation matrix and trigger the gradual replacement of fibrin matrix with a collagen- and fibronectin-rich matrix through proliferation and migration. Accumulating evidence identified distinct origins for the wound fibroblast progenitor cells, such as bone marrow-derived fibrocytes [58] and MSCs [59] and multipotent dermal MSCs [26].

The initial report describing the existence of fibrocytes identified a group of fibroblastic $CD34^+$ and collagen I-positive bone marrow-derived mesenchymal progenitor cells in the peripheral circulation [58]. The phenotypic characterization indicated a distinctive chemokine and cytokine pattern for fibrocytes in addition to expression of fibroblast-specific genes including both collagen I and collagen III genes, vimentin, and fibronectin. Although these cells expressed the leukocyte common antigen CD45 and CD34, a marker shown to be expressed on HSCs, they displayed a fibroblastic morphology with unique projections resembling pseudopodia [58]. When fluorescently labeled peripheral blood-derived mouse fibrocytes were injected into mice with skin wound, secondary lymphoid chemokine (SLC) mediated the recruitment of fibrocytes to the wound sites through cell surface expression of SLC receptor CCR7 [60]. Although the fibrocytes homed to the sites of tissue injury initially express CD34 and CD45, in response to fibrogenic cytokines such as TGFß-1, fibrocytes differentiate into mature fibroblasts and α-smooth muscle actin-expressing myofibroblasts through a poorly characterized process that coincides with the diminished CD34 and CD45 expression and increased prolyl 4-hydroxylase activity [14, 60, 61].

Cell-lineage tracing studies performed on mouse models of skin wound where mice were transplanted with bone marrow from reporter gene-transgenic mice indicated the presence of reporter gene-positive circulating and wound-associated fibrocytes in the host mice thus, identifying the source of fibrocytes as HSCs [59, 62]. Different than resident fibroblasts which could only synthesize collagen I, recruited fibrocytes were shown to produce both collagen I and III and also displayed fibroblast-specific antigen-1 (FSP-1) on the cell surface [59, 62].

Identification of $CD45^-$ fibroblastic mesenchymal cells in the unwounded normal skin of the chimeric mouse fortified the presence of BM-MSCs other than fibrocytes as the source of dermal fibroblast population [59]. These fibroblastic BM-MSCs were shown mostly to be located within the outer and inner root sheath running down to the dermal papilla secondary to the bulge region of the hair follicle and were attained in the healthy dermis in a steady state resourcing the collagen type III production [59]. Another study [11], using a chimeric mouse transplanted with EGP^+ bone marrow, found that BM-MSCs were detectable in the burned wound tissue from the early time to late time points at the granulation tissue making up more than the half of the fibroblasts population [63]. In addition, BM-MSCs could differentiate into endometrial stromal fibroblasts under the necessary hormonal stimuli in vitro, suggesting the involvement of these cells in the renewal of endometrium at each menstruation cycle [64]. In order for BM-MSCs to mobilize to the site of injury, they must respond to the local activation of resident fibroblasts by PGDF-B which results in homing and differentiation of BM-MSCs into myofibroblasts in a bFGF and epithelial neutrophil-activating peptide-78 (CXCL5)-dependent manner [65]. Connective tissue growth factor (CTGF) also appears to be important factor in BM-MSCs transformation into myofibroblasts; it is thought to drive myofibroblasts differentiation and promote fibrogenesis in association with TGFß-1 [66].

BM-MSCs can also contribute to the granulation phase of wound healing by paracrine signaling inducing the differentiation of tissue-derived stem cells into fibroblasts [67]. Isolation of multipotent mesenchymal cells from the dermis that has multilineage differentiation potential indicated the presence of such a target for the paracrine factors released by BM-MSCs [68, 69]. Arising from hair follicle stem cells dermal MSCs have high regenerative capacity and can give rise to multiple cell lineage including myofibroblasts, adipocytes, chondrocytes, osteoclasts, and keratinocytes [26, 68, 69]. Introduction of dye-labeled dermal sheath harboring MSCs into skin wounds compensated for the loss of CTS fibroblasts by inducing the regeneration of these cells from the hair follicle bulb [70].

9.5 Stem Cells in Reepithelialization

The primary source of keratinocytes in the reepithelialized wound is the ESCs repository within the basal layer of IFE and the resident ESCs within the hair follicle bulge [2] (Fig. 9.1; bottom panel). ESC fate decision is controlled by Wnt/β-catenin and c-myc signaling in that the inhibition of Wnt pathways or deregulation of c-myc

results in the depletion of ESC supply in the bulge and IFE [71, 72]. Compared to ESCs of the bulge, ESCs from the basal layer of IFE have limited multipotent character in that with their high self-renewal capacity, they mainly contribute to the transit amplifying (TA) cells of the basal layer [73, 74]. TA cells have short-term proliferative potential with few cycles of cell division before they undergo terminal differentiation through a cross-talk signaling involving the suppression of p63 expression by Notch-1 activation [75]. Transplantation and in vitro studies showed that ESCs isolated from the bulge region of hair follicle have long-term self-renewal potential with the capacity to repopulate multiple layers of the epithelium [10, 76, 77]. However, only 25 % of the cells in a regenerated epithelium were found to have hair follicle origin whose function was mainly to give rise to TA cells which were later on replaced by the nonbulge interfollicular keratinocytes from the basal layer [10, 78]. These findings suggested that bulge-derived cells were essential in the early stages of wound healing to accelerate the wound repair but were not required for the wound closure. Initial studies addressed the hair follicle shaft as the house for the melanocyte stem cells (melSCs) necessary for the repigmentation of the healed wound [79]. Implantation of reporter-dye-labeled intact hair follicles into porcine skin demonstrated labeled melanocytes in the central areas of dark pigmentation [80] confirming that melanogenesis is directed by the melSC-derived melanocytes within the ORS of hair follicle which migrate out to populate the epidermis [79].

9.6 Stem Cells in Revascularization

Revascularization of the wound area is coordinated together with reepithelialization and granular tissue formation [3] (Fig. 9.1; bottom panel). Neovascularization for wound healing involves mainly angiogenesis [81]; however, in case of deeper wounds vasculogenesis has also shown to participate in wound vascularization through de novo generation of blood vessels [15, 82]. Studies in mice indicated that angiogenic progenitor/stem cells participated in angiogenesis were tissue-resident endothelial precursors (TEPs) [83] and pluripotent nestin-positive cells [84]. Transplantation of TEPs into the injured skeletal muscle tissue gave rise to a nascent capillary network that is linked to the preexisting vascularate [83]. Nestin-expressing pluripotent cells residing within the mesenchyme of the hair follicle bulge area [84, 85] have the capacity to differentiate into precursor cells displaying endothelial cell-specific markers and promote the formation of angiogenic vasculature [84, 86]. In transgenic mice model expressing GFP-tagged nestin, microvasculature network formed during the cutaneous wound healing was originated from the fluorescently labeled hair follicle stem cells [84, 87]. Consistently, nestin expression was evident in the neovascularization generated in human myocardium following the transient ischemia and in skin during wound healing [86]. Nestin-expressing stem cells were also shown to have the potential to differentiate into neuroprogenitor lineage fate [88] and mesenchymal such as adipogenic, osteogenic, and chondrogenic lineage fate [49].

Several reports highlight the involvement of bone marrow-derived endothelial precursor cells (BM-EPCs) in the vasculogenesis of deep tissue damage [15]. In response to trauma and ischemia, increase in the systemic levels of angiogenic factors such as VEGF-A, HGF, and nitric oxide results in the activation of metalloproteinases responsible for the release of stem cell-active cytokine "Kit ligand" which in turn leads to the increased stem cell circulation in the peripheral blood and recruitment of BM-EPCs to the side of injury [15, 89, 90]. Increased VEGF levels due to hypoxic nature of wounds induce the chemokine stromal-derived factor-1α (SDF-1) which serves as a homing signal for BM-EPCs to the wound area [41]. In diabetic conditions, delayed wound healing can be restored by the application of SDF-1 that leads to an increase in the population of wound proangiogenic cells in the murine model of ischemia [91, 92].

Isolation of $CD14^+$ and $CD45^+$ cells from peripheral blood mononuclear cell population with endothelial cell markers such as vWF and VEGFR-2 suggested another source for EPCs [93, 94]. Transplantation of culture-expanded $CD14^+$ cells into surgically induced ischemic wounds showed that transplanted EPCs could directly participate in the de novo vasculogenesis and improved the capillary formation [95, 96].

Vasculogenesis, angiogenesis, and endothelial repair were also may be assisted by EPCs derived from multipotent adipose-derived stem cells (ASC) [97]. Although ASCs have been shown to release angiogenic factors and possess induced in vitro differentiation capacity into vWF [98] and VEGFR-2-positive EPCs [99], their direct presence in the wound neovascularization has not been documented [97]. However, studies using ASCs in the cell therapy for ischemic disease models showed that systemic delivery or transplantation of ASCs flourishes the revascularization process in ischemic muscle tissues possibly through stabilizing the formation of nascent blood vessels [98, 100–102]. In line with these findings, recent reports demonstrated that ASCs cells associated with the perivascular regions in microcapillaries [101, 103, 104] and facilitated the in vivo formation of new blood vessels by interacting with endothelial cells in a pericyte manner [105].

9.7 Stem Cell Therapy in Wound Healing

Self-renewing characteristics and multipotent differentiation potential brought the use of stem cells forward as therapeutic agents for tissue repair and homeostasis. Given the role and functional contribution of stem cells in wound healing phases, application of stem cells to wounded area brings about certain benefits to skin regeneration in chronic nonhealing wounds or wounds that are difficult to heal.

In recent years, research about the use of stem cells in the treatment of injury and wound healing has mostly focused on the utilization of adult stem cells, particularly MSCs (Table 9.1). Several studies conducted both in animals and human reported the effectiveness of MSCs in wound healing. In vivo animal studies suggested that exogenous application of MSCs increases the wound closure in rodents through

Table 9.1 Summary of selected key studies

Model	Cell type	Delivery method (number of cells)	Outcomes	Reference
Deep burn wounds in rats	Allogenic and autogenic BM-MSCs	Topical application (2×10^6)	New vessel and granulation tissue formation, decrease in inflammatory cell infiltration	Shumakov et al. [106]
Excisional wounds in diabetic mice	Allogenic BM-MSCs	Topical application (7.5×10^5)	Enhanced epithelialization, granulation tissue formation, and angiogenesis	Javazon et al. [107]
Nude rats with defected skin	Human MSCs	Collagen-derived skin substitute (5×10^6)	Increased wound healing	Nagakawa et al. [108]
Surgical defects in mice	Human BM-MSCs	Fibrin polymer (1×10^6) and intravenous (2×10^6)	Scar-free healing, little immunoreactivity	Mansillla et al. [109]
Excisional wounds in normal and diabetic mice	Allogenic BM-MSCs	Intradermal injection (1×10^6)	Increased angiogenesis and cellularity	Wu et al. [110]
Excisional wounds in mice	Allogenic BM-MSCs	Intravenous injection (1×10^6)	Accelerated wound healing	Sasaki et al. [111]
Human chronic nonhealing wounds	Autologous BM aspirate and cultured MSCs	Subcutaneous injection of BM aspirate, topical application of MSCs	Complete healing	Badiavas et al. [114]
Human acute and chronic wounds	Autologous BM-MSCs	Fibrin spray ($1\times10^6/cm^2$)	Complete healing of acute wounds, increased closure in chronic wounds	Falanga et al. [118]
Human nonhealing wounds	Cultured autologous BM-MSCs	Collagen sponge	Complete closure or increased dermal regeneration	Yoshikawa et al. [121]
Human chronic nonhealing ulcers	Cultured autologous BM-MSCs	Intramuscular injection ($>1\times10^6/cm^2$) and topical application	Reduction in wound size	Dash et al. [122]
Excisional wound in nude mice	Human ASCs	Collagen gel (1×10^6)	Decrease in wound size, accelerated reepithelialization	Kim et al. [123]
Excisional wound in nude mice	Human ASCs	Dermal matrix	Improved wound healing with cellular differentiation	Altman et al. [125]

(continued)

Table 9.1 (continued)

Model	Cell type	Delivery method (number of cells)	Outcomes	Reference
Nude mice	Keratinocyte stem cells, TA cells	10^4 (5×10^5)	Reconstitution of epidermis	Li et al. [127]
Human full-thickness burn wound	Hair follicle	Dermal template	Complete reepithelialization, haired scalp	Navsaria et al. [128]
Excisional wounds in normal and diabetic mice	BM-derived HSCs (side population)	Topical application (8×10^3)	Improved healing	Chan et al. [129]
Excisional wound in mice	Cultured and genetically modified HSCs	Subcutaneous injection (1×10^7)	Enhanced angiogenesis, increased fibroblast proliferation and migration	Templin et al. [130]

BM bone marrow, *MSCs* mesenchymal stem cells, *BM-MSCs* bone marrow-derived MSCs, *ASCs* adipose-derived stem cells, *TA* transit amplifying, *HSCs* hematopoietic stem cells

hastening epithelialization and increasing angiogenesis and granulation tissue formation. For instance, Shumakov et al. reported that MSC topical transplantation onto the burn wounds in rats promotes new vessel and granulation tissue formation in conjunction with a decrease in the inflammatory cell infiltration [106]. Similarly, direct application of stromal progenitor cells isolated from bone marrow resulted in a significant improvement in the epithelialization, granulation tissue formation, and neovascularization of wounds in diabetic mice compared to their controls [107]. Nagakawa et al. used human MSCs along with bFGF in nude rats with defected skin and described an increased wound healing and MSC differentiation into the epithelium [108]. Furthermore, administration of human BM-MSCs both topically with a fibrin polymer and intravenously in a full-thickness skin defect model resulted in scar-free healing with minimal immunoreactivity in the host [109]. In another controlled diabetic mice study, BM-MSCs treatment via local injection into the cutaneous wound area significantly accelerated the closure both in normal and diabetic mice when compared with vehicle control. Evidence of high levels of keratinocyte-specific protein, proangiogenic factors, VEGF, and angiopoietin-1 expression by MSCs also suggested a role for these cells in wound healing through endotheliogenic differentiation supporting angiogenesis [110]. Moreover, transdifferentiation of MSCs into keratinocytes, endothelial cells, and pericytes in wound area was observed following the intravenous injection into the mice maintaining the wound healing [111]. Interestingly, wound treatment with BM-MSCs in rats also showed an improved repair quality with increased tensile strength in healing wounds in addition to the significant acceleration in wound closure [112, 113].

Remarkable clinical studies indicating the MSC application to nonhealing wounds have brought about promising outcomes with the improvement in wound closure. Badiavas et al. demonstrated that topical application and local injection of autologous MSCs isolated from bone marrow to wound area induced complete healing and tissue repair in patients with chronic ulcerations resistant to the conventional therapies [114, 115]. In a single case study, topical application of autologous BM-MSCs on neuro-ischemic wound in patient with type 2 diabetes improved the vascularization and decreased wound size in 7 days after treatment without any systemic response [116]. Similarly, another positive result from a diabetic patient who underwent autologous transplantation of BM-MSCs indicated a total wound closure on the chronic ulcer area with enhanced angiogenesis [117]. Although these reports provided significant data for stem cell therapy in wound healing via direct application, stem cell delivery to the wounded area is another challenging aspect for the therapeutic use. Falanga et al. used a novel fibrin spray approach in the topical administration of autologous MSCs obtained from bone marrow aspirates. The patients suffering from acute wounds after surgical excision for the nonmelanoma skin cancer treatment showed healing in 8 weeks, whereas patients with one year chronic leg ulcer wounds had significant increase in wound closure within 20 weeks [118]. Radiation burn of a patient was in a favorable condition during 11 months after multidisciplinary therapy with MSCs supported with Integra® artificial skin substitute suggesting the improvement of conventional therapeutic approach via stem cell utilization [119]. In another clinical case study, researchers applied the BM-MSCs in combination with a graft containing autologous fibroblasts immobilized on a biodegradable collagen membrane to the diabetic foot ulcer. In this study, MSCs were also directly injected into wound edges on days 7 and 17 which accomplished a decrease in wound size together with an increase in vascularity and dermal thickness on day 29 [120]. In a large cohort study carried out by the participation of 20 subjects with various nonhealing wounds caused by burning, trauma, and pressure ulcers, cultured autologous MSCs on collagen sponge scaffold were used as treatment. Ninety percent of the grafted patients showed wound healing through fibrous and vascular regeneration, thirteen of whom exhibited a complete closure and five of whom displayed improved dermal regeneration [121]. Dash et al. conducted a randomized controlled study of 24 patients with the aim of analyzing the autologous stem cell use in the treatment of chronic nonhealing ulcers. Cultured BM-MSCs were not only intramuscularly injected into the wound edges but also topically applied onto the wounds in the treatment group. After 12 weeks, wound size was reduced significantly up to75 % in the treatment group, whereas there was only 23 % reduction in the wound area in control group subjected to the standard wound treatment procedure [122]. Up to now, this the largest study that demonstrates the benefit of MSCs in the wound healing therapy.

Adipose tissue is another potential source of adult stem cells which possess similar characteristics of BM-MSCs. Given their multipotent nature and easy access from human liposuction aspirates, ASCs might become even more attractive than MSCs derived from bone marrow for therapeutic purposes. Kim et al. indicated in vitro promoting effect of ASC on human dermal fibroblast proliferation through

cellular contact and paracrine signaling. Additionally, in this study, a reduction of wound size and acceleration of reepithelialization were observed after ASC application within collagen gel solution onto excisional wounds in mice [123]. In a recent study, ASC sheets were found to serve as a viable matrix for healing of full-thickness wound defect in a mouse model [124]. Human ASCs were also shown to have capability to differentiate into fibroblastic, endothelial, and epithelial lineages in wound area when delivered within a dermal matrix into mice and were shown to improve the wound healing [125].

The use of cellular therapy based on MSCs in wound healing also brings up the question of the potential use of epidermis resident stem cells as a therapeutic approach. In an example Oshima et al. reported that transplantation of multipotent cells from mice bulge region could generate the hair follicles, sebaceous glands, and epidermis [76]. Likewise profiling analysis of hair follicle stem cells extracted from murine bulge demonstrated their renewal ability to give rise to all the epithelial cell layers of skin [77]. Furthermore, the stem cells isolated from human bulge region exhibited hair follicle differentiation in vitro [126] where both epidermal stem cells and TA cells were able to restore the epidermis in vivo by showing a great capacity for cell renewal [127]. Navsaria et al. reported that graft of hair follicle placed on a skin template increased the healing of full-thickness burn wounds with a complete reepithelialization through restoration of the epidermal stem cell population [128]. Although further research is needed for the use of epidermal stem cells, accumulating evidence suggests that they would become a valuable source of therapeutic applications due to their convenience and easy access to the skin.

HSCs are another conceivable adult stem cell source that can be utilized in cellular therapy of wound healing. In a preliminary study, HSCs isolated from bone marrow were first divided into two populations as c-kit^{+} Sca-1^{+} Lin$^{neg/low}$ CD34^{-} side stem cell population and the remaining bone marrow cells referred as the main population and then topically applied to excisional wounds of wild type and diabetic mice. Interestingly, only the application of side population stem cells demonstrated a significant improved healing in the diabetic mice group suggesting a novel treatment method in wound healing although the mechanism underlying the healing effect of this side population should be further investigated [129]. In 2008, Templin et al. applied a novel hematopoietic progenitor cell line which was genetically transfected with the active form of ß-catenin to the excisional wounds of mice. The application of transgenic HPCs to the wound area enhanced the wound healing by accelerating the migration and proliferation of fibroblasts and angiogenesis which was attributed to the paracrine effects of stem cells [130]. In addition to cutaneous wound healing, HSC administration to the corneal surface also resulted in an acceleration in tissue repair. In this study, topical application of bone marrow cells or CD117^{+} HSCs resulted in an increased reepithelialization on the corneal wounds of mice [131]. Furthermore, Abe et al. showed that HSPCs from the circulating blood contribute to the generation of epithelial cells and fibroblasts in the injured lung [132].

Taken together, stem cell therapy in wound healing has been giving promising results and possible modalities for clinical benefit. However, there are still crucial

points that need to be addressed when it comes to consider adult stem cells as an alternative therapeutic approach for wound healing. First issue arises from the patient's availability for stem cell therapy and most suitable stem cell type for the application should be discussed. Particularly in the application of autologous BM-MSCs, the age of patients becomes important since topical application of stem cells to the wounded area needs to be in adequate number in order to generate the desired therapeutic response, which is hard to achieve as MSCs reside in bone marrow decrease with the age [133]. This creates a problem not only because MSCs reside in bone marrow decrease with the age, but also the isolation of MSCs requires painful and invasive process not suitable for older patients. Another aspect of MSC transplantation is the heterogeneity existence in MSC populations together with the lack of standardized isolation method of MSCs and uncertainty about the characterization of these cells [21]. Further research is also needed to organize more effective treatment approaches through better understanding the mechanism of action of stem cells during skin regeneration and developing delivery methods in the transplantation of cells.

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Chapter 10
Cancer Stem Cells: The Gist of the Matter

Torben Redmer, Dirk Schumacher, Wasco Wruck, Reinhold Schäfer, and Christian R.A. Regenbrecht

Abstract During recent years, circumstantial evidence has suggested that tumor development and cancer heterogeneity are triggered by so-called cancer stem cells (CSCs). These cells comprise a distinct, yet rare population found in both hematological and solid tumors. CSCs initiate tumor formation when ectopically transplanted into immune-deficient mice and share properties of tissue stem cells and partially reprogrammed induced pluripotent stem cells (iPSCs). CSCs are capable of self-renewal and differentiation into every cell type of the primary tumor they reside in. CSCs are responsible for tumor recurrence given that they often develop resistance mechanisms against chemotherapeutic drugs, e.g., an increased expression of ABC transporters or increased DNA-repair activity. Understanding the mechanisms that maintain CSCs in their niche and the development of strategies for specific targeting are essential to improve cancer therapy.

Keywords Tissue-stem cells (TSCs) • Cancer stem cells (CSCs) • Murine model systems

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K. Turksen (ed.), *Stem Cells: Current Challenges and New Directions*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4614-8066-2_10,

Abbreviations

ABL	Abelson-kinase
ADAM	A disintegrase and metalloprotease
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APC	Adenomatous polyposis coli
BCR	Breakpoint cluster region gene
BMI B	Lymphoma Mo-MLV insertion region 1 homolog
BMP	Bone morphogenetic protein
BRCA	Breast cancer gene
BRAF	A serine-threonine kinase participates in receptor tyrosine kinase signaling
CD	Cluster of differentiation
CDH1	Cadherin 1
CKIa	Casein kinaseIa
CML	Chronic myeloid leukemia
c-MYC	Cellular myelocytomatosis
CSC	Cancer stem cell
CTLA-4	Cytotoxic T-lymphocyte 4
DAPT	*N*-[*N*-(3,5-difluorophenylacetyl)-L-alanyl]-*S*-phenylglycine *tert-butyl* ester
DTIC	Dacarbazine
DZNep	3-Deazaneplanocin A
EED	Embryonic ectoderm development
EMT	Epithelial–mesenchymal transition
EGF	Epidermal growth factor
ESA	Epithelial-specific antigen
EZH2	Enhancer of zeste gene 2
FDA	Food and drug administration
FGF	Fibroblast growth factor
FGFR	FGF-receptor
GBM	Glioblastoma multiforme
GSK3b	Glycogen synthase kinase3b
HSC	Hematopoietic stem cell
HGF	Hepatocyte growth factor
HER2/NEU	Human epidermal growth factor receptor 2
hESC	Human embryonic stem cell
HIF1a	Hypoxia inducible factor 1a
IL-2	Interleukin-2
iPSC	Induced pluripotent stem cell
KDR	Kinase insert domain receptor
KLF4	Krueppel-like factor 4
LEF	Lymphocyte enhancing factor

LGR5	Leucine-rich-repeat-containing G-protein coupled receptor 5
LNGFR	Low-affinity nerve growth factor receptor
LRP LDL	Receptor-related protein
MART-1	Melanoma antigen recognized by T-cells 1/Melan-AS
MET	Mesenchymal-to-epithelial transition
MMP	Matrix metalloproteases
MITF	Micropthalmia-inducible transcription factor
MSC	Mesenchymal stem cell
NCSC	Neural crest stem cell
NOD	Non-obese diabetic
NSC	Neural stem cell
PDGFR	Platelet-derived growth factor receptor
PIK3Ca	Phosphatidyl-inositol-3 phosphate kinase catalytic alpha-subunit
PRC1	Polycomb repressive complex 1
RTK	Receptor tyrosine kinase
SAHA	Suberoylanidehydroxamic acid
SCID	Severe combined immunodeficiency syndrome
SDF-1	Stromal-derived factor 1
SSEA-1	Stage-specific embryonic antigen-1
Suz12	Suppressor of zeste 12 homolog
SVZ	Subventricular zone
TCF	T-cell factor
TGFb	Transforming growth factorb
VEGF	Vascular endothelial growth factor
WNT	Wingles (Wg)/int1

10.1 Introduction

In this chapter we intend to discuss two aspects of cancer stem cell (CSC) biology by combining previously published data, recent findings and yet to be published experimental data. First, we pick up the theory of similarity of partially reprogrammed induced pluripotent stem cells (iPSCs) to CSCs. We found new evidence that both cellular entities share pathways already known to be essential for the maintenance of human embryonic stem cells (hESCs) and tissue stem cells (TSCs) alike. In the second part we will give insights into the most important pathways and recently uncovered regulatory circuits, as well as epigenetic mechanisms regulating the CSC population of a given tumor. In addition, we summarize the current knowledge on the most prominent CSC populations identified so far by a characteristic pattern of marker molecules in comparison to their native counterparts, the TSCs, and the possible relevance of marker molecules as therapeutic targets.

During the past decades of experimental cancer research, solid tumors were mainly understood as heteromorphic cell masses that develop in a multistep process. Cancer progression was characterized by accumulation of somatic mutations in

critical genes affecting proliferation, cell survival, migration, invasion, angiogenesis, and metastasis. In colorectal cancer (CRC), for example, the transition of a benign adenoma into a malignant carcinoma is triggered by mutations in the tumor suppressor gene adenomatous polyposis coli (APC). Truncation of APC has been extensively investigated as the early critical alteration in sporadic and hereditary forms (HNPCC, hereditary non-polyposis colon carcinoma or Lynch syndrome) of CRC. Specifically the Apc^{Min} mouse, the genetic model system of human intestinal cancer predisposition, demonstrates that mutations in APC are responsible for a hyper activation of the WNT/b-catenin signaling pathway. While truncations in APC represent a major driving force in carcinoma formation, additional driver mutations like mutations in the KRAS [1] and TP53 genes (reviewed in [2]) are necessary for malignant transformation as well. Besides APC mutations in CRC, other driver mutations like $BRAF^{V600E}$, an activating mutation in the serine/threonine kinase domain with high incidence (66 %) in malignant melanoma or a mutation of phosphoinositide 3-kinase (PI3K), featured in the catalytic alpha-subunit of the kinase (PIK3CA), have been found. The latter mutation, among others, was detected by exon sequencing of glioblastoma and CRCs [3] and leads to an activation of the PKB/AKT pathway. Although a direct correlation of a given driver mutation and the formation or propagation of CSCs has not been found so far, PKB/AKT-mediated signaling is reported to be associated with CSC maintenance (see Sect. 10.4).

Driver mutations are distinct from sporadically occurring bystander-, or passenger mutations, which do not by themselves have transforming capacity, but may be responsible for specific features of given tumors such as therapy resistance, as reviewed in [3]. The identification of new driver mutations of different solid cancers in several cancer entities was enabled by exon sequencing [4]. Interestingly, RAS mutations have been found in a large number of tumor entities and in some tumors with high isoform restriction [5].

The realization of CSCs or tumor-initiating cells is based on an interesting observation. When cells of a specific tumor type are xeno-transplanted into immune-deficient mice, only a small number of transferred cells demonstrate the ability to reconstitute a new tumor with all its heterogeneity. Thus, a tumor does not only consist of cells bearing the ability to reconstitute the cellular variety and plasticity of the whole tumor, but the majority of tumor cells do not seem to be in a tumorigenic state. Only a very small population of cells appears to reside in a precursor-like state with self-renewing and tumor-initiating capacity. The demonstration of the genetic and functional heterogeneity in tumor development was paralleled by an important observation by Kleinsmith and Pierce, who identified pluripotent cells in teratocarcinoma, sporadic embryonic tumors of germ cells [6]. These tumors harbor undifferentiated cells with the potential to renew the tumor mass and to establish a new tumor when transferred into suitable recipients.

With regards to the self-renewing potential and the ability to differentiate into different cell types, two cellular properties of embryonic stem cells, the term CSC was coined to subsume this particular population of tumorigenic cells. Currently, it is proposed that CSCs may reside in every (not only solid) malignant neoplasm and can be identified by the expression of specific markers like

cell surface proteins and transcription factors, that specify the origin of the tumor (as reviewed in [7]).

In developmental processes, stem cells like totipotent blastomeric or pluripotent cells of the inner cell mass have the ability to form the embryo. Multipotent stem cells like adult stem cells reside in tissues and organs, where they can be recruited for tissue regeneration in a stimulus-dependent manner. For example Islet-1 expressing cells were recently found to be the multipotent stem cell of heart tissue [8] and satellite cells, which reside in the muscle in a dormant state and become activated upon injury [9]. Mesenchymal stem cells (MSCs) reside in adipose tissue, bone marrow or cord blood, and can form bone and cartilage-like cells and structures under certain conditions [10].

Since the identification of CSCs in patients with acute myeloid leukemia (AML) bearing markers of hematopoietic stem cells, CSCs were thought to originate from populations of adult stem cells. This concept has already been postulated in the twentieth century by Cohnheim and Rippert in their embryonic rest theory, reviewed in [11, 12]. This theory suggests that CSCs originate from the malignant transformation of tissue- and organ-specific adult stem cells while retaining the expression of the majority of stem cell markers. This widely accepted scenario is supported by the fact that several tumor-specific populations could be isolated from different tumors of specific origin. Tumors can form in any organ of the human body, independent of normal stem cell activity in the adult tissue.

10.2 Malignant Transformation: Partially Reprogrammed iPSCs Meet CSCs

The process of malignant transformation was first reported in 1999 by the group of Robert Weinberg. Hahn et al. transformed human fibroblasts and epithelial cells by overexpression of telomerase-reverse transcriptase (TERT), SV40 LT antigen and a mutant form of HRAS (V12) [13]. Scaffidi and colleagues later complemented this work by interrogating equally transformed cells for CSC characteristics. They demonstrated that these cells begin to express SSEA-1, a marker for embryonic stem cells and neural stem cells (NSCs), thereby acquiring the capability to self-renew and to form tumors when injected in immunocompromised mice [14].

Another artificial system, which was originally developed for de novo formation of embryonic stem cell-like cells, also known as iPSCs, is the reprogramming process. The successful reprogramming of mammalian embryonic fibroblasts has been first reported in 2006 by Takahashi and Yamanaka in their groundbreaking study.

During the stepwise and stochastic process of reprogramming, the (epi)genome of a somatic cell is transformed into a pluripotent cell-resembling state. Most remarkably, the virally transduced core transcription factors OCT4 (POU5f1), SOX2, KLF4, and c-MYC (OSKM) are silenced in the fully reprogrammed state, and only fully reprogrammed iPSCs display the expression of their endogenous counterparts [15].

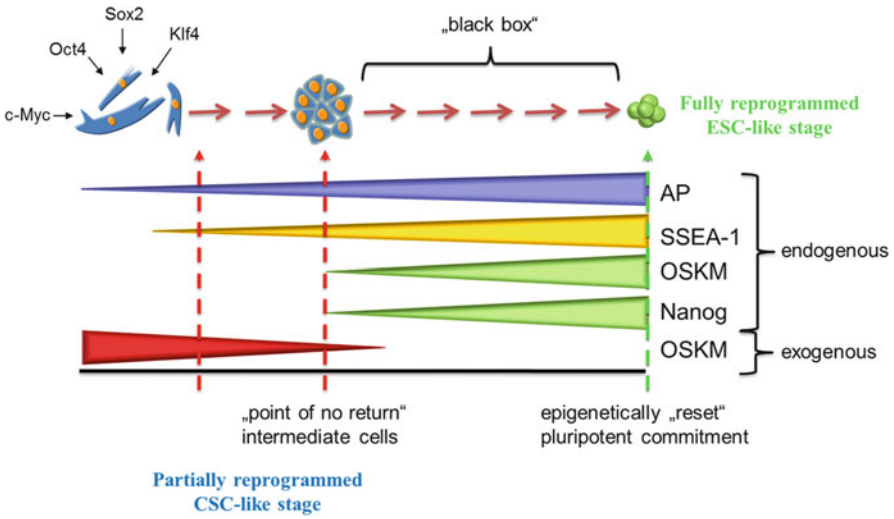

Fig. 10.1 Sequential expression of stem cell markers during the reprogramming process. Reprogramming starts with viral transduction of target cells (fibroblasts) with a viral cocktail of OCT4, SOX2, KLF4, and c-MYC (OSKM). This leads to an intermediate state of early and late partially reprogrammed cells bearing a high or low level of viral transcripts, respectively (*red arrows*) and a low level of their endogenous counterparts (*green arrows*). Although some early markers like alkaline phosphatase (AP, *blue arrow*) and SSEA-1 (*yellow arrow*) are expressed already on a low level, the cells are still in a partially reprogrammed stage. Cells which follow the reprogramming process proper attain the "point of no return," where the viral transcripts become silenced and endogenous factors and their target genes like NANOG are independently expressed. Therefore they do not need the driving force of viral factors anymore. Improper reprogramming results in the formation of un-reprogrammed and partially reprogrammed cells, they are unable to shutdown the viral expression and are likely to be different from intermediate cells. It is most likely that partially reprogrammed cells are unable to reach the fully reprogrammed stage. The multistep process of the transition of intermediate cells towards fully reprogrammed iPSCs which are committed to pluripotency is a black box and not yet understood

The dramatic changes somatic cells undergo during the reprogramming process are poorly understood on a molecular level. However, the establishment of an epithelial cell state seems to be absolutely required and is induced by a mesenchymal-to-epithelial transition (MET) [16]. During this multistep process, transduced cells start to express early marker genes like alkaline phosphatase (AP) or SSEA-1 (SSEA-3/4 in the human system), but the cell fate decision is first determined when the "point of no return" is passed. Only then changes of the (epi)genome, induced by cellular reprogramming, are irreversible and transduced cells stay committed to a pluripotent lineage formation (Fig. 10.1).

Thus, reprogramming not only represents a new technique for the generation of host-specific embryonic stem cell-like cells which, as assumed, have the ability to participate in regenerative processes without immune rejection. It also represents a model system for the study of carcinogenesis and metastasis. MET and its antagonizing process, the epithelial-to-mesenchymal transition (EMT), a hallmark of

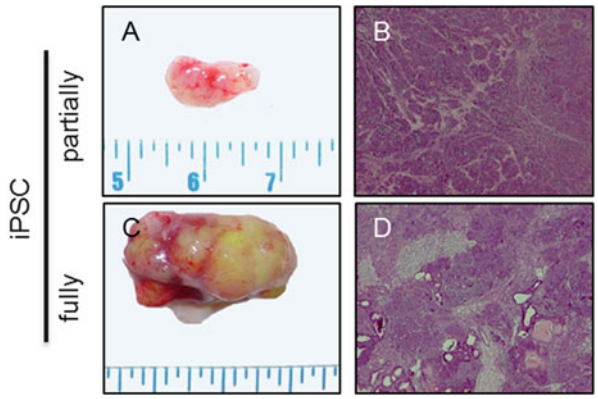

Fig. 10.2 Tumor growth of partially and fully reprogrammed murine iPSCs. Reprogramming of murine embryonic fibroblasts can give rise to partially reprogrammed cells which feature properties of CSCs. Subcutaneous injection of these cells in NOD/SCID mice leads to formation of tumors which are small in size (**a**) and display an undifferentiated phenotype (**b**). Injection of fully reprogrammed iPSCs results in the formation of bigger, well-differentiated tumors (**c**) bearing epithelial, muscular or neuronal structures (**d**)

cancer metastasis, are basic processes occurring in reprogramming or differentiation of ESCs and iPSCs, respectively.

Since it is well known that establishment of an epithelial-cell state requires the expression of the cell adhesion molecule E-cadherin [17], we first investigated the role of E-cadherin in the reprogramming process. In order to our observations we have shown recently that partially reprogrammed iPSCs did not express E-cadherin ($Ecad^{neg}$), or other epithelial markers and still had the viral transcripts expressed, whereas endogenously expressed pluripotency factors like OCT4, SOX2, and NANOG were not detectable in these cells. They appear to be trapped in a state between a transformed fibroblast and a fully committed iPSC.

Nonetheless, $Ecad^{neg}$ cells could be propagated under undifferentiating conditions as stable cells growing in colonies with self-renewing capacity and with remarkable, CSC-like features. Like a metastasizing tumor, detached cells survived in suspension and formed new colonies upon replating (unpublished). Although these cells were strongly restricted in their differentiation potential, they were tumorigenic when injected subcutaneously in NOD/SCID mice (Fig. 10.2), a feature that was most likely associated with the high remaining expression of oncogenes c-MYC and KLF4. However, the cells were unable to develop differentiated tumors (teratoma) or to participate in chimera formation [18]. Because of the generally low efficiency of the reprogramming process (<1 % of transduced cells are able to reach the fully reprogrammed state), the majority of transduced cells remain in a partially reprogrammed, metastable state.

To further uncover relationships between cancer cells, CSCs, and embryonic stem cells or iPSCs, we analyzed patient-specific melanoma cells for expression of OCT4 (POU5f1). Expression of this basic core transcription factor is not only

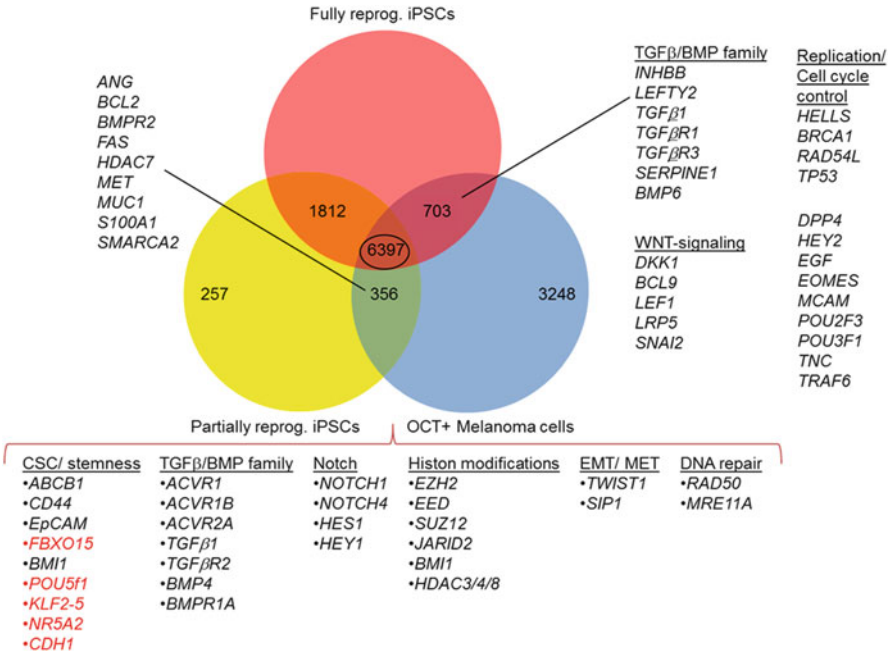

Fig. 10.3 Meta-analysis of partially and fully reprogrammed iPSCs and OCT4$^+$ melanoma cells. Overlaps shown in the Venn-diagram represent genes which are commonly expressed in OCT4$^+$ melanoma cells and partially reprogrammed iPSCs (356) or in OCT4$^+$ melanoma cells, fully and partially reprogrammed iPSCs (6,397), respectively. Among these commonly regulated genes of all three groups are genes typically expressed in stem cells and cancer stem cells (shown in *red*) and are crucial for their self-renewal like members of the TGFb/BMP and Notch pathways, Histone modifiers, regulators of EMT/MET and components of the DNA-repair machinery (summarized in *bracket*). 3,248 genes are exclusively expressed in Oct4$^+$ melanoma cells and 703 genes are commonly regulated in OCT4$^+$ melanoma cells and fully reprogrammed iPSCs

restricted to pluripotent cells, but can also be reexpressed in cancer cells under certain conditions like hypoxia [19]. Stable transfection of melanoma cells ChaMel47 (Charité melanoma 47) with an OCT4-GFP reporter enabled the isolation of a rare (~1 %) OCT4$^+$ subpopulation by fluorescence-activated cell sorting (FACS) analysis. Following isolation, sorted cells were used for expression profiling and a comparative meta-analysis. In this analysis we used expression profiling data sets of partially and fully reprogrammed iPSCs and OCT4$^+$ CSC-like melanoma cells. We found 356 out of several thousand genes to be commonly expressed in OCT4$^+$ melanoma cells and partially reprogrammed iPSCs. Among others, genes like the receptors c-MET and BMP-receptor 2, chromatin modifiers SMARCA2 and HDAC7 and death-receptor FAS and anti-apoptotic protein BCL-2 could be identified. The number of commonly expressed genes was much higher when fully reprogrammed cells were added as third group, adding up to a total of 6,397 commonly expressed genes. Upon close inspection, we found several members of the gene list being involved in

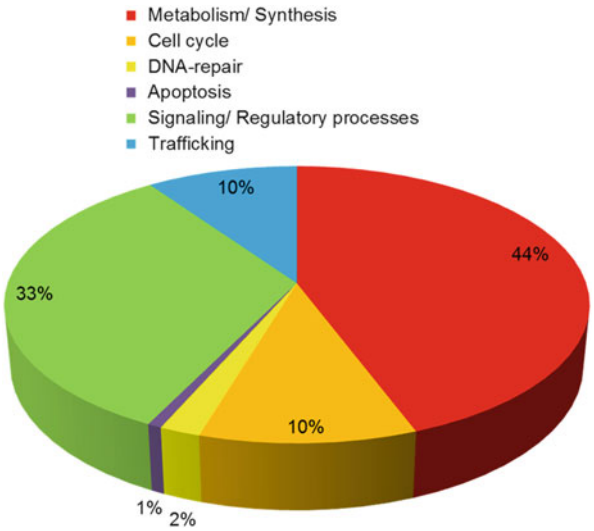

Fig. 10.4 GO statistics. Analysis of intersections of OCT4$^+$ melanoma cells, partially and fully reprogrammed iPSCs revealed a high percentage of genes involved in synthesis and metabolic processes (*red*) but also in signaling/regulatory processes (*green*). Genes involved in trafficking and cell cycle (*blue* and *orange*, respectively) have a minor role as well as those related to DNA repair (*yellow*) and regulation of apoptosis (*purple*)

specific cellular processes like the maintenance of stemness, epigenetic regulation, DNA repair, and activation of several pathways like TGFb/BMP and NOTCH signaling (Fig. 10.3). Remarkably, genes involved in TGFb signaling like INHbB, LEFTY2, TGFb1, TGFbR1, TGFbR3, SERPINE1, and BMP6 are clearly overlapping between fully reprogrammed iPSCs and OCT4$^+$ melanoma cells, and therefore are likely to play a crucial role in both cellular systems. For analysis of gene ontology (GO) statistics, we included genes of all three groups to further reveal the involvement of commonly regulated genes in different cellular processes. The majority of genes participated in metabolism and synthesis (44 %) or signaling events (33 %), but also other processes like cell cycle regulation and trafficking (both 10 %). A minority of genes was associated with apoptosis (1 %) and DNA-repair processes (2 %) (Fig. 10.4).

These results led us to conclude that partially reprogrammed iPSCs and OCT4$^+$ CSCs indeed share a small subset of commonly expressed genes involved in basic cellular processes. With respect to fully reprogrammed iPSCs, the number of genes which are expressed in common in all three groups was significantly higher and uncovered the involvement of typical key molecules and signaling pathways. Based on our previous findings we will now give insights into the main signaling pathways and epigenetic mechanisms which have been identified so far as to be involved in CSC regulation.

10.3 Signaling Pathways and Epigenetic Regulation in Cancer Stem Cells

10.3.1 Receptor Tyrosine Kinase Signaling

The maintenance of the naïve multipotent state of stem cells and CSCs is strongly regulated by signaling pathways, which can be activated or inhibited by soluble factors, secreted by the tumor stroma, or in case of an in vitro system, supplemented with the cell culture medium. The majority of signaling pathways acts via receptor tyrosine kinases (RTK) like members of the epidermal growth factor receptor family (EGFR, HER2/NEU, ERB-B2), FGFR1, the hepatocyte growth factor receptor (c-MET), or the platelet-derived growth factor receptors (PDGFRa/b). These RTKs play a crucial role during tumor cell proliferation and maintenance and differentiation of tumor stem cells. Ligand binding to respective receptors leads to RTK activation and to activation of the small monomeric GTPase RAS (KRAS, NRAS, and HRAS) [20, 21], and subsequently to activation of the extracellular-regulated kinases ERK1 and 2 via the RAF serine/threonine kinases and MEK1/2. In most cases activation of RTK signaling also leads to activation of PI3 kinase (PI3K) and the protein kinase b (PKB)/AKT signaling pathway (Fig. 10.2c) [22]. RTK signaling influences the cellular behavior via its downstream targets like the oncogene c-MYC or an indirect activation of the inducers of EMT, the snail transcription factors Snail (SNAI1) and Slug (SNAI2) [23]. Signaling by FGFR1/FGF-2 appears to have a crucial role not only in the hESC coculture systems but also in the maintenance of glioma stem cells (described in Sect. 10.3.3).

Remarkably, glioma cells cultured in the presence of serum lose their tumor stem cell compartment, whereas in the absence of serum but presence of FGF2, glioma cells express typical neural markers and display clonogenic and tumorigenic properties [24].

10.3.2 Notch and Hedgehog Signaling

Besides RTK-mediated signaling, Notch and Hedgehog pathways play crucial roles for CSC maintenance. Active Notch signaling is mediated via the membrane-bound NOTCH receptors which bind their membrane-bound ligands (DELTA, JAGGED1, JAGGED2, and SERRATE) on opposing cells. Binding of NOTCH ligands to NOTCH leads to cleavage and release of the NOTCH-intracellular domain (NICD) by the A disintegrase and metalloprotease (ADAM)/g-secretase complex. Released NICD translocates to the nucleus and binds to DNA-binding protein CSL, followed by an activation of target genes like HEY or HES1 [25].

Active Notch signaling has been identified recently as an essential pathway for the maintenance of glioma stem cells. In a three-dimensional model system of

glioblastoma multiforme (GBM), Hovinga et al. demonstrate that inhibition of Notch signaling by the use of g-secretase inhibitor DAPT led to a decrease of proliferation and self-renewal of glioma cells and $CD133^+$-glioma stem cells [26]. In line with this study, a decrease of the $CD133^+$ stem cell population of medulloblastoma was observed when miRNA199b-5p, a negative regulator of HES1, was overexpressed [27].

Hedgehog signaling is involved in CSC maintenance as well. The hedgehog signaling is induced by binding of ligands (Sonic hedgehog, SHH; Indian hedgehog, IHH; and Desert hedgehog, DHH) to their receptor patched (human homolog PTCH1). Upon ligand binding, the inhibitory function of patched is disabled and the G-coupled protein smoothened (SMO) is recruited to the membrane. This translocation leads to stabilization of the gli-complex which then translocates to the nucleus and activates target genes like c-MYC, PTCH, or cyclins D and E [28]. In an elegant mouse model, the ablation of Smo and its impact on the maintenance of CML stem cells was investigated. To ablate floxed alleles of Smo, a Cre-recombinase driven by the proto-oncogene VAV was used. Wild type and $Smo^{-/-}$ hematopoietic progenitor cells were used for leukemic transformation by overexpression of the $p210^{bcr/abl}$ protein. This experiment revealed an induction of CML in 47 % and 94 %, when $Smo^{-/-}$ or wild type hematopoietic progenitors were used, respectively [29].

Another signaling pathway that is meaningful in CSC biology is the WNT-pathway. We will discuss the importance of this pathway later in terms of the maintenance of intestinal stem cells and CSCs (see Sect. 10.5). WNT-signaling has already been identified as a determinant for the maintenance of CML stem cells and for sustaining the pool of hematopoietic stem cells. This has been demonstrated in a b-catenin loss-of-function (LOF) mouse model (see also Table 10.1). Vav-Cre-mediated LOF of b-catenin resulted in a strongly reduced Bcr-Abl-dependent transformation of $b\text{-catenin}^{-/-}$ hematopoietic progenitor cells as well as an impaired self-renewal capacity of hematopoietic stem cells.

10.3.3 Regulation of Stemness by the Tumor Microenvironment

Stem cell properties like the capacity to undergo self-renewal and the differentiation capacity in the organism are supposedly maintained by specialized "microenvironments" or "niches" (Fig. 10.5a). The stem cell niche comprises neighboring cells which can directly interact with the stem cells via several cell adhesion molecules like cadherins mediating cell–cell contacts, secreted factors such as soluble ligands and receptors (e.g., soluble frizzled proteins) and integrins mediating interactions of stem cells with extracellular matrix proteins like fibronectins and collagens [30]. The bulb region of hair follicles or the intestinal crypt regions comprise typical stem cell niches. However, the molecular mechanisms triggering maintenance, self-renewal and defined and directed differentiation of stem cells remain elusive. Clearly, these processes depend on a plethora of signaling pathways. In addition,

Table 10.1 Specification of tissue-specific stem cells (TSC), CSCs, and related mouse model systems[a]

Tissue/organ	Tissue stem cell (TSC)	TSC population	Tumor type	CSC population	Cre	Gene (floxed)
Blood	Hematopoietic stem cell	$CD34^+/CD38^-/Lin^-/CD90^+/c\text{-}KIT^+/CD123^-$	AML	$CD34^+/CD38^-/Lin^-/CD90^-/c\text{-}KIT^-/CD123^+$	Vav-Cre	$Smo^{loxP/loxP}$
						$Ctnnb1^{lox(ex3–6)}$
Skin	Melanoblast	DCT, PAX3, SOX10, MITF	Melanoma	$CD271^+$, $ABCB5^+$		
Mammary gland	Mammary stem cell	$CD29^{high}/CD24^+/Lin^-$	Mamma carcinoma	$CD44^+CD24^{-/low}Lin^-$ $ALDH1^+/CD44^+CD24^{-/low}Lin^-$		
Brain	Neural stem cell	CD271	Glioblastoma	$CD133^+$	Gfap-GFP	
Colon	Intestinal stem cell	$LGR5^+$; $BMI1^+$; CD133	Colon carcinoma	$CD44^+/EpCAM^{high}/CD166^+$	$Lgr5^-$ CreERT2	$Apc^{flox/flox}$
					Bmi1-CreER	$Ctnnb1^{lox(ex3)}$
Pancreas	Pancreatic stem cell	$PDX1^+PTFA^+CPA^+$	Pancreas adenocarcinoma	$c\text{-}MET^{high}CD44^+$; $CD44^+CD24^+ESA^+$; CD133		

Expression of specific marker for tissue specific, adult stem cells, and cancer stem cells are indicated as well as mouse model systems used to induce in vivo tumor formation

[a]Genetic model systems

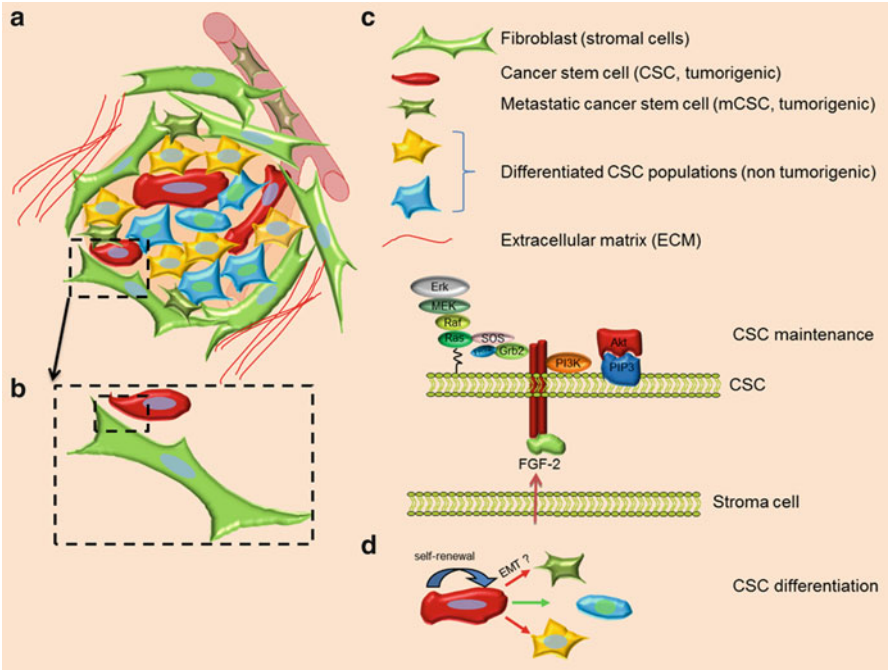

Fig. 10.5 Microenvironment and cancer stem cell niche. The tumorigenic cancer stem cells (CSCs, *red*) reside in solid tumors and are surrounded by non-tumorigenic differentiated cells (*yellow* and *blue*) and by fibroblasts (stromal cells, *light green*). Angiogenesis enables the supply of tumor cells with nutrients and oxygen. Stromal cells interact either directly with tumor cells and CSCs, thereby promoting the maintenance in its undifferentiated and self-renewing state, or via secretion of soluble factors or extracellular matrix proteins (*red* fibers). Metastasizing CSCs (*dark green*) can enter blood vessels to reach distant organs (**a**–**c**). Interaction of CSCs, e.g., with stromal cells may involve an activation of signaling pathways via soluble ligands which bind to respective receptors expressed on the CSCs. FGF-2 which is secreted by stromal fibroblasts, binds to FGFR1, and leads to activation of MEK/ERK signaling and PI3K/AKT signaling which is discussed to support the maintenance of CSCs (**c**). CSCs are able to self-renew and can undergo asymmetric cell division leading to the formation of both a cancer stem cell and a differentiated cell. EMT may be one mechanism involved in conversion of a CSC into a metastasizing CSC (**d**)

little is known about the exact cellular composition of a niche, and its recreation in an "in vitro" cell culture system is challenging, yet does not represent a mission impossible. Stem cell coculture systems were already successfully established for the maintenance of hESCs. This system demonstrated that a direct interaction of fibroblasts, which act as the surrounding stroma, and the hESCs is not obligatory. Rather, soluble factors secreted by fibroblasts in response to FGF-2 are sufficient to keep hESCs in an undifferentiated state. Beside FGF-signaling, hESC maintenance requires the activation of the Activin/Nodal-TGFb pathway and the subsequent activation of Smad2/3, whereas inhibiting BMP/Smad1,5,8 signaling leads to hESC differentiation [31, 32]. Also, in the absence of fibroblasts the system requires the presence of Matrigel which provides an artificial extracellular matrix.

Interestingly, the hESC-microenvironment is able to reprogram metastatic melanoma cells. The transfer of metastatic melanoma cells to a three-dimensional matrix, which was preconditioned by hESCs, led to phenotypic changes and a strong reduction of metastatic potential [33]. Although well established for hESC maintenance, it still remains elusive whether TGFb signaling plays an essential role for CSC maintenance per se (see Sect. 10.4.4).

In analogy to adult stem cells maintained in their niche, it is likely that tumor stem cells also require a niche which is built up by cells of the tumor mass and tumor surrounding stroma cells, such as fibroblasts (Fig. 10.5a, b). Interactions of tumor stem cells with differentiated progeny or stromal cells seem to be crucial for the existence of CSCs. These interactions are mediated in a paracrine or autocrine fashion by secreted factors (like FGF-2), and their membrane-bound receptors or adhesion proteins such as cadherins or CAMs (cell adhesion molecules). Some secreted factors and their associated receptors of tumor stroma cells have been identified already. For example, the SDF-1 (CXCL12)/CXCR4 system plays a crucial role in breast cancer [34]. Stromal cell-derived factor-1 (SDF-1) is secreted by the stromal cells and activates the G-protein-coupled receptor CXCR4. The activated receptor signals through AKT/PKB and ERK pathways and plays important roles in the homing of adult stem cells and CSCs, as reviewed in [35].

Human mesenchymal stromal cells (hMSCs) are known to express FGF-2 (basic FGF, bFGF) and SDF-1 (unpublished data). Further, it is known that FGF-2 is responsible for the proliferation and tumorigenesis of glioblastoma cells [36]. So, one may speculate about the functional role of FGF-2 as a tumorigenesis promoting soluble factor secreted by the tumor-associated stromal cells. However, FGF-2 has also been identified as an inducer of proliferation, colony formation, and anchorage-independent growth of cutaneous melanoma over-expressing FGF-2 and FGF receptors. The dependence of melanoma cells on FGF-signaling has been confirmed as inhibition of FGF-2/FGF-4 triggered FGF-receptor 1 (FGFR1) signaling by the small molecule inhibitor SU5402 and simultaneous application of the BRAF inhibitor Sorafenib, led to apoptosis of melanoma cells [37]. Insufficient oxygen supply, owing to rapid tumor growth, leads to the constitution of yet another niche, the eponymous hypoxic niche. Tumor cells are able to fully satisfy their need for energy by glycolysis which is independent from oxygen. These hypoxic niches have been identified to support the maintenance of CSCs. A very recent study has shown that HIF-1a signaling is active in AML CSCs and that inhibition of HIF-1a activity eliminates AML CSCs [38]. Another recent study revealed that hypoxia induced the expression of OCT4 and a hESC specific signature in several cancer cell lines. Additionally, overexpression of HIF-1a in combination with OSLN (OCT4, SOX2, the microRNA binding protein Lin28, and NANOG) led to formation of hESC-like colonies in A549 cells [39]. In addition, hypoxia led to an enrichment of $ALDH^+$ stem/progenitor cells in human breast cancer xenografts. The enrichment was partly dependent on the activation of the WNT/b-catenin pathway in an AKT-dependent manner. Additionally, the increase of CSCs was induced by application of angiogenesis inhibitors Sunitinib and Bevacicumab to mouse xenograft models [40].

10.3.4 Epigenetic Regulation of Cancer Stem Cell Markers

Stemness is not only regulated on the transcriptional level, but epigenetic markers are equally important to determine the native state of stem cells and CSCs. The regulation of gene expression is facilitated by changes of the chromatin structure that involve condensation and de-condensation of the chromatin by specific methylation "flags", mostly at DNA CpG motives, governed by DNA-methyltransferases DNMT1, DNMT3A, and DNMT3B in mammalian cells. These enzymes catalyze the transfer of a methyl group from *S*-adenosyl methionine to cytosine reviewed in [41]. Silencing of gene expression by methylation of specific promoter elements has been well understood for stem cells, but how epigenetic mechanisms contribute to the formation and maintenance of a given CSC population remains elusive. A correlation of DNMT1 and DNMT3B activity and expression of the CSC marker CD133 which marks glioma stem cells and hepatocellular cancer (HCC) stem cells [42] have been observed by You et al. Activity of both DNMTs decreased upon activation of TGFb signaling. This decrease was accompanied in HCC cells by an increase in the $CD133^+$ population, while the CD133-promoter methylation was decreased [43]. As it has been shown that TGFb1 is able to function as a demethylating agent by blocking DNMTs, one may speculate if this holds also true for other genes, like pluripotency-associated genes which could be reactivated by this mechanism, therefore enabling the formation and maintenance of other CSC populations. Furthermore, as the demethylating agent Decitabine is in clinical use for the treatment of the myelodysplastic syndrome [44], one should bear possible detrimental systemic side effects in mind.

Posttranslational modifications like methylation, acetylation, or phosphorylation of the N-terminal tail of histones present further mechanisms to regulate gene expression. Especially the methylation of lysine (K) residues at positions 4, 9, and 27 of histone H3 are important for regulatory processes. These modifications facilitate the active chromatin state by tri-methylation at K4 (K4me3) or the inactive, repressive state by the methylation at K9 and K27. The methylation of K27 is mediated by the polycomb repressive complex 2 (PRC2). This complex consists of the histone *N*-lysine methyltransferase (HMT) EZH2 and cofactors SUZ12 and EED. The HMT activity of the PRC2 is negatively controlled by the binding of JARID2/Jumonji which co-occupies PRC2 binding sites [45].

In line with previous observations concerning the inhibition of DNMTs 1 and 3B, resulting in an increase of CD133 gene expression, Suvá et al. have shown the involvement of EZH2 in the regulation of self-renewal in glioma stem cells. Down-regulation of EZH2 or inhibition of its histone methyltransferase activity by DZNep (an *S*-adenosylhomocysteine hydrolase inhibitor) led to a strong reduction of self-renewal and in vivo tumor-initiating capacity [46]. Remarkably, the same inhibitor has been used in another study for the treatment of MCF-7 breast cancer cells. Here, the inhibitor directed a decrease in EZH2 expression and growth inhibition [47]. Supplementary, a sustaining role of EZH2 for $CD133^+$-glioma stem cells has also been found in a later study in dependence of histone deacetylase (HDAC)

inhibition. Treatment of glioma stem-like cells (GSC) with the HDAC inhibitor SAHA led to a decrease of EZH2 and CD133 [48]. Taken together, these data clearly demonstrate that not only signaling pathways are important for the regulation of the CSC-compartment, but epigenetically processes are crucial as well.

Some of the most important regulatory circuits have been discussed lately in terms of the activation of specific signaling pathways or epigenetic events. Now we will give insights into some CSC populations with regard to their marker profile which has been used for their identification and isolation. As shown above, some of these markers have distinct functions; they not only represent a cell surface molecule, but are essential for the features of a given CSC population. This raises the question of what constitutes the organ-specific stem cell niche in the tumor. We will address this topical question in the following section—also with regard to the TSC pendant of a given CSC population and putative treatment regimens.

10.4 Tissue-Specific Stem Cells and Cancer Stem Cells

10.4.1 Hematopoietic Stem Cells (HSC)

HSCs reside in the bone marrow and are responsible for the formation of myeloid and erythroid derivatives like lymphocytes and erythrocytes, respectively. They have been identified by isolation of a population of naïve, CD38 (marks T- and B-cells) negative cells expressing high levels of CD34 ($CD34^+$), the ligand for the cell adhesion molecule L-selectin, which is necessary for the attachment of HSCs in the bone marrow. HSCs do not express mature cell lineage markers (Lin^-). Isolated cells were capable of repopulating the bone marrow of non-obese diabetic (NOD) mice with severe combined immunodeficiency syndrome (SCID) [49]. Based on these markers Dick et al. were able to isolate leukemia stem cells (LSCs) from patients suffering from AML. $CD34^+CD38^-$ cells were present in varying proportions (0.1–1 %) of AML cells in a donor-dependent manner and could be further subdivided and separated from HSCs upon selection for Thy-1 (CD90) expression and the absence of c-Kit, features which have been found only in LSCs, as reviewed in [50]. Injection of these cells in NOD/SCID mice lead to formation of human AML following a clonal hierarchy. Cells of this population homed to the bone marrow and displayed an extensive proliferation potential. Also, populations with a differential expression of CD34 and/or CD38 ($CD34^+CD38^+$ and $CD34^-$) did not show any of these properties [51]. Anyway, the differences between HSCs and LSCs are not sufficient for a targeted therapy aimed solely at LSC populations. In 2000 Jordan et al. identified the IL-3 receptor a (CD123) as a promising marker with clinical relevance. CD123 is expressed on $CD34^+CD38^-$ cells to 98 %, but absent in normal bone marrow cells [52]. Recently it has been shown that targeting CD123 expressing LSCs by a neutralizing antibody in a murine model system led to blocking of CD123 signaling and impaired LSC homing to and engraftment in the bone marrow [53].

10.4.2 Neural Crest Stem Cells (NCSCs)

Neural crest stem cells comprise an embryonic population of multipotent cells formed at the gastrulation stage of embryonic development. The formation of NCSCs is controlled by simultaneous activation of WNT and bone morphogenetic protein (BMP) signaling pathways [54]. Differentiation of NCSCs can give rise to a diversity of cells like smooth muscle cells, melanocytes, neurons, and glia cells (reviewed in [55]). The formation of neurons, astrocytes, and glia cells is based on the differentiation of NSCs which have been found in the hippocampus and the subventricular zone (SVZ) [56]. NSCs are marked by cell surface molecules like CD133 [57] and the carbohydrate motive LeX antigen (CD15; stage-specific embryonic antigen-1, SSEA-1), reviewed in [58]. SSEA-1 was originally found to be expressed on the cell surface of murine blastomeres and murine embryonic stem cells [59] and has also been identified as a marker of glioma initiating cells (gliomastem cells) [60]. In the hierarchical CSC model, glioma stem cells originate from the malignant transformation of glia cells, a population of nonneuronal cells that produce myelin and surround the neurons, supporting them with oxygen and nutrients. They were identified as yet another CSC population in glioma as cells expressing the cell surface marker Prominin-1 (CD133) [61]. The treatment of glioma with chemotherapeutic drugs is limited by the permeability of the blood–brain barrier. Therefore, the treatment option for glioma is mostly a radiation therapy in combination with suitable drugs able to pass the blood–brain barrier like the alkylating agent Temozolomide or the topoisomerase 1 inhibitor Irinotecan. Worryingly, the population of $CD133^+$ CSCs becomes enriched in glioma during radiation therapy and seems to escape from radiation damage by an increased DNA-repair capacity [62]. Interestingly, Temozolomide treatment has been reported to specifically deplete the $CD133^+$ glioma stem cells which were negative for MGMT (O^6-methyl-guanine DNA-methyltransferase) expression [63]. Currently, the treatment of glioma with a combination of the humanized monoclonal antibody Bevacizumab that binds to vascular endothelial growth factor (VEGF) [64] and Temozolomide was reported as a potent treatment strategy [65].

In addition, a vascular endothelial-cadherin (CD144)-expressing side population of $CD133^+$ CSCs gives rise to endothelial cells and appears to be a derivative of a $CD133^+/CD144^+$ precursor cell. The differentiation of this precursor towards endothelium is independent of the differentiation of tumor endothelial progenitors that can give rise to angiogenesis and cannot be blocked by application of Bevacizumab [66].

Glioma stem cells display an overlap in their expression profile with melanoma stem cells due to the ancestry of glia cells and melanocytes in development.

10.4.3 Melanocyte Stem Cells (Melanoblasts)

Melanocytes are specialized cells that originate from the differentiation of NCSCs (Fig. 10.6). They are responsible for melanin production from tyrosine and thus for the pigmentation of, e.g., hair, eye, and skin in vertebrates and invertebrates.

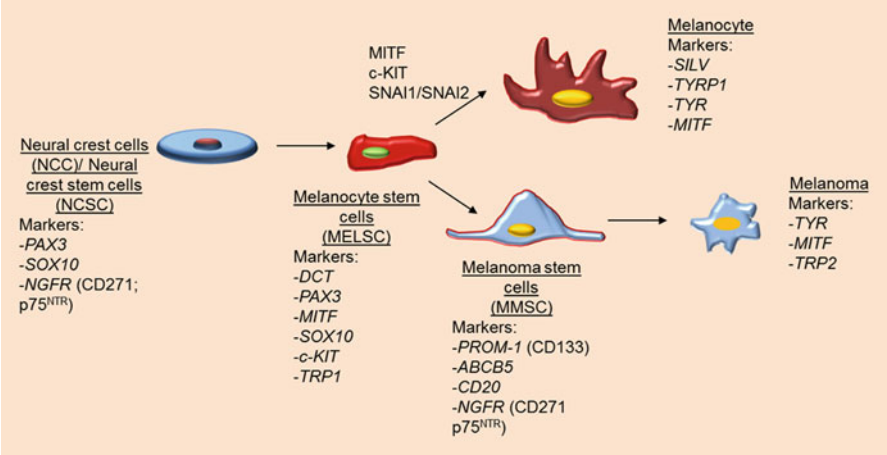

Fig. 10.6 Derivation of melanocytes, MELSCs, MMSCs, and Melanoma cells. Melanoblasts (Melanocyte stem cells, MELSCs) deduce from neural progenitor cells the neural crest cells (NCC) or neural crest stem cells (NCSCs) which are marked by the expression of PAX3, SOX10, and low-affinity nerve growth factor receptor NGFR (CD271; $p75^{NTR}$). MELSCs differentiate under the control of MITF, c-KIT, and Slug/Snail into melanocytes. Melanocytes specifically express Tyrosinase (TYR), an enzyme which catalyzes the synthesis of melanin from tyrosine, the transcription factor MITF and the Tyrosinase-related protein 1 (TYRP-1) which is also involved in melanin synthesis as well as the SILV (Melanosomal protein Pmel 17) gene. Mutations occurring in the Melanoblast drive the formation of MMSCs. MMSCs bear markers of both, Melanoblasts/Melanocytes and NCSCs like NGFR, the ATP-binding cassette subfamily B member 5, ABCB5, the B-lymphocyte antigen (CD20) [67], and Prominin-1 (CD133). Melanomas represent a heterogeneous cell system as they bear not only typical melanoma cells but also melanoma stem cells. Therefore although there are some markers which specify the melanoma cells like MITF [68], TYR [69], and TRP-2 (Anthranilate synthase), these markers are in part common with their progenitor cells

Skin pigmentation is a critical step as it protects keratinocytes from UV radiation damage by an interaction of melanocytes and keratinocytes that, like fibroblasts, reside in close proximity in the skin [70]. Melanocytes derive from progenitor cells, melanoblasts which in turn are derivatives of a glial-melanoblast progenitor [71]. Melanoblasts express markers like PAX3, MITF, SOX10, and c-KIT [72] and migrate from the neural crest into the epidermis. There they localize in the bulge region of newly formed hair follicles [73], constitute their niche.

10.4.4 Melanoma Stem Cells

These cells have been found in malignant melanoma (a type of skin tumor that is derived from the malignant transformation of melanocytes or melanocyte stem cells, respectively) and share common markers with melanocytes and neuronal stem cells (Fig. 10.1). Melanoma-initiating cells were identified as $ABCB5^+$ cells, residing predominantly in primary or metastatic melanoma, whereas the amount of $ABCB5^+$

expressing cells was lower in benign naevi [74]. Lately, cells expressing the neural crest cell marker $p75^{NTR}$ (low-affinity nerve growth factor receptor LNGFR, CD271) were identified as cells initiating melanoma with metastatic behavior when injected in $Rag2^{-/-}gc^{-/-}$ mice deficient in T-, B-, and natural-killer cells. Interestingly, these cells lack expression of the melanoma markers tyrosinase and MART-1 [75].

Although the hierarchical CSC model is widely accepted, a recent study contradicted the hierarchical order in patient-derived melanoma cells. All cells of the isolated melanoma cell population were able to induce primary tumors and to metastasize. The malignant properties were not correlated with expression of marker proteins like CD271 or ABCB5 [76].

Treatment of late stage melanoma (stages III and IV) is difficult as the tumor metastasizes to distant organs like the lymph nodes, lung, liver, or brain [77]. After resection of affected organs, different chemotherapeutics, e.g., alkylating agents like Dacarbazine (DTIC) leading to DNA hypermethylation and gene silencing, or specific signaling pathway inhibitors, like PLX4720 (Plexxikon) [78], are in clinical use. Although this inhibitor specifically targets the mutated form of BRAF ($BRAF^{V600E}$), its application is restricted to those patients bearing this mutation. Further, it is unknown whether this or another mutation is correlated with CSC maintenance or differentiation. The existence of melanoma stem cells is, although many hinds are given by those studies already mentioned here, still a matter of controversy [79]. However, targeting cells expressing the marker $p75^{NTR}$ by a small molecule inhibitor may be a road to follow as these cells seem to be dependent on NGF, or more general, neurotrophin signaling, and fulfill criteria of CSCs. This signaling involves receptors $p75^{NTR}$, Sortilin, TRKs A, B, and C and ligands NGF, BDNF, NT-3, and NT-4/5. Remarkably, the involvement of neurotrophin signaling in metastasis of malignant melanoma to the brain is established [80].

10.4.5 Multipotent Mammary Stem Cells (MaSCs)

The mammary gland is composed of ductal and alveolar structures that allow the production of milk during pregnancy. The gland comprises a heterogeneous mix of alveoli, mammary epithelial cells, cuboidal cells, fibroblasts, and adipocytes. Transplantation of fragments of the mammary gland rich in epithelial cells into cleared fat pads of recipient mice revealed a regenerative capacity due to MaSCs which express distinct cell surface markers. The skin stem cell marker CD29 (b1-Integrin) was used in combination with CD24, a marker which is also enriched in NSCs [81], to isolate an MaSC population from mouse mammary glands [82]. This $CD29^{high}$ $CD24^{+}$ Lin^{-} population had the capability to reconstitute the whole mammary gland. In contrast, the first breast CSCs were identified as a $CD44^{+}CD24^{-/low}$ Lin^{-} population with high tumorigenic capacity, as shown by tumor formation by less than 100 cells injected in immune compromised mice [83]. In a later study Ginestier et al. used Aldehyde dehydrogenase 1 (ALDH1) as an additional marker to isolate a very rare subpopulation (<1 %) with $ALDH1^{+}/CD44^{+}CD24^{-/low}$ Lin^{-} phenotype, which had a small overlap with previously defined breast cancer CSCs.

Although ALDH1 is expressed in normal mammary epithelium and malignant mammary stem cells, the incidence of this population has been associated with poor prognosis [84] and has been shown to highly metastasize in the bone [85]. This behavior was blocked by treatment of cells with a dimer of BMP2 and BMP7 activating BMP-signaling while blocking the TGFb pathway [85].

10.4.6 Multipotent Intestinal Stem Cells

Intestinal stem cells reside in the base of the crypts of the colon and are capable to differentiate towards all intestinal cell types of the colon. In 2007 the leucine-rich-repeat-containing G-protein-coupled receptor 5 (LGR5) was identified as a marker that specifies the stem cell population in the small intestine and the colon [86]. A mouse model (see also Table 10.1) demonstrated that Lgr5 expressing cells can undergo a neoplastic transformation when APC, a negative regulator of the WNT-pathway [87], is ablated [88]. Therefore, this transformation is based on an aberrant regulation of the WNT/b-catenin signaling pathway; a hyperactivation is most commonly associated with mutations in the APC gene, representing a key step in the development of colon cancer [89]. For a detailed review of the WNT/b-catenin signaling pathway see [90].

Several genes known to be crucial for stem cell and CSC maintenance have been identified as targets of WNT-signaling. The intestinal stem cell marker LGR5, which has been mentioned here quite recently, binds to the WNT/Frizzled/LRP complex and regulates WNT-signaling extracellularly; down-regulation of LGR5 and its homologue LGR4 results in the inhibition of WNT-signaling [91]. Pluripotency factors OCT4, NANOG [92], and SOX2 [93] are regulated by WNT-signaling as well. Other well-described WNT-targets are c-MYC [94], the micropthalmia-inducible transcription factor (MITF) [95] which is expressed in malignant melanoma and CD44 [96], which has been used to identify colon CSCs as a $CD44^{+}/EpCAM^{high}/CD166^{+}$ population [97]. Therefore WNT-signaling is involved in a plethora of cellular functions and embodies a major player in stem cell biology.

Hyperactivation of the WNT-pathway has also been linked to expression of BMI1, a subunit of the Polycomb repressive complex 1 (PRC1). BMI1 is expressed in discrete cells located near the bottom of crypts in the small intestine. In a mouse model (see also Table 10.1), Bmi1-expressing cells have been identified as cells of origin of intestinal cancer. Overexpression of a stabilized form of b-catenin in intestinal Bmi1-expressing cells by using a Bmi1-driven Cre-recombinase (deletion of destabilizing phosphorylation sitesSer45/Ser33/Ser37 and Thr41 of b-catenin) rapidly induces adenoma formation [98].

10.4.7 Multipotent Pancreatic Stem Cells

Pancreas carcinomas or pancreas adenocarcinomas arise from the pancreatic duct cells and are often attributed with poor prognosis and represent one of the most aggressive

cancers worldwide. During pancreas development, a multipotent progenitor cell expressing the transcription factors pancreatic and duodenal homeobox1 (PDX1), pancreas-specific transcription factor (PTFA), and the pancreas-specific enzyme CPA (crucial for pancreatic development) gives rise to all exocrine and endocrine cell types. At later stages this self-renewing, multipotent progenitor differentiates towards acinal and ductal cells or forms a- and b-cells (reviewed in [99]). A very recent study by Li et al. involved the signaling of the hepatocyte growth factor (HGF) receptor c-MET as a marker for pancreatic CSCs [100]. The researchers identified a stem cell population which displayed a high expression of c-MET and demonstrated that these cells were capable to form tumors in NOD/SCID mice. Tumorigenicity of these c-MET^{high} cells was even increased in cells with a simultaneous expression of CD44. Instead of c-MET/CD44^{-} cells, c-MET^{high}/CD44^{+} cells were able to undergo sphere formation and had a high tumorigenic potential comparable with the CSC population CD44^{+}CD24^{+}ESA^{+}, which has been identified earlier [101]. Beside c-MET^{high} CD44^{+} CSCs, other markers for putative pancreatic CSCs like CD133 have been analyzed. Interestingly the differences in tumorigenicity of CD133^{+} and CD133^{-} cells were only marginal [100] (for further information see review [102]). Also, a recent report describes a dependence of CD133^{+} pancreatic CSCs on Activin/Nodal signaling. The authors also observed a decrease of the CD133^{+} cell population and a sensitization to gemcitabine upon treatment of patient-derived pancreatic cancer cells with the ALK4/7 inhibitor SB431542 [103].

CSCs represent a family of tumor-specific cells capable of regrowing the tumor mass. They reside in hematological but also in solid tumors and are marked by characteristic cell surface molecules which are used for their isolation. However, the impact of these markers on CSC maintenance and behavior in their niche still remains elusive, leaving several questions yet to be answered. For example, how reliable is the expression of a combination of markers for the identification of a specific CSC population? Is a specific CSC population able to interconvert into another CSC population in a microenvironment-dependent manner? Are driver mutations linked with CSC occurrence? And the most important question is, how can we specifically target CSCs with a therapeutic drug, thereby permanently eradicating the tumor and preventing its recurrence? Our knowledge about CSCs is growing steadily, but we still have to learn a lot about this fascinating and yet weird type of cells.

10.5 Material and Methods

10.5.1 Data Analysis of OCT4^{+} Samples from Melanoma Cell Lines

Expression profiling of three technical replicates of OCT4^{+} melanoma cells was performed with HT12 BeadChip (Illumina). Bead summary data were saved with the Illumina BeadStudio software without background correction. Follow-up processing was done in the Bioconductor environment applying the packages lumi [104], limma [105], *q*-value, and biomaRt.

10.5.2 Analysis of Expression Profiling Data Sets of Partially and Fully Reprogrammed iPSCs

In this study, expression profiling data of partially and fully reprogrammed murine iPSCs deposit in NCBI GEO (GSE10781) was performed [106]. For a comparison with our data acquired from human sample, probes were mapped to human gene symbols. Illumina probe-IDs were used as provided or mapped to human ortholog gene symbols (Affymetrix) via Biomart version 62. Intersections of genes expressed in $Oct4^+$ human melanoma cells and partially and fully reprogrammed murine iPSCs were calculated. *p*-Values were calculated with limma package or Bioconductor MAS5 implementation. Gene expression levels were considered to be significant for *p*-values of 0.05 or lower.

10.5.3 Gene Ontology Enrichment Analysis

Gene Ontology (GO) analysis was done with overlapping data sets of all three cellular entities. Significantly enriched GO Biological Process (BP) terms were determined via the hypergeometric test implemented in the R/Bioconductor package GOstats. The Bioconductor package biomaRt was employed to map gene symbols to Entrez gene IDs which were used for the hypergeometric test. As background set for the tests all gene symbols mappable to EntrezIDs were used. Significant GO terms were filtered with a threshold of $p = 0.05$.

Acknowledgements C.R.A.R. and W.W. were supported by ERASYS (BMBF); T.R. was supported by the TREAT20 Consortium (BMBF); and D.S.s was supported by OncoTrack. We also thank the Wilhelm Sander-Stiftung for funding part of this work.

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Chapter 11
Filling the Gap in the Relationship Between Cancer and Stem Cells

Erdal Karaoz and Belma Akpinar

Abstract Mesenchymal stem cells which are the group of cells that can be isolated from various tissues having the ability of morphing into distinct tissue types and self-renewal have constituted the most popular topic for the stem cell research. Mesenchymal stem cells and cancer cells share common properties like high level of telomerase enzyme activity, deprogramming and proliferation, immortalization, self-renewal, and invasion. As a result of these, common properties have been suggested that some embryonic genes are reexpressing in cancer cells. Additionally, of these properties, MSCs have been shown to have a remarkable tropism towards tumors, so that stem cells might be the key factors of the cancer formation and propagation. There are some studies about the interactions between cancer and stem cells. As a result of these studies, MSCs, with their immunosuppressive activities, vasculogenic supports, anti-apoptotic properties, and being a component of tumor tissue, could affect cancer cells as a promoter, or they could affect tumor formation and propagation as an inhibitor.

Keywords Stem cells • Mesenchymal stem cells • Inflammation • Vasculature • Cancer stem cells

Abbreviations

MSC	Mesenchymal stem cell
B.C.	Before Christ
hESC	Human embryonic stem cell
HSC	Hematopoietic stem cell

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K. Turksen (ed.), *Stem Cells: Current Challenges and New Directions*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4614-8066-2_11,

BM-MSC	Bone marrow-derived mesenchymal stem cell
UCB	Umbilical cord blood
WJ	Wharton's jelly
AT	Adipose tissue
ISCT	International Society for Cellular Therapy
HLA	Human leukocyte antigen
GvHD	Graft-versus-host disease
ALS	Amyotrophic lateral sclerosis
TNF	Tumor necrosis factor
NSC	Neural stem cell
IDO	Indoleamine 2,3-dioxygenase
PGE2	Prostaglandin E2
EC	Endothelial cell
FGF	Fibroblast growth factor
PDGF	Platelet-derived growth factor
SDF-1	Stromal-derived factor-1
CAF	Carcinoma-associated fibroblast
ECM	Extracellular matrix
HGF	Hepatic growth factor
TGF	Transforming growth factor
hASC	Human adipose tissue-derived mesenchymal stem cell
hCEC	Human corneal epithelial cell
STZ	Streptozotocin
α-SMA	α-Smooth muscle actin
LPA	Lysophosphatidic acid
shRNA	Short hairpin ribonucleic acid
CFU-F	Colony-forming unit fibroblast
STAT3	Signal transducer and transcription activator
JAK	Janus kinase
IL-6	Interleukin-6
EMT	Epithelial–mesenchymal transition
EGF	Epidermal growth factor
DP-MSC	Dental pulp-derived mesenchymal stem cell

11.1 Introduction

Throughout the history, one of the utmost goals of mankind was to discover the cures for the diseases and to find a way to prolong human lifetime. There are evidences which reveal that the potions extracted from diversity of herbals had been used as medications thousands of years ago. In a papyrus which is thought to remain from 1534 B.C. (before Christ), there are statements about several diseases and their ancient cures, as well as illustrations about the change of the sick viscerals in ancient Egyptian monuments and hieroglyphs.

The information from early 1700 B.C. reveals that Babylonians were performing operations using the knives. Despite the unclear situation of the organ transplantation in that time, great efforts of human have been continuing to overcome the diseases and to delay the aging process up until now. These efforts from history become a background and impetus for the improvement of the evidence-based medical science.

Upon completion of global program for mapping the human genome, i.e. Human Genome Project, gave the opportunity for researchers around the world to decipher 99.99 % of the genes where the secret of life is hidden. Decrypting of the genetic code would be able to open the way to find the permanent and ultimate cures for the diseases which take their origin from the impairment of the gene structures. However, there are a number of life-threatening diseases that may not have causal relationship with the impairment of the genetic codes. In regard to this sort of diseases, medical treatment can be possible; nevertheless, in most of the cases, tissue or organ insufficiency may be the inevitable outcome. In these conditions, cell–organ–tissue transplantation comes to fore as effective treatment option. However, transplantation cure inevitably brings along the immunological problems such as rejection and infection. In addition to this, appropriate donor shortage has continued to be a dramatic issue that lies in front of the transplantation treatment. The strategy including the transplantation of cells and tissues derived from stem cells of the patient can be a promising therapy alternative ruling out both the donor shortage and the transplant rejection. Besides, it has been suggested by the scientists that the cell-based therapy methods may cure a number of diseases, such as Type I diabetes, multiple sclerosis, and severe impairment of the medulla spinalis, which have no ultimate treatment in today's world.

The main purpose of the cell-based therapy, in other words cell therapy, is to succeed the replacement of the biological function of the damaged cell, tissue, or organ, to restore and/or to improve these. This can be possible when the stem cells which are isolated and defined in adequate quality and quantity to restore the biological functions are transplanted into the target organ. Stem cells have been showing great potential for use in this area so-called regenerative or reparative medicine.

One of the main common specialties between the cancer cells and the stem cells is high level of telomerase enzyme activity. Human embryonic stem cell (hESC) colonies express high level of telomerase activity and maintain having the potential to differentiate into the all three germ layer-derived cells. hESCs lead to the generation of teratoma 7–8 weeks after the injection to the severe combined immunodeficient (SCID) mice [1].

The common features of stem cells and cancer cells, such as reprogramming, proliferation, self-renewal, migration and their immortile status suggest that some embryonic genes could be re-expressed or reactivated also in cancer cells. A number of reports have been available showing that Oct-4 and other embryonic genes are also expressed in cancer cells. As these researches suggested that there is a relationship between the Oct-4 and the tumorigenesis, it would be reasonable to think that understanding the Oct-4 function in the biology of the stem cells could facilitate in defining the new treatment alternatives in some cancers [1].

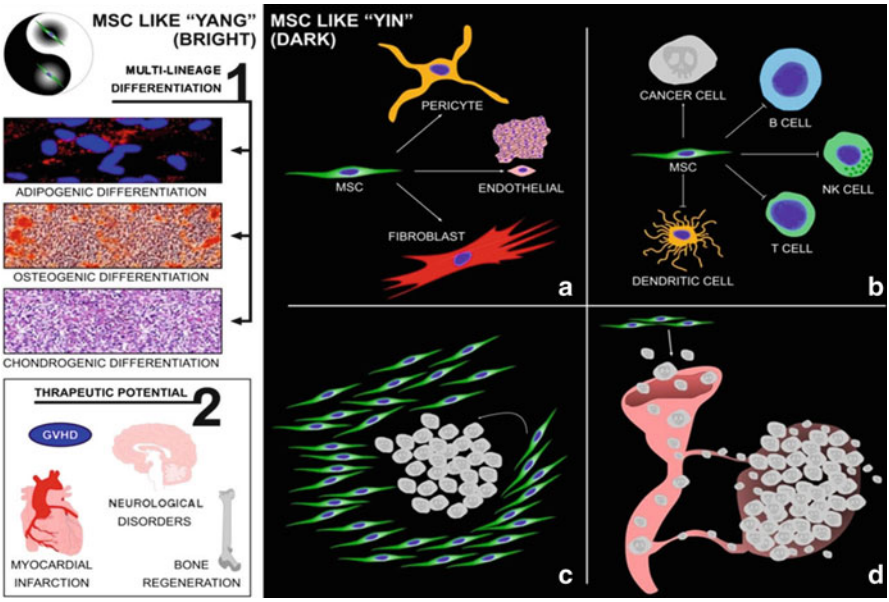

Fig. 11.1 (**1**) MSCs isolated from multiple resources are the multipotent cells which have the capacity of forming the multilineage (adipogenic, osteogenic, chondrogenic) precursor cells. (**2**) Using their anti-apoptotic, immunosuppressive, proliferative, and regenerative features, it is possible to use these cells in the treatment of several diseases. (**a**) MSCs can support the development of tumor stroma by differentiating into neo-pericytes, neo-endothelial cells, and neo-fibroblast cells. (**b**) MSCs suppress the warrior cells and help the tumor cells escape from immune system. (**c**) They provide a convenient environment for the cancer cells by secreting the growth factors and cytokines. (**d**) They induce neoangiogenesis and lead new vascularization and metastasis

In this chapter, we attempt to compile the available scientific information about the extremely interesting cross talk between the stem cells and tumor cells emphasizing the "good" and "evil" sides of this interaction to shed a light for future considerations of using these cells in cancer research and treatment.

11.1.1 Discovery of the Mesenchymal Stem Cells and Their Use in Clinics

MSCs which are the group of cells that can be isolated from various tissues having the ability of morphing into distinct tissue types and self-renewal have constituted the most popular topic for the stem cell research (Fig. 11.1, Panel 1). In the early 1960s, the exciting exploration of the stem cells began. While preparing suspension culture of the hematopoietic cells, A.J. Friedenstein, one of the first researchers in this area, observed a group of plastic-adherent cells with fibroblast-like morphology

with differentiation ability into chondrocytes and osteoblasts, which were named later as "colony-forming unit fibroblasts" [2, 3]. Because of their differentiation capacity into mesenchymal-derived cells, these cells were later renamed as "mesenchymal stem cells" [4]. It is worth to remember that prior to Friedenstein, who was not the first to use the "stem cell" term, a lot of scientists reported several study results which can be considered as the historical milestones for the stem cell research. The idea of "stem cell" was first proposed by A.A. Maximow due to the differentiation of hematopoietic cells into a variety of cell types [5]. Maximow's "unitarian theory of hematopoiesis" was developed from the idea that all blood cells originated from a single mother cell in regard to hematopoiesis process. On the other hand, Ernest A. McCulloch and James E. Till are the pioneer scientists who profounded the clonal structure of the hematopoietic cells by their research experiments. These scientists identified that "spleen colonies" have been seen in the spleen of the irradiated mice after hemopoietic cell injection with a comparable colony-forming rate of the injected cells quantity; then they observed these colonies take their origin from one progenitor hemopoietic cell [6–8].

In our current thinking, it is well known that bone marrow contains two types of stem cells: the hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). The bone marrow-derived mesenchymal stem cells (BM-MSCs) have been shown in vitro to play a crucial role in proliferation, differentiation, and survival of HSCs [9]. It has been known that BM-MSCs constitute the 0.0001 of all bone marrow cells and can be isolated by Ficoll density gradient centrifugation or by their plastic-adherent features [10]. MSCs also exist out of bone marrow in locations of umbilical cord blood (UCB) [11], Wharton's jelly (WJ) [12], adipose tissue (AT) [13], synovial fluid [14], amniotic fluid [15], placenta [16], and dental tissues [17, 18] and could be isolated from these locations with similar methods. Several markers are expressed in the phenotype of MCSs. According to International Society for Cellular Therapy (ISCT), MSCs are defined by the following criteria:

1. Their property of adherence to plastic
2. Their phenotype: CD14− or CD11b−, CD19− or CD79α−, CD34−, CD45−, HLA-DR−, CD73+, CD90+, and CD105+
3. Their capacity to be differentiated into three lineages: chondrocyte, osteoblast, and adipocyte [19]

Among the diseases where the MSCs are used as curatives, the most important ones are cardiac diseases, diabetes, Crohn's disease, graft-versus-host disease (GvHD), amyotrophic lateral sclerosis (ALS), neurodegenerative diseases, and cartilage renewal.

The multilineage differentiation capacity of the MSCs has been enabling the usage of them in tissue engineering and new treatment option for several diseases. Tissue engineering has been considered as promising alternative way to find the suitable tissues, organs, and adequate numbers of donors for the transplantation overcoming the hurdles such as immunological rejection. Using the methods of tissue engineering, it is possible to from a particular tissue seeding them on biodegradable scaffoldages. Tissue defects resulting from any trauma or disease can be healed with

usage of engineered tissues. In addition, the ex vivo engineering of the tissue by culturing of three-dimensional bioscaffolds with mature cell or stem cells cultivated in bioreactors would provide the generation of tissues or organs, e.g., liver, hearts, cartilage, or kidneys [20]. MSCs have been widely used in tissue engineering because of their capacity of proliferation and differentiation [20].

The discovery of MSCs and the evidence of their involvement in the tumorigenesis obtained from a lot of studies enhanced the interest to these cells in the last decade. Particularly, recent preclinical studies showed that the MSCs possess the below-listed characteristics:

(a) Immunosuppression and immunomodulation
(b) Anti-apoptotic function
(c) Anti-fibrotic function
(d) Anti-inflammatory function
(e) Chemoattractive characteristics
(f) Promoting the angiogenesis

The use of stem cells in some clinical trials in human subjects with these proven characteristics has already been initiated. For instance, the interventions with the purpose of the prevention of the alloimmunity in tissue and/or organ transplantations have reached to the Phase III trial stage. Besides, the interventions to reanimate the ischemic tissues have been increased with stem cells' neoangiogenesis potential; the cell therapy where the MSCs were used proves that the reanimation target has been met in the diabetic feet and Buerger's disease cases in clinical trials [1].

Understanding more the features of the stem cells and with the help of genetic research could rise new opportunities for treatments like:

- The signals inducing the neoplastic cell differentiation
- Oncolytic factors
- Biofusion agents
- Agents inducing apoptosis
- Agents improving the immunity
- Antiangiogenesis factors
- Cell cycle modulators
- Signal transduction mediators of cancer cells [1]

The scientists have been making efforts to elaborate the interaction between the MSCs and cancer cells by using several tumor models [1]. Interestingly, there are some conflicting results claiming that MSCs could induce the tumorigenesis, whereas some emphasize that these cells may inhibit this process. In order to provide the basis for the observations, there are some mechanisms raised to discussion such as chemokines, apoptosis modulation, vascular support, and immunomodulation. More understanding of the roles of MSCs in tumorigenesis and metastasis under certain conditions could clarify their involvement in carcinogenesis, and will make the use of these cells safer as therapeutical agents.

MSCs have been shown to have a remarkable tropism towards tumors [1]. MSCs residing in tumor's fibrovascular plexus were observed to differentiate into tumor-associated fibroblasts and vascular pericytes. The tumoral tropism of the MSCs makes these cells the unique cellular mediators that could carry the anti-tumoral agents such as interferon-β, cytosine deaminase, tumor necrosis factor (TNF), and oncolytic viruses towards the target tumor cells. Aboody et al. showed in their study that when the neural stem cells (NSCs) expressing cytosine deaminase gene that works with 5-fluorocytosine were transplanted into the rodents having experimental intracranial glioma, the cytotoxic 5-fluorouracil and its metabolites diffuse quickly into the tumor, and tumor tissue decreased 2 % as a result of the mechanism associated with oncolysis [21].

11.2 Effects of the MSCs in Tumorigenesis

11.2.1 MSCs Promote Tumor Formation: Evil Side

11.2.1.1 With Their Immunosuppressive Activities

The immunosuppressive activities of MSCs, derived from various sources, were demonstrated on the T cells, B cells, natural killer cell (NK cell), and dendritic cells (DCs) recently [22–48]. How is the mechanism of immunosuppressive activities of the MSCs? To get the answer for this question, Chang et al. designed a study where he reported that DCs, effector T cells, and NK cells changed their cytokine secretion profiles in order to encourage the anti-inflammatory effect or tolerant phenotypic under the condition when the MSCs were cocultured with the T cells [26]. It was found that the MSCs were activated by interferon-γ (IFN-γ) released from the cells, like the T cells and natural killer (NK), and by interleukin-1 (IL-1) like cytokines, from the monocytes, whereas by releasing cytokines like interleukin-10 (IL-10) [22, 27] and interleukin-6 (IL-6) [22, 28] and soluble factors, including the transforming growth factor-β (TGFβ) [22, 29, 30], hepatocyte growth factor-β (HGFβ) [22, 31], indoleamine 2,3-dioxygenase (IDO) [32], prostaglandin E2 (PGE2) [28], human leukocyte antigen-G (HLA-G), and nitric oxide (NO) [22, 33], those cells showed the inhibitory effect.

As reported in the recent articles, when the MSCs was indirectly cocultured with lymphocytes (with paracrine mechanism-soluble factors), the immunosuppressive effect of MSCs derived from human has happened [22, 27, 29, 34–37]. On the other hand, in vivo immunosuppressive effects of MSCs were first shown in monkeys. The infusion of donor-derived MSCs delayed the rejection of tissue-inharmonious skin grafts [44]. It has been reported that the mouse MSCs suppressed the lymphocyte proliferation that was started by the allogeneic splenocytes and caused to increase the skin grafts' lifetimes with in vivo immunosuppressive effects [23]. It was indicated that the human MSCs changed the cytokine secretion profiles of the DCs, effecter T cells, and NK cells in order to encourage the anti-inflammatory

effect or tolerant phenotype when they are cocultured with the immune cells. When the MSCs were transported together with HLA-identical hematopoietic stem cells, acute and chronic GvHD decrease was observed [45]. It was reported that there was a rapid engraftment for the acute leukemia patient whom the MSC and peripheral blood stem cell transplantation was donated from his father with the HLA haploidentical via the standard immunosuppressive treatment and the patient survived without any acute or chronic GvHD until 31 months after the transplantation [46]. Similarly, the haploidentical MSCs were used for a 9-year-old male child with GvHD who was resistant to treatment in the intestine, skin, and liver after the allogeneic HSC transplantation because of acute lymphocytic leukemia (ALL). The transplantation from his mother completed and the child's GvHD and related clinical pathologic findings were improved [41]. Fang et al. performed two studies on severe acute GvHD disease and observed that the disease has developed after a blood transfusion for a 15-year-old and a 12-year-old child who suffered from acute myeloid leukemia (AML) and acute lymphocytic leukemia, respectively, and any response was not obtained for the pharmaceutical therapy [48]. In these two reports, it was declared that the MSCs have considerably positive results in treating and/or preventing the GvHD [47, 48]. The adipose tissue MSCs were implemented to the children by plastic surgery by obtaining the subcutaneous abdominal adipose tissue as a second treatment method and attained a very important success by reviving both of them in 15 days. After a donor-derived MSC infusion to 14 children besides the HLA-inharmonious peripheral blood HSC transplantation, Ball et al. observed that the graft failure was not detected in this group; however, such failure was previously observed at 15 % on 47 transplantations [47].

In order to be able to focus more on the biological pathways of cell transformation, the tumorigenic potential of MSCs has been studied using experimental protocols where MSCs are injected as cell suspensions into immunodeficient recipient animals. Based on the results obtained, the crucial matter now is to determine whether or not those known properties of the MSCs could display adverse effects in certain circumstances on the promotion of tumorigenesis. Djouad et al. analyzed the immunosuppressive function of MSCs by injecting into animals to see whether they showed any side effect-related with systemic immunosuppression that could lead tumor growth [49]. They first showed in vitro that the murine C3H10T1/2 (C3) MSC line and primary MSCs exhibit immunosuppressive properties in mixed lymphocyte reaction through production of soluble factors. These researchers showed in a murine melanoma tumor model that the immunoregulatory properties of MSCs favor the development of tumors as evaluated in allogeneic immunocompetent mice after co-implantation of MSCs and B16 melanoma cells [49]. Nevertheless, it has been shown in another study that low MSC numbers (10^2 MSC with 10^4 tumor cells) induced unexpected tumor rejection [50].

Patel et al. demonstrated that MSCs have been protecting breast cancer cells by expanding Treg cells, with concomitant decrease of Th1 and increase of Th2 cytokines. This effect was largely mediated by TGF-ß1 [51].

Karnoub et al. coinjected bone marrow-derived human MSCs with green fluorescent protein-labeled human breast cancer cells (MCF/Ras, MDA-MB-231,

MDAMB-435, and HMLER) in a ratio of 3:1 into immunocompromised mice [52]. The MSCs accelerated tumor growth in one of the four cell lines (MCF/Ras) but did not affect local tumor growth in the other cell types. Coinjection with MSCs increased the number of breast cancer metastases that formed in all cell lines investigated. The tissue engineering approaches where MSCs are used bring the presence of a bioscaffold to mold and facilitate the new development of a functional tissue [53]. The MSCs and bioscaffold that are in mutual effect could change the oncogenic potential of MSC [54] or influence the host's response to the cell graft. This is of crucial importance as MSC are frequently used in the clinical arena for tissue repair in combination with suitable bioscaffolds [53, 55], and the range of potential therapeutic applications is expanding [53, 56, 57]. Tumor and the tumor cells in microenvironment could be involved in providing the factors to block this immune reaction. There are evidences that different molecular and cellular pathways induce the differentiation of tumor-specific CD4+CD25+ Tregs that are able to block the antitumor-specific immune response [58, 59].

Tasso et al. [53] used a modified model of ectopic bone formation in mice by subcutaneously implanting porous ceramic seeded with murine MSC with the purpose of showing development of sarcomas in mice implanted with mesenchymal stem cells seeded onto bioscaffolds. They showed that host-derived sarcomas developed when implanted MSC/bioscaffold constructs into syngeneic and immunodeficient recipients. These researchers concluded that the bioscaffold provided a tridimensional support for MSC to aggregate, producing the stimulus for triggering the process eventually leading to the transformation of surrounding cells and creating a surrogate tumor stroma. Tasso et al. suggested that the immunoregulatory function of MSC contributed to tumor development. Implanted MSC expanded clones of CD4+CD25+ T regulatory lymphocytes that suppressed host's antitumor immune response [53].

Glioblastoma multiforme is a very agressive poor prognosis tumor type that hardly responds to therapy. Glioblastoma multiforme cancer-initiating cells have been shown to mediate resistance to both chemotherapy and radiation; however, it is unknown to what extent these cells contribute to the profound immunosuppression in glioblastoma multiforme patients [60]. Wei et al. [60] used two different experimental approaches to investigate the immune properties of glioma-associated cancer-initiating cells. In the first approach, the supernatants from glioma-associated cancer-initiating cells were used in immunologic assays with T cells from healthy donors to determine the effects of glioma-associated cancer-initiating cells in the absence of preexisting T-cell immunosuppression while avoiding allogeneic responses that could confound the interpretation of the data [60]. In the second experimental approach, using glioblastoma multiforme patients' T cells and the respective patients' glioma-associated cancer-initiating cells, allogeneic interactions would not confound the data, allowing for analysis of direct cell-to-cell contact; however, preexisting immunosuppression in the patient T cells and secreted factor from autologous cancer-initiating cells might dampen the extent of cell-to-cell contacting immunosuppression exerted by the glioma-associated cancer-initiating cells [60]. In both different experimental

designs, noncontradictorily, the data showed that the glioma-associated cancer-initiating cells mediate immunosuppression by several redundant mechanisms. They found that the cancer-initiating cells markedly inhibited T-cell proliferation and activation, induced regulatory T cells, and triggered T-cell apoptosis mediated by costimulatory inhibitory molecule B7-H1 and soluble galectin-3 that is constitutively expressed in glioma cell lines [60].

11.2.1.2 With Their Angiogenic Contributions

Among the very crucial processes, we can mention the vasculature for both embryonic development and differentiation and homeostasis of adult tissues. In a series of pathological conditions such as wound repair and some metabolic diseases, angiogenesis also plays an active role [61, 62]. The hypothesis that tumor growth is dependent on the formation of new blood vessels and inhibition of tumor angiogenesis would be an effective strategy to treat human cancer was reported in 1971 by Folkman [63]. The antiangiogenic drugs are being more and more used for the treatment of cancer; and the development of compounds that interfere with different angiogenic pathways have emerged from the past. VEGF-A/VEGFR2 signaling pathway, PDGF-B/PDGFR-β, Angiopoietins/Tie2 receptor and DII4/Notch 1 pathway are considered as four major pathways regulating proliferation and vascular growth of endothelial cells (EC) that received great attention [64]. Recently, antiangiogenic strategies for cancer treatment are mainly focused on compounds blocking the VEGF-A/VEGFR2 pathway.

Recent findings suggest that MSCs could also have an adverse effect that favors tumor growth: For example, when tumor cells mixed with MSCs are transplanted subcutaneously, the MSCs exhibit elevated capability of proliferation and rich angiogenesis in tumor tissues [65]. Recent studies suggest that MSCs contribute to tumor vasculogenesis mainly by producing proangiogenic factors and by transdifferentiating into endothelial-like and pericyte-like cells [66]. The first mechanism is supported by the fact that MSCs secrete specific proangiogenic factors such as VEGF, PDGF, FGF, and CXCL12 acting on tumor and/or endothelial cells [67]. In this respect, MSCs, coimplanted with cancer cells in syngeneic animals, accelerate tumor appearance, probably by favoring an angiogenic switch [68, 69]. MSCs can also differentiate into pericytes and endothelial-like cells and contribute to mature tumor vasculature formation [68, 70].

The evidence reported by Direkze et al. suggested that BM-MSCs might contribute to fibroblasts in tumor stroma area [71]. About 25 % of α-SMA-positive myofibroblasts and some vimentin-expressing fibroblasts were found to derive from the BM in pancreatic insulinoma in mice [71]. MSC-derived fibroblasts have also been shown to increase tumor growth in mice [71]. According to the results of Ramasamy et al. [72], 75 % of the mice injected with a mixture of tumor cells and cultured MSCs developed tumor, whereas 12 % of the animals receiving tumor cells alone showed signs of tumor growth. MSC-derived fibroblasts could also promote tumor growth directly by the production of proangiogenic factors, such as VEGF,

platelet-derived growth factors (PDGF), fibroblast growth factor (FGF), and stromal-derived factor-1 (SDF-1) [67, 73].

A number of experimental evidence has been indicating that MSCs are involved in the tumor angiogenesis by providing a supportive role as carcinoma-associated fibroblasts (CAFs) [56, 74–77] or perivascular mural cells [78]. MSCs are shown to express typical CAF markers, such as extracellular matrix (ECM) proteins (tenascin-c and thrombospondin), ECM remodelling enzymes and growth factors (hepatic growth factor (HGF), EGF, VEGF, transforming growth factor (TGF)-ß, and IL-6) [76]. These cells express α-smooth muscle actin [75], Tie-2 [79], and other pericyte markers [80] as well. Jeon et al. [81] demonstrated that conditioned medium from A549 human lung adenocarcinoma cells induces differentiation of human adipose tissue-derived mesenchymal stem cells (hASCs) to CAFs expressing α-smooth muscle actin, vascular endothelial growth factor, and stromal cell-derived factor-1. Using a murine xenograft transplantation model of A549 cells, in the same study, the researchers showed that co-transplantation of hASCs with A549 cells stimulated growth of A549 xenograft tumor, angiogenesis, and differentiation of hASCs to carcinoma-associated fibroblasts (CAF) in vivo. Knockdown of LPA1 expression in hASCs abrogated hASC-stimulated growth of A549 xenograft tumor, angiogenesis, and differentiation of hASCs to carcinoma-associated fibroblasts. Moreover, A549 conditioned medium-treated hASCs stimulated tube formation of human umbilical vein endothelial cells by LPA1-dependent secretion of vascular endothelial growth factor. These results suggest that A549 cells induce in vivo differentiation of hASCs to carcinoma-associated fibroblasts, which play a crucial role through an LPA–LPA1-mediated paracrine mechanism in tumor angiogenesis within tumor microenvironment [81].

It was demonstrated that most of the tumor vessels were non-hematopoietic, tissue-resident cells from the local environment rather than bone marrow-derived cells from the circulation [82]. Prantl et al. reported that ASCs are involved in tumor angiogenesis in a murine breast cancer model [83]. Moreover, it was found by Lin et al. [84] that PC3 prostate cancer cells recruited adipose tissue-derived stem cells (ADSC) by the CXCL12/CXCR4 axis. This study revealed that tumors from ADSC-treated mice had twice as much CD31 staining as tumors from PBS-treated mice and that FGF2 was expressed at a significantly higher level in the tumors of ADSC-treated mice. It has been suggested that the ADSC-induced upregulation of FGF2 was probably responsible for the increased vascularity and tumor growth. Recently, Prantl et al. [85] injected MDAPCa 118b cells mixed directly with GFP-labeled human adipose tissue-derived stem cells (hASCs) into athymic Swiss nu/nu mice. At 3 weeks after injection, they observed that the mean tumor volume in the MDAPCa 118b/hASC coinjection group was significantly higher than that in the MDA PCa 118b only group. Engrafted hASCs exhibited the nuclear marker of proliferation Ki67 and expressed markers for endothelial differentiation, indicating their engraftment in tumor vessels.

In summary, MSCs promotes the tumor angiogenesis either directly by transforming the pericytes and endothelial cells or indirectly by secreting the angiogenic growth factors such as VEGF, FGF, PDGF, and SDF-1. This process promotes the cytokine secretion and stimulates the growth of the blood vessels, consequently.

11.2.1.3 With Their Anti-apoptotic Properties

There is a consensus that the MSCs show their therapeutic effects both by direct differentiation into injured tissue and by production of paracrine and autocrine factors. In cases of tissue injury, MSCs can promote the secretion of a variety of cytokines and growth factors that have both paracrine and autocrine activities in the tissue environment [86, 87]. In investigating treating effects of MSCs transplanted into the infracted heart, several researchers noticed that MSCs undergoing hypoxia environments stimulated the infracted heart local microenvironment to secrete more amounts of cardioprotective vital growth factors to inhibit cardiomyocytes' apoptosis compared with MSCs in vitro cultured under normoxia [88]. It was shown that human MSCs secreted small amount of IL-6 while secreting large amount of TGF-β1 into the culture medium [89]. In addition, increased IL-6 secretion was demonstrated when hMSCs were cocultured with chemically damaged human corneal epithelial cells (hCECs). This has also been supporting the studies reporting upregulation of IL-6 in stimulated MSCs [30, 90]. It has been suggested that suppressive effects of MSCs can be through IL-6 by inhibiting lymphocyte apoptosis [91]. These findings showed that MSCs inhibit apoptosis of lymphocytes and that soluble factors, mainly IL-6 secreted by MSCs after direct interaction with lymphocytes, play an important role in their anti-apoptotic function. In another study, it was shown that coculturing with rBM-MSCs might have a significant potential to protect streptozotocin (STZ)-induced injured pancreatic islets, through paracrine actions such as cytoprotective, anti-inflammatory, and anti-apoptotic effects [92]. These results suggested that the underlying mechanisms modulating pancreatic islet viability might be attributed to paracrine mediators, IL-6, TGF-β1, osteopontin, and fibronectin secreted by MSCs.

A number of clinical evidences and experimental models with cancer pathogenesis supported the effect of the stromal microenvironment in the development of a wide variety of tumors [93–96]. It has been shown that tumor cells actively recruit stromal cells, including inflammatory cells, vascular cells, and MSC, into the tumor and that this recruitment is very important for the generation of a microenvironment that actively enhances tumor growth [97–99]. In the bone marrow, MSCs play a crucial role in supporting hematopoiesis by providing hematopoietic progenitor cells the necessary cytokine and cell contact-mediated signals to self-renew and differentiate [100]. It seems that malignant hematopoiesis is similarly affected by the presence of MSC providing tumor cells with a better survival by preserving their proliferative capacity and self-renewal ability. Leukemic cells were shown to grow and accumulate in close association with bone marrow MSC which might regulate their differentiation [101]. In acute lymphoblastic leukemia, there is evidence that MSCs regulate response to cytotoxic agents by directly interfering with their mechanisms of action [102]. Ramasamy et al. [72] showed that human MSCs preserve the proliferative capacity and self-renewal ability of tumor cells by inhibiting cell cycling and protecting the tumor cells from apoptosis. Vianello et al. [103] studied the effect of MSC on the intrinsic resistance of leukemic cells to imatinib. In their study, they reported evidence that a cell contact-mediated interaction between MSC

and chronic myeloid leukemia (CML) cells effectively protects leukemia progenitors from imatinib-induced cell death. When CML cells are exposed to imatinib while in contact with MSCs, their ability to engraft NOD/SCID mice is preserved, thus indicating that MSCs protect SCID leukemia-repopulating cells from the effect of imatinib. The authors suggested that protection from imatinib-induced cell death by MSCs involves caspase-3 activation in a CXCR4-dependent manner.

11.2.1.4 As a Main Component of Tumor Stroma

Tumor stromal cells including pericytes, endothelial cells, fibroblasts, myofibroblasts, macrophages, inflammatory cells, and other immune cells have been shown to contribute to the malignant progression of tumors [66]. MSCs are thought to modulate the tumor microenvironment by different mechanisms. Some evidence indicate that MSCs are recruited in large numbers to the stroma of developing tumors [72, 104] and they act enhancing the motility, invasion, and metastasis ability of adjacent cancer cells [52]. Carcinoma-associated fibroblasts (CAFs, also known as myofibroblasts or cancer stroma), which express α-smooth muscle actin (α-SMA) as a phenotypic marker, have been shown to play important roles during cancer progression and metastasis [105, 106]. They stimulate tumorigenesis, angiogenesis, and invasion in a variety of solid tumors, including prostate, breast, ovarian, and hepatocellular carcinomas [98, 107–111] by secretion of various extracellular matrix proteins, proteases, chemokines, and angiogenic factors [112]. Co-transplantation of CAFs has been shown to stimulate invasiveness of prostate and breast tumors in a xenograft tumor model [98, 110]. According to the recent evidence, CAFs originate from various cell types, including tissue-resident fibroblasts, cancer cells or epithelial cells undergoing epithelial-to-mesenchymal transition, or mesenchymal stem cells [107].

Moreover, recruitment of bone marrow-derived MSCs into the stroma of developing tumors has been reported [104]. MSCs constitute a large proportion of non-neoplastic stromal cells within the tumor microenvironment [107]. In addition, MSCs coinjected with human breast carcinoma cells into a subcutaneous site by xenograft transplantation stimulated metastatic potency of breast carcinoma [52]. Furthermore, human bone marrow-derived MSCs exposed to tumor-conditioned medium have been reported to exhibit phenotypic characteristics and ability of CAFs in promotion of tumor cell growth in vitro and in an in vivo co-implantation model [75, 76]. These results suggest that paracrine factors secreted from cancer cells can induce differentiation of MSCs to CAFs within the tumor-associated stroma [113].

Lysophosphatidic acid (LPA) is a small bioactive phospholipid produced by activated platelets, mesothelial cells, fibroblasts, adipocytes, and some cancer cells [114–116]. According to Aoki [115], it has been suggested that LPA is implicated in tumorigenesis and metastasis. Jeon et al. [81] reported that LPA induced migration of human adipose tissue-derived MSCs (hASCs) and stimulated differentiation of cells to α-SMA-positive CAFs [117, 118], suggesting a pivotal role of LPA in

generation of CAFs within the tumor microenvironment. Co-transplantation of hASCs with A549 human lung adenocarcinoma stimulated growth of xenograft tumors in vivo, and short hairpin RNA (shRNA)-mediated silencing of LPA receptor 1 (LPA_1) in hASCs abrogated hASC-stimulated in vivo growth of A549 xenograft tumors [81]. In addition, conditioned medium from A549 cells (A549 CM) contained significant levels of LPA and elicited differentiation of hASCs to α-SMA-positive CAFs through an LPA_1-dependent mechanism in vitro [81]. These results suggest that hASCs can be differentiated to α-SMA-positive CAFs through an LPA–LPA_1-dependent mechanism within the tumor microenvironment [113]. Lastly, Jeon et al. [81] demonstrate that LPA secreted from tumor cells is responsible for the differentiation of hASCs to CAFs through LPA1-dependent mechanism using an in vivo xenograft co-transplantation model.

Periostin, originally named osteoblast-specific factor-2, is a disulfide-linked 90-kDa secretory protein that functions as a cell adhesion molecule. It shares a structural homology to insect fasciclin I and supports adhesion of osteoblasts, thereby functioning in recruitment and attachment of osteoblasts to the periosteum [119]. In addition, accumulating evidence has demonstrated involvement of periostin in tumor growth and survival, angiogenesis, and metastasis [120]. High expression of periostin has been associated with tumor size, lymph node metastasis, disease stage, and lymphatic invasion in non-small cell lung cancer patients [121]. Furthermore, overexpression of periostin in patients with lung cancer has been correlated with clinicopathological findings, including squamous cell carcinoma type, higher stage, vessel infiltration, and tumor relapse [122]. Ectopic overexpression of periostin promoted proliferation and migration of A549 cells in vitro [123]. It was reported on high expression of periostin in CAFs of epithelial ovarian cancer tissues [124]. In addition, conditioned medium from ovarian cancer cells stimulated expression of not only α-SMA but also periostin in hASCs through an LPA_1-dependent mechanism [124], implying a possible role of periostin as an hASC-derived paracrine factor. In order to explain the paracrine mechanisms involved in the cross talk between cancer cells, A549, and hASCs, Heo et al. showed the role of secreted periostin of hASCs in tumor growth by xenograft co-transplantation [113]. In this study, it has been shown that periostin plays a pivotal role in adhesion and proliferation of A549 xenograft tumors within the tumor microenvironment as a paracrine factor secreted from hASCs [113].

Certain models of metastatic progression propose that cancer cell invasion and metastasis from the primary tumor site are strongly influenced by contextual signals emanating from the stroma of the primary tumor. It has been found that mouse stroma prepared from developing human MCF7/Ras or MDA-MB-231 breast cancer xenografts is rich in cells with an ability to generate fibroblastoid colony-forming units (CFU-F) in vitro, a hallmark of MSCs [49, 52]. The absence of such colonies from control Matrigel plugs or from neighboring tissues suggested that endogenous murine MSCs localize specifically to sites of neoplasia [52].

It was noted that CCL5, which is prominent in the stromal gene expression signature associated with poor prognosis of breast cancers [88], is also enriched in the leukocyte- and endothelial cell-free stroma of primary invasive ductal carcinomas,

specifically in the CD10-positive compartment [89]. When considered collectively, these observations suggest strongly for a significant association between stromal CCL5 levels, MSCs, and human invasive breast cancers. Studies have focused here on CCL5 in the MSC–MDA-MB-231 cell interactions; CCL5 seems to have an equally critical involvement in the functional interaction of MSCs with MDA-MB-435 human BCCs. CCL5 levels accumulate synergistically when the two cell types are cocultured together and MSCs in which CCL5 expression was compromised by shRNA knockdown failed to promote metastasis by MDA-MB-435 cells to which they were admixed. It is worth to note that they have observed that MSCs induce the metastasis of cells to the lung that are, on isolation and reinjection into recipient mice, no more metastatic than their predecessors in the primary tumor. This indicated that acquisition of increased metastatic powers by these tumor cells was reversible and suggested that the maintenance of this phenotype depends on continuing contact with stromal cells.

It was found by Karnoub et al. [52] that CCL5 released from the MSCs is involved in prometastatic effect in the mammary breast cancer cells. In addition, there is evidence that suggests that when mammary breast cancer cells coinjected with MSCs, there is a two- to sevenfold increase in lung cancer development [52]. Moreover, the similar prometastatic effect was not observed when the MSCs were injected in a more distance area: This suggests that the prometastatic transformation occurs by the contact with the MSCs or the paracrine factors released by these cells.

Bone marrow-derived MSCs have been shown to promote the colon cancer lymphoma and melanoma cancer cells, in vivo [49, 62, 125]. When the adult and fetal-derived MSCs coinjected with colon cancer cells in a murine model, the enhancement of the tumorigenesis was observed. Both adult and fetal MSCs had similar growth-promoting effects, but adult MSCs appeared to favor tumor incidence more than fetal MSCs. Muehlberg et al. [83] reported the evidence that there is a larger and more rapidly growing tumor existence when the adipose tissue MSCs and mammary breast cancer cells were coinjected in a syngeneic murine model. Moreover, there are similar results available when adipose tissue MSCs coinjected with lung and glioma cancer cells in murine [83].

Interleukin-6 (IL-6) is known as a multifunctional cytokine which is generally involved in regulation of inflammatory and immune response and the major activator of the Janus kinase (JAK)–signal transducer and transcription activator 3 (STAT3) signal pathway [126]. In addition to these general functions, it is also known that IL-6 signaling is involved in tumorigenesis [126]. STAT3, playing a role in this signaling, is considered as oncogen in a variety of cancer types and has been shown to cause malign cellular transformation in various experimental models.

Recent studies suggested that the anti-inflammatory signals released from tumor microenvironment promote the tumor growth and maintenance. In this context, we designed a study to investigate the effects of the stromal cell-derived IL-6 on mammary breast cancer cells (unpublished data). Stromal cells that have origin from healthy and malign mammary gland were cocultured with MCF-7 (human breast adenocarcinoma cell line). The preliminary results showed that IL-6 levels which is involved in the tumorigenesis in plate has dramatically increased after

coculture of stromal cells that derived from tumor tissue and MCF-7 cells. In addition, we showed that IL-6 and relevant gene (STAT3 and LIF) expressions dramatically increased in malign stromal cells after coculture. These results have been supporting our study where the recombinant IL-6 was used in coculture. In return for increase in IL-6 gene and protein levels, increased STAT3 protein expression in MCF-7 cells was identified after coculture with malign stromal cells. On the other hand, STAT3 increase was determined in MCF-7 cells cultured with IL-6. The increase in malignant stromal cell-derived IL-6 levels after coculture with MCF-7 cells apparently activates STAT3, and these signaling pathways promote tumorigenesis. As a result of our own more recent effort, we suggested that tumor microenvironment could promote tumorigenesis and taking the tumor microenvironment as primary target during cancer treatment might be a good alternative in treating this disease [127].

MSCs also can be involved in the modulation of the epithelial–mesenchymal transition (EMT) which can be defined as the process resulting in acquisition of more aggressive phenotype of the tumor cells [128]. After coculture with MSCs, the expression of EMT-specific markers (N-cadherin, vimentin, Twist and Snail) in mammary breast cancer cells increased [129]. Similar effect was observed in prostate cancer cells as well [130].

It is known that mesenchymal cells secrete SDF-1 [75] and VEGF [131] and are recruited to the sites expressing VEGF, platelet-derived growth factor (PDGF), and epidermal growth factor (EGF) [131]. It was also reported that intravenous injection of human mesenchymal stem cells expressing interferon-β to MDA231 metastases bearing mice significantly prolonged the overall survival of the animals [132].

In Table 11.1, the studies suggesting that MSCs promote tumorigenesis were summarized.

11.2.2 MSCs Inhibits Tumorigenesis: Good Side

Several studies reveal that MSCs have promoting effect, but also they possess cytotoxic effect on cancer cells. More recently, there are reports addressing the new horizons in the perplexing interaction between cancer and the stem cells. In one of these studies, MSCs were shown to inhibit the tumorigenesis when coinfected with murine colon carcinoma cells in equal or tenfold amounts [133].

It was reported that MSCs derived from human fetus skin prevented tumorigenesis both in vivo and in vitro by inhibiting the colony forming and oncogen expression and by decreasing the liver cancer cells proliferation [134]. When coinjected with MSCs, cancer cells were shown to cause delay in tumorigenesis and decrease in tumor volume. Similarly, it was suggested that the fetal skin-derived MSCs inhibited the in vitro tumorigenesis of the mammary breast cancer [135]. Researchers revealed that the cancer cells cultured in conditional supernatant medium showed decrease in the expression of the viability factors such as β-catenin, c-Myc, and survivin [135]. This happens via the DKK-1 which is a β-catenin signal inhibitor

Table 11.1 The studies suggesting that MSCs promote tumorigenesis

Source of MSC	Experiment model	Findings	Suggested affect	References
hBM-MSC	Breast cancer cell lines	Increased size in one cell line (MCF/Ras) and increased metastasis	Chemokine secretion (CCL5)	Karnoub et al. [52]
Fetal and adult BM-MSC	Colon cancer cell line	Enhanced proliferation and angiogenesis	Increased incidence	Zhu et al. [65]
Mouse BM-MSC	Melanoma (B16)	Increased incidence	Immunologic	Djouad et al. [49]
Human/mouse AT-MSC	Breast (4T1 and MDA231)	Increased size	Paracrine factor (SDF1/CXCR secretion)	Muehlberg et al. [83]
mAT-MSC	Breast (BB1)	Increased incidence and size	Vasculogenic	Galie et al. [69]
hAT-MSC	Prostate (PC3)	Increased incidence and size	Vasculogenic and modulation of tumoral CXCR4	Lin et al. [84]
hAT-MSC	Prostate	Increased size	Vasculogenic with differentiation into endothelial cells	Prantl et. al. [85]
hAT-MSC	A549	Increased tumor volume	Stimulate angiogenesis	Jeon et al. [81]
hBM-MSC	Hepatocellular carcinoma cell line (MHCC97-H)	Tumor volume increased	Modulation of TGFβ	Li et al. [147]

secreted by MSCs [135]. Adipose tissue-derived MSCs were shown to inhibit the leukemia cell proliferation [136]. In another study, it has been shown that MSCs have a pivotal role as suppressors of tumorigenesis in pancreas cancer by changing the cycles of cancer cells; it was also observed that MSCs-cocultured pancreas cancer cells ceased in G1 phase of cycle [137].

We also focused on the cytotoxic effects of the human dental pulp-derived MSCs (DP-MSCs) on K562 (human chronic myeloid leukemia) cells in vitro by using several experimental tools. DP-MSCs were stimulated with IL-2 to activate their apoptotic signal pathways; then K562 cells cultured in coculture system with stimulated and unstimulated DP-MSCs. When the viability/proliferation capacities and apoptosis status were examined, it was observed that K562 cell proliferation was suppressed in indirect coculture after stimulation, whereas in direct culture, this effect was shown in more dramatic levels. Although similar interaction was seen in unstimulated culture environment, IL-2 stimulation has increased the cell's proliferative capacity. It was determined that anti-apoptotic effects of stimulated cells is more significant than those of non-stimulated ones, whereas direct cultures

Table 11.2 The studies revealing that MSCs suppress the tumorigenesis

Source of MSC	Experiment model	Findings	Suggested affect	References
MPC1cE MSC (the immortalized mesenchymal progenitor cell line)	Rat colon carcinoma cells (H1D1)	Tumor size smaller	Increased inflammatory infiltrate	Ohlsson et al. [133]
Human fetal skin	Human hepatoma cell line (H7402 and HepG2)	Tumor size smaller	Wnt signaling	Qiao et al. [134]
Human fetal skin	Breast cell line (MCF-7)	Increased latency, reduced tumor size, and metastasis	Wnt signaling	Qiao et al. [135]
hAT-MSC	Pancreatic cancer cells	Tumor size smaller	G1 arrest	Cousin et al. [137]
hBM-MSC	SK-MES-1, A549	Decreased tumor incidence	Downregulation of VEGF expression	Li et al. [148]
hUCB-MSC	MDA-MB-231	Decreased tumor volume	Suppressing WNT pathway	Sun et al. [149]

are more effective compared to indirect cultures. Thus, stimulation of stem cells with IL-2 represents novel treatment approach to cure cancer [138].

In Table 11.2, the studies revealing that MSCs suppress tumorigenesis were summarized.

11.2.3 Claim of No Apparent Effect

Some studies reveal that MSCs may not have any effect on tumorigenesis. This was reported in an ovarian cancer experimental model, where intraperitoneally established xenografts were subsequently injected with bone marrow MSCs, and also of human adipose tissue-derived MSCs (AT-MSCs), which did not modify colon cancer cell growth in vitro.

Human MSCs seem also to have no effect in most cases on tumor growth of breast cancer cells implanted s.c. in athymic mice. In fact, it is not easy to interpret the action of MSCs on cancer cell growth as they can have opposite effects in vitro and in vivo [139].

The MSCs, when cocultured with hematopoietic and non-hematopoietic cancer cells, have been showing antiproliferative effect by triggering the cell cycle of cancer cells in G1 phase.

A tumorigenic potential of human and mouse MSCs was reported in both in vitro and in vivo experimental designs using cell populations expanded for five to six passages in plates [140–142]. Aneuploid karyotypes were observed in human and

mouse MSC cultures after several (P9–P15) in vitro passages [143, 144]. There is a possibility that the spontaneous transformation of MSC is due to the relative resistance of these cells to the telomerase-dependent mechanisms controlling the cell proliferation [145, 146].

11.3 Conclusion

In conclusion, the stem cells have the capacity of both promoting and treating certain cancer types. Thus, there is a very elaborative and complex interaction between the cancer and the stem cells. Recent studies have been providing the results in regard to changing behaviors of the stem cells depending on the existing conditions. In other words, the stem cell can act in a way in stimulated immune system cell and in another way in injured tissue localization. These fascinating features of stem cells raised more questions for the scientific research that still remain to be answered. It is not yet fully clear whether or not the experimental models are sufficient to reflect what is actually happening in the natural milieu of this devastating and lethal disease. The more understanding of the MSC involvement in microenvironment of the tumor would shed a light for the paradigmatic remarks of today's science of cancer formation and evolution.

Potential use of the MSCs in cell-based anticancer therapies in different cancer types and localizations is one of the primary scientific focuses nowadays. Promising cancer curative features of MSCs should be more investigated particularly in precise mechanism of action, and their origin, considering the possible side effects simultaneously.

Acknowledgments We thank Duygu Irmak for help in proofreading the manuscript. We wish to acknowledge Nurcan Erarslen for creating the artwork in Fig. 11.1.

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Chapter 12
Neural Stem Cells as Therapeutic Delivery Vehicles for Malignant Brain Tumors

Tugba Bagci-Onder

Abstract Malignant brain tumors are nearly untreatable due to their highly infiltrative nature and resistance to existing therapies. The main reason for recurrent tumor growth is believed to be the presence of tumor cells that migrate great distances into the brain tissue. In addition, poor delivery of therapeutics to the tumors due to blood–brain barrier limits the clinical success of currently available systemically delivered antitumor therapies. Recently, a different mode of therapeutic delivery, whereby therapeutic biomolecules are expressed by tumor-tropic neural stem cells (NSCs), has gained considerable attention. Exploiting the intrinsic tumor-homing ability of NSCs, the past decade has witnessed significant advances in the discovery and development of NSC-based therapies for malignant brain tumors. Prodrug converting enzymes, immunomodulatory cytokines, pro-apoptotic (tumouricidal) agents, growth-inhibiting factors, anti-angiogenic agents, and viral particles have been among the most commonly studied antitherapeutic molecules produced by NSCs. While the mechanisms of tumor-directed NSC migration and fate of NSCs after engrafting are still not truly understood, the results from current preclinical tumor models have demonstrated promising utility for NSCs as "armed vehicles" in treatment of aggressive brain tumors. Indeed, the first clinical trial with NSC-delivered antitumor agents is now in progress for recurrent gliomas.

Keywords Neural stem cells • Malignant brain tumors • Tumor-tropism • Targeted therapy • Cell carrier

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K. Turksen (ed.), *Stem Cells: Current Challenges and New Directions*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4614-8066-2_12,

Abbreviations

GBM	Glioblastoma multiforme
TMZ	Temozolomide
BBB	Blood–brain barrier
NSC	Neural stem cell
CNS	Central nervous system
ES	Embryonic stem
SVZ	Subventricular zone
Fluc	Firefly luciferase
Rluc	Renilla luciferase
MRI	Magnetic resonance imaging
BLI	Bioluminescence imaging
FE-Pro	Ferumoxide protamine sulfate complex
SF/HGF	Scatter factor/hepatocyte growth factor
SDF-1	Stromal-derived factor
CXCR4	CXC chemokine receptor 4
VEGF	Vascular endothelial growth factor
PI3K	Phosphoinositide 3 kinase
TMEM18	Transmembrane protein 18
ECM	Extracellular matrix
PCE	Prodrug converting enzyme
HSV-TK	Herpes simplex virus-thymidine kinase
CD	Cytosine deaminase
CE	Carboxylesterase
GCV	Ganciclovir
5-FC	5-Fluorocytosine
5-FU	5-Fluorouracil
CPT-11	Camptothecin-11
CPA	Cyclophosphamide
CYP2B6	CPA-activating enzyme cytochrome p450 2B6
TSP	Thrombospondin
OV	Oncolytic virus
CRAd	Conditionally replicating adenovirus
MMP	Matrix metalloproteinase
EGFR	Epidermal growth factor receptor
ENb	EGFR targeting nanobody
IL	Interleukin
IFN	Interferon
BM-NSC	Bone marrow-derived NSC
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
GMP	Good manufacturing practice
iPSc	Induced pluripotent stem cell

12.1 Introduction

Malignant brain tumors, especially Glioblastoma Multiforme (GBM), are practically untreatable due to their highly infiltrative nature and resistance to existing therapies. GBM is the most common and aggressive brain tumor, with a mean survival of 12 months after diagnosis [1]. This dismal survival rate has remained unchanged over the past 20 years in spite of the significant improvements in imaging and surgical techniques, as well as post-operative therapies [2]. There are currently few approved chemotherapeutic agents for GBM patients; the most widely used is Temozolomide (TMZ), a DNA alkylating agent that can only prolong patient survival by 2–3 months [3]. Therefore, innovative therapies that help eradicate tumors and prolong patient survival are still very much needed.

One of the major challenges in GBMs is the extraordinary ability of tumor cells to infiltrate into the brain parenchyma [4], making surgical resection an inadequate monotherapy. The tumor cells that can disseminate into great distances in the brain are believed to be the main reason for the recurrent tumor growth near the resection site and at further distances in the brain. In addition, poor delivery of therapeutics to the main tumor site as well as distant lesions due to blood–brain barrier (BBB) limits the clinical success of currently available systemically delivered anti-GBM therapies [5]. The emerging approaches that aim to overcome the problem of insufficient drug delivery to brain tumors are controlled release systems that utilize the implantation of drug-releasing materials on-site of the tumors, and convection enhanced delivery systems that diffuse the therapeutic agents to the surroundings of the tumors [6]. Despite the promise of these approaches, limited half-life of the delivered biological agent can still be an obstacle for successful therapies. Recently, a different mode of delivery has gained considerable attention, where therapeutic biomolecules are expressed by tumor-targeting neural stem cells. The past decade has witnessed significant advances in the discovery and development of neural stem cell-based therapies for malignant brain tumors.

12.2 Neural Stem Cells

Neural stem cells (NSCs) are multipotent stem cell populations that continuously self-renew and possess the capacity to generate neurons and glia of the nervous system [7]. These cells are abundantly present in the developing mammalian central nervous system (CNS) and limitedly present in the adult CNS. As the adult CNS has very limited capacity to replace damaged neural tissue and repair neural connections endogenously, external cell sources to replenish missing cell types are greatly needed for treatments of CNS diseases. Due to their ability to generate mature cell types such as neurons, astrocytes, and oligodendrocytes, NSCs have recently received tremendous attention in regenerative medicine. There are excellent reviews on the use of NSCs in regenerative medicine, which comprehensively describe the recent advances in NSC-based therapies in preclinical models of neurological diseases [7–10], as well as their translational path to the clinics [11].

12.2.1 NSC Sources

For any preclinical study or therapeutic application, it is of utmost importance to have a steadily growing source of cultured NSCs that maintain the capacity to give rise to different subpopulations of cells with specified biological functions. In addition, such NSCs should be derived from reliable sources, be safe in clinical applications, and recapitulate diverse developmental potentials in response to environmental cues after engrafting. To date, many different NSC sources have been utilized, particularly in preclinical mouse models of neurodegeneration or CNS injury. These multipotent NSCs are abundant during various stages of embryonic development; however, their numbers change as development progresses due to differentiation and lineage restriction. Therefore, the fetal CNS tissue is a rich source for generating and expanding NSC lines in vitro [7]. Another common way of producing NSC lines is to differentiate them from embryonic stem (ES) cells, which are pluripotent with the capacity to give rise to all cell types [12]. Through directed differentiation under specific culture conditions, different subtypes of NSCs can be derived from ES cells [13]. Another less-utilized source of NSCs is the adult CNS tissue. Indeed, the discovery of the presence of endogenous stem cells in the adult CNS is relatively recent [14]. The adult progenitors are located in two neurogenic niches of the brain, the subventricular zone (SVZ) of the lateral ventricular wall, and the subgranular zone of the dentate gyrus. The main function of these multipotent cells is to maintain neurogenesis and gliogenesis in the adult brain [7]. However, their fairly low abundance in these restricted locations makes NSCs unsuitable for routine isolation and derivation.

It is still a question whether the different cultured NSCs that continuously self renew and maintain multipotency in vitro are identical to the NSC cell populations that are found in vivo [15]. In addition, there is heterogeneity among the currently used NSC lines, which are derived from diverse sources and reported to grow in very different culture conditions. Furthermore, some of the commonly used NSC lines that are referenced here are generated from NSCs immortalized with various oncogenes, such as human telomerase (hTERT), simian vacuolating virus 40 (SV40) large T-antigen, and most commonly, v-myc [16]. While the immortalized lines offer several advantages in vitro, due to their ease of continuous growth in culture, they pose safety concerns for clinical settings, due to their potential to give rise to tumors. Given the heterogeneity of the NSC lines available, one needs to be careful while interpreting the results of the studies employing NSCs, since “NSC” is a rather generalized term given to these cells based on their operational features of multipotency and directed differentiation. These NSC lines might possess several intrinsic differences in their ability to give rise to specialized cells types of interest in regenerative applications. Despite such potential differences, accumulating evidence suggests that NSCs can be utilized in a new clinical application: as potential delivery vehicles for malignant brain tumors. The list and description of NSC types used as therapeutic vehicles in brain tumors are depicted in Table 12.1.

Table 12.1 Source and description of NSCs used in brain tumor therapies

Name of NSC line	Source	Description	Reference
c17.2	Mouse	Derived from neonatal cerebellum, immortalized with v-myc	[19, 25, 44, 60, 65, 97, 100]
HB1.F3	Human	Derived from fetal telencephalon, immortalized with v-myc	[10, 20, 45–47, 49–56, 63, 69, 70, 72, 81, 82]
mNSC	Mouse	Derived from embryonic mouse cortical tissue	[75, 79, 103, 104, 106, 108]
hNSC	Human	Derived from embryonic diencephalon and telencephalon, immortalized with v-myc	[26, 75]
hNSCs/ReNCell	Human	Derived from fetal ventral mesencephalon, immortalized with v-myc	[66–68, 105]
NT2RA2	Human	Derived from N2 cells, selected under NSC culture conditions	[61]
BM-NSC	Mouse	Derived from bone marrow, selected under NSC culture conditions	[78]
primary mouse NSC	Mouse	Derived from newborn forebrain	[62]
primary mouse NSC	Mouse	Derived from fetal frontoparietal region	[77, 96]
primary mouse/rat NSC	Mouse/rat	Derived from newborn cortex	[76]

12.3 NSCs' Potential as Tumor-Chasing Therapeutic Delivery Vehicles

As malignant brain tumors are known to be extremely invasive, complete surgical excision of these tumors without leaving behind any residual tumor cells is practically impossible [2]. Therefore, tumor recurrence occurs very frequently, where the recurring tumors are also refractory to conventional therapies. In the last decade, NSCs have been shown to home to brain tumors when engrafted in the vicinity of the tumors in preclinical models. Their intrinsic ability to home to tumors brought the novel concept of "delivering antitumor agents from tumor-tropic NSCs" turning these NSCs into "armed vehicles" (Fig. 12.1). Attesting to the potential utility of this approach in clinical settings, the first clinical trial with NSCs as antitumor agent delivery vehicles has been launched [17].

12.3.1 Engineering NSCs with Genetic Manipulation

In most studies that use NSCs in brain tumor models as therapeutic delivery vehicles, NSCs are modified to express markers of cell identity, such as fluorescent or bioluminescent proteins, and/or the therapeutic gene of interest. These modifications are

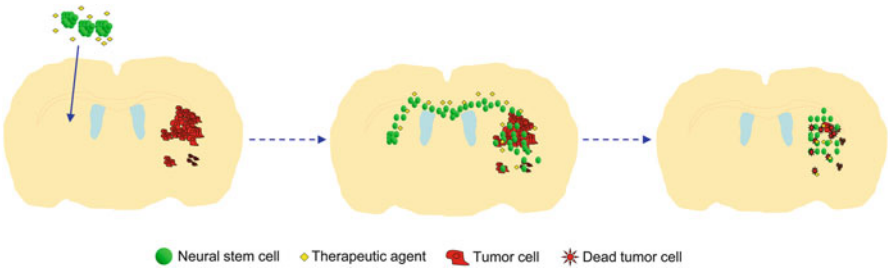

Fig. 12.1 Schematic representation of NSC-based therapies for malignant brain tumors. Neural stem cells (NSCs) can home to brain tumors when engrafted in close vicinity or at a greater distance away from the tumor site. NSCs can also colocalize with hard-to-reach disseminated tumor cells. Exploiting the innate tumor-directed migration ability of NSCs for therapeutic purposes is a novel and promising approach in brain tumor therapies. Accordingly, NSCs can be engineered to produce tumor-specific therapeutic agents, turning NSCs into "armed delivery vehicles"

made possible by transfection, or more commonly, viral transduction. Retrovirus, lentivirus, adenovirus, or herpes simplex virus-mediated gene transduction are the most frequently used methods to deliver genes of interest into NSCs [18]. Most of these genetic manipulations and their functional outcomes are discussed in the following sections and depicted in Table 12.2.

12.3.2 NSCs as Tumor-Chasing Cells

The first evidence for NSC tropism for brain tumors emerged in 2000, where an immortalized mouse NSC line, C17.2, was examined in brain tumor-bearing adult nude mice. In this model, NSCs, marked with β-galactosidase, were extensively migratory towards tumors. NSCs were distributed along the tumors and also at distant sites away from the main tumor mass, where they were "chasing" the invading tumor cells. Even when implanted into contralateral hemisphere, NSCs ended up in the tumors growing distant from the NSC implantation site [19]. These observations led to several new studies that together aimed to monitor this unique tropism and develop NSC-based delivery of anticancer agents targeted towards malignant tumors that infiltrated the brain (Table 12.2). Most of the current knowledge on the tumoritropic behavior of NSCs comes from in vivo studies that employed orthotopic tumor models and NSC lines introduced into the vicinity of tumors at various distances. Histological examination of brain sections after implantation of NSCs to tumor-bearing brains has been a major approach to assess NSC-tumor interactions. X-Gal staining in brain sections was the first method to reveal the presence of the b-galactosidase-expressing C17.2 cells, infiltrating and surrounding the tumors [19]. Another commonly employed and now clinically approved cell line is HB1.F3, which is a human NSC line originally derived from human fetal telencephalon and then immortalized using a retroviral vector encoding v-myc [20].

Table 12.2 List of antitumor therapeutic agents produced from NSCs

Therapeutic agents	Molecules expressed	NSC type	Method to engineer NSCs with therapeutics	Disease model	Reference
Prodrug converting enzymes	CD	C17.2	Retroviral transduction	Intracranial glioma	[19]
				Extracranial tumors	[44]
		HB1.F3	Retroviral transduction	Intracranial medulloblastoma	[45]
				Leptomeningeal medulloblastoma	[46]
				Brainstem glioma	[47]
				Brain metastasis from melanoma	[49]
				Brain metastasis from breast cancer	[50]
				Syngenic GBM	[51]
	CE	HB1.F3	Adenoviral transduction	Neuroblastoma	[52]
				Subdural medulloblastoma	[53]
				Intracerebellar medulloblastoma	[54]
				Brain metastasis from breast cancer	[55, 56]
	TK	Primary rat NSCs	Retroviral transduction	Rat C6 glioma	[57–59]
		C17.2	Stable transfection	Glioma	[60]
		NT2RA2 cells	Lentiviral transduction	Intracranial glioma	[61]
	CYP2B6	Primary mouse NSCs	Retroviral transduction	Intracranial glioma	[62]
Pro-apoptotic agents	Human trail	Primary mouse NSCs	Adenoviral transduction	Orthotopic glioma	[96]
	S-TRAIL	C17.2	HSV mediated transduction	Subcutaneous glioma	[97]
				Orthotopic glioma	[25, 100]
		mNSC	Lentiviral transduction	Subcutaneous glioma	[104]
				Orthotopic glioma	[75, 79, 103, 106, 108]
	mTRAIL	hNSC/ReNCell	Lentiviral transduction	Intracranial glioma	[105]

(continued)

Table 12.2 (continued)

Therapeutic agents	Molecules expressed	NSC type	Method to engineer NSCs with therapeutics	Disease model	Reference
Immunomodulatory agents	IL-14	Primary mouse and rat NSCs	Retroviral transduction	Syngeneic GBM, rat glioma	[76]
	IL-12	Primary mouse NSCs	Adenoviral transduction	Intracranial glioma	[77]
	IL-23	BM-NSCs	Adenoviral transduction	Intracranial glioma	[78]
	IL-24 (MDA-7/SM7L)	mNSC	Lentiviral transduction	Intracranial glioma	[79]
	IFN-β	HB1.F3	Adenoviral transduction	Disseminated neuroblastoma	[81, 82]
				Brainstem glioma	[47]
Antiangiogenic agents	Tsp	hNSC	Lentiviral transduction	Intracranial glioma	[64]
Viruses	HSV	C17.2	With replication conditional HSV-1 mutant	Intracranial glioma	[65]
	Oncolytic adenovirus	hNSC/ReNCell	With conditionally replicating adenovirus	Intracranial glioma	[66–68]
		HB1.F3	With conditionally replicating adenovirus	Orthotopic glioma (mouse, hamster, rat)	[69]
Naturally occurring antitumor agents	PEX	HB1.F3	Stable transfection	Glioma	[70]
Antibodies	Anti-HER2	HB1.F3	Transfection, lentiviral transduction	Metastatic breast cancer	[72]
	Anti-EGFR	mNSC, hNSC	Lentiviral transduction	Glioma, GBM	[75]
Multiple bioagents	CD and TK	HB1.F3	Retroviral transduction	Metastatic brain tumor	[63]
	S-TRAIL and EGFR nanobody	mNSC	Lentiviral transduction	Orthotopic glioma	[75]
	S-TRAIL and IL-24	mNSC	Lentiviral transduction	Orthotopic glioma	[79]
	IFN-β and CD	HB1.F3	Retroviral transduction	Orthotopic glioma	[107]

With stereological and histological examination of tumor bearing rodent models, HB1.F3 cells were reported to be extremely migratory towards main tumor mass as well as distant tumor deposits [21]. Similarly, using confocal microscopy and mathematical modeling, the tumor-centric distribution of DiI-labeled HB1.F3 cells was demonstrated in a mouse model of orthotopic glioma [22]. Using a different clone of HB1 cells, HB1.F5, in a rat glioma model established from intracranial implantation of C6 cells, the tumor tropism of NSCs was demonstrated in situ [23]. While these examinations were necessary to confirm the interaction of engrafted NSCs with resident tumors, more advanced noninvasive imaging methods provided more clues about the temporal distribution of tumor-chasing NSCs in addition to spatial information. This approach was first demonstrated by Tang et al. [24], where the mouse NSCs (C17.2 cells) transfected with Firefly luciferase (Fluc) were administered intraparenchymally, intravenously, or intraperitoneally to brain tumor-bearing mice and the distribution of NSCs was monitored with serial bioluminescence imaging. Accordingly, Fluc-expressing NSCs moved towards the tumor-site over time. The intraparenchymal injection of NSCs was the most efficacious in populating the tumors where the NSCs were able to move through the corpus callosum from contralateral site to the opposite hemisphere containing the tumors [24]. In a more advanced study, Shah et al. demonstrated the tumor-tracking ability of mouse NSCs by engineering tumor cells and NSCs with separate luciferase reporters. In this first study to employ dual bioluminescence imaging, Fluc-labeled NSCs migrated towards Renilla luciferase (Rluc)-labeled tumors that were growing in the opposite hemisphere [25]. In a follow-up study, Shah et al. utilized state-of-the art bimodal reporters, where the NSCs and tumor cells were engineered with different bioluminescent and fluorescent reporters and monitored with bioluminescence imaging and intravital microscopy in vivo [26]. The experimental details of these studies are comprehensively described elsewhere [27, 28]. Despite the important insights bioluminescence and fluorescence imaging provides in animal models, these techniques cannot be used in clinical settings to track the fate of engrafted stem cells. However, other imaging modalities such as magnetic resonance imaging (MRI) would be better suited to identify the location of NSCs in clinical applications. To this end, fate of engrafted NSCs has been monitored in several brain disease animal models, most commonly in hypoxic-ischemic injury model, upon labeling NSCs with ferromagnetic materials [29]. The first study to monitor NSC fate in orthotopic xenograft glioma models with MRI was performed by Thu et al., where human NSCs (HB1.F3 cells) were iron-labeled using ferumoxide–protamine sulfate complex (FE-Pro) and characterized in comparison with nonlabeled NSCs. As a result, the tumor-tropism, viability, and multipotency of the engrafted NSCs were validated with this clinically relevant tracking modality [30].

12.3.3 Mechanisms of NSC Migration Towards Brain Tumors

The mechanism for the unique tropism of NSCs towards malignant brain tumors is not completely understood. It is most likely that there are multiple mechanisms at

play for this migratory behavior. Most of our knowledge on the mechanisms of NSC tumoritropic migration comes from in vitro studies, where the NSCs are tested for their migration abilities in modified Boyden chamber chemotaxis assays. Briefly, either tumor cells or their conditioned media are cultured in lower chamber compartments and the NSCs are induced to migrate towards tumor cell-derived factors. One important factor identified with these assays was scatter factor/hepatocyte growth factor (SF/HGF), where blocking SF/HGF signaling reduced NSC migration towards tumor-conditioned medium [31]. A commonly implicated mechanism in NSC tumor-tropism involves chemokine signaling. Release of chemokines by tumors that can activate a migratory response in resident and/or engrafted stem cells is most likely to play a significant role in this behavior, similar to the activation and mobilization of NSCs in inflammatory conditions. For example, the secretion of stromal-derived factor (SDF-1) in response to CNS injury was shown to induce the directed migration of NSCs that expressed CXC chemokine receptor 4 (CXCR4) [32]. Given that tumors are considered "unhealed wounds," secreting inflammatory cytokines such as SDF-1, and that NSCs express a set of chemokine receptors such as CXCR4 [33], implication of SDF-1/CXCR4 paracrine signaling in the directed migration of NSCs to tumors is not surprising. However, more functional experiments are needed to identify and characterize these interactions between engrafted NSCs and tumor cells. Another mediator of the NSC movement towards tumors is the secretion of pro-angiogenic factors that are produced in brain tumor microenvironments. For example in GBMs, where the core of the tumor is highly hypoxic, neo-angiogenesis gets activated through the upregulation of hypoxia-inducible genes such as Vascular Endothelial Growth Factor (VEGF) [34]. Indeed, VEGF signaling was shown to activate NSC migration, in one of the first reports of this tropism [35]. Similarly, hypoxia-mediated induction of SDF-1 was implicated in NSC trafficking providing a link between SDF-1 and hypoxia-mediated NSC migration. This link was strengthened by the comprehensive in vitro and in vivo analysis of the effects of hypoxia on the tumor-tropic migration of HB1.F3 cells [36]. Accordingly, HB1.F3 cells were found to be preferentially distributed in hypoxic regions in tumor xenografts. Also, hypoxic conditions induced the expression of SDF-1, as well as VEGF and SF/HGF signaling, providing a link between hypoxia and chemokine induced NSC migration [36]. A potential downstream mediator of the soluble factor signaling between NSCs and tumors is Phosphoinositide 3-kinase (PI3K) signaling, as its inhibition led to a significant reduction of NSC migration suggesting that PI3K pathway serves as critical convergence point for the growth factor induced directed NSC migration [37]. Besides these soluble growth factors and chemoattractants, a novel gene encoding transmembrane protein 18 (TMEM 18) was identified to be a potential mediator of NSC tumor-tropism using a cDNA expression library screen. Briefly, overexpression of TMEM18 in C17.2 cells markedly increased their migration capacity towards tumor cells in vitro and in vivo [38]. Conversely, siRNA-mediated silencing of TMEM28 reduced this migration. TMEM18 was shown to upregulate CXCR4 expression as one possible mechanism of its-promigratory action and link to chemokine signaling [38]. Besides these

factors, the role of tumor-derived extracellular matrix (ECM) proteins in NSC migration was also examined. Accordingly, purified ECM components from tumor microenvironment, such as Laminin and Tenascin-C, strongly induced the NSC migration in vitro, suggesting a permissive role for tumor-derived ECM for NSC tumor-tropism [39]. A large-scale screen to examine the factors expressed by tumor cells with a role in inducing NSC migration would be very beneficial to identify novel mechanisms involved in NSC tumor-tropism. An et al. utilized such a strategy assessing protein and gene expression profiles of a panel of tumor cells and identified tumor-derived Annexin A2 as a potential inducer of NSC migration [40]. Further understanding of the mechanisms of these interactions will provide additional strategies to be utilized for clinical applications.

12.3.4 NSCs as Therapy-Carrying Vehicles in Aggressive Brain Tumors

Due to their unique tumor-tropic properties, NSCs can be utilized in a novel therapeutic function: that is to deliver tumor-specific therapies to aggressive brain tumors. It has been demonstrated by several laboratories that NSCs "armed" with antitumor agents can provide therapeutic efficacy [41]. The therapeutic molecules produced by NSCs can be classified as prodrug converting enzymes, immunomodulatory cytokines, pro-apoptotic (tumouricidal) cytokines, growth-inhibiting factors, anti-angiogenic agents, and viral particles [42] as depicted in Table 12.2.

12.3.4.1 Prodrug Converting Enzymes/Suicide Gene Therapy

Majority of the NSC-based tumor therapies involved the incorporation of prodrug converting enzymes (PCEs) into NSCs. These enzymes function through converting systemically administered inactive/nontoxic prodrugs into toxic metabolites. One of the important properties of this system, which is also known as "suicide gene therapy," is the "bystander effect," where not only the cells that carry the transgene but also the surrounding tumor cells are affected by the therapy. The mechanism for this "therapeutic spread" is thought to involve the expression of gap junctions between the affected and neighboring cells [43]. Given the intratumoral localization of NSCs, toxic metabolites are only produced in the vicinity of the tumor cells by PCE-expressing NSCs, thereby affecting specifically the tumor cells while preserving normal tissue. Several PCEs have been used for this approach, such as Herpes simplex virus-Thymidine kinase (HSV-TK), cytosine deaminase (CD), and carboxylesterase (CE) as also discuseed in other reviews [16–18, 41, 43]. HSV-TK converts ganciclovir (GCV) into the toxic form GCV-triphosphate; CD converts inactive 5-fluorocytosine (5-FC) into toxic 5-fluorouracil (5-FU); and CE converts camptothecin-11 (CPT-11/irinotecan) into active metabolite SN-38.

Among the PCEs, CD was the first to arm the NSCs. The product of CD conversion, 5-FU, is a pyrimidine analogue that can inhibit DNA/RNA synthesis and thereby kill the cells that receive it or surrounding it due to bystander effect [18]. Accordingly, NSCs (C17.2) were stably transduced to express CD and implanted into tumor bearing brains with different methods: intratumorally, short distance away from tumor bed, in contralateral hemisphere, intravascularly, and intraventricularly. Regardless of the route of administration, CD-expressing NSCs (NSC-CD) migrated similarly to the untransduced NSCs retaining their tumor-homing properties. Upon systemic administration of 5-FC, NSC-CD caused a marked reduction in tumor volumes compared to control animals that received untransduced NSCs or 5-FC only [19]. The same group later showed that NSC-CD was efficacious in targeting both intracranial and extracranial tumors when administered through peripheral vasculature [44]. Several other studies also showed the utilization of CD-expressing NSCs in not only gliomas but also other brain cancers such as medulloblastomas [45, 46], brainstem gliomas [47], melanoma [48] and secondary brain metastases from melanoma [49], or breast cancer [50]. It is recently reported that human NSCs (HB1.F3) engineered to express CD were not only efficacious in targeting brain tumors specifically but also elicited a mild immune response participating in reduction of tumor volumes in syngenic mouse glioma models [51].

Another PCE that was widely used in NSC engineering is CE, whose toxic product (SN-18) is a potent topoisomerase I inhibitor. In most of the published studies with CE, rabbit CE was introduced into HB1.F3 cells through replication-deficient adenovirus-mediated transduction. In one of the earlier studies, NSCs expressing CE (NSC-CE) were tested in a disseminated neuroblastoma model, where the tumor cells were introduced by intravenous injection and caused multiple anatomic tumors at several sites including kidney, liver, lung, and ovaries. In this model, where NSC-CE was also given intravenously, NSC-CE and CPT-11 treatment led to a significant increase in survival rates with 90 % of the mice surviving 1 year later with no evidence of detectable tumors [52]. More recently, NSC-CE was characterized in a subdural medulloblastoma model, where human tumor cells were implanted into the subdural space and NSC-CE was injected intravenously. Upon CPT-11 administration, NSC-CE treatment led to increased survival of mice [53]. Comparable efficacy of NSC-CE was later demonstrated in intracerebellar medulloblastoma models as well [54], as well as in a breast-to-brain metastasis model, where the breast cancer cells were intraparenchymally implanted and the NSC-CE was implanted into opposite hemisphere. Survival was improved attesting to the migratory and therapeutic ability of CE-expressing NSCs [55]. Very recently, the therapeutic efficacy of NSC-CE was assessed in a metastasis model, where distant metastases from breast cancer into lymph nodes, liver, lung, and bone were established from breast cancer cell injection into mammary fat pad, and NSCs were administered through the tail vein. Accordingly, the presence of NSC-CE and CPT-11 reduced the metastatic burden significantly, suggesting the NSC-CE can be a useful therapy for hard-to-treat systemic metastasis in addition to primary brain tumors [56].

HSV-TK/GCV system is another commonly studied viral-mediated suicide gene therapy, where GCV is converted into its toxic metabolite GCV-triphosphate, a DNA

synthesis inhibitor targeting the readily proliferating cells. The efficacy of NSC delivery of HSV-TK/GCV system is mostly assessed in rodent models of glioma, where C6 rat glioma cells are orthotopically implanted in rat brains to establish intracranial tumors. Instead of the immortalized NSC lines such as C17.2 and HB1.F3, most of the work done with HSV-TK system utilized rat-derived primary NSCs isolated from 14-day-old rats and transduced with HSVtk encoding retroviruses [57]. In these studies, the bystander effects of NSCtk were comprehensively analyzed in NSC-tumor cocultures and the efficacy of intratumorally injected NSCtk was demonstrated in in vivo conditions in the presence of GCV [58]. The same group later tested the migratory ability of NSCtk in the same model, where they implanted NSCtk at several different sites away from the main tumor mass. Accordingly, NSCtk cells targeted deeply infiltrating and disseminating tumor cells in the brain, and caused marked tumor reduction in the presence of GCV [59]. In one study, C17.2 cells were engineered to express HSVtk using stable transfection and tested for their bystander efficacy in established glioma cell lines in vitro [60]. Another cell source to be engineered with HSV-TK was human NT2 neural precursor-derived tumor tropic cells, which were cultured in the presence of retinoic acid and then isolated based on their migratory capacity towards glioma cells in vitro. These cells, called NT2RA2-tk, were successful in homing to and eradicating gliomas [61].

A recent study employed a less well-known PCE in NSC-mediated glioma targeting. Engineering primary NSCs with cyclophosphamide (CPA)-activating enzyme cytochrome p450 2B6 (CYP2B6), Mercapide et al. showed that engineered NSCs (NSC-CYP2BA) elicited tumor-tropic properties and substantial reduction in tumor growth upon administration of CPA [62]. This study added to the growing list of preclinical studies with NSC-mediated prodrug converting enzymes activation in brain tumor therapies. A very recent work from Seung U Kim's group showed a different perspective in utilization of PCEs in stem cell-based therapies. They engineered HB1. F3 cells with both CD and HSVtk and showed that the combination of both suicide therapies led to intensified tumor eradication in a lung cancer metastasis model. Upon administration of GCV and 5-FC, tumor bearing mice displayed increased survival and less metastatic burden [63]. Together, genetic engineering of NSCs with PCEs has been employed in several intracranial and extracranial tumor models and offers immense towards clinical applications.

12.3.4.2 Anti-angiogenic Agents

Angiogenesis, which is the process of growth of new blood vessels from preexisting vessels, is a fundamental hallmark of tumors and a very important target of therapy in cancers. There are several clinical trials testing anti-angiogenic agents in brain tumors, particularly targeting the vascular endothelial growth factor (VEGF) pathway [5]. However, delivering anti-angiogenic agents to the brain through systemic chemotherapies would still be subject to the challenge of BBB. Therefore, delivery of such agents locally through the use of stem cells would be well suited for successful therapies. The number of studies on NSC-delivered

anti-angiogenic agents is comparably small, with one important study performed by Khalid Shah's group [64]. In this study, van Ekkelen et al. first created and characterized multiple variants of an anti-angiogenic protein, thrombospondin (TSP) (aaTSP-1). Then human NSCs were transduced with aaTSP-1 were tested in malignant glioma models as well as for their effects on glioma-initiating cells. Accordingly, NSC-aaTSP-1 markedly reduced tumor vessel density and inhibited tumor progression [64]. This study provided a platform for future use of NSCs as anti-angiogenic agent delivery vehicles.

12.3.4.3 Viruses/Oncolytic Viral Particles

Oncolytic virus (OV) therapy, where the viruses are genetically modified to selectively replicate in tumor cells, deliver a cytotoxic therapeutic agent and kill the replicating tumor cells. However, tumor-targeting problems and immune clearance are obstacles for successful OV therapies making NSC-based OV therapy an alternative strategy [16]. Loading NSCs with viruses for antitumor therapies is a different but most commonly used approach, where the NSCs serve as producer cell lines for replication-incompetent viral vectors that can transduce oncolytic genes into tumor cells very efficiently due to their tumor tropism [16, 43]. The first proof-of-concept study by Herrlinger et al. demonstrated the feasibility of producing replication conditional HSV-TK from NSCs (C17.2 cells). These cells retained their migratory capacity and distributed throughout the tumor tissue [65]. A pioneer study to comprehensively test OV therapy by NSCs was performed by Maciej S Lesniak's group, using conditionally replicating adenoviruses (CRAds) [66]. Using commercially available human NSCs, Tyler et al. first produced CRAds under survivin promoter, which was specifically expressed in GBM cells assuring the specific production of viruses only in tumor cells. NSCs loaded with CRAds were able to migrate towards tumors. The therapeutic efficacy of NSC-CRAds was tested in a subcutaneous tumor model, where the volume of tumors that received virus loaded NSCs reduced significantly compared to tumors that received viruses alone [66]. Another study from the same group showed a similar effect in orthotopic glioma model, where NSC-CRAd inhibited tumor growth, and increased the median survival by 50 % [67]. In a comparative study by the same group, the potential of NSCs and other stem cells types (MSCs) as carriers for CRAds was assessed and NSC-CRAds that were administered intracranially in an orthotopic glioma model significantly prolonged the survival of tumor bearing animals and displayed superior therapeutic efficacy in intracranial tumors [68]. Very recently, Maciej S Lesniak's group assessed the pharmacokinetics and biodistribution of NSC-CRAds (generated from HB1.F3 cells) injected intracranially in tumor-bearing mice, as well as in other animal models, such as Syrian hamster and cotton rats. This study revealed the specificity of cell-based CRAd delivery and suggested NSCs as an optimized carrier system with minimal toxicity for anti-glioma OV therapies [69].

12.3.4.4 Naturally Occurring Antitumor Agents/PEX

A naturally occurring fragment of matrix metalloproteinase-2, called PEX, was also evaluated in NSC-based therapies for gliomas. PEX is expressed in malignant gliomas, and it has an inhibitory function on angiogenesis and tumor cell proliferation. Exploiting the antitumor properties of this protein, Kim et al. transfected HB1.F3 cells with PEX-encoding expression vectors and confirmed the retention of migratory ability of these PEX-producing NSCs. When injected inratumorally, NSC-PEX resulted in reduction of tumor microvessel density, proliferation rate, and a reduction of tumor volumes by 90 % [70]. Given the importance of matrix metalloproteinases (MMPs) in tumor biology, targeting extracellular matrix components or MMPs via NSC-delivered agents can be utilized as an alternative strategy in NSC-based therapies.

12.3.4.5 Recombinant Antibodies

Recombinant antibodies have recently gained considerable attention and are currently being evaluated in several clinical trials [71]. However, their large size is considered to be an obstacle in their tumor tissue penetration as well as crossing the BBB. Therefore, engineering NSCs to release such recombinant antibodies on site of brain tumors would be very beneficial in delivering sufficient amounts of these therapeutic agents to tumor cells and overcoming the obstacles created by BBB. Karen S Aboody's group demonstrated the proof-of-concept example of expressing antitumor recombinant antibodies from NSCs in a breast cancer model. They focused on Herceptin (Trastuzumab), a monoclonal antibody used to treat HER2-expressing breast cancers. They genetically modified HB1.F3 cells to secrete anti-HER2 immunoglobulin molecules, and showed that these cells were able to deliver antibodies in breast cancer xenografts [72]. Besides full length recombinant antibodies, therapies that involve smaller antibody fragments such as Fabs, ScFvs, and nanobodies have been emerging [73]. For example nanobodies, single-domain antibodies that consist only of the antigen-specific domain, are significantly smaller in size and potentially provide higher tissue dispersion than their counterparts [74]. In a very recent study from Khalid Shah's group, van de Water et al. engineered different bivalent EGFR targeting nanobodies (ENbs) and their imageable and pro-apoptotic immuno-conjugates for extracellular release from NSCs [75]. In this study, we utilized tumor models of malignant and CD133 primary invasive GBM, and first showed ENb pharmacokinetics with state-of-the-art bioluminescent imaging techniques and then showed that NSC-Enb was more successful than systemic injection of anti-EGFR antibdoes, such as Cetuximab in vivo. This study demonstrated the feasibility of expressing recombinant antibodies from NSCs in orthotopic models of GBM and provided a novel platform for NSC-antibody targeted therapies.

12.3.4.6 Immunomodulatory Agents

Immunotherapy by using immune-stimulating cytokines aims to activate immune response against cancer cells and therefore has been a major interest for therapies. Indeed, immunomodulatory agents such as interleukins (IL-4, IL-12) are among the most commonly delivered therapeutic agents using NSCs. The first study to report the use of IL-4 secreting NSCs was performed by Benedetti et al. In this study, primary mouse neural progenitor cells were engineered to express IL-4. These cells were first tested in a syngenic mouse GBM model, where there was marked increase in mice survival upon NSC-IL-4 administration. A similar result was observed using a different model, where rat-derived NSCs were first immortalized, transduced with IL-4, and then tested in established C6 rat glioma models using MRI and histological examinations of brain sections [76]. The second study to test NSC-secreted interleukins was performed by Ehtesham et al., where fetal mouse brain-derived NSCs were transduced with adenoviral vectors encoding IL-12. In a murine glioma model established from GL26 cells, NSC-IL-12 were injected intratumorally, which prolonged survival compared to treatment with control NSCs. This was associated with enhanced T-cell infiltration in primary tumor and microsatellites suggesting that NSC-IL-12 treatment could provide long-term antitumor immunity in this model [77]. Another study delivered IL-23 from a different cell source that exhibited NSC-like characteristics. These cells were derived from bone marrow, selected under NSC growth conditions to form neurospheres and then engineered with adenoviral vectors encoding IL-23. Delivery of IL-23 from these BM-NSC-IL-23 cells led to inhibition of growth of tumors established from GL26 cells. This study showed the necessity of CD8+ T cells in the observed phenotype and also showed that IL-23 expressing BM-NSCs induced antitumor immunity given the resistance of BM-NSC-IL-23 treated mice to tumor rechallenge [78]. A very recent study from Khalid Shah's group showed that engineering primary mouse NSCs with different secretable variants of IL-24 (MDA-7) with enhanced secretion and diagnostic properties, called SM7L, led to marked attenuation of tumor progression in an orthotopic GBM model. The results of this study promised a new approach of targeting aggressive GBMs using the optimized interleukin variant SM7L delivered by NSCs [79].

Another frequently used immunomodulatory agent is Interferon-β (IFN-β), which is known to have antitumor effects by inhibition of tumor cell proliferation and angiogenesis, as well as induction of apoptosis. However, its short half-life and systemic toxicity are thought to be the root causes for its limited success in clinical trials [17] making NSC-delivered IFN-β a strong alternative that can possibly overcome these obstacles [80]. The first example of NSC-delivered IFN-β came from Dickson et al. in a disseminated neuroblastoma model that was established by tail-vein inoculation of tumor cells. In this model, HB1.F3 cells overexpressing IFN-β were also administered intravascularly which then disseminated to tumor tissue and markedly reduced tumor growth as judged by BLI imaging [81]. Another group tested the use of NSC-IFN-β in conjunction with cyclophosphamide (CTX) aiming to exploit IFN-β's function as a modulator of vascular maturation in the tumors. Targeted delivery of IFN-β enhanced the effect of adjuvant CTX treatment and

resulted in effective tumor therapy [82]. A study specifically targeting gliomas was performed by Lee et al., where the utility of HB1.F3-IFN-β was tested in a brainstem glioma model in rats [47]. These cells displayed tropism, targeted brainstem gliomas efficiently, and reduced tumor volumes significantly. The potential of NSC-IFN-β in orthotopic aggressive GBM models remains to be tested.

12.3.4.7 Pro-apoptotic Agents/TRAIL

While most therapeutic agents are directed to keep tumor cell proliferation under control, very few agents aim to reactivate the dormant or inactive apoptotic pathways in tumor cells. After the initial discovery of Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo2L) as an apoptotic molecule [83, 84], its promise as a therapeutic agent has become evident by its tumor-specific action [85–90]. Therefore, TRAIL has emerged as a prime candidate for the treatment of several cancers [91]; and soluble recombinant human TRAIL ligand (Apo2L/TRAIL/dulanermin), and TRAIL receptor agonist monoclonal antibodies (mapatumumab, lexatumumab) [91–95], have shown promise in preclinical studies of solid tumors and are currently being evaluated in clinical trials [91]. However, in brain malignancies such as GBM, the presence of BBB is a major obstacle in delivering bio-agents like TRAIL, therefore NSC-mediated delivery would be the most suited in targeting infiltrating glioma cells. The first study to engineer NSCs with TRAIL was performed by Ehtesham et al., where primary fetal mouse brain-derived NSCs were transduced with an adenoviral vector encoding human TRAIL. These NSCs were then intratumorally inoculated into orthotopic brain tumors established with U343 glioma line [96]. There was a marked reduction in tumor volumes in NSC-TRAIL inoculated tumors compared to controls, showing for the first time that NSC-TRAIL might be a feasible approach in targeting brain tumors. Most of the assessments of NSC-TRAIL were performed by Khalid Shah's group. As opposed to using full length TRAIL, which is a type-2 membrane protein and its cleavage is required for its activity, Shah et al. engineered a soluble/secretable form of TRAIL (S-TRAIL) to be used as an optimal anticancer agent. Briefly, N-terminal extracellular domain of TRAIL was fused to extracellular domain of a ligand for Flt3 tyrosine kinase receptor [97–99] to generate S-TRAIL, which demonstrated higher cytotoxicity than TRAIL in vitro. S-TRAIL's in vivo efficacy was first revealed by delivering it to subcutaneous tumors using HSV amplicons [97]. Later, NSC-TRAIL was generated by engineering C17.2 cells to secrete S-TRAIL. These NSCs retained their migratory capacity and caused a substantial reduction in tumor volumes in a highly malignant glioma model, as judged by dual luciferase imaging [25]. Khalid Shah's group expanded NSC-TRAIL-based glioma therapies further by combining NSC-delivered TRAIL with different treatment modalities and state-of-the-art imaging tools. For example, suppression of a tumor-promoting microRNA, miR-21, with the use of locked nucleic acid (LNA)-anti-miR-21 oligonucleotides and delivering TRAIL by NSCs cooperated to reduce to glioma growth substantially [100]. Another example to exploit NSC-TRAIL in a combinatorial approach was to downregulate

an anti-apoptotic protein, Bcl-2, using lentiviral short hairpin RNAs in a highly malignant glioma model in vivo. Bcl-2 inhibition and NSC-TRAIL was more efficacious in glioma models compared to each treatment alone [101]. In addition to genetic manipulation of glioma cells to inhibit tumorigenic pathways, Khalid Shah's group exploited other NSC-TRAIL-based combinations using small molecule inhibitors. For example, combining the standard-of-care DNA alkylating agent TMZ with NSC-TRAIL induced dramatic cell killing by augmenting apoptosis [102]. In another study that combines a chemical inhibitor with NSC-TRAIL in the same group, we used primary mouse NSCs to deliver TRAIL in a highly malignant glioma model. In this pioneering study to show the feasibility of NSC-TRAIL and systemic treatment combination, we showed that systemic administration of a PI3K inhibitor, PI-103, cooperated with NSC-delivered TRAIL in orthotopic glioma models [103]. We also recently showed that combining NSC-TRAIL with a histone deacetylase inhibitor, MS-275, was also very efficacious in not only TRAIL-sensitive gliomas but also malignant TRAIL-resistant gliomas [104]. A similar approach was exploited by expressing a membrane bound version of TRAIL, mTRAIL, from NSCs and combining NSC-TRAIL with a proteosome inhibitor, bortezomib, in orthotopic glioma models [105].

12.3.5 Multifunctional Agents: Killing Multiple Birds with One Stone?

Engineering NSCs with multiple or multifunctional molecules can provide several advantages in NSC-based therapeutics. First, genetic engineering of a therapeutic agent to also possess trackable properties would allow for simultaneous monitoring of the therapeutic efficacy as well as the fate of stem cells or the therapeutic agent. A great example to this came from a study by Hingtgen et al., where a new variant of TRAIL was generated by adding a diagnostic luciferase domain to TRAIL moiety. This new molecule retained its therapeutic function and, when introduced into NSCs, provided a thorough in vivo assessment of pharmacokinetics of TRAIL secretion and localization of NSCs [106].

Another advantage of genetic engineering of NSCs is to be able to deliver multiple biologically active therapeutic molecules from an engineered NSC line. This would be the most ideal strategy, as it would minimize the need for additional therapeutic modalities such as systemic chemotherapy. As discussed above, engineering NSCs to express more than one PCE resulted in efficient tumor eradication [63]. In addition to expressing molecules with similar modes of action, such as PCEs, it is also possible to engineer NSCs to express multiple bioactive molecules that target different effectors on tumor cells. For example, combined expression of IFN-β and CD from HB1.F3 cells resulted in marked therapeutic efficacy in orthotopic glioma models and prolonged survival [107]. Our very recent study documented the success of simultaneously expressing a multifunctional TRAIL variant, specifically, a

fusion of Epidermal Growth Factor Receptor (EGFR) antagonist (EGFR-Nb) and TRAIL to target cell proliferation and induce apoptosis by a single molecule. In this study, NSC expression of EGFR-Nb and TRAIL from a single vector caused enhanced killing of tumor cells as opposed to expression of each molecule from NSCs separately [75]. Similarly, combining the stem cell-based expression of a growth modulating cytokine IL-24 and TRAIL caused a significant reduction in tumor volumes in animal models [79]. Taken together, expressing multifunctional therapeutic agents from NSCs, which can act simultaneously on multiple targets on tumor cells, offers great therapeutic potential as an ideal NSC-based antitumor therapy approach.

12.4 Guiding Principles for NSC-Based Brain Tumor Therapies

There are several considerations in translating NSC-based therapies to clinical settings, which are also discussed elsewhere [16]. First and foremost, the safety of NSCs should be comprehensively analyzed before their use in antitumor therapies. This is not as big of an issue for preclinical studies as it is for translational settings, where high-quality NSC lines need to be produced in GMP-grade conditions. Having a generalized protocol for the isolation and culturing of NSC lines for preclinical studies would allow the translation of this novel technology into the clinic efficiently. Second, the NSCs should be amenable to genetic modification to express high levels of therapeutic modality of interest. They should retain their multipotency and tumor-tracking abilities after genetic manipulation. These genetic manipulations are mostly achieved through retroviral or lentiviral transduction because of their high efficiency. However, these gene transfer methods have the potential of inducing insertional mutagenesis in NSC genome. In this case, it would be important to screen for DNA integration sites and select safe clonal NSC lines. Third, the therapeutic modalities should not affect the NSC viability; they rather need to be tumor-specific. The sustainability of NSCs in culture, as well as in vivo should be assured after genetic manipulation. Fourth, if NSC-based therapies will be used in conjunction with other therapeutic modalities, such as systemic injection of anticancer drugs (e.g, TMZ, MS-275, bortezomib), the potential of these treatments to cause collateral damage to NSCs should be ruled out. Fifth, the fate of NSCs after engrafting needs to be carefully examined. Especially when using immortalized NSC lines in preclinical models, it will be of interest to conduct long-term studies to survey the potential formation of de novo tumors. One way of circumventing this problem could be through incorporating suicide genes (e.g, CD, TK, CE) into NSC genome, in addition to other therapeutic genes, and killing all the residual NSCs after they perform their therapeutic delivery to tumors. Together, these safety and practical measures can accelerate the approval of NSC-based therapeutic delivery for malignant brain tumors.

12.5 Current State and Future Directions

Exploiting NSC's inherent tumor-tropic properties for delivering tumor-specific therapies to disseminated, hard-to-reach, brain tumors has been a novel method of the last decade demonstrating great success in numerous preclinical tumor models. There are still unanswered questions about the mechanisms of NSC's tumor-tropism, and choice of NSC sources and genetic manipulation methods, which are currently under investigation by several laboratories. However, the existing evidence suggests that NSCs are very promising delivery agents in highly malignant brain tumors, where the survival is extremely limited due to the failure of conventional therapies. Indeed, the first clinical trial to utilize NSCs as delivery vehicles in gliomas has been launched [17]. In this pilot trial (NCT01172964) conducted by Karen S. Aboody's group at City of Hope Medical Center (Duarte, CA), the HB1.F3 cell line engineered to carry suicide gene therapy (HB1.F3.CD) will be injected into the resection cavity of recurrent glioma patients at the time of surgery. The clinically approved HB1.F3 line has previously been characterized in great detail by Seung U Kim's group and shown to be safe in various rodent tumor models. However, one needs to keep in mind that NSC survival and immune rejection in human brain can be an issue in this first in-human test with this line. If they survive, these NSCs are expected to distribute along the primary tumor site as well as colocalize with the residual infiltrated cells for 5 days. The patients will then orally receive 5-FC for 7 days. The prodrug 5-FC will be converted into toxic metabolite 5-FU, by the CD-expressing NSCs on the tumor site specifically targeting the malignant cells. The results of this pilot study are extremely important in assessing the feasibility of the approach and will greatly contribute to the design of future therapies.

In order to circumvent the survival problem of NSCs, Khalid Shah's group has recently published an interesting article on the use of biodegradable gels as a matrix for NSC engraftment. Kauer et al. showed in a novel imageable mouse glioma resection model that, NSCs encapsulated in biodegradable gels were more successful than infusing the NSCs directly [108]. This might be an important future direction of administering the therapeutic NSCs into patient brains. In order for overcoming the immune rejection issue, use of allogeneic NSCs would be the most ideal system. However, currently available methods to isolate and culture autologous NSCs from patients are limited. Due to very low abundance of endogenous NSCs in adult CNS, it is currently practically impossible to isolate and expand sufficient quantities of patient-specific NSC material in vitro. However, with the recent advances in stem cell biology, it is possible to generate NSCs through differentiation from pluripotent stem cell sources [109]. Indeed, it is technically possible to generate one's own ES cell-like cells (induced pluripotent stem cells, iPSCs) through cellular reprogramming [110], therefore deriving NSCs from iPSCs might be one way of generating allogeneic NSC sources. All in all, NSCs delivering therapeutic modalities in malignant brain tumors can be a possible solution to these devastating cancers and improve patient survival considerably.

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Chapter 13
Therapy Resistance in Prostate Cancer: A Stem Cell Perspective

Sandra Klein, Fiona M. Frame, and Norman J. Maitland

Abstract Primary human prostate cancers are frequently treated with radiotherapy and subsequently with compounds which block the response to male sex hormones. After the failure of these treatments, the tumours are notoriously resistant to standard chemotherapies. Such therapy resistance has been ascribed to the heterogeneous cellular nature of the tumours and the ability of tumours to rapidly sustain mutations to counteract therapy. This is inconsistent with the low rate of mismatch repair seen in prostate cancer. An alternative and complementary explanation lies in the existence of a therapy-resistant core of cells within the heterogeneous tumour mass. These cells, frequently termed cancer stem cells, exhibit a less-differentiated, basal phenotype, which is resistant to therapies directed against the majority luminal cell population in prostate cancers. The cancer stem cells are largely quiescent, rendering them resistant to cell cycle and proliferation-based therapies.

Keywords Cancer stem cells • Therapy resistance • Prostate cancer

13.1 Prostate Cancer

Prostate cancer is the second most commonly diagnosed cancer in men worldwide, with more than 200,000 men per year diagnosed in the USA alone [1, 2]. Indeed, the disease is most prevalent in North America and Europe. Apart from its higher prevalence in the Western World, prostate cancer risk factors include family history, age, ethnic origin and nutrition [3–7]. Clinical evidence of prostate cancer in young men

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K. Turksen (ed.), *Stem Cells: Current Challenges and New Directions*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4614-8066-2_13,

is uncommon, but almost one-third of men over 50 years of age already have histologically identifiable prostate tumours, and the incidence rises to at least 80 %, for men in their 80s [8–10]. Concerning the ethnic origin, the highest incidence of prostate cancer is diagnosed in African American men [11]. Diet appears to play a key role; for example when men from Japan, a country of low prostate cancer risk, move to the USA and change to an American diet, their incidence of prostate cancer increases. This review will describe current treatment strategies and reflect on the different mechanisms that prevent successful eradication of prostate tumours. There will be a particular focus on prostate cancer stem cells as one of these mechanisms. Cancer stem cells provide a mechanistic explanation for both tumour heterogeneity and treatment resistance. The therapy-resistance mechanisms that will be discussed have to be overcome in order to develop effective treatments, and as a consequence are targets for novel therapeutic strategies.

13.2 Current Treatments for Prostate Cancer

Therapy modality is dependent upon cancer staging, which for prostate cancer, is based on tumour progression assessed by MRI (magnetic resonance imaging) or CT (computer tomography) scan, Gleason grade [12] and PSA (prostate-specific antigen) score [13]. The Gleason grading system was established in the 1960s and remains the most significant clinical prognostic factor. The PSA test is widely used, however it cannot distinguish between aggressive and non-aggressive cancers, thus false positive results can result in patients undergoing unnecessary surgery. Current treatments for low-grade prostate cancer are either by active monitoring of disease progression, surgery to remove the entire prostate gland (radical prostatectomy) or radiotherapy in the form of external-beam radiation or brachytherapy (implantation of radioactive seeds) [14–16] (see Table 13.1). Surgery has the potentially serious side effects of incontinence and impotence. In cases where there is evidence of prostate capsular penetration, or metastases (typically to the bone), these treatments have only a limited efficacy and the cancer frequently returns.

At this more advanced stage, prostate cancer can be treated with a variety of chemical agents designed to block the response to male sex hormones (androgen deprivation therapy—ADT), which are essential for the proliferation and survival of the cancer [17]. Due to their relevance in PSA production, cell survival and growth, AR signalling pathways are considered to be important oncogenic drivers, and therefore present a critical target for prostate cancer treatment [18]. ADT can be conducted either by chemical castration through gonadotropin-releasing hormone analogues, direct inhibition of the androgen receptor activity or by surgical castration (bilateral orchiectomy). ADT is initially effective in most patients with metastatic prostate cancer.

However, ADT can be time-limited and the tumours become castration resistant (CRPC), after which life-expectancy is rarely more than 2 years, even with optimal chemotherapy [19]. Chemotherapy is a last line of treatment, following failure of

Table 13.1 Treatment options for different stages of prostate cancer

Stage of the disease/Treatment options	Early cancer	Advanced cancer/ Metastatic cancer	Hormone resistant
Watchful waiting	✓		
Surgery	✓		
EBT	✓		
Brachytherapy	✓	✓	
Androgen ablation		✓	
Chemotherapy	✓	✓	✓
Protontherapy	✓	✓	
Cryotherapy	✓		
High-intensity focused ultrasound (HIFU)	✓		
Photodynamic therapy (PDT)	✓	✓	
Immunotherapy	✓		

It is apparent that as disease progresses, treatment options diminish

ADT, and typical drugs used include docetaxel, paclitaxel, vinblastine and gemcitabine as well as novel androgen ablation therapies. However, these drugs typically only extend life by a matter of months [20, 21]. With docetaxel, only half of the patients showed a PSA response and 25 % showed an improvement in quality of life. The topoisomerase II inhibitor mitoxantrone was the first drug approved for the treatment of CRPC but is now more usually given as a second-line chemotherapeutic drug, where it can have palliative advantages for patients who progress after treatment with docetaxel. Next generation therapies such as abiraterone, which decreases testosterone levels by inhibition of the enzyme CYP17, also extend the mean life time expectancy of a patient with CRPC by a few months [22].

To conclude, there is no current therapy that eliminates treatment-resistant secondary tumours and metastatic cancer. Hence, further research is needed to find new therapies and therapeutic targets.

13.3 Alternative Treatment Strategies

As well as optimisation of the current treatments along with trials of combination therapies, several new treatments are being developed with some success in clinical trials. These include photodynamic therapy (PDT), high intensity ultrasound

(HIFU), cryotherapy, gene therapy and immunotherapy, including vaccines. It is paramount that such novel strategies are researched and tested because not only is CRPC a significant problem, but radiorecurrent prostate cancer also poses a serious issue for clinicians and patients, with 30 % of patients relapsing [23–26]. HIFU, cryotherapy and PDT are focal therapies which aim to specifically target cancer areas, with minimum harm to normal tissue and reduced side effects [27]. However, these strategies are in the early stages of testing and there are mixed opinions as to their efficacy. There are also concerns over potential side effects and a requirement for longer follow-up data. Immunotherapy and gene therapy are outwith the scope of this article, but current status of these treatments have been reviewed extensively elsewhere [28–30].

13.4 Mechanisms of Therapy Resistance and Treatment Strategies

There are multiple therapy-resistance mechanisms that apply to many cancers and indeed some that apply specifically to prostate cancer (shown in Fig. 13.1).

13.4.1 Tumour-Initiating Cell/Cell of Origin/Cancer Stem Cell

The recent identification and characterisation of cancer stem cells in haematopoietic and solid tumours has provided sound experimental evidence for the cancer stem cell hypothesis, which states that not all cells within a tumour have equal tumorigenic potential [31–42]. Cancer stem cells are thought to form a rare subpopulation of primitive cancer cells within the tumour mass. Indeed, evidence exists to show that cancer stem cells are resistant to therapies and therefore give rise to therapy-resistant secondary tumours [43–47].

The first evidence for the existence of cancer stem cells was produced in 1997, for acute myeloid leukaemia (AML), where $CD34^{++}CD38^{-}$ cells initiated AML in NOD/SCID mice, whereas committed progenitors cells failed to engraft. Furthermore, the $CD34^{++}CD38^{-}$ cells showed major hallmarks of stem cells, such as self-renewal and a differentiation and proliferative potential [48]. In the following years, cancer stem cells were also identified in many solid tumours types including; breast, lung, head and neck, pancreas, liver, kidney, colon, ovarian, glioblastoma, medulloblastoma, bladder, endometrial and prostate [43, 49–63].

13.4.1.1 Identification of Prostate Cancer Stem Cells

The existence of normal prostate stem cells was first shown using experiments in a rat model where the prostate gland involuted following castration, but could be

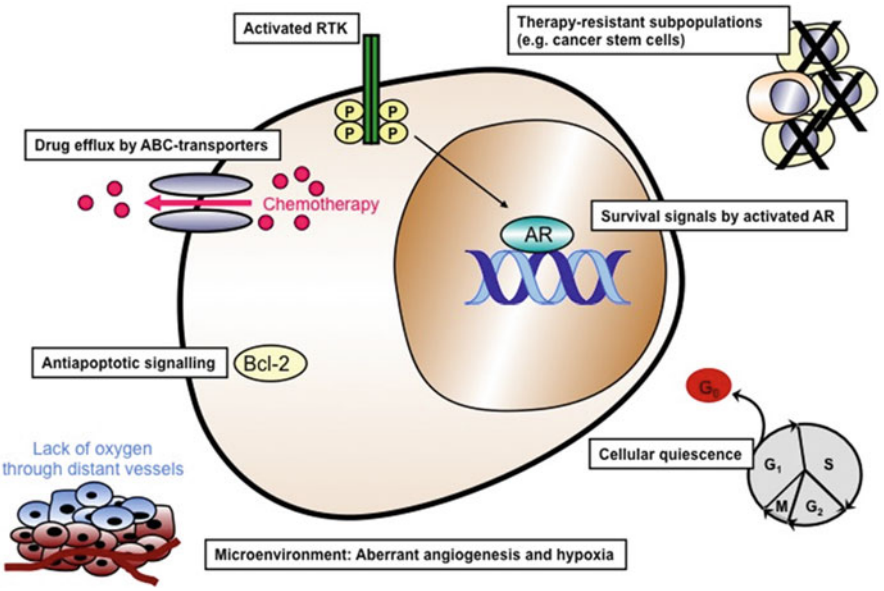

Fig. 13.1 Mechanism of therapy resistance in prostate tumours. Mechanisms of resistance to prostate cancer treatment. The resistance to treatment can be achieved by various mechanisms. Some of these are specific for prostate cancer, such as continued androgen receptor signalling as a consequence of activated RTK, which results in the promotion of proliferation and cell survival. General mechanisms of resistance that can also apply to other types of cancer include drug efflux by ABC-transporters, aberrant angiogenesis, anti-apoptotic signalling, cellular quiescence and therapy-resistant subpopulations. Some of these mechanisms can overlap. Aberrant angiogenesis can lead to hypoxic regions causing cellular quiescence and impaired drug delivery. Cancer stem cells have been shown to express ABC-transporters and are also often in a quiescent state. Adapted from [162]

restored by hormone induction [64, 65]. This cycle could be repeated multiple times, and strongly suggested the survival of a castration-resistant stem cell population. Subsequently, normal human prostate epithelial stem cells were identified in gland regeneration experiments in normal/benign prostates, where a basal epithelial phenotype of cells expressing CD133 and high levels of α2β1-integrin showed the highest clonogenicity and gland regeneration potential in immuno-compromised mice [66]. As is the case in many other cancer types, the same markers were exploited by Collins et al. [60] to sub-fractionate epithelial cells from human prostate cancers. Cells with this phenotype came from (High Gleason grade) tumour cultures with invasive properties and had self-renewal, proliferative and differentiation properties and high secondary colony forming efficiency.

Prostate cancer stem cells probably develop from a deregulation of normal stem cells that reside in the prostate epithelium, which most likely occurs through mutations and epigenetic changes. Alternatively, they could originate from differentiated

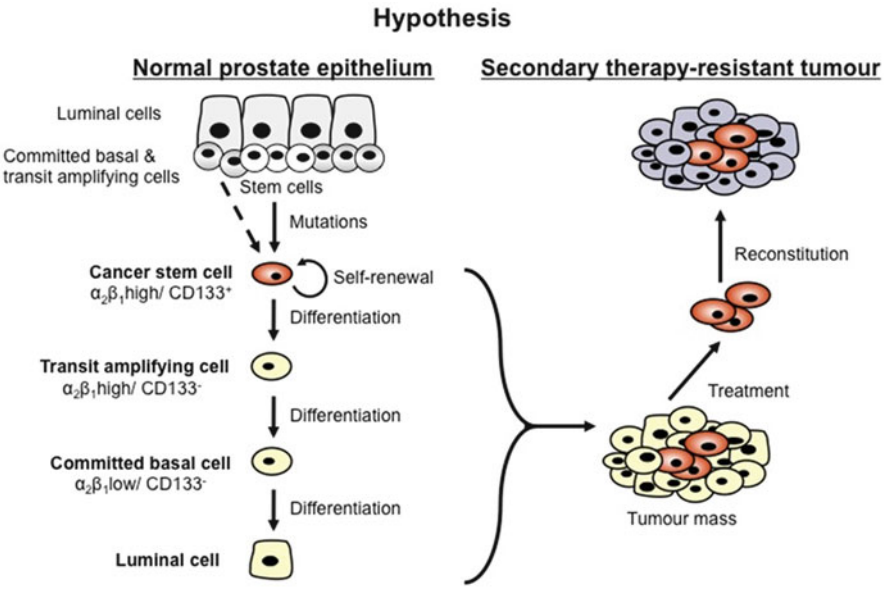

Fig. 13.2 The prostate cancer stem cell hypothesis. The normal prostate epithelium consists of stem cells that give rise to transit amplifying, committed basal and luminal cells. Cancer stem cells can develop from mutations in normal stem cells (or transit amplifying cells) that reside in the normal prostate epithelium and give rise to malignant transit amplifying, comitted basal and luminal cells, which make up a heterogenous tumour mass. It is likely that in a heterogenous tumour mass not all cells respond to the treatment in the same way. According to the cancer stem cell hypothesis, cancer stem cells survive the treatment and are then responsible for the generation of secondary tumours that are resistant to any form of treatment

progeny that revert to a stem-like phenotype [46, 67–70]. Prostate cancer stem cells, like their healthy counterparts, have the ability to both self-renew and differentiate [60] (see Fig. 13.2). The hierarchical and heterogeneous organization of the malignant tissue suggests that CSCs give rise to the whole tumour mass [69, 71]. Similarly to normal stem cells it has been proposed that cancer stem cells are likely to harbour better protective and repair mechanisms against DNA damage, making them harder to kill with conventional therapeutic strategies [44, 72, 73].

Upon microarray analysis to compare transcription patterns in stem cells ($CD133^{+}/\alpha2\beta1integrin^{hi}$) and committed basal cells ($CD133^{-}/\alpha2\beta1integrin^{lo}$) from both benign and malignant prostate samples [74], a distinct stem cell gene expression signature emerged, and more crucially, gene expression differences were noted between normal and cancer stem cells. The profiles indicated that expression of 581 genes were significantly different in prostate cancer stem cells. A number of these genes had previously been associated with carcinogenic changes, including promotion of an invasive phenotype in prostate and other cancer types. By exploiting bioinformatics tools such as the Gene Ontology and the KEGG pathway database, functional associations and the activation of a number of signalling pathways were identified in CSCs relative to normal tissues. For example, the malignant SC

expressed clusters of genes associated with inflammatory response such as IL-6 and NFκB activated genes, and showed evidence of Wnt pathway activation. Also, the JAK-STAT pathway and adhesion signalling pathways were upregulated in the cancer stem cells.

Other markers recently proposed to define prostate cancer stem cells include TRA-1-60/CD166/CD151 cells [75]. Like $CD133^{+}/\alpha2\beta1integrin^{hi}$ cells, this cell fraction did not express markers associated with differentiated secretory luminal cells, androgen receptor or prostate-specific antigen [75]. They did however possess stem cell characteristics and multipotency as shown by in vitro sphere-formation and in vivo tumour-initiation in the CWR22 mouse model. In this study the authors did not directly correlate the expression of TRA-1-60/CD166/CD151 to CD133 expression. However, from the Birnie et al. [74] data we know that the $CD133^{+}/\alpha2\beta1integrin^{hi}$ stem cell populations also expressed high levels of the TRA-1-60/CD166/CD151 genes. Therefore they constitute potentially the same stem cell population. Significantly, Birnie et al. as well as a study by True et al. showed clear gene expression differences between high and low grade cancer, with gene expression from cells displaying Gleason pattern 3 being distinctly different from cells displaying Gleason pattern 4 or 5 [76]. Alternative or additional prostate cancer stem cell markers have been collated elsewhere [46].

13.4.1.2 Are Prostate Cancer Stem Cells the Origin of Prostate Cancer?

Historically, prostate cancer was considered to be a disease of luminal cells. Luminal cells were also thought to be the origin of the disease, in particular due to the ratio changes of luminal: basal compartments from ~1:1 in normal prostate compared to a vast reduction of basal cells in prostate cancer and the majority of cells having a luminal phenotype [70, 77]. However, the origin of prostate cancer is still debated. Several findings agree that the cell of origin resides in basal cells or stem cells within the basal compartment [66, 78–83]. Logically, as stem cells persist during the lifetime of the host, the risk for the accumulation of mutations leading to malignancy is higher than in more differentiated short-lived cells. Furthermore, crucial pathways required for stem cell maintenance such as Notch, Sonic hedgehog and Wnt-Signalling are associated with carcinogenesis [84]. In the literature, there remains considerable discussion on nomenclature comparing cell of origin, cancer stem cell and tumour-initiating cell, which have been defined in [71] as follows: (1) The cell of origin of cancer is the cell that sustained the initial mutagenizing hit, (2) the cancer stem cell has similar characteristic properties of a normal stem cell and can give rise to a tumour with hierarchical organisation and (3) a tumour-initiating cell can initiate a tumour but is not necessarily a cancer stem cell.

In support of the basal compartment as an origin of prostate cancer, $CD133^{+}/\alpha2\beta1\ integrin^{hi}$ basal stem cells from malignant tissue have the ability to self-renew, differentiate, form primary and secondary colonies, and are sourced from cells that are highly invasive [60]. One piece of evidence missing from this study is the direct proof of tumour-initiation in vivo. However, work in this area is beginning to emerge [69].

In support of these findings, a basal origin for prostate cancer has been demonstrated with mouse basal cells, which formed tumours with a luminal phenotype after induction of ERG expression and activation of PI3K signalling [78]. A similar outcome was revealed with human benign prostate cells transduced with lentiviruses to introduce AKT, ERG transactivator and AR genes in both luminal and basal cells [79]. In immunocompromised mice, only the basal cells initiated tumour growth. Interestingly, the histological analysis of the tumours revealed a reduced basal compartment and an increased mass of luminal cells, typical of human prostate cancer.

In contrast, castration-resistant NKx3-1-expressing cells (CARN) have been suggested as a luminal cell type responsible for the initiation of the disease [85]. In mice, CARN cells do not express the basal marker p63, but are positive for cytokeratin 18 and AR. CARN cells remain after androgen deprivation and are able to form tumours. However, it is important to distinguish between mouse models and studies using human cells because the mouse prostate and human prostate are significantly different in structural arrangement and cell content [86]. It is yet to be determined if there is more than one kind of cancer stem cell in prostate cancer, as seems to be the case in breast cancer [87].

13.4.1.3 Do Cancer Stem Cells Mediate Treatment Resistance in Prostate Cancer Stem Cells?

In a similar manner to that in normal stem cells, it has been proposed that cancer stem cells are likely to harbour better protective and repair mechanisms against DNA damage, making them harder to kill with conventional therapy strategies [44, 72, 73] (Fig. 13.2). The most common non-surgical treatment for organ-confined prostate cancer is radiotherapy, and studies from other tumours, including glioblastoma and breast carcinoma, have suggested that the CSC population displays increased radioresistance, as compared to the replicative tumour mass [47, 88, 89]. As discussed earlier, chemotherapy is not successful in the context of advanced prostate cancer, and this resistance might be mediated by resistant cancer stem cells repopulating the tumour mass.

In our laboratory, treatment of primary prostate basal epithelial cultures with potentially therapeutic molecules revealed a unique property of the stem cells, which would not have been predicted from cultures of established cell lines. A number of cytotoxic agents, which targeted the transit-amplifying and committed basal cells had the unexpected effect of enriching or even stimulating expansion of the stem cell population (Frazier A., unpublished data). If one considers that the normal function of stem cells is to repair and repopulate a tissue after wounding, this is perhaps not so surprising. Therapies which reduce tumour mass can be thought of as initiating a wound, to which the CSC responds by proliferating. In normal tissues this is a positive effect, but in a tumour, the same response results in an increase in the tumour initiating (or even metastasis establishing) cell population, with the potential to increase tumour number and spread.

In man, this is precisely the pattern of disease seen after salvage chemotherapy observed in CRPC: a multifocal metastatic relapse consisting of clonally related tumours, following an initial reduction in primary tumour(s) volume. In this case the short-term positive effect as indicated by a drop in serum PSA for example, could be envisaged as ultimately and negatively changing the natural history of the disease. This observation has been seen in a breast cancer model where chemical treatment stimulated cancer stem cell growth [90]. More recently, the FDA in the USA has issued a warning about chemoprevention strategies for prostate cancer using inhibitors of 5-alpha reductase (which targets AR+ luminal cells). Studies now suggest that these agents prevent non-aggressive prostate growths while at the same time promoting potentially fatal poorly differentiated tumours when applied to patients who had a pre-existing tumour. Again, this is precisely the pattern of response predicted by elimination of differentiated luminal cells, which results in the promotion of basal-like tumour cell expansion.

These findings raise the issue of why and how cancer stem cells are better protected against treatment than more differentiated tumour cells. The stem cell niche (microenvironment), increased drug efflux, detoxifying enzymes, microRNAs, cell cycle checkpoints, increased DNA repair, the inhibition of apoptosis and stem cell maintenance are all believed to be potential factors in treatment resistance [68]. Some of these mechanisms are also general therapy-resistance mechanisms that may apply to other cells in the tumour mass.

13.4.2 Microenvironment, Vascularisation and Hypoxia

The conditions within the tumour microenvironment influence treatment response and tumour survival, and successful tumours can only establish when supported by an appropriate “niche”. This requires adaptation to inflammation, oxidative stress and hypoxia. Furthermore, in order to undergo invasion and metastasis cancer cells have to go through an epithelial-to mesenchymal transition (EMT) [91–93].

Chemotherapeutic agents reach solid tumours through the blood vessels and must penetrate through extracellular matrix (ECM) to reach the cancer cells. Tumours and host cells enhance the development of new vessels through angiogenic growth factors. However, blood vessels in malignant tissues can be distinguished from those in normal tissue on the basis of a more disorganized arrangement, and by being further apart from each other. As a consequence, delivery of oxygen is decreased, leading to the development of hypoxic regions. Due to the deficient growth of the vascular system, the delivery of anticancer drugs is also impaired, as they reach their targets through the bloodstream.

Further factors that lead to the failure of drug delivery are increased interstitial fluid pressure that originates from the leaky and disorganized vascular system, and decreased lymphatic drainage. The poor oxygen supply observed in malignant tissues (tumour hypoxia) is a common feature of aggressively growing tumours and their metastases. Such hypoxic areas arise from a lack of blood supply that is

believed to occur in the early development of the tumour [94, 95]. The hypoxic conditions usually remain, even after neovascularization, leading to a constantly poor oxygenation within the tumour [96].

In many cancers, including prostate cancer, hypoxia is associated with a poor clinical outcome, since hypoxia provides protective mechanisms against a variety of therapeutic strategies e.g. radiation, chemotherapy and androgen deprivation [97]. Thus, elucidating the tumour cell response under the influence of oxygen deprivation is paramount for our understanding of treatment resistance. It is perhaps significant in the context of the cancer stem cell phenotype, that the expression of the common CD133 marker is strongly upregulated under hypoxic conditions [98–101]. The lack of vasculature in hypoxic tumours regions might also select for these more malignant tumour cells. Hypoxia-inducible transcription factors such as HIF1α are activated by hypoxia and trigger the expression of genes that increase survival and metastasis of prostate cancer cells and other cancer cells [102, 103]. HIF1α overexpression has been observed in different cancer types, including prostate cancer [104]. HIF1α has anti-apoptotic properties and its expression has been linked to less sensitivity to hypoxia-induced apoptosis [105]. The low oxygen pressure selects for malignant cells with disrupted mitochondrial pathways and increased expression of the anti-apoptotic protein Bcl-2. Bcl-2 regulates Pim-1 under hypoxic conditions, which may be critical for anti-apoptotic responses via inactivation of BAD [106]. Cells in hypoxic regions are therefore a target for therapy but novel strategies are required [107]. Drugs that are activated under hypoxic conditions are one possible solution. Hypoxia is also associated with radioresistance, and therefore reduces the efficacy of radiotherapy [108–110].

Gene therapy treatment strategies can take advantage of the hypoxic conditions and cytotoxic genes can be placed under the control of hypoxia-activated promoters, thus specifically targeting hypoxic tumours [111]. Another strategy has been to harness the natural propensity for macrophages to home to hypoxic tumour sites and use them to deliver oncolytic adenoviruses to kill the cancer cells [112].

13.4.3 Aberrant Androgen Receptor Signalling

The androgen receptor is a transcription factor that is usually activated by binding of its androgen ligand, resulting in the transcription of target genes necessary for growth and survival and the upregulation of the marker gene PSA [113]. Hence, androgens and AR signalling are considered to be the main oncogenic factors in the development of prostate cancer and therefore present a critical target for the treatment of prostate cancer [114]. However, whilst ADT can usually lead to the prolongation of the patients´ life, it doesn't cure the disease, which frequently progresses to CRPC [115, 116]. There are several alternative mechanisms of AR activation in androgen-independent prostate tumours, which allow cells to escape the therapeutic implication of ADT and to resist growth inhibition. Briefly, the known mechanisms of resistance to ADT are AR amplification, and AR mutations resulting in activation by

other steroids such as oestrogen or other ligands, AR hyperactivation without androgen binding, AR activation by tyrosine kinase signalling (in presence of only low or even undetectable levels of androgens), resistance to castration-induced apoptosis and the presence of AR^- cancer stem cells [116]. Androgen amplification or mutations in the AR can enhance the sensitivity to DHT and non-androgenic steroids or anti-androgens thus leading to continued AR signalling. Further mechanisms include the recruitment of AR by ligand-independent modifications, for example the phosphorylation of AR. Additionally, the interaction of AR with activated tyrosine kinase receptors such as EGFR or deregulation of coactivators and corepressors (mediators of AR signalling) are important AR-dependent mechanisms. Indeed, preclinical trials and studies of human tumours indicate that an overexpression of steroid receptor coactivators is linked to the development of CRPC [117–120].

The androgen receptor expression in normal and malignant stem cells remains a matter for debate, although there is a weight of opinion in favour of a basal AR negative phenotype as discussed earlier (reviewed in [71]). Thus, AR inhibitors are likely to be ineffective in treating prostate cancer stem cells.

13.4.4 Growth Factor Signalling

The ErbB1 family (EGFR/ErbB1, ErbB2/HER27neu, ErbB3HER3 and ErbB4/HER4) of receptor tyrosine kinases is known to be involved in initiation and progression of prostate cancer. Hence, the ErbB1 family represents another logical therapy target. However, inhibitors against ErbB1 and ErbB2 showed only limited effects in clinical trials [121–126]. Recent studies suggest ErbB3 as a critical target in castration-resistant prostate cancer [127]. Some therapeutic strategies to inhibit the mechanism of ErbB3 include antibody-based therapy to prevent ligand binding to ErbB3, inhibition of ligand-induced phosphorylation of ErbB3, small molecule tyrosine kinase inhibitors and siRNA strategies [127].

13.4.5 ABC Transporters

The ATP binding cassette (ABC) transporter superfamily is widely known to play a critical role in chemoresistance for many cancer types. ABC transporters provide protective mechanisms to cells by effluxing toxic substances such as metabolic byproducts, natural compounds or drugs. Hence, they protect tumour cells from chemotherapeutic agents thus rendering them ineffective [128].

The ABCC (multidrug resistance protein) family contains most of the known drug transporters, for example ABCB1 (MDR1, P-glycoprotein), ABCC1 (MRP1) and ABCG2 (BCRP or MXR) [129]. ABCG2 was first identified in the breast cancer cell line MCF-7 [130, 131]. It is highly expressed in human endothelium and plays a crucial role in the blood–brain barrier [132–134]. However, ABCG2 is

rarely expressed in most differentiated cell types [135]. Its expression is mainly associated with a stem-like subset of cells termed the "side-population" (SP). The SP—identified by the extrusion of Hoechst dye—has been found in several tumours such as breast cancer, ovarian cancer, sarcomas, neuroblastomas the hematopoietic system and adenocarcinomas of the prostate [136–142]. Furthermore, an ABCG2-expressing SP that demonstrated efflux of the cell membrane permeable fluorescent substance Dye Cycle Violet has been identified in the human prostate cancer cell lines CWR-R1, DU-145, RWPE-1 and in Transgenic Adenocarcinoma of Mouse Prostate (TRAMP) cells, human cells from benign and malignant prostate tissue, in xenografts of benign and malignant prostate tissue and in rat prostate progenitor cells [142, 143].

ABCG2 and ABCB1 have been shown to efflux steroids such as cholesterol metabolites, dihydrotestosterone and oestrogen [143–145]. Immunohistochemical analysis of normal and malignant ABCG2+ cells revealed that they are androgen-receptor negative and it is likely that the ABCG2+-mediated extrusion of androgens is important for the maintenance of a stem-like character [143]. Hence, in addition to their role in the clearance of chemotherapeutic drugs they might mediate therapy-resistance in prostate cancer stem cells against androgen ablation therapy.

13.4.5.1 Targeting ABC-Transporters

Due to their role in drug and steroid efflux, ABC transporters were initially thought to be a promising therapy target, but clinical trials with inhibitors to block drug efflux have failed to produce striking results. Whereas in patients with ovarian cancer, the risk of a relapse following treatment with paclitaxel/carboplatin was correlated to distinct polymorphisms, ABCB1 genotyping in patients with breast and prostate cancer patients treated with taxanes revealed only inconsistent results [146]. Polymorphisms in the ABCB1 gene have been shown to influence the levels of the ABCB1 expression and drug efflux. The ABCB1 genetic variation influences the toxicity and clinical outcome of patients with androgen-independent prostate cancer treated with docetaxel [147]. Further studies have related ABCC1 to a poor clinical outcome in treatment. However, none of these studies provided sufficient evidence for a critical role in treatment resistance. The inhibition of ABC-transporters remains challenging. New approaches aim to target ABC-transporters with nanoparticles, antibodies, siRNA, antisense nucleotides and transcriptional regulation [148].

13.4.6 Anti-Apoptotic Mechanisms

Mutations leading to the inhibition of apoptotic mechanisms are critical for the development of malignancy and are considered to result in treatment resistance, as many types of cancer treatments fail in the presence of blocked apoptotic pathways.

The members of the Bcl-2 family are crucial regulators of apoptosis and comprise anti-apoptotic proteins such as Bcl-2 itself, Mcl-1 and Bcl-cL, as well as pro-apoptotic molecules such as Bak, Bax, Bim, Bid and Bad [149–151]. In early prostate cancer, Bcl-2 is overexpressed in up to 60 % of the patients, and in CRPC high levels of Bcl-2 can be detected in almost all patients [152, 153]. The overexpression of Bcl-2 has been linked to a failure of radiation, chemotherapeutic drugs (e.g. docetaxel) and androgen deprivation. Indeed, apoptotic resistance has been suggested as a marker for the radio-responsiveness of prostatic tumours. Another crucial factor in the failure of apoptotic responses is Akt activation, which phosphorylates the pro-apoptotic proteins BAD and BAX leading to an anti-apoptotic mechanism by Bcl-Xl and failure of mitochondria-mediated apoptosis [154]. Since Bcl-2 and BclxL are likely to be key factors in the progression to androgen-independence and resistance of hormone refractory cancer to chemotherapeutics, they are thought to be promising therapy targets [155]. Blocking Bcl-2 with anti-sense oligonucleotides boosts the chemosensitivity of (prostate) cancer cells in vitro and in vivo in various tumour models [156–160]. However, although promising results have been revealed by model systems, delivering anti-sense oligonucleotides to patients remains a challenge [149].

There is evidence for resistance to apoptosis in prostate cancer stem cells. A differential expression of genes related to inflammation, cellular adhesion, and metastasis was identified in $CD133^{+}/\alpha2\beta1integrin^{hi}$ prostate cancer stem cells [74]. In particular, a decrease in expression of the PTEN phosphatase, linked to Akt activity, and an increased expression of the anti-apoptotic factor NF-κB was observed in the cancer stem cell population. Further evaluation of differential expression at the protein level demonstrated nuclear localization of NF-κB in progenitor cells and cancer stem cells. Blocking NF-κB with parthenolide induced apoptosis in primary tumour cells, but not in normal cells [74]. Similar to these findings in $CD133^{+}/\alpha2\beta1$ prostate stem cells, TRA-1-60/CD166/CD151 prostate cells are characterized by an increased NF-kB signalling at mRNA and protein level. Inhibition of NF-kB with Parthenolide, Celastrol, PHA and 48 1407 led to a decrease of sphere formation and a reduction of tumour size. Following the administration of NF-kB inhibitor 48 1407 the stem-like sphere cells undergo apoptosis [75].

13.4.7 Cell Cycle Status

The stage of the cell cycle is critical in cancer therapy, as many therapies are designed to target highly proliferative cells. The stage of the cell cycle can also be influenced by hypoxia, as hypoxic tumour cells, distant from functional blood vessels, tend to proliferate more slowly and are therefore able to escape anti-cancer drugs [161]. In a heterogeneous tumour mass, not all cell types are at the same stage of the cell cycle. In particular, cancer stem cells are likely to be quiescent and therefore be less susceptible to drugs targeting cell proliferation. Indeed, we have shown that normal and cancer prostate stem cells cycle less than progenitor cells (Frame

et al., unpublished results). Normal adult stem cells are also known to be quiescent, to maintain genomic integrity. Also, the cell cycle status of stem cells is known to have an effect on the type of DNA damage response that is initiated following insult. More quiescent cells, such as normal adult stem cells, are less likely to repair damage through homologous recombination and more likely to use the error-prone non-homologous end-joining pathway [89]. Interestingly, this could be the cause of tumour-initiating mutations. Perhaps paradoxically, quiescence can therefore be a therapy-resistance mechanism, or conversely may even increase the possibility of tumour formation.

13.5 Conclusions

A new generation of cancer therapeutics should not only strive for enduring treatment response and tumour shrinkage, but also prevention of metastases from a primary tumour and ultimately towards total tumour eradication. There is a need for new detection, with diagnostic and therapeutic markers to assess the efficacy of any new treatment. In order to develop new treatment strategies, we must have a complete understanding of the mechanisms of resistance that are employed by the prostate cancer cells. One of these potentially key therapy-resistance mechanisms is the existence of cancer stem cells. In order to eliminate the tumour it is likely that a combination treatment will be most effective, with the goal of eradicating the bulk tumour while simultaneously precisely targeting the cancer stem cells. The timings of such therapies would be critical, and further experimentation using in vivo models more characteristic of the original tumour, using primary human tissue models, rather than xenografts from cell lines, will be required to assess efficacy of novel treatments. Areas that could be explored in terms of developing novel therapeutics for cancer stem cells, are signalling pathways that are related to stem cell maintenance e.g. Wnt/β-catenin, Notch and Hedgehog. However, these pathways are also active in normal stem cells, where they regulate self-renewal, differentiation and proliferation, which may limit their use as a therapeutic target. The role of detoxifying enzymes, DNA damage response and micro-RNAs such as *miR-34* in prostate cancer stem cells have not been fully explored, but have been shown to play a role in glioblastoma and breast cancer.

The complexities of treatment regimes must now reflect the tumour cell heterogeneity and the multiple cell types present within a single tumour to have any chance of obtaining a sustained response, or even a cure. This is certainly not the case for current therapies for metastatic prostate cancer. The existence of cancer stem cells is principal amongst several therapy-resistant mechanisms that need to be addressed in terms of designing new treatments.

Acknowledgments We wish to thank Yorkshire Cancer Research for the provision of programme and project support to the Cancer Research Unit, Mark Meuth (Univ of Sheffield) for discussion and input on DNA repair mechanisms, and the White Rose Studentship Network for support of S.K.

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Chapter 14
Mechanisms of Somatic Cell Reprogramming

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Abstract Generation of induced pluripotent stem cells (iPSCs) from somatic cell types has revolutionized the field of stem cell biology and opened the way for production of disease- and patient-specific stem cells which have tremendous potential for regenerative medicine. Despite the rapid progress and improvement in iPSC-derivation techniques, transcription factor-based reprogramming remains an inefficient and poorly understood process. Successful reprogramming requires the completion of a number of rate-limiting steps that include avoiding senescence, mesenchymal–epithelial transition, and activation of endogenous pluripotency genes. It has also become clear that the global epigenetic landscape of the somatic cell types is completely overhauled during acquisition of pluripotency. The epigenetic state is largely determined by the deposition of chromatin marks which include histone tail modifications and DNA methylation. These marks are not only indicative of a given cell state; they are also functionally important during reprogramming. In this chapter I will review our current understanding of the mechanism of reprogramming and the role chromatin marks and the associated chromatin-modifier proteins play in this process.

Keywords iPSC • Reprogramming • Epigenetics • Chromatin • Histone modifications

14.1 Introduction

To date, reprogramming of somatic cells to a pluripotent, embryonic stem cell-like state has been achieved by three methods: somatic cell nuclear transfer (SCNT), cell fusion with pluripotent cells, and overexpression of pluripotency-associated

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K. Turksen (ed.), *Stem Cells: Current Challenges and New Directions*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4614-8066-2_14,

transcription factors [1]. In the last method, the four pluripotency-associated transcription factors are Oct4, Sox2, Klf4, and c-Myc (OSKM) as determined by Takahashi and Yamanaka [2]. Introduction of these factors into both mouse and human somatic cells yields induced pluripotent stem cells (iPSCs) [3–8]. IPSCs are highly similar to embryonic stem cells (ESCs) in terms of gene expression pattern, global epigenetic state, and differentiation and developmental potential [9–15]. Generation of iPSCs from adult somatic cells thus provides an unrestricted source of pluripotent stem cells that can easily be expanded, modified, and differentiated in vitro to produce autologous therapeutic cells or to model diseases [16, 17]. To date, iPSCs have been generated from a growing number of genetic and complex diseases [18]. In addition to being a fascinating biological process, the tremendous potential of patient-specific stem cells in regenerative medicine makes understanding the molecular basis reprogramming an important area of research.

14.2 Steps in Reprogramming

Transcription factor-based reprogramming is able to reset the entire gene expression program of the somatic cells to that of a pluripotent cell. How OSKM expression is able to achieve this feat is one of the key mechanistic questions of this field. The fact that only a small fraction of the starting cells can achieve full reprogramming suggests that there is a degree of stochasticity to this process [19, 20]. The inefficiency of the reprogramming is thought to be due to the presence of multiple rate-limiting steps, each of which is overcome by progressively fewer cells [21].

Retrospective analyses of live-cell imaging experiments indicate that the first discernible change in the somatic cells that will achieve full reprogramming upon OSKM expression is a decrease in cell size and increased proliferative rate [22]. In mouse fibroblasts this is followed by downregulation of Thy1 and upregulation of alkaline phosphatase activity [23, 24]. In human fibroblasts, loss of CD13 and gain of the Tra-1–60 marker is observed with the emergence of first cell clusters that will become bona fide iPSC colonies [25]. Not all the somatic cells that receive the OSKM factors exhibit these changes in morphology, marker expression, and cell proliferation. It seems that overexpression of OSKM results in cell cycle arrest, apoptosis, or senescence in a significant proportion of the starting cells. The evidence for this notion comes from experiments where inhibition of these processes results in increased reprogramming events. For example, upon deletion of key executers of apoptosis and senescence, such as p53, p21, and p16/INK4A, iPSC generation is accelerated, the efficiency of reprogramming is increased, and Klf4 and c-Myc can be dispensed with [26–31].

Gene expression profiling in the first few days of reprogramming has revealed that a large number of epithelial cell-associated genes are turned on while regulators of the mesenchymal cell lineage such as Snail and Zeb1/2 are downregulated [32]. This change in cell state has been termed a mesenchymal–epithelial transition (MET). Because ESCs are known to have epithelial characteristics and grow as

colonies with tight cell–cell adhesion, it is not surprising that starting mesenchymal somatic cell types such as fibroblasts have to go through the MET process during reprogramming. Oct4, Sox2, and c-Myc are able to suppress regulators of the mesenchymal state such as *Snai1* and *Tgfb1*, while Klf4 enhances the expression of the epithelial cell adhesion molecule E-cadherin [33]. Preventing MET either by TGF-beta1 or activin A addition, Snail overexpression or E-cadherin suppression impairs iPSC formation. For mesenchymal cell types such as fibroblasts, MET is a necessary process to achieve reprogramming; however, that is not the case for epithelial cell types such as keratinocytes, hepatocytes, and gastric epithelial cells. The fact that all of these epithelial cell types can be reprogrammed at higher efficiencies than fibroblasts lends credence to the notion that MET is rate-limiting step in transitioning to pluripotency [34, 35].

The last major hurdles to be overcome to achieve full reprogramming are activation of the endogenous pluripotency network and silencing of the exogenous OSKM transgenes. In ESCs and fully reprogrammed iPSCs, Oct4, Sox2, and Klf4 bind to the promoters of a large set of highly active genes that are specific to the pluripotent state, such as Nanog and Lin28, and activate their transcription [36–39]. These factors also bind to their own promoters, thereby establishing a positive feedback loop. Gene expression analyses show that many of the pluripotency-related genes are activated late during reprogramming [32]. And in partially reprogrammed cells that emerge at intermediate stages of reprogramming, these genes are not turned on at all, and there is no detectable binding of OSK to their promoters [39]. One possibility for the inability of OSK to bind to their targets is the absence of a cofactor that is not included in the original reprogramming cocktail [21]. Nanog is a good candidate for such an additional coactivator, because it interacts extensively with Oct4 and Sox2 in ESCs and co-binds to many promoters with them [36, 40]. Overexpression of Nanog is able to rapidly convert partially reprogrammed cells to fully reprogrammed iPSCs and, if expressed along with OSKM from the start, increases the overall reprogramming efficiency [19, 41].

Transcriptional activation is also regulated by the local chromatin structure surrounding promoters and enhancers. This local chromatin state can have a strong impact on transcription factor binding. Therefore, another likely explanation for why OSK are not be able to bind to their entire set of targets during intermediate stages of reprogramming may be the presence of repressive chromatin structures surrounding these targets. Remodeling of these somatic cell-associated chromatin domains, which are presumably formed during development, is a major barrier to successful reprogramming. Evidence for this notion and the impact of chromatin modifications on reprogramming are discussed below.

14.3 Chromatin and Reprogramming

The fundamental building block of chromatin is the nucleosome, which is composed of 147 base pairs of DNA wrapped around an octamer of the four core histones (H3, H4, H2A, H2B). Histone H1 acts a linker between two adjacent nucleosomes.

Both the DNA and histone components of the chromatin can be modified in a variety of ways including methylation of DNA and posttranslational modifications of histones [42]. Changes to the chromatin structure that are stably maintained through cell divisions, and which do not affect the underlying DNA sequences, are defined as epigenetic modifications. Each of these modifications or chromatin marks is associated with or is functionally important for a transcriptional outcome. Generally, the tri-methylation of lysine 4 in H3 (H3K4), together with histone acetylation, marks the binding of RNA polymerase II and transcriptional activation. On the other hand, DNA methylation, histone deacetylation, and H3K27 and H3K9 tri-methylation signal repressive transcriptional states.

The most well-studied histone methyltransferases belong to the Trithorax- and Polycomb-group proteins. First identified in Drosophila, these proteins regulate homeotic gene expression during development. Trithorax-group (TrxG) proteins perform H3K4 tri-methylation which is generally associated with gene activation. The human homolog of Trx is the mixed-lineage leukemia (MLL) gene. TrxG-mediated gene activation is antagonized by the action of Polycomb-group proteins. Polycomb repressive complex (PRC2) consists of Ezh2, Eed, and Suz12 and catalyzes H3K27 tri-methylation. This methyl-mark is then recognized by the PRC1 complex followed by Ring1A- and Ring1B-mediated mono-ubiquitylation of histone H2A and gene repression [43].

14.3.1 Establishing an Active Chromatin

Genome-wide maps of different chromatin marks recently constructed on the basis of chromatin immunoprecipitation and next-generation DNA-sequencing (ChIP–Seq) experiments revealed that pluripotent cells have a unique chromatin structure. Compared to somatic cells such as fibroblasts, ESCs have reduced numbers of repressive domains that are marked by H3K27me3 and K3K9me3 [44, 45]. In addition, pluripotent cell chromatin has an “open” structure with high amounts of H3K4me3, hyperdynamic core histone proteins, and widespread transcription [46, 47]. Consistent with this notion, one of the first chromatin-based events after OSKM expression is the acquisition of H3K4me2 at more than a thousand loci which include a large number of pluripotency-related genes [48]. Furthermore, WD-repeat protein-5 (Wdr5), which is a core member of the mammalian TrxG complex, is required early on for efficient reprogramming of somatic cells by the OSKM factors [49]. Wdr5 has also recently been shown to be a regulator of ESC self-renewal [49]. It interacts with Oct4 and is localized to highly expressed genes in undifferentiated ESCs. These findings suggest a necessary role for recruitment of Trithorax complex to specific genes during reprogramming.

Additional support for the importance of opening up of the somatic cell chromatin during reprogramming comes from the study of Chd1, which is a chromatin-remodeling enzyme. Chd1 is associated with active transcription and contains an ATPase SNF2-like helicase domain and two chromodomains which recognize

tri-methylated H3K4 [50]. RNAi against Chd1 in MEFs significantly reduces reprogramming efficiency without affecting the overall cell proliferation rate [51]. Chd1 knockdown in ESCs has minimal impact on self-renewal and the pluripotency transcriptome but impairs the ability of ESCs to differentiate. Under steady-state conditions, Chd1 associates with euchromatin, and upon its loss, heterochromatin marks such as H3K9me3 and HP1 are increased throughout the ESC chromatin [51]. Although how Chd1 antagonizes heterochromatinization is not clear, the fact that Chd1 is required for efficient iPSC formation provides strong evidence for a shift towards more open chromatin during reprogramming.

A further determinant of transcriptional activation is nucleosome density and the availability of nucleosome-depleted regions upstream of the transcription start sites to which transcription factors can bind. In closed promoters, nucleosomes cover key *cis*-regulatory sequences and block access to the transcription factors [52]. Therefore, chromatin-remodeling complexes which displace or insert nucleosomes by an ATP-dependent mechanism play important roles in regulation of gene transcription. One of the most well-studied chromatin-remodeling complexes belongs to the switch–sucrose non-fermenting (SWI–SNF) family which can both slide and eject nucleosomes. This family of proteins can bind acetylated histone tails and further promote gene activation. Members of the ESC-specific Brahma-associated factor (esBAF) complex belong to this family and have been identified in a recent proteomics screen as facilitators of reprogramming [53]. Overexpression of BAF complex members Brg1 and Baf155 significantly increases the efficiency of four factor-mediated reprogramming and can replace c-Myc. Consistent with a proposed role in gene activation, co-expression of these two proteins increases deposition of the active H3K4me3 mark on pluripotency-related genes such as Sall4, Tcf3, and Dppa4 and increases binding of Oct4 to these loci [53]. On the other hand, Klf4 and the SWI–SNF chromatin-remodeling complex catalytic subunit SMARCA2–BRM has been shown to interact with each other in ESCs [54]. This finding suggests that reprogramming factors utilize chromatin-remodeling complexes to promote gene activation. Finally, liver progenitor cells have been shown to be very amenable to reprogramming by OSKM which is due, in part, to high endogenous expression of BAF complex members [55].

14.3.2 Repressive Chromatin as a Barrier to Reprogramming

A major determinant of transcriptional activity is histone acetylation and deacetylation. Acetylation, which is catalyzed by histone acetyltransferases (HATs), is a mark of active transcription and is found throughout gene bodies, promoters, and enhancers in euchromatin. On the other hand, histone deacetylases (HDACs) remove this modification which results in chromatin compaction and gene repression. The pluripotent state is generally associated with a preponderance of histone acetylation and relaxed chromatin [56]. Consistent with this state in ESCs, various inhibitors of HDACs which increase overall acetylation levels have been shown

to facilitate reprogramming of somatic cells. These HDAC inhibitors include suberoylanilide hydroxamic acid (SAHA), trichostatin A (TSA), butyrate, and valproic acid (VPA) [57–60]. All of these small molecule inhibitors increase overall reprogramming efficiency, and VPA, in particular, enables iPSC generation in the absence of c-Myc from mouse fibroblasts and in the absence of both c-Myc and Klf4 from human cells. Gene expression and DNA methylation analyses of butyrate-treated cells at intermediate stages of the reprogramming process revealed that HDAC inhibition leads to enhanced histone H3 acetylation, promoter DNA demethylation, and the expression of endogenous pluripotency-associated genes [57]. These findings support the notion that increasing overall histone acetylation levels allow for a more permissive chromatin structure for OSKM factors to act on.

One of the most well-characterized repressive histone marks is tri-methylation of H3K9. Among the H3K9 methyltransferases, the first to be implicated in somatic cell reprogramming is G9a. In fusion experiments between ESCs and neural stem cells, knockdown of G9a inhibits the reactivation of the Oct4 promoter in the NSCs, suggesting a necessary role in reprogramming [61]. Interestingly, overexpression of the H3K9 demethylase Jhdm2a promotes this process. On the other hand, during ESC differentiation, G9a participates in the silencing of the Oct4 locus by depositing H3K9 methylation which allows for the binding of the heterochromatin factor HP1 and subsequent de novo DNA methylation [62]. While the precise targets of G9a-mediated repression remain to be determined, these studies show that H3K9 methylation plays an important role in the transitions to and from pluripotency. Direct evidence for G9a's involvement in transcription factor-based reprogramming comes from the use of BIX-01294 (BIX) which is a small molecule inhibitor of this enzyme. BIX treatment lowers global H3K9me2 levels, improves reprogramming efficiency of neural progenitors and MEFs, and can replace Sox2 in the reprogramming cocktail [63–65].

An shRNA screen to identify positive and negative regulators of reprogramming among chromatin modifiers revealed a role for additional H3K9 methyltransferases as well (Fig. 14.1). Euchromatin-related H3K9 methyltransferases EHMT1 and Setdb1 were shown to be necessary for efficient reprogramming of human fibroblasts [66]. In ESCs, Setdb1 has been implicated in silencing of retroviral elements and a subset of developmental regulators. Its knockdown results in the loss of the ES cell state [67–69]. On the other hand, another H3K9 methyltransferase, Suv39H1, was identified to be a suppressor of reprogramming such that its inhibition resulted in increased efficiency of iPSC generation. Suv39h1 is the principal enzyme responsible for methylatingH3K9 in heterochromatin, especially in pericentric regions [70, 71]. It may also act in de novo gene silencing [72, 73]. Suv39h1-mediated H3K9 tri-methylation provides a docking site for HP1 proteins which are implicated in chromatin compaction and gene silencing [74]. Attesting to the specificity of Suv39h1 in this process, inhibition of a closely related enzyme, Suv39h2, did not have an effect on reprogramming of fibroblasts, presumably because this enzyme is primarily expressed in a testis-specific manner [66, 75]. The fact that H3K9-modifying enzymes are associated with both inhibition and enhancement of reprogramming suggests that these enzymes have distinct sets of functionally important

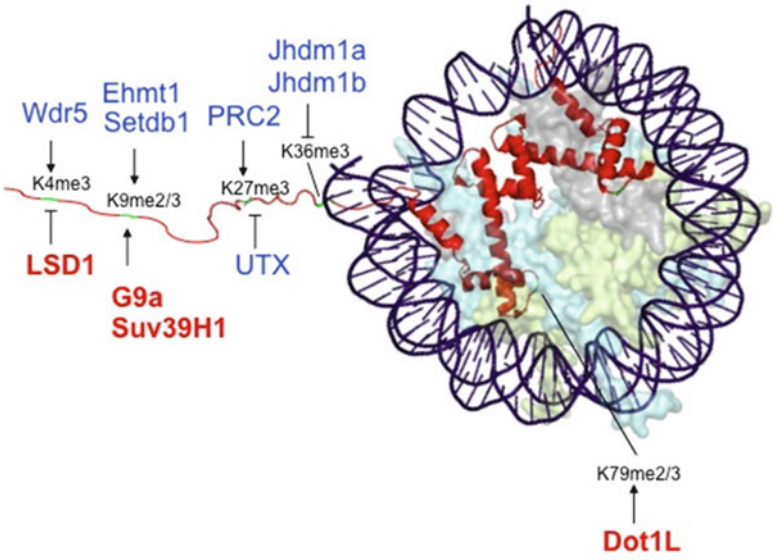

Fig. 14.1 The role of Histone H3 lysine methyl-marks and associated chromatin modifiers in reprogramming. Enhancers or necessary proteins are shown in *blue* and suppressors are shown in *red*

target genes. The precise identification of these targets through a combination of loss-of-function, genome-wide localization and gene expression analyses will contribute significantly to our understanding of the role of H3K9 methylation during reprogramming.

14.3.3 Histone Demethylases in Reprogramming

The first histone demethylase to be discovered was LSD1 (KDM1) which has been shown to demethylate mono- or di-methylated H3K4 and H3K9 [76]. LSD1 has a role in reprogramming as its inhibition by either lithium or RNAi increases the overall reprogramming efficiency [77]. LSD1 is also a target of mir-302 which is an miRNA that has been shown to facilitate reprogramming [78–80]. In ESCs LSD1 binds to Rcor2 which functions as a corepressor. While suppression of Rcor2 abrogates reprogramming, its overexpression improves reprogramming efficiency and can even replace Sox2 in this process [81]. Interestingly, LSD1 is not essential for the maintenance of self-renewal, but ESCs lacking LSD1 cannot differentiate fully [82]. LSD1 binds enhancers of active genes that are co-bound by Oct4, Sox2, and Nanog in ESCs. Together with the NuRD (nucleosome remodeling and histone deacetylase) complex, LSD1 facilitates the shutting down of these enhancers during differentiation [83]. In the absence of LSD1, these active ESC enhancers cannot be

repressed through H3K4me1 demethylation. Taken together, these observations suggest that LSD1 is required for both the acquisition of pluripotency and exit from this state.

In addition to H3K4–K9 demethylation, H3K36 demethylation also plays a role during reprogramming. H3K36 demethylation is carried out by jumonji family proteins Jhdm1a–1b (KDM2A–B). Vitamin C acts as a cofactor for these enzymes and has been shown to promote reprogramming at least in part by facilitating H3K36 demethylation [84, 85]. Jhdm1a–1b play necessary roles in reprogramming as their suppression by shRNAs inhibits formation of iPSCs from MEFs. On the other hand, overexpression of Jhdm1b improves reprogramming efficiency and can even enable iPSC generation by Oct4 alone in the mouse system [85]. While earlier work had shown that knockdown of Jhdm1b inhibits cell proliferation and induces cellular senescence by binding to and derepressing the p15(Ink4b) promoter [86], its effect on iPSC generation seems to be independent of this function [87]. Instead, Jhdm1b has been shown to bind and demethylate gene promoters which are activated early during the reprogramming process such as the E-cadherin promoter [87]. How H3K36 demethylation leads to gene activation remains an open question. Nevertheless, these findings provide strong evidence for a functional role of H3K36 demethylation in resetting the epigenetic landscape upon OSKM expression.

Finally, Mansour et al. has recently observed that the H3K27 demethylase UTX is required for efficient reprogramming [88]. UTX contributes to reprogramming by facilitating the derepression of potent pluripotency-promoting genes such as Sall1, Sall4, and Utf1. UTX has been shown to bind the promoters of these genes in combination with OSK and promote H3K27 demethylation. Interestingly, ESCs lacking UTX could execute lineage commitment and contribute to adult chimeric animals suggesting that H3K27me3 demethylation by UTX is not required for proper differentiation.

14.3.4 Silencing of the Somatic Gene Expression

In pluripotent cells, promoters of key developmental regulator genes contain both the active H3K4me3 and the repressive H3K27me3 mark; this configuration has been termed as a bivalent structure [89, 90]. These bivalent structures resolve upon differentiation and, conversely, are reestablished during reprogramming [91]. PRC2 components which generate the repressive H3K27me3 mark are dispensable for maintaining ESC self-renewal, but they are required for differentiation. Lack of PRC2 results in impaired silencing of pluripotency genes during lineage commitment. PRC2 has also been shown to be required for both fusion- and transcription factor-based reprogramming, highlighting the importance of repressing the somatic cell program during this process. In cell fusion experiments, Suz12 and Eed knockout mouse ESCs were impaired in their ability to reprogram human B cells [92]. ShRNA-mediated suppression of any of the core PRC2 complex members (Ezh2, Eed and Suz12) abrogated OSKM-based reprogramming of human fibroblasts [66].

In mouse ESCs, PRC2 has three additional highly expressed proteins: JARID2, MTF2, and esPRC2p48. Co-expression of these three proteins has been shown to facilitate repression of lineage-associated gene expression and enhance reprogramming of MEFs [93]. PRC2-catalyzed H3K27me3 is recognized by the PRC1 complex whose components Bmi1 and Ring1A ubiquitylate H2A and facilitate chromatin compaction [94]. Bmi1 and Ring1A are necessary for reprogramming, and overexpression of Bmi1 increases reprogramming efficiency and can substitute for Sox2 and Klf4 [66, 95]. Taken together, these findings point to crucial roles for Polycomb-mediated silencing of the somatic gene expression program during reprogramming.

In support of this notion, factors that prevent the silencing of the somatic cell program seem to suppress reprogramming. The best example of this is Dot1L which is the only H3K79 methyltransferase identified to date (Fig. 14.1). Inhibition of Dot1L in somatic cells either by shRNAs, a small molecule inhibitor, or Cre-mediated excision of conditional alleles increases reprogramming efficiency and can substitute for Klf4 and c-Myc [66]. First identified in the yeast, Dot1L acts to antagonize gene repression [96]. Dot1L-catalyzed H3K79 methylation is found in gene bodies of almost all actively transcribed genes in fibroblasts [97]. Interestingly, loss of H3K79 methylation does not have an overt effect on gene expression under normal culture conditions indicating that even though H3K79 methylation is a mark of active transcription, it is not required for transcription itself. The effect of Dot1L inhibition becomes apparent only when reprogramming is initiated by OSKM factors and widespread transcriptional changes take place. During this process, expression of a subset of fibroblast-specific genes such as *Snai1* and *Tgfb1* is turned off with concomitant loss of H3K79 methylation and increased H3K27 methylation. Dot1L inhibition seems to facilitate the repression of these fibroblast-specific genes through decreased H3K79 methylation. As such, H3K79 methylation acts as a barrier to efficient repression of the somatic program by the reprogramming factors [66]. Whether other active chromatin marks and enzymes that generate them, such as the H3K4 methyltransferases, act in a similar manner during reprogramming remains to be determined.

14.4 DNA Methylation

Methylation of cytosines in the DNA is another major chromatin mark which can have profound effects on chromatin structure and gene expression. DNA methylation is found throughout the genome with the exception of unmethylated regions called CpG islands which are frequently found in the promoter regions [98]. Cytosine methylation is established by de novo DNA methyltransferases Dnmt3a–b and maintained through DNA replication and cell division by Dnmt1. CpG island methylation may obstruct transcription factor binding and serves as a docking site for methyl-CpG-binding domain proteins. MBD proteins can then recruit additional chromatin remodelers and histone-modifying enzymes such as HDACs to the methylated locus. Thus, DNA methylation results in a closed chromatin state and leads

to transcriptional silencing. In ESCs, pluripotency factors, "housekeeping" genes, and developmental genes that contain bivalent domains are devoid of CpG methylation [90]. In contrast, in somatic cells, promoters of key pluripotency factors such as Oct4 and Nanog are heavily DNA methylated. In all cases of cellular reprogramming including transcription factor-, oocyte-, and fusion-based methods, demethylation of these pluripotency gene promoters is essential [14, 99, 100]. Failure to do so results in partially reprogrammed cells that are characterized by incomplete repression of lineage-specifying transcription factors and DNA hypermethylation at pluripotency-related loci. Treatment of such cells with an inhibitor of Dnmt1 (5-azacytidine) or silencing of Dnmt1 by RNAi results in a stable conversion to a fully reprogrammed state [14]. Another Dnmt inhibitor (RG108) also enhances reprogramming efficiency [65]. Therefore, DNA demethylation seems to be a rate-limiting step in transitioning to pluripotency.

In addition to DNA demethylation at pluripotency genes, resetting DNA methylation patterns throughout the genome during reprogramming is also important. Residual DNA methylation at key lineage-specific regulators has been shown to skew the differentiation potential of the resulting pluripotent cells [101–105]. For example, fibroblasts which are generally the staring cell types for reprogramming have high levels of promoter methylation at hematopoietic-specific regulators. Failure to reset methylation levels at these loci during iPSC formation prevents the efficient activation of these genes later during iPSC differentiation to blood cells [101, 103, 104]. Treatment of such fibroblast-derived iPSCs with Dnmt and HDAC inhibitors such as 5-azacytidine and TSA leads to more efficient blood differentiation indicating a direct functional link between aberrant DNA methylation and differentiation potential.

A remaining question is how DNA demethylation occurs during transcription factor-based reprogramming. One possibility is passive loss of methylation through cell divisions by suppression of Dnmt expression. This would require multiple cell divisions which certainly take place during the relatively long reprogramming process. However, in SCNT- and fusion-based reprogramming, demethylation happens without any cell divisions. Therefore, active enzymatic demethylation of cytosines during OSKM-mediated reprogramming is another possibility [99, 100]. Several enzymes have been implicated in this process. One of these is the activation-induced deaminase (AID) which converts a methylated cytosine to a thymidine that then gets repaired by base excision repair. In interspecies heterokaryons (fusion between mouse ESCs and human fibroblasts), AID has been shown to be required for promoter demethylation and induction of Oct4 [99]. Whether AID acts in a similar manner during OSKM-based reprogramming remains unaddressed. Another class of enzymes that could potentially facilitate active DNA demethylation during transcription factor-based reprogramming are the ten-eleven translocation (TET) proteins. These enzymes convert 5-methyl-cytosine to 5-hydroxymethyl-cytosine (5hmC) by an oxidative mechanism [106, 107]. 5hmC is further processed to 5-formylcytosine and 5-carboxylcytosine which are specifically recognized and excised by thymine-DNA glycosylase, thus leading to the loss of the original DNA methylation mark [108, 109]. TET proteins have been shown to be involved in DNA

demethylation that occurs in the paternal pronucleus after fertilization, mouse preimplantation embryos, and in SCNT [110–112]. Although not experimentally demonstrated, this family of proteins is likely to play a role in active DNA methylation that may occur during transcription factor-based reprogramming.

14.5 Conclusions

Although the contribution of particular chromatin states to somatic cell reprogramming is only beginning to be determined, there is increasing evidence that certain chromatin modifiers are functionally important for somatic cell reprogramming. However, a complete understanding of the epigenetic changes that accompany reprogramming will only be possible with the development of methods which can combine analyses of specific chromatin states at a single cell level with reprogramming systems that have very high efficiency. In addition, through loss- and gain-of-function approaches, a broader systematic assessment of the entire spectrum of chromatin-modifier proteins in reprogramming needs to be made. Finally, we need to understand how these chromatin modifiers work in conjunction with the OSKM factors and get recruited to their specific targets. A comprehensive understanding of the epigenetic changes that occur during transcription factor-based reprogramming is likely to generate new tools to enhance existing reprogramming methods or even enable completely novel ways to achieve reprogramming. Thus, developments in this field can have a direct impact on the derivation of iPSCs for future clinical use as well.

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Chapter 15
Disease Modeling and Drug Discovery Using Human Pluripotent Stem Cells

Christian Honoré and Mattias Hansson

Abstract Cellular reprogramming and genome editing have enabled the generation of disease-specific human pluripotent stem cells for disease modeling and drug discovery. Although these cells have been heralded as a novel and invaluable tool for these application and numerous proof-of-principle studies have demonstrated their potential, there are still several challenges that have to be addressed in order for them to efficiently translate into drug discovery programs. In this chapter, we review some of the recent progress made in the field and also highlight the challenges that must be overcome in the use of human pluripotent stem cells to recapitulate human disease phenotypes and to screen for therapeutic agents in vitro.

Keywords Pluripotent stem cells • Induced pluripotent stem cells • Embryonic stem cells • Disease modeling • Drug discovery

Abbreviations

ALS	Amyloid lateral sclerosis
AMPK	AMP-activated protein kinase
ARVD/C	Arrhythmogenic right ventricular dysplasia/cardiomyopathy
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
DA	Dopaminergic
DCM	Familial dilated cardiomyopathy
FD	Familial dysautonomia
FAO	Fatty acid oxidation

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K. Turksen (ed.), *Stem Cells: Current Challenges and New Directions*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4614-8066-2_15,

hESCs	Human embryonic stem cells
hiPSCs	Human-induced pluripotent stem cells
hPSCs	Human pluripotent stem cells
HTS	High-throughput screening
IGF1	Insulin-like growth factor 1
IKBKAP	I-κ-B kinase complex-associated protein
iPSCs	Induced pluripotent stem cells
LNS	Lesch-Nyhan syndrome
LQTS	Long QT syndrome
MJD	Machado-Joseph disease
PD	Parkinson's disease
RTT	Rett syndrome
RSK	Ribosomal S 6 kinase
Serca2a	Sarcoplasmic reticulum calcium^{+} ATPase
SCZD	Schizophrenia
SNCA	α-Synuclein
SMA	Spinal muscular atrophy
T1D	Type 1 diabetes
XCI	Inactive X chromosome

15.1 Introduction

Target-specific drug discovery is typically performed using high-throughput cell-based biochemical assays, screening large molecule libraries to find novel compounds that modulate a disease-specific pathway. Alternatively, if the active molecule is known, the drug development process may focus on improving the natural compounds performance, such as efficacy, specificity, and stability. The existing assays for drug discovery are often associated with several drawbacks that can translate to expensive attritions at later stages in the drug development process (Fig. 15.1). The leading cause for failing clinical phase II and III studies is insufficient efficacy [1, 2]. Cell-based assays that better reflect the clinical geno- and phenotypes are highly desired as they will increase predictability and accuracy in the discovery phase and thus reduce the number of failures at later stages. Furthermore, such models can help reduce or replace suboptimal animal studies in drug discovery.

Another major cause for failure in drug development is safety concerns [1, 2]. Although the success rates vary between therapeutic areas and between small molecules and biologics [3], the current trend is that the rates are falling. There are numerous examples of drugs that have failed during development or had to be withdrawn from the market due to unanticipated toxicity that was not observed in the preclinical safety studies or during the clinical trials. Currently, preclinical safety evaluations are dependent on animal studies, which may fail to reflect the human (disease) physiology. Novel in vitro assays for toxicological evaluation in specific cell types and safety pharmacology assessments based on human cells could potentially improve this outcome.

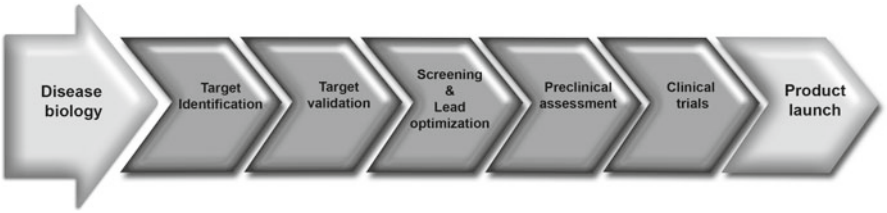

Fig. 15.1 The drug discovery process. Insight in the biology underlying a disease is critical for identifying pathways and phenotypes relevant to the disease. Building on this knowledge, potential targets for treatment can be identified and validated using cellular and animal models. High-throughput screening of libraries or use of already known modulators of targets is then applied to identify compounds that can ameliorate the disease phenotype in in vitro models. Preclinical assessment of toxicity and efficacy of identified compounds is then performed in cellular and animal models before translating into clinical trials. Disease-specific hPSCs and their differentiated progeny offer a novel tool that can improve many of the steps in the drug discovery process, ranging from improved insight in the disease mechanisms to the cellular source for high-throughput screening and toxicity assessment

In general, current in vitro drug discovery and toxicology assays have several limitations. Many assays are based on immortalized cell lines that can provide an unlimited source of cell material needed for high-throughput assays. However, the genetic manipulation to immortalize a cell as well as the accumulation of genetic alterations during prolonged cell culture influences the cells phenotype and may ultimately alter the response to a given substance. On the other hand, the use of primary cells that may assure an accurate drug response presents other challenges. Primary cells are often difficult to access and may be of shifting quality, leading to inconsistent results. Furthermore, primary cells may have a limited in vitro proliferation potential, which further restricts their accessibility and use in high-throughput assays. In many cases, scientists are dependent on animal cells as the human counterparts are inaccessible, which may lead to species-specific responses not relevant to humans.

These challenges may potentially be addressed by utilizing human pluripotent stem cells (hPSCs) as starting material to generate human cell types relevant for drug discovery and preclinical development. hPSCs can provide large quantities of quality-controlled cells for multiple assays, which will increase predictability and accuracy. Furthermore, hPSC can provide disease-relevant cell types for drug screening and lead discovery.

15.2 Human Pluripotent Stem Cell

Human pluripotent stem cells (hPSCs) are characterized by their ability to self-renew indefinitely in vitro while maintaining the capacity to differentiate into well-defined cell types of the three germ layers (endoderm, mesoderm, and ectoderm) [4].

These two characteristics make hPSCs particularly attractive to the research community, since they represent a potential unlimited source of specialized cells and tissues useful to human developmental biology, drug discovery, and cell replacement therapy. Mammalian pluripotent stem cell lines have been established from multiple cellular sources such as teratocarcinomas [5], postimplantation epiblast cells [6, 7], and primordial germ cells [8, 9]. However, the scope of this review focuses on human embryonic stem cells (hESCs) established from the preimplantation blastocysts of in vitro fertilization (IVF) embryos and induced pluripotent stem cells (iPSCs) generated by reprogramming of somatic cells to pluripotency.

15.2.1 Embryonic Stem Cells

The first hESCs lines were established in 1998 [10], seventeen years following the derivation of the mouse embryonic stem cells in 1981 [11, 12]. The considerable gap between the derivation of mouse and human ESCs was primarily a result of species-specific differences in ESCs [13] and poor culture conditions for human IVF embryos [14]. However, building on the work of establishing nonhuman primate ESCs [15, 16], Thomson and colleagues were able to derive multiple hESC lines from the inner cell mass of human IVF embryos cultured to the blastocyst stage. These cell lines were karyotypically normal, could be expanded and maintained undifferentiated for multiple passages, and spontaneously differentiated into derivatives of all three germ layers [10]. Following this initial derivation of hESCs, extensive research resulted in the elucidation of the signaling pathways involved in the maintenance of undifferentiated hESCs [17] and survival upon single-cell dispersion [18]. Thus, hESCs can now be cultured in defined serum and feeder-free conditions and clonally expanded from single cells. The pluripotent nature of hESCs is maintained at the molecular level by the key pluripotent transcription factors Oct4, Sox2, and Nanog that integrates external signals into a core pluripotent transcriptional network that regulates the expression of many genes, including developmentally important transcription factors [19]. While these transcription factors are critical for sustaining pluripotency, there is also evidence suggesting that they are involved in determining early lineage commitment of embryonic stem cells [20–22].

hESCs have the unique ability to differentiate into all adult cell types, and harnessing this potential could provide the starting material for development of cell replacement therapies. In addition, hESCs offer a unique resource for basic research of early human development. However, research using hESCs are burdened with ethical concerns and the likely risk of immune rejection of hESC-derived tissues following transplantation [23–25]. These limitations motivated the search for alternative source of hPSC ideally containing the genetic material derived from somatic cells as this would enable the generation of patient-specific hPSC.

15.2.2 Induced Pluripotent Stem Cells

In 1997, a groundbreaking study reported the cloning of the first mammal, Dolly the sheep, from an adult somatic cell using somatic cell nuclear transfer [26]. The cloning of Dolly established that an adult somatic cell could be reprogrammed to a totipotent state, thus generating cells that can develop into any part of the animal and that differentiation of cells were not accompanied by irreversible silencing of specific genes. Subsequent studies demonstrated that the somatic genome could be reprogrammed to an embryonic state by fusion of somatic cells with ES cells [27, 28]. Together, these findings suggested that the oocyte and ES cells contained factors capable of reprogramming a somatic genome to pluripotency and that in theory it would be possible to derive pluripotent stem cells directly from somatic cells of humans.

Theory quickly became reality in 2006 when Shinya Yamanaka reported the generation of pluripotent stem cells from embryonic and adult mouse fibroblasts [29]. This remarkable feat was achieved by introducing four transcription factors, Oct4, Sox2, Klf4, and c-Myc, known to be involved in maintenance of pluripotency in ES cells to fibroblasts under ESC culture conditions. These induced pluripotent stem (iPS) cells displayed an ESC-like morphology, expressed many of the markers characteristics of ESCs, and could differentiate into derivatives of all three germ layers. Yamanaka and others extended these findings the following year using human fibroblasts as starting material, hereby generating the first human-induced pluripotent stem cells (hiPSCs) [30, 31]. The significant potential that hiPSCs presented for the fields of regenerative medicine and disease modeling spurred an overwhelming effort across academia and industry to repeat and extend the original work on iPSCs, eventually culminating with Shinya Yamanaka along with Sir John Gurdon being rewarded the 2012 Nobel Prize in Physiology or Medicine.

Following their discovery, initial efforts were directed towards describing the similarities and differences between hiPSCs and hESCs. Numerous studies have tried to address this issue using various experimental approaches, but the conclusions reached so far have been somewhat contradictory. Microarray gene expression profiling revealed that hiPSCs were highly similar to hESCs and also quite different from the fibroblasts from which they were derived. However, Chin et al. compared the gene expression profile of several hiPSC lines with hESC lines and found several genes differentially expressed between the two cell types [32, 33]. Other groups reported persistence of DNA methylation patterns in the iPSCs that appeared to originate from their parental somatic cells [34, 35] and demonstrated that such "epigenetic memory" could influence the potential of the iPSCs to differentiate towards different cell fates [36–38]. In contrast, an extensive comparison of 12 hiPSC lines with 20 hESC lines by Bock et al. revealed significant variation in both global gene expression and DNA methylation across all pluripotent stem cell lines, but no distinct pattern that distinguished hiPSCs from hESCs was observed [39]. Another study examining histone modifications and the gene expression profile of several hiPSC and hESC lines reached a similar conclusion that no consistent differences could distinguish the two cell types from each other [40].

Inadequate reactivation of the X chromosome in female hiPSC lines [41, 42], accumulation of mutations and chromosomal abnormalities during and after reprogramming of iPSCs [43–46], and increased immunogenicity of iPSCs [47] are additional examples of reported differences between iPSCs and ESCs. However, other studies addressing similar questions have reached different and often contradicting conclusions [48–52], again highlighting the challenges that lie in comparing iPSCs with ESCs. There are several likely explanations for some of the discrepancies in the conclusions reached in the aforementioned studies. Cell culture conditions, passaging technique, passage number, and iPSC derivation methods can all influence the results obtained. Indeed, a recent study demonstrated that hiPSCs derived using non-integrating modified RNA molecules encoding the reprogramming factors had a gene expression profile more similar to hESCs compared to virally derived hiPSCs [53].

Another caveat in assessing any possible differences between hiPSCs and hESCs is that the most stringent assay available to define pluripotency for human cells is the generation of teratomas. This assay might not be adequate to define truly hPSC. Hochedlinger and colleagues demonstrated that aberrant silencing of the imprinted *Dlk1-Dio3* gene cluster correlated with the developmental potential of mouse iPSCs evaluated by blastocyst complementation [54]. Furthermore, inclusion of ascorbic acid during the reprogramming improved the derivation of mouse iPSCs with developmental potential equivalent to mouse ESCs [55]. For ethical reasons, chimeric contribution and tetraploid embryo complementation assays are not available to human pluripotent stem cells. Thus, it will be important to develop novel functional assays that can evaluate any potential molecular difference between hiPSCs and hESCs with regard to pluripotency. Perhaps even more relevant when considering applying pluripotent stem cells to disease modeling is whether any of these potential differences between undifferentiated hiPSCs and hESCs are manifested in their differentiated progeny. A recent study suggested that although transcriptional differences were observed between undifferentiated hiPSCs and hESCs, the differentiated progeny of these pluripotent stem cells were nearly identical [56].

Altogether, hESCs and hiPSCs are very similar cell types with regard to morphology, gene expression profile, and ability to differentiate into specialized cell types of all three germ layers. Whether the two cell types represent identical pluripotent stem cells is a topic of intense debate as highlighted by the studies mentioned above. As the methods for reprogramming somatic cells to pluripotency and the knowledge of the process continue to improve at a breathtaking pace, it seems very likely that soon one will be able to generate hiPSCs' biological equivalent to hESCs.

15.3 Disease Modeling Using Human Pluripotent Stem Cells

A significant limitation for studying human diseases has been the inaccessibility of live tissue from patients. hPSCs offer a unique opportunity to address this shortcoming as virtually any tissue can be derived from these cells. In addition, the ability to

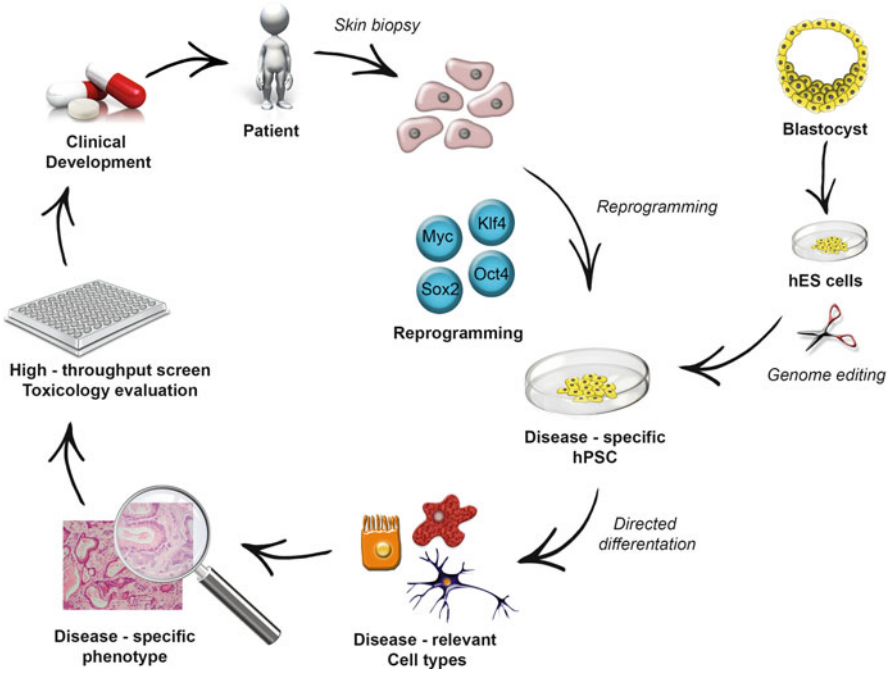

Fig. 15.2 Disease modeling and drug discovery strategy using hPSCs. Disease-specific hPSCs are generated in two ways; somatic cells (usually a skin or blood sample) are derived from a patient with the disease of interest. Forced overexpression of key pluripotent transcription factors (Oct4, Klf4, Sox2, and c-Myc) reprograms the somatic cells to hiPSCs. Alternatively, specific disease-causing mutations can be introduced or corrected in hPSCs through the use of genome-editing techniques. These techniques also allow for the generation of reporter cell lines useful for high-throughput screening. Disease-specific hPSCs are then differentiated towards the relevant tissue by mimicking embryonic development using small molecules and growth factors. Differentiated cell types are analyzed across a broad range of technological platforms to identify disease phenotypes. High-throughput therapeutic screens are then performed to identify compounds that can ameliorate the disease phenotype. This approach can produce leads for drug discovery strategies and also generate novel insight in the pathogenesis of a given disease

derive patient-specific hiPSCs or genome-modified hESCs, hPSCs containing the genetic component of diseases can now be generated. Together, this offers an unprecedented tool for modeling human diseases and developing screens for therapeutic agents that might slow or halt disease progression in affected tissues (Fig. 15.2). Following the initial studies, which demonstrated the reprogramming of adult somatic cells to a pluripotent state, the derivation of hiPSC from patients with various genetic diseases was described [57, 58] (see Table 15.1 for examples). The hiPSCs retained the disease-specific genotype from the patients and could be differentiated into disease-relevant tissues, demonstrating its potential for modeling human diseases.

Table 15.1 An incomplete list of disease-specific iPSCs and disease models, their affected tissues, and disease-relevant phenotypes observed

Disease	Tissue	Molecular defect	Phenotype	Reference
ADA-related severe combined immunodeficiency	Lymphocytes	Mutations in ADA	Not determined	[58]
Alpha1-antitrypsin deficiency	Hepatocytes	Point mutation in *A1AT* gene	Misfolded A1AT protein and reduced enzymatic inhibitory activity. Genetic correction of mutation rescues phenotype	[76, 101]
Alzheimer's disease—familial and sporadic	Neurons	Unknown/duplication of *APP* gene	Increase amyloid-β secretion, elevated phospho-tau levels, and GSK3-β activity	[81]
Amyotrophic lateral sclerosis	Motor neurons	Mutations in *SOD1* gene	Selective motor neuron toxicity to glia expressing mutant *SOD1* gene	[57, 88, 89]
Amyotrophic lateral sclerosis	Motor neurons	Mutations in *TARDBP* gene	Accumulation of TDP-43 protein and reduced survival of motor neurons	[112]
Arrhythmogenic right ventricular dysplasia	Cardiomyocytes	Mutation in *PKP2* gene	Exaggerated lipogenesis and apoptosis and impaired Ca^{2+} handling	[111]
β-Thalassemias	Red blood cells	Mutations in beta globin	Not determined	[113, 114]
Cystic fibrosis	Lung airway epithelial cells	Mutation in *CFTR* gene	Misfolded CFTR protein and impaired CFTR channel activity	[79]
Down syndrome	Multiple tissues	Trisomy 21	Trisomy 21 anomaly observed in derived iPS cells	[58]
Duchenne muscular dystrophy		Mutation in *DMD* gene	Not determined	[58]
Familial dilated cardiomyopathy	Cardiomyocytes	Mutation in *TNNT2*	Heterogeneous sarcomeric pattern, compromised handling of calcium, and lowered contraction force	[70]
Familial hypercholesterolemia	Hepatocytes	Mutation in LDL receptor	Impaired LDL uptake	[76]
Familial dysautonomia	Sensory and autonomic neurons	Mutation in *IKBKAP* gene	Reduced levels of IKBKAP expression. High-throughput screen identified several small molecules that alleviated disease phenotype	[82, 83]
Fragile X syndrome	Neurons	Silencing of *FMR1* gene	Not determined; *FMR1* remained inactive upon reprogramming	[115]
Glycogen storage disease type 1A	Hepatocytes	Absent hepatic glucose-6-phosphatase enzyme	Accumulation of glycogen and lipids and excessive lactic acid production	[76]

Huntington's disease	Neurons	CAG expansion in the huntingtin gene	Increased cell death, mitochondrial deficits, and altered TGF-β signaling. Genetic correction of CAG repeats rescues phenotype	[116, 117]
LEOPARD syndrome	Cardiomyocytes	Mutation in *PTPN11* gene	Increase in cell size, sarcomeric organization, and nuclear NFATC4 localization	[118]
Lesch-Nyhan syndrome	Neurons	Mutation in *HPRT* gene	Reduction in size and number of neurons	[41]
Long QT syndrome	Cardiomyocytes	Mutations in *KCNH2* or *KCNQ1* gene	Prolonged action-potential duration and arrhythmia	[72, 73]
Machado-Joseph disease	Neurons	Expansion of CAG repeats in *ATXN3* gene	Neuron-specific aggregation of ATXN3 protein	[66]
Timothy syndrome	Cardiomyocytes	Mutation in *CACNA1C* gene	Prolonged action-potential duration and arrhythmia	[74]
Timothy syndrome	Neurons	Mutation in *CACNA1C* gene	Impaired calcium signaling and neuronal differentiation	[119]
Parkinson's disease—sporadic	Dopaminergic neurons	Unknown	Not determined	[60]
Parkinson's disease—familial	Dopaminergic neurons	Triplication of *SNCA* gene	Increased expression of *SNCA* mRNA and α-synuclein	[63, 65]
Parkinson's disease—familial	Dopaminergic neurons	Mutation in *LRRK2* gene	Increased expression of α-synuclein and susceptibility to oxidative stress	[61, 120]
Parkinson's disease—familial	Dopaminergic neurons	Mutation in *Parkin* gene	Increased oxidative stress due to elevated monoamine oxidases expression	[64]
Pompe disease	Cardiomyocytes	Mutation in *GAA* gene	Increased glycogen content and mitochondrial defects	[121]
Rett syndrome	Glutamatergic neurons	Mutations in *MeCP2* gene	Reduced glutamatergic synapses. Impaired spontaneous excitatory and inhibitory postsynaptic currents	[50]
Schizophrenia	Neurons	Multifactorial	Decrease in neuronal connectivity and neurite number. Identification of several differentially expressed genes	[80]
Spinal muscular atrophy	Motor neurons	Mutation in *SMN1* gene	Progressive loss of motor neurons	[59]
Type 1 diabetes	Beta cells	Multifactorial	Not determined	[58, 86]

15.3.1 Modeling Neurological Disorders

Using hiPSCs to model neurological disorders is of particular interest due to the poor accessibility of neuronal cells. In one of the first studies that reported a disease phenotype in hiPSC-derived tissue, Ebert et al. generated hiPSCs from a spinal muscular atrophy (SMA) patient and an unaffected individual [59]. The SMA-derived hiPSCs displayed progressive loss of motor neurons upon differentiation to neuronal lineages, a phenotype that might be similar to loss of this cell type seen in patients with SMA. More recently, hiPSCs have been derived from patients with familial and sporadic Parkinson's disease (PD) [60–64]. In two studies, hiPSCs were derived from PD patients with a triplication in the α-synuclein gene (*SNCA*). The hiPSC-derived dopaminergic (DA) neurons expressed significantly higher levels of *SNCA* mRNA and α-synuclein protein compared to DA neurons derived from healthy control hiPSCs, hereby recapitulating the cause of PD seen in the patients [63, 65]. In another study, in patients with mutations in the leucine-rich repeat kinase-2 (*LRRK2*) gene, the most commonly associated PD-mutation, hiPSC-derived DA neurons showed increased expression of key oxidative stress response genes and α-synuclein protein and were more sensitive to stress-induced cell death [61].

Rett syndrome (RTT) is a neurological disorder caused by mutations in the *MeCP2* gene. RTT is classified as an autism spectrum disorder, and patients display several autistic characteristics. Marchetto et al. derived hiPSCs from several RTT patients and differentiated these towards neuronal lineages [50]. A significant reduction in synapses and spines along with reduced soma size was observed in glutamatergic neurons derived from RTT hiPSCs. In addition, the RTT hiPSC-derived neurons displayed altered calcium handling and electrophysiological defects compared to control neurons. Insulin-like growth factor 1 (IGF1) had previously been shown to promote a partial reversal of RTT phenotype in a mouse model, and administration of IGF1 to the RTT neurons increased the number of glutamatergic synapses in culture [50].

Machado-Joseph disease (MJD) is a rare late-onset inherited neurodegenerative disorder leading to progressive cerebral ataxia. The disease is caused by a mutation in the *ATXN3* gene which results in an expansion of CAG trinucleotide repeats consequently leading to aggregation of the ATXN3 protein. Brüstle and colleagues derived hiPSCs from MJD patients and healthy controls and differentiated these towards the neuronal lineage [66]. No difference in the differentiation propensity or function of neurons was observed; however, expression of the expanded ATXN3 protein was observed in MJD-derived neurons. Stimulating the neurons with L-glutamate resulted in calcium influx and cleavage of the ATXN3 protein. Interestingly, formation of insoluble ATXN3 aggregates was specifically observed in MJD-derived neurons following stimulation. The authors hypothesized that temporary activation of a calcium-dependent protease might represent an early event in the aberrant processing of ATXN3 in MJD neurons. In agreement with this, the authors showed that ATXN3 aggregation was specific to neurons, dependent on calcium influx, and mediated by calpain proteases [66]. While the link between ATXN3

aggregation and the formation of late-stage neurodegeneration remains elusive, this study shows how features of a late-onset disease can be modeled in a cell type-specific manner using hiPSCs.

15.3.2 Modeling Cardiac Diseases

Several disease models using hiPSCs have recently been described from patients with inherent genetic disorders leading to impaired heart function (Table 15.1). Protocols for directing the differentiation of hPSCs to cardiomyocytes are well defined [67–69] and have certainly aided the development of these hiPSC disease models. Sun et al. derived hiPSCs from a cohort of patients with familial dilated cardiomyopathy (DCM) [70]. These patients carry a mutation in *TNNT2*, a gene specifically expressed in cardiomyocytes, resulting in a weakened and enlarged heart that is unable to pump blood sufficiently. The hiPSCs were differentiated towards cardiomyocytes and their functional properties analyzed. Cardiomyocytes derived from DCM hiPSCs exhibited a heterogeneous sarcomeric pattern, compromised handling of calcium, lowered contraction force, and were more susceptible to stress compared to cardiomyocytes derived from control hiPSCs. The disease phenotype observed in DCM hiPSC-derived cardiomyocytes was improved by overexpression of sarcoplasmic reticulum calcium^{+} ATPase (Serca2a) or beta-adrenergic blockers, two interventions that have shown clinical promise for treatment of DCM.

Long QT syndrome (LQTS) is a rare inherited hearth condition manifested by a delayed repolarization, prolongation of the QT interval in the electrocardiogram, and ventricular tachyarrhythmias known as torsade de pointes. The genetic cause of LQTS results from mutations in cardiac ion channels and membrane proteins and can be classified into at least ten subtypes based on the various mutations [71]. Two recent studies demonstrated the feasibility of modeling this disorder using hiPSCs derived from patients with LQTS [72, 73]. hiPSCs derived from patients diagnosed with type 1 and 2 of LQTS caused by mutations affecting cardiac potassium channels were differentiated towards the cardiac lineage. Compared to cardiomyocytes derived from healthy donors, the LQTS cells exhibited the prolonged QT interval characteristic for LQTS and susceptibility to tachyarrhythmias. Both disease models identified defects in the cardiac potassium currents, thus providing novel insight in the pathogenesis of type 1 and 2 LQTS. In a similar study using hiPSCs derived from patients with Timothy syndrome, a disorder associated with developmental delay, autism and LQTS, the resultant cardiomyocytes displayed cellular defects that were consistent with the cardiac defects observed in the patients [74]. More importantly, all the studies mentioned above identified or validated therapeutic compounds that ameliorated the disease phenotype demonstrating that hiPSC-derived cardiomyocytes may provide a useful platform to screen for drug candidates.

15.3.3 Other Disorders

Disease models based on hPSCs have predominantly focused on disorders arising within neuronal and cardiac cell types, most likely a consequence of the relatively well-defined and efficient protocols for differentiating hPSCs towards these lineages. However, there are examples of hPSC disease models based on cell types from other lineages (Table 15.1). Vallier and colleagues developed a protocol for efficiently deriving hepatocyte-like cells from hPSCs [75]. While this protocol did not produce fully mature hepatocytes, the differentiated cells did express several hepatocyte-specific markers and shared several functional characteristics with hepatocytes, such as storage of glycogen and low-density lipoprotein, drug metabolism via the CytP450 pathway, and secretion of albumin. Building on this protocol, hiPSCs derived from patients with various liver disorders were differentiated to hepatocyte-like cells, and several disease-relevant phenotypes were observed, hereby demonstrating the feasibility to model various aspects of liver diseases [76].

Disease modeling of lung disorders has also been limited by efficient protocols to specify lung airway epithelium. However, several recent studies systematically dissected the developmental pathway of the lungs and applied it to directed differentiation [77–79]. As a proof of principle for disease modeling of a lung disorder, hiPSCs derived from cystic fibrosis (CF) patients were differentiated towards lung tissue. Airway epithelia derived from control hPSCs expressed cystic fibrosis transmembrane conductance regulator (CFTR) protein at the plasma membrane, whereas CF-derived epithelia showed no expression of CFTR, a phenotype that was partially corrected with a chemical compound known to rescue the trafficking effect of CFTR mutants [79].

As all of these hPSC-based disease models emphasize, hPSCs holds a tremendous potential to generate cell types with a disease-relevant phenotype. Such models will be very useful for studying the pathogenesis and progression of diseases and also provide a valuable cellular resource for screening for therapeutic drugs and pharmacotoxicology.

15.4 Drug Discovery Using Human Pluripotent Stem Cells

The ability to generate large numbers of specialized cell types affected by relevant diseases illustrates the potential usefulness of disease-specific hiPSCs or genetically modified hESCs for high-throughput screening (HTS) of small molecules. However, there are several challenges that must be addressed in order to use disease-specific hiPSCs for HTS. First, disease-specific hiPSCs must be derived from patients and validated. As discussed in details below, thorough characterization of the hiPSCs and validation of a disease-specific phenotype is critical. Secondly, an efficient differentiation protocol must be developed that reproducible enables large-scale production of the relevant cell type to perform the HTS. Obtaining a pure

population of the disease-relevant cell type is desired in order to eliminate any potential off-target/non-cell autonomous effects. Finally, a robust disease-relevant readout must be identified that will be applicable for the HTS, which could be changes in gene/protein expression levels, cell function, or survival (Fig. 15.2).

Several recent disease modeling studies have demonstrated the use of disease-specific hiPSC-derived tissues to validate small sets of drug candidates [59, 72–74, 79–81], but none of these studies performed HTS to identify additional therapeutic compounds. Familial dysautonomia (FD) is a rare autosomal recessive disease characterized by loss of sensory and autonomic neurons. The disease is caused by a single point mutation in the in I-κ-B kinase complex-associated protein (*IKBKAP*) gene, leading to a mis-splicing of the *IKBKAP* transcript and reduction of IKAP protein levels. Lee et al. derived hiPSCs from several FD patients and differentiated these to neural crest precursors. Several disease-relevant differences were observed when comparing neural crest precursors derived from FD and healthy hiPSCs among those a significant decrease in the wild-type *IKBKAP* gene transcript [82]. Building on these findings, a HTS was designed to identify small molecules that could increase expression of wild-type *IKBKAP* gene. This approach identified several small molecules that increased *IKBKAP* expression and ameliorated some of the observed disease-relevant phenotypes in FD hiPSC-derived neural crest precursors [83]. One limitation to this screen was the use of neural crest precursors instead of the neural crest-derived peripheral neurons affected by the disease. The reasoning for this is most likely a result of the reduced neurogenesis observed in FD hiPSC-derived neural crest precursors [82]. Thus, it remains to be shown whether the identified compounds will improve the survival of postmitotic sensory and autonomic neurons. Nonetheless, these studies provide a notable first glimpse of the potential use of disease-specific hiPS cells for identifying novel clinical relevant compounds using HTS.

Drug development is an expensive and laborious process that more often than not results in early termination of potential therapeutic compounds due to efficacy, safety, or commercial concerns [1–3]. Improved in vitro assays developed to screen for drug efficacy and toxicity will undoubtedly aid the transition from preclinical to clinical trials and likely also increase the chances of clinical approval. Identifying unwanted toxicity of therapeutic compounds in preclinical trials is of particular interest in order to increase the safety of clinical trials. hPSCs offer a novel avenue to generate tissues and specialized cells useful for preclinical trials, where it can complement existing cellular and animal models. Sunitinib is a tyrosine kinase inhibitor approved for clinical use for treatment of metastatic renal cell carcinoma and gastrointestinal stromal tumors, but concerns about its cardiac toxicity have been reported [84]. To explore the mechanism of clinical toxicity, Cohen et al. derived cardiomyocytes from hiPSCs and tested the effect of sunitinib on these. Sunitinib displayed cardiotoxicity observed by loss of cellular ATP, increase in oxidized glutathione, and apoptosis in the hiPSC-derived cardiomyocytes [85]. AMP-activated protein kinase (AMPK) and ribosomal S 6 kinase (RSK) have previously been suggested as causative based on animal models. While the study did not identify the underlying cause for the sunitinib-induced cardiotoxicity, the authors

were able to test this hypothesis and rule out AMPK and RSK as primary contributors. More importantly, this study highlights the use of hiPSC-derived cardiomyocytes to study the underlying mechanism of drug-induced toxicity. Highly pure populations of hPSC-derived hepatocytes, cardiomyocytes, and other cell types could potentially provide a more stringent assay for investigating drug efficacy and toxicity than the current models available. As protocols for differentiating hPSCs become more refined, it is assumed that hPSCs will begin to be incorporated in the preclinical toxicology evaluation of potential therapeutic compounds.

15.5 Challenges

Compared to monogenic disorders, which show complete penetrance of the disease phenotype in specific cell types, modeling complex and polygenic diseases in which the underlying genetic cause is unknown or several cell types and/or environmental factors are involved in the pathogenesis will be particularly challenging. Nonetheless, several recent examples have highlighted the feasibility of modeling more complex disease using hiPSCs. Israel et al. generated hiPSCs from patients with familial (caused by duplication of amyloid-beta precursor protein gene) and sporadic Alzheimer's disease. Several disease-relevant phenotypes were observed in the hiPSC-derived neurons from the familial Alzheimer's patients: increased secretion of amyloid-beta protein, higher levels of phospho-tau, and glycogen synthase kinase-3 beta activity. Interestingly, hiPSC lines from one of the two sporadic Alzheimer's patients also recapitulated several of these disease phenotypes upon neuronal differentiation [81]. Clearly, a larger cohort of sporadic Alzheimer's patient will have to confirm these findings, but it does suggest that the genome of some of the sporadic Alzheimer's patients will generate a robust neuronal phenotype and the hiPSC-derived neurons will provide a useful tool to elucidate the genetic variant(s) involved in the pathogenesis. Another recent example of a disease model that identified a specific molecular phenotype from a complex disease comes from a study using hiPSCs to examine schizophrenia (SCZD) [80]. In this study, fibroblasts from several schizophrenia patients were reprogrammed to hiPSCs and subsequently differentiated to neurons. Phenotypic analysis revealed lower neuronal connectivity in SCZD neurons compared to control neurons and a reduction in neurites and amount of postsynaptic density protein 95 in the dendrites. Using gene expression profiling of SCZD and control neurons, this study identified several differentially expressed genes previously associated to SCZD but also pathways that had yet to be linked to this disease [80].

Modeling diseases that involves multiple cell types and non-cell autonomous interactions also presents a great challenge for hPSCs research. While hiPSCs have been derived from patients with type 1 diabetes (T1D) and differentiated towards the pancreatic lineage [86], modeling T1D would require the generation of not only pancreatic beta cells but also other cell types involved such as T cells and thymus epithelium and the establishment of advanced coculture systems [87]. There are few

examples suggesting that developing disease models involving several cell types should be feasible. In a model for amyloid lateral sclerosis (ALS), hESC-derived neurons were cocultured with primary glia. Specific toxicity towards motor neurons was observed when the glia overexpressed an ALS-causing mutant form of the superoxide dismutase 1 gene, a phenotype that appears in agreement with the disease phenotype [88, 89]. Despite these few examples, the practicality of hPSCs to model more complex diseases involving several cell types and polygenic diseases still remains to be determined.

As highlighted earlier, cellular reprogramming is still a work in progress, and incomplete or aberrant induction of pluripotency might affect the subsequent phenotypic analysis of hiPSC-derived tissues. One striking example of this comes from studies trying to model X-linked diseases. Several recent studies reported that during reprogramming, female hiPSCs retained an inactive X chromosome (XCI). The female hiPSCs displayed a nonrandom XCI in that the X chromosome that was inactive in the donor fibroblast population retained an inactive state following reprogramming [41, 42, 90]. During passaging of female iPSCs, Mekhoubad et al. observed a transcriptional derepression of the inactive X chromosome. This erosion of the XCI had significant ramifications in their disease model of Lesch-Nyhan syndrome (LNS). When using early passage hiPSCs, fewer and smaller neurons were derived from LNS hiPSCs. However, this phenotype was lost when using the higher passage LNS hiPSCs that had reactivated their XCI [41]. These findings underscore the importance of monitoring the epigenetic variations when using hPSCs for disease modeling.

The variation in genetic background among individuals also presents a concern for disease modeling using hPSCs due to the uncontrolled influence of functional differences in genes and noncoding sequences [91–93]. This genetic variation might also account for the observed variability in differentiation efficiencies among various hPSCs [39, 94–97]. For monogenic diseases, genetically rescued cell lines represent the ideal control. Zinc-finger nucleases and transcription activator-like effector nucleases have emerged as valuable tools for editing specific gene loci of hPSCs [98, 99]. Introducing or correcting mutations in hPSCs enables the generation of isogenic controls that differ exclusively at the disease causing point mutation [100–102]. An alternative approach, especially suitable for more complex or multifactorial diseases, would be to derive control hiPSCs from healthy first-degree relatives, hereby minimizing the genetic background variation. If none of these options are available, comparison between panels of disease-specific hiPSC lines with healthy, unrelated control hiPSC lines should be applied to ensure that none of the phenotypic differences observed is a consequence of variation between hiPSC lines.

The lack of efficient differentiation protocols for certain disease-relevant cell types has also hampered the development of disease models with hPSCs. Modeling various forms of diabetes would be greatly facilitated by a robust differentiation protocol that can generate functional mature beta cells in vitro. Cells expressing insulin and other endocrine hormones of the pancreas have been derived through directed differentiation [103, 104]; however, these cells are poly-hormonal and nonfunctional and resemble an immature phenotype. In fact, many differentiation

protocols produce immature cell types with an embryonic phenotype rather than adult cells [69, 75, 78, 103, 105]. A possible explanation for the relative immature phenotype observed in most of the cells derived using directed differentiation protocols could be that the relevant cell type may require additional signals or proper three-dimensional settings to generate a fully mature phenotype. Indeed, several studies have demonstrated that transplantation of immature cells generated by directed differentiation of hPSCs into a mouse can promote their maturation in a period of months [75, 78, 106, 107]. Other studies have demonstrated the feasibility of applying three-dimensional culture systems to generate tissues that display self-organizing properties [108–110]. The immature phenotype could also be a result of the fact that most of these differentiation protocols involve culturing the cells for weeks, whereas the development of fully mature cells in humans requires much longer time. Thus, induction of more mature cell types by directed differentiation could require extended culture periods and modulation of culture conditions. Arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C) is an inherited heart disease characterized by pathological fatty infiltration and increased cardiomyocyte apoptosis primarily in the right ventricle. The mean age of ARVD/C manifestation is 26 years. To model this adult-onset disease, Kim et al. derived hiPSCs from ARVD/C patients with mutations in the desmosome component *PKP2* and differentiated these towards cardiomyocytes using a standard cardiogenic differentiation protocol [111]. Consistent with previous models of ARVD/C, abnormal nuclear translocation of plakoglobin and reduced beta-catenin activity were observed in the *PKP2* hiPS cell-derived cardiomyocytes. However, no increase in lipogenesis and apoptosis was observed following several months of culturing. Because the major metabolic difference between embryonic and adult cardiomyocytes is that embryonic cardiomyocytes primarily use glycolysis for energy production, whereas adult cardiomyocytes use fatty acid oxidation (FAO), the authors hypothesized that an induction of FAO was required to induce a disease phenotype. A cocktail of adipogenic factors and peroxisome proliferator-activated receptor-gamma (PPAR-gamma) activators was used to induce a more mature metabolism. This resulted in exaggerated lipogenesis and pronounced apoptosis in mutant hiPSC-derived cardiomyocytes, both hallmarks of ARVD/C pathogenesis [111]. This study provides an example of the challenge of modeling adult-onset diseases using hPSCs but notably demonstrates how culture conditions can be modified to promote maturation of the disease-relevant tissue and acceleration of disease pathogenesis.

15.6 Summary

Despite the limitations described above, hPSCs have already been applied to model a broad range of diseases. Considering that hESC lines were derived for the first time only 15 years ago and the generation of the first hiPSC lines was described 6 years ago, this research field has been moving at a breathtaking pace. Continued improvements of reprogramming techniques, differentiation protocols, and culture

conditions will undoubtedly be reflected in the development of more disease models, both of monogenic and complex disorders, hereby providing novel platforms to study the pathogenesis and screen for therapeutic agents that can ameliorate the disease.

Acknowledgements The authors are members of the StemBANCC—Stem Cell for Drug Discovery consortium (www.stembancc.org). StemBANCC receives research funding from the Innovative Medicines Initiative Joint Undertaking under grant agreement no. 115439, resources of which are composed of financial contribution from the European Union's Seventh Framework Programme (FP7/2007–2013) and EFPIA companies in kind contribution. www.imi.europa.eu. We thank Agnieszka Cecha for helping with the graphical design and Ole Dragsbæk Madsen for critical reading of the manuscript.

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Chapter 16
Stem Cell Research: Ethical Considerations

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Abstract Stem cell research promises great hopes for treatment of chronic degenerative diseases and permanent disabilities as well as bringing important ethical problems to the agenda as well. These problems can be discussed as the sources of stem cells and the method to derive them, stem cell research processes, the use of stem cells at the clinical phase, and the ethical challenges afterwards. A wide consensus in the international arena has not been provided on the subject of stem cell researches. This situation arises from different sociocultural, religious, and political points of views as well as the dynamism of the stem cell researches. Even so, a common attitude towards the continuity of the studies on this field with different levels of restrictions and prohibition of cloning for the purpose of reproduction can be mentioned. On one hand adhering to general principles of research ethics in stem cell researches has a great importance; on the other hand in order to prevent gaps in the field, international regulations should be made, and such regulations should be updated by reviewing the studies in this field at regular periods in the light of scientific and social developments.

Keywords Stem cell research • Ethics • Embryos • Legal regulations

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K. Turksen (ed.), *Stem Cells: Current Challenges and New Directions*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4614-8066-2_16,

16.1 Introduction

While medical practices in the past were restricted to treat symptoms of most diseases, thanks to today's advanced diagnosis and treatment methods efforts to eliminate the causes of diseases and to heal them exactly have become prominent. In this sense, stem cell researches represent "revolutionary" developments in medicine. Stem cell treatments based on the principle of replacement of non-functional cells in the body with functional cells promise great hopes for treatment of chronic diseases or permanent disabilities. Some arguments are accepted to be precursor of a new era in medicine; namely treating some diseases such as diabetes, myocardial infarction, Parkinson's disease, Alzheimer's disease, ALS, spinal cord injury, osteoarthritis, rheumatoid arthritis, progressive muscular diseases, infertility, cancer, deafness, and blindness and generating new organs instead of non-functional organs, like liver failure, by means of stem cell technologies. Offering tailor-made cellular treatments in the near future is foreseen [1, 2]. Despite a rapid increase in accumulation of knowledge in the field of stem cell, great expectations especially related to the treatment have overtaken the developments achieved by researches. Yet, nowadays bone marrow transplantation is the most reliable therapy with its standards and its practice conditions in the therapeutic use of stem cells. Significant developments have been provided in recent times also in the skin and cornea repair. In spite of being promising, experimental studies on other treatments have still been under developmental process [3].

Emerging of opportunities to benefit from stem cell researches in treatment of some diseases has changed the contents of the ethical debates sustained on the issue. In this respect, problems such as embryo experiments, artificial tissue, and organ generation, the philosophical meaning of the embryo, the embryo's rights, the use of spare embryos for treatment and research, and the need to create non-embryonic stem cell have been brought to agenda.

The fact that stem cells have the capacity to differentiate into the required tissue has led to emergence of a new treatment approach in the medicine. "Regenerative medicine," which is based on the regeneration of tissues and organs in the treatment of diseases developing as a result of cellular degeneration or tissue damage, has found a broad field of discussion and practice in the dominant medical paradigm of today. Regenerative medicine has been a source of hope for the treatment of such diseases, complete treatment of which is not possible in general and which requires various treatments throughout life.

The fact that human stem cells were first derived from human embryos and kept alive under laboratory conditions for the first time by James Alexander Thomson and his team in Wisconsin University in 1998 opened the door to an important new era of biomedical research [4]. As a result of the studies that have been conducted, some standard information on cell development, maturation, and functions almost entirely changed. For instance, the view that cell differentiation is a one-way and irreversible process disappeared; differentiation was shown to be reversible in transforming adult stem cells back to undifferentiated stem cells [5].

16.2 Stem Cells and Their Properties

Stem cells are parent cells that form all the tissues and organs in the body. These cells that have been undifferentiated yet have capability of infinite divisibility, regeneration, and transforming into organs and tissues by generating differentiated cells [4]. Although other organ cells such as nerve cells and heart cells have particular functions, stem cells are not differentiated and they remain so, unless they receive a stimulus to turn into a specific cell. Undifferentiated cells' capabilities to divide and transform cells that have particular functions like muscle cells or nerve cells are their properties making them unique [1].

Biological development of the human is a process of transformation starting with single-cell zygote to a multicellular organism with functions of regeneration, differentiation, and development. The first ring of this chain is fertilized ovum. Developmental potential of the cells during this process is different from each other, yet generally in a decreasing direction. When zygote has the capacity to give a gestation product (totipotent), it transforms nearly 200 cells that form blastocyst fetus (pluripotent). Upon the implantation of these cells onto uterus wall, it has the capacity to transform into fewer cells (multipotent) [6]. With this developmental process, stem cells are divided into three types as totipotent, pluripotent, and multipotent.

16.2.1 Totipotent Stem Cells

These cells form embryo, non-embryonic membranes, and all tissues and organs in the body, and they have capacity of infinite division and differentiation. All blastomeres are totipotent up to eight cells in early embryonic stage.

16.2.2 Pluripotent Stem Cells

These cells can transform into all cell types in the organism and have potential of infinite proliferation. Embryonic stem cells are derived from inner cell mass in blastocyst which forms 5–8 days after fertilization, and they are pluripotent.

16.2.3 Multipotent Stem Cells

These cells are the ones that have differentiated to produce the same tissue type in the following stages of fetal development. Adult stem cells are multipotent cells as bone marrow cells [7, 8].

16.3 Sources of Stem Cells

16.3.1 Embryonic Stem Cells

Embryonic stem cells are undifferentiated and pluripotent cells having theoretically capability of infinite division in culture. Human embryonic stem cells are derived from embryos that have reached to blastocyst phase during pre-implementation of early development period. They are derived from embryos remaining after IVF applications and from embryos developed from the ovums which were donated for research purposes [9]. Apart from these methods, human embryonic stem cells can also be obtained in alternative ways.

16.3.1.1 Somatic Cell Nuclear Transfer (SCNT)

Pluripotent stem cell is obtained from a blastocyst that is created by transferring a nucleus taken from somatic cell of an individual into an enucleated egg cell. The major advantage of this method is the fact that, in the event of transferring these stem cells into the person whose somatic nucleus was taken, it does not cause reject reaction [10].

16.3.1.2 Altered Nuclear Transfer (ANT)

The same technique with SCNT is used; however, embryo development capacity of somatic cell nucleus that is to be transferred into enucleated egg cell (ovum) is genetically deteriorated. Since the embryo created in this way does not have the chance to survive, creating stem cell by taking inner cell mass out of blastocyst has less ethical problems [7].

Similarly, the usage of embryos with dead organism or embryos with abnormal chromosomal structure to create embryonic stem cells is in question as well. It is also possible to create stem cell series by applying single blastomere biopsy, which is used for preimplantation genetic diagnosis, without damaging the embryo [11].

Somatic cell dedifferentiation is a quite interesting method that aims at reversing the developmental process of cell. Degrading adult somatic cell, which has completed its differentiation, back to pluripotent phase offers a wide working area for stem cell researches for therapeutic purpose. Tissue rejection reaction will be overcome technically, and also ethical arguments on embryo will come to an end [11].

16.3.2 Fetal Stem Cells

Fetal stem cells exist in amniotic fluid, umbilical cord, and product of pregnancy after abortion. It is possible to obtain multipotent hematopoietic stem cell from umbilical cord and neural stem cell from product of pregnancy [7].

16.3.3 Adult Stem Cells

Development of these cells begins during fetal period, 8 weeks after fertilization. They are found undifferentiated in many tissues; however, their plasticity is lower compared to embryonic stem cells. Adult stem cells have functions like maintaining continuity of the tissues they are in and repairing. Adult stem cells have been used with the purpose of bone marrow transplantation for many years [11].

Human stem cell researches are essential for both basic medical sciences and clinical sciences. Benefits expected from these researches are to specify the mechanisms, which organize cell differentiation, and to transform stem cells into special type cells to be used for some disorders, which are not able to be treated completely today [12].

16.4 Ethics of Stem Cell Research

Basic challenges of stem cell researches base on the research methods and the concerns about possible outcomes. Most debated issues are the facts that these researches involve destruction of human embryo and they may result in practices, which may even be inhuman, with an uncertain outcome in future. The fact that outcomes, which do not accord with human dignity, especially such as cloned babies, embryo farms, and the use of fetuses for spare parts, are potential dangers has been discussed in the context of slippery slope argument [2]. Furthermore, the research methods are evaluated in terms of ethical aspect. Can unacceptable methods, such as violation of fundamental rights, to achieve good results be approved as a choice? Human stem cell research includes a wide group of challenges to be scrutinized ethically. It is possible to investigate these related ethical challenges under three groups:

- Ethical issues on deriving stem cells and their source
- Ethical issues on research process
- Ethical issues on clinic phase and afterwards

16.5 Ethical Issues on Deriving Stem Cells and Their Source

16.5.1 The Use of Human Embryonic Stem Cells for Research or Therapeutic Purposes

Since human embryonic stem cells are derived from human embryos, using them in researches or for therapeutic purposes brings ethical issues forward. There is no common consensus on the moral status and legal definition of embryo [2]. Being one of the oldest sets of problems in medical ethics, moral status of embryo has been a

debate which is basically held on abortus issue. Destruction of the embryo is in question in deriving embryonic stem cell despite different from purpose of abortus.

Owing to different sociocultural and religious perspectives, different values are attributed to human embryo. Is the fetus an individual? At which phase of the process from fertilization to delivery we can mention embryo or fetus as an individual? Can embryos destructed during research process be considered equivalent to killing a human? Those who base these discussions on religious beliefs argue that God gives soul to embryo with fertilization and every human being starts life as an embryo. Therefore, if an adult person deserves respect to his/her life and privilege, those at any phase of development also deserve the same respect. Stem cell researches on embryos are ethically unacceptable applications for those who think in this way. However, opponents argue the fact that every individual starts the life as an embryo does not prove their individuality. They put forward that the embryo has the potential; however, human life develops step by step. Moreover, it is also emphasized that since the embryo does not have chance to live out of uterus, environmental conditions are important in this process as well. It is argued that an embryo in a petri dish would not be in the same moral status as an individual. Assessing stem cell researches over embryo's moral status would lead the ethical debates into the dead end easily, as this is an irreconcilable field. Hence, it is believed to be beneficial to take other options, which are already on the agenda as stem cell source, into consideration [2, 11].

16.5.2 The Use of Surplus/Spare Embryos Formed During IVF Applications

When the facts that embryos are created under in vitro conditions during IVF applications and surplus embryos which are not placed uterus are used during researches are taken into consideration, the necessity for special protection of these embryos "that are cell mass which are not going to be able to reach human status" will disappear. As embryos out of uterus do not have potential to grow up and become an adult individual, the use of these embryos seems less problematic ethically. Using embryos, otherwise to be destroyed, for the sake of humanity and for the suffering patients, may be proved to be right in the sense of "preventing wastage" and being beneficial [2, 11]. To make sure that the use of these embryos for stem cell researches is not without consent, it is required to take the woman's (/parent's) informed consent.

16.5.3 The Use of Fetal Tissue That Is Obtained as a Result of Pregnancy Termination (Miscarriage/Intentionally Induced)

Abortion has been defined as "the spontaneously or purposely removal of the gestational product (embryo or fetus) as dead from the mother dead from the mother

prior to viability" [13]. In this sense the ethical discussions about embryo become unfounded since the product of gestation is dead after a completed procedure. The use of a fetal tissue, which would be destroyed anyway, for research purposes after taking woman's consent, in accordance with principles of wastage prevention and providing benefit, can be legitimized. However, creating pregnancy and terminating this pregnancy with abortion for research and treatment purposes is a procedure which cannot be acceptable ethically. As in the instances of directing women to abortion for money and selling the embryos obtained in some countries or taking the infants away from their mothers just after delivery and making women become pregnant, taking neonatals from these women, and destroying them for stem cell trading, the process may move to very dangerous directions [14, 15]. Oviedo Convention (a. 22) prohibits organ and tissue trading [16].

16.5.4 Creating Embryos to Be Used in the Stem Cell Researches

Production of embryos for research purpose is an unacceptable procedure within the context of instrumentalization of the embryo and fundamentally human being. Considering an embryo as an instrument/meta is refused, because of being against human dignity [17]. Oviedo Convention (a.18) has given green light to conduct research on embryo; however, strictly prohibited is producing embryo to conduct research [18].

16.5.5 The Use of Somatic Stem Cells

It seems that the use of somatic stem cells will resolve the challenges derived from using embryonic or fetal tissues and discussions about the moral status of embryo. Concentrating the researches on this axis is suggested.

By means of nucleus transfer technique, embryonic stem cells that are compatible with the individual, who is donor of nucleus, can be developed. This situation brings up the hope of the use of tailor-made tissues and organs, which eliminate rejection issue of immune system, for patients who are in need of tissue and organ transplantation in the future. If this comes true, it will be no necessity of organ transplantation from a living donor anymore, and so related ethical issues will be gone away [6, 11]. Similarly, implementation of dedifferentiation, namely, transforming differentiated somatic cell back to pluripotent cell, is still the last point achieved. In this way, ethical problems related to histoincompatibility, rejection issue, need for living donor, and embryonic stem cell will be resolved. These types of researches are believed to be the most acceptable ones in the future [7, 11].

16.6 Ethical Issues on Research Process

16.6.1 Ethical Issues in the Context of Women's Rights/Reproductive Rights

Status of the woman becomes crucial since ovum is used in the embryonic stem cell researches. Because of the critical roles of women, as a vulnerable group, during research processes, women being under health risk to some extent, being oppressed, and being considered as "meta" due to being a donor/provider of ovum can be in question in the researches. In these researches women should be provided to decide with her free will without a pressure, forcing, monetary motivation, and cheating for the matters of abortion, IVF applications, embryo donation, and ovum donation by taking women's sensitivity into consideration. Prior to the removal of ovum, women need to take hormone treatment for over stimulation. After the removal operation of ovum, infertility and even fatal risks come into question [11, 19]. According to Declaration on Rights of Mother and Newborn, "Every woman has the right to be included in the decision-making mechanism (diagnosis and treatment) which will affect herself and the fetus. Every decision should be made independently and expectant mother should be informed about the subject" [20]. Factors such as social status of woman and social gender inequality, her sociocultural and economic condition, and her educational status affect women's opportunity to benefit from healthcare services and her right to have a voice over her body [21]. As long as these factors change towards the negative way, woman becomes vulnerable to the probable risks of stem cell researches that are aimed at research and treatment. A woman may participate in stem cell researches that have approval of ethics committee due to monetary issues as well as she may be involved in illegal applications in this field. Providing protection of all volunteers' rights seems a main ethical duty for doctors/researchers. United Nations Declaration on Human Cloning [22] makes call for the member countries to take measurements for this purpose by specially referring to the prevention of women abuse.

16.6.2 Informed Consent from Volunteers During Research Process

Informed consent is the prerequisite of all medical interventions. Using and protection of every part of human body require patient's consent [23]. Universal principles pointed out by Amsterdam Declaration are also valid for stem cell researches. Volunteers should be provided with all information about the research completely during informed consent despite being too technical and complicated. The individual should be provided to make the decision about himself/herself at the cellular and tissular level.

While being produced under in vitro culture conditions, stem cells sometimes may be exposed to unwanted genetic mutations that may be harmful for the

organism. It is unknown whether cells that are reproduced in the existence of serum, chemical substances, and medium, all of which are used in researches, contain potential harm for human health or not [6]. During informed consent period, all of these risks should be mentioned honestly, like the possible benefits, and voluntariness should be provided. When a new situation is encountered during stem cell treatments, informed consent should be received from the volunteer again.

16.6.3 Confidentiality of Privacy/Hiding of Information During Research Process

During research process, volunteers' private lives should be respected and protected carefully; information that belongs to these people should be prevented from being learned by unauthorized people. In order for the information transferred to electronic environment to be protected, these computers should not have access to the Internet, and authorized people should have the access to them only with a personal password. Oviedo Convention (a. 23) highlights on the conservation of personal information and on providing its security [16].

16.7 Ethical Issues on Clinical Phase and Afterwards

16.7.1 Problems Caused by Commercial Concerns' Being Leading and Determinant in Researches

Commercial expectations' being dominant in stem cell researches should be accepted as an ethical problem. The fact that commercial tendencies determine the course of researches instead of scientific facts leads the results to be evaluated according to profit and loss criteria. Yet the results of these researches, which all humanity expects with great hopes, should not be sacrificed to commercial interests.

16.7.2 Advertisement/Misinforming the Society/Miraculous Treatments/Sharing Research Results, Whose Hypothesis Is Not Proven, with the Public

It is generally accepted that advertisement/promotion which creates unfair rivalry and aims at generating demand in offering healthcare services cannot be made; only informing that is protective and promotive for health can be made [24]. Presenting stem cell researches with exaggerating and wrong information in the media stimulates the expectations in this field and serves the issue to be commercialized. This issue which is required to be discussed also in terms of the media ethics will gain more

importance with the expansion of stem cell treatments. As well as introducing successes on this subject to the public, research results without anticipated outputs should also be shared with the public. Society's surrealistic expectations should be brought to the realistic borders by presenting associations', which are authorized in the field of stem cell, and science people's views objectively and clearly.

16.7.3 Reflecting Some Treatment Methods, Efficiency of Which Has Not Been Proven, as Miraculous Treatments During Research Process

This approach may lead to exploitation of patients morally and materially as well as causing iatrogenic harms. Especially patients in the terminal phase can easily approve to take stem cell treatment with no proved efficiency. With these non-standardized treatment trials, patient can experience unnecessary pain and nuisance and also encounter life-threatening risks. The negative environment to be created by these events may strengthen arguments of those who oppose stem cell researches, and it may cause difficulties in conducting the real researches [25].

16.7.4 Copyrights/Patent Problem

When Jonas Edward Salk invented polio vaccine, he answered the question on the patent of vaccine as follows: "There is no patent. Could you patent the sun?" [26] Could a beneficial "thing," a common heritage of humanity, be the subject of the patent? Stem cell issue is a little more complicated from this aspect, and it does not fit the traditional patent concept. Biomedicine Convention (a. 21) prohibits gaining financial and similar incomes out of human body and body parts [16]. The European Patent Office (EPO)'s opinion on unpatentability of human embryonic stem cells is not clear [27]. Discussions on patent issue in stem cell studies keep their currency.

16.7.5 Problem of Cost and Accessibility of Treatments to Be Developed as a Result of Stem Cell Studies

It is argued that stem cell researches promise for high cost treatments that a few rich people may get benefit from and mostly intend for the disorders experienced during elderliness [28]. Whole society contributes to the research process via volunteers, healthcare personnel, and public funds, whereas there are restrictions regarding the use of probable treatments to be developed in terms of patients. This situation may be discussed in the sense of justice principle. It is necessary for the burden and the benefit to be shared equally and fairly at the social level.

Regarded as a birthright, healthcare should be services that are needed to be provided to everyone equally in the society within the sense of social justice [29]. When stem cell treatments are brought to agenda in the future, all patients who are in need should be served with this perspective. Socioeconomic, cultural, and geographical factors should not restrict the accessibility of the services.

16.7.6 Ignoring Other Local and Global Health Problems Not Associated with Stem Cell Researches

Sources that are allocated for healthcare issues should be directed to the fields that are in need, and they should be distributed fairly. However, due to high cost of stem cell researches and directing healthcare investments towards this field, basic healthcare services can be pushed to the background. On United Nations Declaration on Human Cloning, member states are called upon to take into account the global emergency issues such as HIV/AIDS, tuberculosis, and malaria, which are serious problem for in particular the developing countries, on their research budgets [22].

16.8 Basic Approaches About Stem Cell Researches in Various Countries

Different countries in the world exhibit various attitudes towards the issue of stem cell researches. However, these can be basically classified into three approaches: restrictive, permissive, and moderate.

16.8.1 The Restrictive Option

Prohibits human embryonic research; does not explicitly permit research with existing hESC lines. Countries of this option are Ireland, Poland, Austria, Slovakia, Latvia, Germany, and Italy.

16.8.2 The Permissive Option

Accepts the production of human embryos for research purposes through in vitro fertilization and/or nuclear transfer (cloning). Countries of this option are Spain, the UK, Northern Ireland, Belgium, Sweden, Finland, South Africa, Israel, Japan, China, India, South Korea, and Australia.

16.8.3 The Moderate Option

Permits the derivation of new hESC lines but only through the use of remaining embryos from infertility clinics. Countries of this option are France, Portugal, the Netherlands, Denmark, Iceland, Norway, Russia, Czech Republic, Hungary, Slovenia, Estonia, Lithuania, Greece, Turkey, Iran, New Zealand, Brazil, and Canada [30].

16.9 International Regulations and Countries' Approaches Towards Stem Cell Researches

There has been still no explicit consensus on international platform in terms of stem cell researches. Stem cell issue which had been discussed for a long time under the roof of United Nations seems to be resulted in a text called *United Nations Declaration on Human Cloning* [22].

> Member States are called upon to adopt all measures necessary to protect adequately human life in the application of life sciences
>
> Member States are called upon to take measures to prevent the exploitation of women in the application of life sciences
>
> Member States are called upon to prohibit all forms of human cloning in as much as they are incompatible with human dignity and the protection of human life

In contrast to 84 countries that voted in favor of the Declaration, total number of countries giving abstention votes 37 and counter votes 34 is nearly equal to number of above-mentioned countries. This result indicates the deep differences of opinions at the international level in this field. Declaration prohibits all forms of human cloning and makes an important reference to the protection of human life. In this period, it lays specific emphasis on the prevention of the abuse of women and fair distribution of financial sources. "Member States are further called upon, in their financing of medical research, including of life sciences, to take into account the pressing global issues such as HIV/AIDS, tuberculosis and malaria, which affect in particular the developing countries".

It is possible to state that Oviedo Convention of Council of Europe, another international structure like United Nations, also allocates a considerable amount of place for this issue. Article 18 of the Convention, which has also been involved in legislations of each member state of the Council, is required to be included in this context.

Article 18 of the Oviedo Convention on Human Rights and Biomedicine (Oviedo, 1997) [18].

1. Where the law allows research on embryos in vitro, it shall ensure adequate protection of the embryo.
2. The creation of human embryos for research purposes is prohibited.

Only the creation of embryos for research purposes is prohibited. In vitro embryo researches are allowed when adequate protection related to embryo is provided. The convention gave green light to studies to be conducted on this subject and leaded national regulations.

Another international intervention is ISSCR Guideline [31]. It was prepared with the participation of scientists, ethicists, and legal experts from 14 different countries. This guideline aims at facilitating the international cooperation on human embryonic stem cell research. It was thought that developing a set of standard applications would encourage the researchers and institutions.

According to the guideline, three main factors should be taken into consideration in human embryonic stem cell researches:

1. Working transparently
2. Looking after social interests
3. Conserving sense of trust of the society

Basic principles specified by the guideline are as follows:

1. Call for oversight
2. Permissible and impermissible research
3. Requirement for explicit consent
4. Financial considerations
5. Encouraging compliance
6. Accessible research material

In this context, the guideline (Guidelines for the conduct of Human Embryonic Stem Cell Research, Version 1; Dec. 21, 2006) includes 14 subsections of justification, mission of task force, comment on scientific terminology, scope of guidelines, responsibility for conduct, statement on reproductive cloning, issues pertinent to international collaborations, recommendations for oversight, mechanisms for enforcement, categories of research, procurement of materials, principles for derivation, banking, distribution of human stem cell lines, dispute resolution, and ongoing review of guidelines.

There are five samples of informed consent forms in the section of annexes of the guideline:

1. Sample research consent form; egg procurement for SCR (p.22)
2. Sample research consent form; egg donation for SCR (p.36)
3. Sample research consent form; embryo donation for SCR (p.38)
4. Sample research consent form; somatic cell donation for SCR (p.45)
5. Sample research consent form; sperm donation for SCR (p. 52)

Also, human embryonic stem cell-related materials transfer agreement form (p. 59–64).

Consequently, the guideline seems to be intended to have the researches on human embryonic stem cell, which is a new pioneer field of medicine and biological sciences, conducted within borders of ethical acceptability and by being aware of social responsibilities as well.

16.10 Conclusion

Quick developments about stem cell constitute a quite striking example of scientific developments. However, it is early for evaluation of results of both scientific studies on the subject and clinical practices, and the number of patients who are in need of stem cell treatment and in a strong effort towards healing is quite high; therefore, some unwanted problems and consequences in terms of medical ethics are resulted. The fact that the society is not informed sufficiently and the decision mechanisms based on social participation is not operated especially in developing countries with dominance of a traditional sociocultural structure prevents people, who would get benefit from these applications, from examining the developments in detail. Legal regulations' following scientific developments dilatorily or existence of legal gaps provides an environment for violations of right at different levels in a field where rights and values, such as human health and dignity, are in question. This unfavorable picture in which healthcare is abandoned to decisiveness of market dynamics brings out an extremely complicated attitude towards medical applications in society. On the one side, there is a feeling of insecurity against medicine environment, on the other hand adopting every new development at once without questioning enough. Based on tendencies of those requesting service with self-confidence provided by the increase in medical information with a logarithmic speed and the opportunities of application based on technology, performing brave interventions, which are sometimes considered as unethical, can be in question in medical environments.

Stem cell researches, on the other hand, are highly dynamic, with many questions and "unknowns" [32]. It is a wide studying field scientifically as well as ethically. As it covers deep-rooted problems—moral status of embryo—it includes three generations of ethical problems with currently ongoing and possible future ethical challenges. As a result of scientific developments obtained in this field, it is put forward that some ethical problems can lose their importance—organ donation from a living donor—and be pushed out of the list.

Although there is a comprehensive discussion on human stem cell research in the world, generally there is also a consensus on the continuity of such studies and prohibition of cloning for reproduction. Despite existence of different guidelines in this field, sufficient international regulations have not been made yet. This situation has arisen from different sociocultural, religious, and political perspectives, as well as from the dynamism of the field. A "wait-and-see policy" can be mentioned in the meaning of waiting for the developments and deciding and creating regulations according to their outcomes. In this case, adhering to general principles of research ethics is of vital importance. However, in order to prevent gaps in the field, the requirement for a regulation, on which a considerable extent of consensus is provided under roof of United Nations, has been increasing with each passing day. Such regulations should be updated by reviewing the studies in this field at regular periods in the light of scientific and social developments.

When great hopes and optimism stem cell researches create on patient and their relatives are induced with the rivalry in science world, the wish to get a result as

soon as possible lessens ethical sensitivity in research processes. Establishing ethical standards should not be assessed as handicaps before scientific studies; on the contrary, it is necessary to prevent misusage of researches, protect volunteers' rights, and increase reliability of the study. Ethicist, embryologist, medical biologist, and clinicians as well as patient representatives (representatives of nongovernmental organizations related to patients or diseases) should be included in independent stem cell ethics committee founded for this purpose. Ethics committees not only evaluate researches from ethical aspect but also assess the purpose, method, and expected benefits of the research from the scientific aspect. The question, in the stem cell researches, of what the expected result is or whether getting this benefit is possible or not may not be clear/explicit all the time. Therefore, it is crucial for ethics committees to perform follow-up and supervision duties efficiently, after giving approval for the research.

Researches conducted on human beings have a great contribution at the backstage of medical developments. The most important difference of stem cell researches from other clinical trials is the fact that they have more unknown risks. Even if they start essentially with good intentions, there are risks of having unpredictable, unwanted, and ethically unacceptable—human lives' becoming commodity—consequences. So it is necessary to make the required legal regulations and provide high ethical standards in researches.

Although stem cell researches provide positive developments for treatment of important chronic disorders, they are also argued due to having been still at the early phase and in terms of their efficiency, cost, accessibility, and possible risks they may bring. Even if stem cell treatments become a part of daily life of medicine in future, they represent sense of "therapeutic" of healthcare services. Holistic view over health is not just focusing on treatments of diseases or considering healthcare services only as therapeutic services.

Preventing the occurrence or progress of diseases is more efficient, easier, and cheaper than treating them. Protecting should always be more privileged than treatment. Giving importance and priority to preventive medicine in healthcare services is a more right approach from ethical aspect [33].

Giving priority to preventive medicine services may be able to prevent occurrence of some diseases that are targeted by stem cell treatments. For instance, precautions such as adequate and balanced nutrition, prevention of obesity, regular exercise, and avoiding stress may prevent most chronic diseases or restrain them from reaching to clinical level. Similarly, traffic safety and accident prevention works decrease or prevent spinal cord injuries associated with accidents.

According to World Health Organization (WHO), millions of deaths associated with chronic disorders in the world can be prevented. WHO estimates that approximately 400 million people would die between 2005 and 2015 because of heart diseases, diabetes, and other chronic diseases. However, WHO, making a striking determination, states that these deaths can be prevented with early and simple therapies as well as changes in life style [34].

Public health strategies [33] such as clean drinking water, enough sanitation, waste disposal in a safe way, adequate and balanced nutrition, health education,

vaccinations, alcohol, prevention of tobacco and substance addiction, regular exercise, avoiding from stress, healthy environment, precautions for house and occupational health, traffic safety and prevention of accidents, premarital consultancy, and reproductive health workings prevent diseases and disabilities which require stem cell treatment and provide benefit to individual and public health.

Health problems' being irrecusable and irreplaceable may cause diseases with easy and cheap treatment to be ignored, while looking for cures for incurable diseases. While humankind pursues sophisticated stem cell treatments, child deaths due to illnesses that can be prevented with vaccine, mother deaths during pregnancy and delivery processes, and a great number of deaths associated with wars, hunger, and poverty are our world's reality, which needs to be questioned ethically. In this sense, for the purpose of providing a healthcare service which is worthy to human dignity and which also addresses the necessities of stem cell researches and their clinical practices within the framework of scientific data and ethical principles, the society should be informed properly; every kind of interventions, which create hope mongering and mislead the public, should be prevented by efficient methods; science and ethics should be the cornerstones in applications of healthcare workers; related detailed legal regulations should be formed; and laws should be implemented.

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K. Turksen (ed.), *Stem Cells: Current Challenges and New Directions*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-4614-8066-2,