Stem Cell Biology and Regenerative Medicine

Maria Gazouli
George E. Theodoropoulos *Editors*

Digestive System Diseases

Stem Cell Mechanisms and Therapies

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Stem Cell Biology and Regenerative Medicine

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Our understanding of stem cells has grown rapidly over the last decade. While the apparently tremendous therapeutic potential of stem cells has not yet been realized, their routine use in regeneration and restoration of tissue and organ function is greatly anticipated. To this end, many investigators continue to push the boundaries in areas such as the reprogramming, the stem cell niche, nanotechnology, biomimetics and 3D bioprinting, to name just a few. The objective of the volumes in the Stem Cell Biology and Regenerative Medicine series is to capture and consolidate these developments in a timely way. Each volume is thought-provoking in identifying problems, offering solutions, and providing ideas to excite further innovation in the stem cell and regenerative medicine fields.

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This book is dedicated to our families

Preface

Digestive System Diseases: Stem Cell Mechanisms and Therapies

The gastrointestinal tract has a rapid epithelial cell turnover, which remains throughout life. Stem cells play a key role in the regulation and maintenance of this process and give rise to all the gastrointestinal epithelial cell lineages. The identification of specific markers for the gastrointestinal stem cells, along with the technological advantages to track their endogenous activity and to exploit their ability to generate new epithelia, has significantly improved our understanding of stem cell-driven homeostasis and pathogenesis of gastrointestinal diseases. These exciting new insights in the implication of stem cells into the gastrointestinal system pathologies might lead to the potential development of stem cell-based therapies.

This book places the current developments in the gastrointestinal stem cell field clearly in context. It will hopefully serve as a useful tool, concentrating current knowledge on this "hot" topic, which has been currently attracting researchers' and clinicians' interest. Additionally, this book is a referral textbook for whoever would like to enhance his/her knowledge on stem cells.

The authors focused on digestive diseases and analyzing stem cell contribution on each of the digestive system's parts. Whether you are a student, researcher, clinician, or patient, or just interested in digestive diseases, we hope you enjoy this book as much as we have enjoyed researching and organizing it!

Athens, Greece

Maria Gazouli
George E. Theodoropoulos

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About the Editors

Maria Gazouli, PhD, Biologist is Associate Professor of the Department of Biology at the National and Kapodistrian University of Athens (NKUA), School of Medicine. She performed her first PhD training in the Department of Biology, School of Science of NKUA in partnership with the Hellenic Pasteur Institute; her second PhD training in the Department of Microbiology, School of Medicine of NKUA; and her postdoctoral work in the USA (1997–2000) in the Cell Biology and Pharmacology Department, Georgetown University Medical Center, Washington, DC. In 2007, she joined the NKUA Medical School as Lecturer of Biology; in 2012, she was promoted to Assistant Professor of Molecular Biology; and in late 2016, she was promoted to Associate Professor of Molecular Biology. Dr. M. Gazouli's work refers mainly to the molecular basis of diseases (mainly autoimmune diseases and cancer), to molecular detection of pathogens, and to the investigation of the pathogenesis of the diseases they cause in humans. She worked on stem cell implication in mucosal healing and is responsible for the e-learning program of the NKUA Stem Cells and Regenerative Medicine. Recently, Dr. Gazouli was involved in the incorporation of nanotechnology into targeted cancer detection, imaging, and drug delivery. She was honored with a Fulbright Scholarship for the Development of Nanotechnology-Based Biosensor Arrays for the Detection of Circulating Colorectal Cancer Cells at the University of Maryland, College Park, MD, USA. Dr. Gazouli's work is reflected in more than 230 publications that have received more than 8900 citations and

an h-index of 48 (Google scholar, 1/10/2018). She owns one granted international patent and three European patents. She has given more than 50 invited lectures at international and national conferences and universities and has trained several junior scientists. She has served as ad hoc reviewer for various high-impact scientific journals and is regularly invited to serve on review panels as an expert evaluator by prestigious organizations, such as the National Research Grant Funding Agencies of Greece; Broad Medical Research Program Inflammatory Bowel Disease Grants; the National Science Centre (Narodowe Centrum Nauki), Krakow; the PISCOPIA Fellowship Programme on behalf of the University of Padova, Italy; the Czech-Norwegian Research Programme; the Qatar National Research Fund; the Danish Council for Independent Research for DFF, YDUN Research Project; the Italian Ministry of Education, Universities and Research (MIUR); and the evaluation of research products.

George E. Theodoropoulos was born in Greece in 1969 and graduated from Athens Medical School in 1992. His PhD research in Tumor Markers of Gastrointestinal Malignancies was completed in 1994. He completed a 6-year residency program in General Surgery and a fellowship in Colon and Rectal Surgery in the USA. Following a 4-year course as a Private Surgeon in Athens Medical Center, he was elected as a Lecturer of Surgery in Athens Medical School in 2007. He is currently holding an academic post as an Associate Professor of Surgery at the same university. He is a Diplomat of the American Board of Surgery and of the American Board of Colon and Rectal Surgery and a Fellow of the American College of Surgeons (FACS) and of the American Society of Colon and Rectal Surgeons. Twenty PhD theses have been completed under his supervision. He has also completed a 6-month research fellowship in the Department of Colorectal Surgery, Cleveland Clinic Florida, Weston, FL, USA. He has set up and guided the function of a clinic of surveillance of health-related quality of life and oncologic process of postoperative colorectal cancer patients; has been supervising the Colorectal Unit of

the First Department of Propaedeutic Surgery of Athens Medical School at Hippokration Hospital, Athens, Greece; and has recently established and has been coordinating, along with the Radiology and the Academic Gastroenterology Department of the hospital, a "Lower Digestive Tract Study Unit," aiming at the multidisciplinary approach of large bowel diseases. He has presented at more than 200 national and international meetings and invited talks at 90 meetings. He is the author/coauthor of 120 internationally cited, peer-reviewed journal publications. His research work has international recognition, and there are more than 4,000 citations of his publications related to his research (h-index 34).

Introduction: Gastrointestinal Stem Cells in Human Health and Disease

Maria Gazouli and George E. Theodoropoulos

Histologically, the human gastrointestinal tract is composed of a series of epithelial cells (ECs) highly compartmentalized in terms of morphology and function. These epithelia are renewed on a periodic basis for the homeostasis to be preserved. Regeneration can also occur following tissue damage so as for the tissue integrity and compartmentalization to be retained. However, there are conditions that can lead to the formation of lesions, including metaplasia and dysplasia. The former refers to the replacement of one differentiated cell type by another type of differentiated cell; these lesions are related to a high risk of intestinal cancer [1]. Dysplasia refers to an abnormality of development, growth, or differentiation. Since intestinal epithelial cells are renewed by local intestinal stem cells (ISCs) and the regulation of their functions, most importantly proliferation and differentiation, is associated with these lesions, the regulatory mechanisms related to these cells need to be enlightened.

Recently, the identification of specific markers for ISCs resulted in a better understanding of the regulation of homeostasis and regeneration of the small intestine. Its histological structure includes a mono-stratified epithelium that forms two anatomical structures: the crypts and the finger-like protrusions known as villi in which different cellular types can be found. The crypts harbor stem cells and Paneth cells and transit amplifying cells, and the villi harbor ECs, goblet cells, and enteroendocrine cells (EEs). Paneth cells are located at the crypt's base, closely associated with stem cells and secreting antimicrobial substances and lysozyme. Stem cells

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serve a dual purpose: self-renewal and generation of TA cells which will generate all the differentiated cell types of the villi, maintaining the epithelial homeostasis. The newly identified markers can divide these stem cells into two subpopulations: the crypt base columnar stem cells (CBC) which as their name suggests lie on the base crypt and the +4 cells which can be found four cellular diameters apical of the crypt's base. CBC cells express the Leu-rich repeat-containing G protein-coupled receptor 5 (LRG5) which is a gene targeted by Wnt. Follicle epithelial stem cells are also labeled by Wnt, a fact that suggests that LGR5 could serve as a marker for Wnt-activated stem cells [2]. Epithelial cells from the base of colonic crypts have been cultivated in vitro and behave as multipotent stem cells [3], while Lgr5-GFP positive cells may be able to generate uniform intestinal organoids which is a characteristic ISC property [4].

The esophagus, the tube that connects the throat to the stomach, consists of a stratified epithelium without the distinct structures of the small intestine (including the crypts) and multiple layers of squamous keratinocytes. Here, the proliferating cells are the basal cells which are attached to the basal membrane. From there, the cells migrate to the upper layers and eventually shed inside the lumen. However, it remains unclear whether all the basal cells or just a subpopulation have stem cell characteristics; the results of the available studies are rather conflicting [5–9].

The human stomach consists of three regions: the cardiac, the corpus, and the pylorus. The corpus is composed of an epithelium with gastric units and structures that resemble the small intestine's crypts and project deep inside the mucosa. The four different cell types that can be found lead to the subdivision into four regions. The pit region which is close to the lumen contains mucous cells. Beneath these, the isthmus harbors stem cells which proliferate rapidly. Below the isthmus, the neck region can be found, in which gland mucous cells are contained. In its base, there the last category of cells, the chief cells, is responsible for the secretion of several digestive enzymes. Parietal cells which produce acid can be found in all these regions. The first stem cells to be recognized are in the isthmus zone; these stem cells can regenerate all the differentiated cell types. Another rare stem cell population has been tracked along the gland in the pylorus. Typically, these cells are dormant, but during injury they can regenerate all different cellular types. Studies have revealed more stem cell sites (in the pylorus and corpus near the isthmus and at the bottom of the gastric unit in the corpus), a fact that suggests the gastric's epithelium plasticity.

In the human small intestine, ISC functions are fine-tuned by a plethora of factors that derive from the stem cell niche. This formation comprises adjacent epithelial cells, myofibroblasts, neurons, lymphocytes, and the basal membrane. Of note is that the Wnt activity shows different activity inside the crypt with the most increased at the crypt's base. This gene is vital for ISC proliferation and determines cell fates within the crypt; if the Wnt signaling is lost, the intestinal crypts are ablated.

Another important regulatory mechanism is the Bone Morphogenetic Protein (BMP) signaling which exhibits its highest activity toward the villus and its lowest at the crypt's base. Depletion of its receptor 1a leads to opposite effects from those

that occur when Wnt signaling is lost. Progenitor and proliferative stem cells expand, and the inhibition of BMP signaling leads to the formation of ectopic crypts. This indicates that ISC proliferation is negatively regulated by the BMP signaling. These signaling molecules are affected by Hedgehog (Hh) signaling which increases BMP signaling and reduces Wnt signaling activity; epithelial precursor cells are reduced. The inhibition of Hh signaling leads to defective villus formation and results in a hyperproliferative epithelium [10].

As knowledge around the regulation of gastrointestinal stem cells evolves, the origin and progress of epithelial lesions become clearer. Metaplasia typically occurs in epithelial tissues exposed to the environment (esophagus and stomach) [1]. The most common metaplasias reported in humans are Barrett's esophagus and intestinal metaplasia (affecting the gastric region). Normally, the esophagus is lined by multiple squamous cell layers which during this condition are replaced with cells that form an intestine-like columnar epithelium. This disease is an important risk factor for esophageal adenocarcinoma. Treating options include the inhibition of acid production, anti-reflux surgery, chemoprevention, and ablation therapy. Several studies have been made to clarify the mechanism of the disease with some of them suggesting that either this condition involves stem cells from the cardiac region of the stomach or that the normal esophageal stem cells change their identity leading to the disease.

Another type of metaplasia, the intestinal metaplasia, occurs when intestinal epithelial cells are present in the stomach. This condition includes two stages: the complete intestinal metaplasia which occurs in the early phase (the metaplastic epithelium is like the mucosa of the small intestine and has ECs and goblet cells) and the incomplete intestinal metaplasia which occurs in later stages (the metaplastic epithelium resembles the morphology of the large intestine and includes only goblet cells). Both stages express an intestinal-specific marker mucin 2 (MUC2), and at the same time, the gastric-specific marker mucin 6 (MUC6) is lost. Furthermore, intestinal metaplasia may result in gastric dysplasia, a precancerous state that can lead to gastric cancer.

Helicobacter pylori infection, a major cause of gastric cancer, is believed to enhance cellular proliferation [11] and to negatively affect the maturation of precursor cells. Bone marrow-derived cells transform into metaplastic cells acting as a source of intestinal metaplasia and possibly gastric cancer, but the mechanism remains unclear [12].

Dysplasia is a common finding at neoplastic stages, and the progression to an invasive cancer phenotype is rapid both in the stomach and the colon [13–15]. The initialization of the invasive gastric cancer occurs when dysplastic cells cross the barrier of the basal membrane. Histologically, based on the degree of the cellular abnormality, dysplasia can be characterized as either high- or low-grade dysplasia. Precancerous metaplastic sites can transit to dysplasia with a varying progression rate. Inflammatory bowel disease (IBD) which includes ulcerative colitis and Crohn's disease is associated with colorectal cancer. Inflammation boosts cancer progression though the secretion of growth and survival factors which limit apoptosis

and increase cell proliferation. Additionally, inflammation cells release reactive oxygen species which disturb genome integrity [16, 17].

Of notice is that both lesions (metaplasia and dysplasia) require the transformation or trans-differentiation of epithelial cells; these events most likely involve changes in the transcriptome which is responsible for the cell's identity. Several transcription factors have been related to these transitions including the homeodomain transcription factors Cdx1 and Cdx2 which define prospective intestinal cells but not cells from the gastric region. Metaplasia has been also associated with ectopic expression of CDX genes which eventually forms intestinal tissues in the stomach [18–20]. Furthermore, the development of these lesions is linked to the deregulation of gastrointestinal stem cells, and thus a better understanding of their regulatory mechanisms (which include cellular identity) is vital for these pathologies to be treated.

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Introduction to Stem Cell Principles and Biology

Maria G. Roubelakis

Embryonic Stem Cells (ESCs)

Embryonic stem cells (ESCs) are derived from the inner cell mass of a blastocyst, which is formed 4–5 days after fertilization and exhibit the potential to self-renew without limit in culture. In more detail ESCs exhibit a high proliferation potential in vitro; maintain high levels of Oct-4 expression, telomerase activity, and a normal karyotype; and retain the potential to differentiate into cell types of all three lineages [1, 2].

Established human ESC lines were typically derived from in vitro fertilized embryos destined for destruction at in vitro fertilization units. In order to generate a single ESC line, 30–34 cells of the inner cell mass of blastocyst are isolated and expanded in vitro. Human ESC lines are cultured in growth medium supplemented with animal sera and maintained usually on mouse feeder layers (i.e., mouse embryonic fibroblasts) [3]. Furthermore, ESCs are pluripotent with a great differentiation potential to various cell types. The differentiation potential of human ESCs can be evaluated either in vivo or in vitro, whereas ESCs can be cultured in vitro under certain culture conditions to induce differentiation into the desired cell type [1, 4, 5]. The in vivo models involve injecting cells into immunocompromised mice and analyzing the teratoma formation. However, it is notable that established ES lines may display some genomic instability [5]. Thus, the use of ESCs for regenerative medicine is questioned, as ESCs appear to be tumorigenic and form teratomas that contain cell types representing all three primary germ layers in vivo [5]. It is evident that, prior clinical use, it will be important to exclude undifferentiated stem cells from cell types or products derived from ESCs. Another important issue that remains

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unsolved and must be addressed is the immune rejection that these cells may provoke. It has been generally assumed that due to the fact that human ESCs and their differentiated derivatives can express high levels of major histocompatibility complex (MHC) class I antigens, any ES cell-based product will be subjected to graft rejection [5].

Induced Pluripotent Stem Cells (iPSCs)

In 2006, Yamanaka et al. managed to reprogram mouse skin fibroblasts into stem cells, similar to embryonic stem cells, by overexpressing four transcription factors OCT4, SOX2, KLF4, and c-MYC; these cells were characterized as induced pluripotent stem cells (iPSCs) [6]. By using this approach, adult cells can be genetically reprogrammed to an embryonic stage by switching the expression of the necessary genes for the embryonic stem cell properties [6]. The iPSCs have the ability to further differentiate into various types of cells, like ESCs, without the presence of concomitant ethical problems associated with the destruction of the blastocyst. Accordingly, Yamanaka and et al. managed to reprogram the "biological clock of the cell." Since then, iPSCs have been generated from human somatic cells by using a variety of protocols. In subsequent studies, researchers replaced the original transcription factors with other combinations, but always in the presence of OCT-4, which represents an essential transcription factor for reprogramming somatic cells. The iPSCs resemble but are not identical to ESCs, as detailed genomic analysis reported the existence of epigenetic memory in iPSCs [7, 8].

To this end, iPSCs have been shown to possess some specific features or properties that can be acquired during the reprogramming process or are remnants of epigenetic modifications of the DNA derived from the parental tissue or cell that influence gene expression [7, 8]. These residual signatures of epigenomes and transcriptomes of the somatic tissue or cell of origin were termed as "epigenetic memory." It has been reported that residual DNA methylation signatures derived from the somatic tissue of origin may favor their differentiation potential into lineages related to the donor cell while restricting alternative cell fates [9].

The advantages and disadvantages of iPSCs can be summarized as follows:

Advantages: (i) iPSCs are undifferentiated with unlimited differentiation potential into all cell types. In addition, iPSCs can be expanded in vitro to a high passage. These properties are allowing them to be used as a potential therapeutic tool in all tissues and organs [8]. (ii) Studying iPSCs derived from pathological or normal tissue can offer a better understanding of a disease and the relevant molecular pathways. iPSCs are often termed as a "disease in a dish" [10]. (iii) No ethical considerations are related to iPS generation.

Disadvantages: (i) The efficiency or reprogramming is very often low and depends on the donor tissue and the reprogramming method. (ii) Prior transplantation into patients, it is needed to ensure that iPSCs are *fully differentiated* into the required

specialized cells. (iii) iPSCs, like ESCs, are reported to form teratomas in vivo after transplantation [7]. (iv) Epigenetic memory in iPSCs influences the gene expression [9].

Fetal Stem Cells (FSCs)

Fetal stem cells represent a relative new source of stem cells. These cells can be derived either from the fetus or from the supportive extraembryonic structures. FSCs have been recently isolated from several tissues such as amniotic fluid, amnion, umbilical cord blood, Wharton's jelly, placenta, fetal liver or fetal bone marrow [11–14].

Recent reports describe fetal stem cells as ideal cell types for regenerative medicine because they (i) are easily accessible as these cells are usually derived from tissues that are normally discarded following birth, such as umbilical cord, placenta, or amnion, (ii) exhibit high proliferation rates in vitro, (iii) do not form teratomas when injected to immunosuppressed mice in vivo, (iv) do not present ethical reservations like embryonic stem cells (ESCs) and (iiv) exhibit functional features indicating that they represent intermediates between ESCs and adult stem cells (e.g., amniotic fluid stem cells express the pluripotency marker Oct-4 in high levels [15, 16]). Another important issue is that early fetal stem cells appear to have pre-immune status and can be used with limited implications compared to adult stem cells in allogenic transplantations. In particular, these cells do not express HLA-class II, but express HLA-class I antigens, and they do not elicit lymphocyte proliferation in vitro [11–13].

However, these cells have a limited differentiation potential compared with ES cells, as they cannot give rise to all cell types of the three germ layers. It will remain necessary to show that fetal stem cells can differentiate into fully functional committed cells in vivo in order to evaluate better their therapeutic potential [11, 14].

In the following sections, fetal sources such as amniotic fluid, umbilical cord blood and extraembryonic tissues will be analyzed in detail.

Amniotic Fluid (AF)

AF serves as a protective liquid for the developing embryo, providing mechanical support and the required nutrients during embryogenesis. Amniocentesis has been used for many decades as a routine procedure for fetal karyotyping and prenatal diagnosis, allowing the detection of a variety of genetic diseases.

AF also represents a rich source of a stem cell population deriving either from the fetus or the surrounding amniotic membrane. Additional investigations by several groups have been recently focused on the cellular properties of amniotic-derived cells and their potential use in preclinical models and in transplantation therapies [12, 16–19].

The amniotic fluid cells (AFCs) represent a heterogeneous population derived from the three germ layers. These cells share an epithelial origin and are derived from either the developing embryo or from the inner surface of the amniotic membrane, which are characterized as amniotic membrane stem cells. The AFCs are mainly composed of three groups of adherent cells, categorized based on their morphological, growth, and biochemical characteristics. Epithelioid (E-type) cells are cuboidal to columnar cells derived from the fetal skin and urine, amniotic fluid (AF-type) cells are originating from fetal membranes, and fibroblastic (F-type) cells are generated mainly from fibrous connective tissue. Both AF- and F-type cells share a fibroblastoid morphology, and the dominant cell type appears to be the AF-type, co-expressing keratins and vimentins. Several studies have documented that human amniotic fluid stem cells (AFSCs) can be easily obtained from a small amount of second trimester AF, collected during routine amniocenteses, a procedure with spontaneous abortion rate ranging from 0.06% to 0.5%. Up to date, a number of different cultivation protocols have been described, leading to enriched stem cell populations [12, 16, 20, 21]. The isolation of AFSCs and the respective culture protocols were summarized in a review by Klemmt et al. [22] and can be categorized as follows: (i) a single-step cultivation protocol, where the primary culture was left undisturbed for 7 days or more until the first colonies appear; (ii) a two-step cultivation protocol, where amniocytes, not attached after 5 days in culture, were collected and further expanded; (iii) cell surface marker selection for CD117(c-kit receptor); (iv) mechanical isolation of the initial mesenchymal progenitor cell colonies formed in the initial cultures; and (v) short-term cultures to isolate fibroblastoid colonies. The majority of the AFSCs, isolated following these methodologies, shared a multipotent mesenchymal phenotype and exhibited higher proliferation potential and a wider differentiation potential compared to adult MSCs [15]. The AFSCs exhibit a typical mesenchymal marker expression profile, being positive for markers such as CD90, CD73, CD105, CD29, CD166, CD49e, CD58, and CD44, as determined by flow cytometry analyses. Additionally, these cells expressed the HLA-ABC antigens, whereas the expression of the hematopoietic markers CD34 and CD45, the endothelial marker CD31, and the HLA-DR antigen was undetected. More importantly, the majority of cultured AFSCs expressed pluripotency markers, such as the octamer-binding protein 3/4 (Oct-3/4), the homeobox transcription factor Nanog (Nanog), and the stage-specific embryonic antigen 4 (SSEA-4) [16, 18, 19].

It was also reported that amniocyte cultures contain a small population of CD117 (a tyrosine kinase specific for stem cell factor present primarily in ESCs and primordial germ cells)-positive cells that can be clonally expanded in culture. The differentiation properties of $CD117^+$ AFSCs were tested for the first time in vivo, proving in this way their stem cell identity. Experimental evidence suggested that AFSCs are derived from spindle-shaped fibroblastoid cells [15].

In an attempt to analyze the AFSCs subpopulations, our group recently identified two morphologically distinct populations of AFSCs of mesenchymal origin, with different proliferation and differentiation properties, termed as spindle shaped (SS) and round shaped (RS). Both subpopulations were expressing mesenchymal stem cell markers at similar levels. However, it was identified that SS colonies expressed higher levels of CD90 and CD44 antigens compared to RS colonies [18].

Umbilical Cord Blood

Umbilical cord blood was first seen as biological waste product post birth. In 1980, Di Landro et al. reported the colony-forming capacity of cord blood to be similar to bone marrow [23]. In 1988, the first successful cord blood transplant took place in France for Fanconi's anemia with the donor being an identical human leukocyte antigen (HLA)-matched sibling by Eliane Gluckman [24]. Most importantly, the patient is reported to be alive and well 18 years after the transplantation [25].

In 1990, three more patients had been transplanted for Fanconi's anemia, and it was reported that cord blood transplantation may also be applicable to other diseases with a possibility of transplanting adults. The cord blood cellular product represented an advantageous source of hematopoietic stem/progenitors cells for transplantation.

In 1991, the first report of a Public Cord Blood Bank for unrelated cord blood transplants and in 1992 was published, reporting the characterization of cord blood by flow cytometry by Dr. Gluckman's team. This study demonstrated that the cord blood graft cells represented both suppressive and naive cells [25].

Cord blood cells are considered a gold standard product for hematopoietic transplantation and reconstitution and a potential product for regenerative medicine [26]. In addition, hematopoietic cell transplantation is a gold standard worldwide for a long list of hematopoietic diseases, which includes leukemia; myelodysplastic syndrome; myeloproliferative and lymphoproliferative disorders; inherited metabolic, immune, or platelet disorders; and other malignancies. Transplantation of umbilical cord blood is characterized by low immunogenicity as indicated by reduced acute GVHD [14, 27–29].

Cord blood in the past was considered as a product for transplantation mainly in children due to the low number of cells that could be harvested from a single cord blood collection. However, recently adult cord blood transplantation has been successfully studied using double cord blood unit transplantation. Further it has been demonstrated that UCB can provide long-term hematopoietic reconstitution in adults [26–28].

Stem Cells Derived from Extraembryonic Tissues

Amniotic Membrane (AM)

The amniotic membrane, lacking any vascular tissue, forms most of the inner layer of the fetal membrane and is composed of (i) an epithelial monolayer consisting of epithelial cells, (ii) an acellular intermediate basement layer, and (iii) an outer mesenchymal cell layer, rich in mesenchymal stem cells and placed in close proximity to the chorion [13]. AM was used in clinic for many decades for wound healing in burns, promoting epithelium formation and protecting against infection. Recently, the use of AM has been evaluated as a wound dressing material for surgical defects of the oral mucosa, ocular surface reconstruction, corneal perforations, and bladder

augmentation. Amniotic membrane stem cells (AMSCs) include two types, the amniotic epithelial cells (AECs) and the amniotic membrane mesenchymal stromal cells (AM-MSCs) derived from the amniotic epithelial and the amniotic mesenchymal layers, respectively [13, 14]. Both cell types are originated during the pregastrulation stages of the developing embryo, before the delineation of the three primary germ layers, and are mostly of epithelial nature. A variety of protocols has been established for AECs and AM-MSCs isolation, primarily based on the mechanical separation of the AM from the chorionic membrane and the subsequent enzymatic digestion. AM-MSCs exhibited plastic adherence and fibroblastoid morphology, while AECs displayed a cobblestone epithelial phenotype. AM-MSCs shared similar phenotypic characteristics with the ones derived from adult sources. More interestingly, AM-MSCs exhibited a higher proliferation rate compared to MSCs derived from adult sources and a multilineage differentiation potential into cells derived from the three germ layers [13, 14, 30].

Placenta

The placenta serves the functions of all organs of the developing embryo by working in association with the mother. During pregnancy, it functions as the embryo's lungs, kidneys, digestive system, liver, and immune system. It is evident that due to the placenta, an embryo can survive until birth, even when one or more vital organs fail to develop.

The placenta also serves to protect the developing embryo from an attack by the mother's immune system, since the embryo and the placenta are genetically unique and distinctly different from the mother [13, 14].

Several stem cell populations are derived from the placenta with the best studied the placenta mesenchymal stem/stromal cells (MSCs). Placenta MSCs express markers of pluripotency such as SEEA-4, Oct-4, Stro-1, and Tra 1-81, and also they have a wide range of differentiation potential. It has been reported that placenta MSCs are capable of in vivo differentiation into neuronal, glial, and insulin-positive cells and hepatocytes and the generation of heart valves seeded in scaffolds [13, 14, 17, 20, 21].

Adult Stem Cells

Adult stem cells are found, in small percentage, in almost all tissues after birth and are able to self-renew and differentiate, in most cases, into cell types of the tissues that they originate [14]. However, recent studies have identified adult stem cells with a greater range of potential than that originally believed. The most well-characterized adult stem cell types are the ones derived from human bone marrow (BM). However, adult stem cells have been also isolated from the blood, brain, fat, liver, muscle or pancreas [14].

Since adult stem cells are often a very small percentage of the total cells of a tissue or organ, isolation and expansion are considered difficult and time-consuming. In many cases, investigators isolate adult stem cells based on their surface antigen expression or by examining their differentiation potential. In some cases, the lack of a single marker for their characterization leads to the isolation of a heterogeneous population with questioned stem cell identity [14].

As standardized protocols develop for adult stem cell isolation, more rigorous criteria will develop for determining true stem cell populations and their differentiation potential.

BM stem cells represent the most well-characterized example of adult stem cells, are fairly easy to isolate, and have been the most thoroughly investigated, with several reports demonstrating their contribution to regenerative medicine. BM stem cells consist of hematopoietic stem cells (HSCs), which can give rise to blood cell lineages and endothelial progenitor cells (EPCs), and mesenchymal stromal cells (MSCs), which have been shown to differentiate into mesodermal phenotypes (adipocytes, chondrocytes, and osteocytes). HSCs and MSCs can also be derived in high numbers from umbilical cord blood and Wharton's jelly, respectively. Although adult stem cells may represent a valuable tool for autologous transplantations, their proven multilineage differentiation potential is limited [14]. As examples of adult stem cells, HSCs, MSCs and neural stem cells are described in more detail in the following sections.

Hematopoietic Stem Cells (HSCs)

Hematopoietic stem cells (HSCs) represent the stem cell population responsible for the development, maintenance and regeneration of the blood-forming tissue during life. Because HSCs can reconstitute and restore the hematopoietic system of a myeloablated host, they have been used for treating hematologic disorders, starting in 1945 [31].

HSC presence has been shown in adult mouse bone marrow as a cell population marked by c-kit^{pos}, thy-1^{low} and sca-1^{pos} [32]. Human HSCs are characterized by c-kit^{pos}, Thy-1^{pos} and CD34^{pos} expression. HSCs from mice and humans are being isolated, starting with a lineage depletion step in which lineage-specific cells (B220, CD3, 4, 8, 11b Mac-1, Gr-1 and Tcr-119 for mice and CD10, 14, 15, 16, 19, and 20 in human) are depleted. The resultant population, termed as Lin^{neg}, can be enriched and is able to repopulate the bone marrow of a lethally irradiated host [33, 34].

HSCs can be in vitro expanded by co-culturing them with bone marrow-derived stromal cells. Several subpopulations within Lin^{neg} HSCs are existing. One such homogenous population is characterized as side population (SP) cells based on their unique ability to exclude Hoechst dye. This subpopulation can be examined by FACS analysis and SP cells fall within a separate population to the side of the rest of the cells on a dot plot of emission data. These cells are also able to home and engraft to the BM of a lethally irradiated host [33].

HSCs, which primarily reside in the BM, maintain blood formation and replenish themselves throughout the adults' lifespan. The activity of bone marrow HSCs was discovered in 1960s, identifying a robust contribution of donor BM cells in lethally irradiated recipient mice. After 30 years of work, the contribution of donor hematopoietic cells in recipients had been demonstrated to derive from a few specific "clones," suggesting the existence of HSCs [35]. HSCs was isolated for the first time in 1988 when Weissman et al. enriched HSCs from the murine BM using a fluorescent-activated cell sorter [32]. Since then, numerous groups have demonstrated that HSCs possess stem cell properties including the ability to self-renew as well as to differentiate into all of the hematopoietic lineages [33, 34]. HSCs are committed to a differentiation program, in that they exclusively create all of the cells of the hematopoietic origin. Clinical trials of HSC transplantation for the purpose of restoring hematopoiesis have been widely performed for treating leukemia, severe autoimmune disease, and severe combined immunodeficiency. In addition, HSC treatment has been used in adjunct to chemotherapy for other cancers such as breast cancer, neuroblastoma and testicular cancer. Currently, HSC research is studying the development of novel ways to improve the transplantation success by reducing graft-versus-host disease and infection during recovery and accelerating hematopoiesis after transplantation [14, 33, 34].

Mesenchymal Stromal Cells (MSCs)

The first descriptions of fibroblastic cells that could be isolated and grown from bone marrow and also retained the ability to differentiate to bone tissue were presented by Alexander Friedenstein in the 1960s, using guinea pig bone marrow as the source. Friedenstein used the term "osteogenic cell" in order to describe the properties of these cells. MSCs can differentiate into osteocytes, adipocytes and chondrocytes, but they can also exhibit multilineage in vitro differentiation depending on their source of origin (fetal sources of MSCs have been characterized during the last 20 years and are described earlier in this chapter) [36].

Human MSCs were firstly isolated from BM by their adherence to tissue culture plastic vessel and were expanded through multiple passages in medium containing high concentrations of fetal calf serum (FCS). However, the proliferation rates and other properties of the cells gradually change during expansion. The cells are cloned as single-cell-derived colonies, but both the colonies and the cells within a colony, are heterogeneous in morphology, rates of proliferation and efficacy with which they differentiate [36–38].

Because of their heterogeneous phenotype, the International Society of Cell Therapy (ISCT) published in 2006 a position paper on defining minimal criteria on MSCs, such as [39]:

1. *Adherence to plastic in standard culture conditions (expandability of these cells without losing their differentiation potential)*

2. *Phenotype: Positive for CD105, CD90, and CD73 and negative for CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR*
3. *In vitro differentiation: osteoblasts, adipocytes, and chondroblasts (demonstrated by staining of in vitro cell culture)*

The current ISCT definition recommends to use the term "multipotent mesenchymal stromal cells" (MSC) instead of "mesenchymal stem cells." However, literature review showed that after 2006, ISCT members (including the authors of MSC position paper) themselves frequently use terms "mesenchymal stromal cells" or "mesenchymal stem cells."

MSCs produce a number of secreted factors such as vascular endothelial growth factor (VEGF), stem cell factor (SCF-1), leukemia inhibitory factor (LIF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukins (IL-1, IL-6, IL -7, IL -8, IL -11, IL -14, and IL -15), stromal cell-derived factor (SDF-1), Flt-3 ligand, and others. The expression of these factors may be modulated through interactions with other cell types [40]. Due to the secretion of the various types of factors, Caplan in 2011 used the term "drug store" to describe these cells [41].

MSCs also exhibit homing properties to sites of tissue injury, particularly ischemic regions of the heart where the MSCs may prevent deleterious remodeling. In addition, MSCs also have the ability to alter immune responses and engraft in allogeneic recipients, and it has been reported that the MSC treatment has been used to clinically treat graft-versus-host disease (GVHD) [14, 40].

Neural Stem Cells (NSCs)

Neural stem cells (NSCs) represent the most primitive and uncommitted cells of the nervous system. Evidence support that these cells give rise to the vast majority of more specialized cells of the central nervous system (CNS) and peripheral nervous system (PNS). The term "neural stem cell," is in contrast to the term "progenitor" cell (i.e., describes cells that are lineage committed to give rise to only one category of neural cell type, such as glial cells, neurons, etc.) [42].

Neural stem cells must be capable of (i) generating all neural lineages (neurons, astrocytes and oligodendrocytes), (ii) having limited capacity for self-renewal and (iii) being able to give rise to cell types in addition to themselves through asymmetric cell division [14, 40].

Neural Stem Cells in the Developing Brain

During embryogenesis, CNS development initiates with the induction of the neuroectoderm, which forms the neural plate and then the neural tube. Within these neural structures, a complex and heterogeneous population of neuroepithelial progenitor cells (NEPs) represents the earliest neural stem cell type. As CNS development proceeds, NEPs generate distinct neural stem/progenitor populations. NEPs also undergo symmetric divisions to expand NSCs. In the later stage of neural development, it has been reported that NSCs undergo asymmetric divisions and differentiate into lineage-restricted progenitors. Thus, intermediate neuronal progenitor cells are formed that subsequently differentiate into neurons. Previously it was stated that neurogenesis in the adult mammalian CNS was complete, implying incapability of mitotic divisions in order to generate new neurons and therefore lacking in the ability to restore or regenerate damaged tissue caused by diseases (such as Parkinson's disease, multiple sclerosis) or injuries (such as spinal cord and brain ischemic injuries). However, recent strong in vivo and in vitro evidence support that NSCs exist in the mature mammalian CNS [14, 42].

Regenerative Medicine

Mason and Dunnill in their review in 2008 [43] summarized the clear distinction between organ regeneration and organ repair. Regenerative medicine includes activities such as surgery, surgical implants, and biomaterial scaffolds. Thus, regenerative medicine integrates human cell therapy, gene-based methods, biomaterials and molecular medicine. To this end, regeneration is described as "the process in humans, whereby lost specialized tissue is replaced by proliferation of undamaged specialized cells", whereas repair is "the replacement of lost tissue by granulation tissue, which matures to form scar tissue" [43]. Consequently, repair is an adaptation to loss of normal organ mass leading to restoration. It is evident that regeneration restores the normal structure and function of the organ, whereas repair does not. Therefore, regenerative medicine replaces or regenerates human cells, tissues or organs to restore or establish normal function [43].

The major aim of regenerative medicine is to establish novel therapies for severe injuries or chronic diseases in which patients' own responses do not suffice to restore functional tissue. Recent reports describe several major medical needs, which might be addressed by regenerative technologies. Such examples include severe burns, spinal cord injuries, congestive heart failure, diabetes, Alzheimer's and Parkinson's diseases and others [44].

The areas of specialty of regenerative medicine continue to change rapidly; however, the main focus of regenerative medicine therapies is the use of stem/progenitor cells into clinical applications for both allogenic and autologous transplantations. The field now integrates a wide area of scientific fields and technologies such as stem cells, genetic reprogramming, gene therapy, nuclear transfer, genomics,

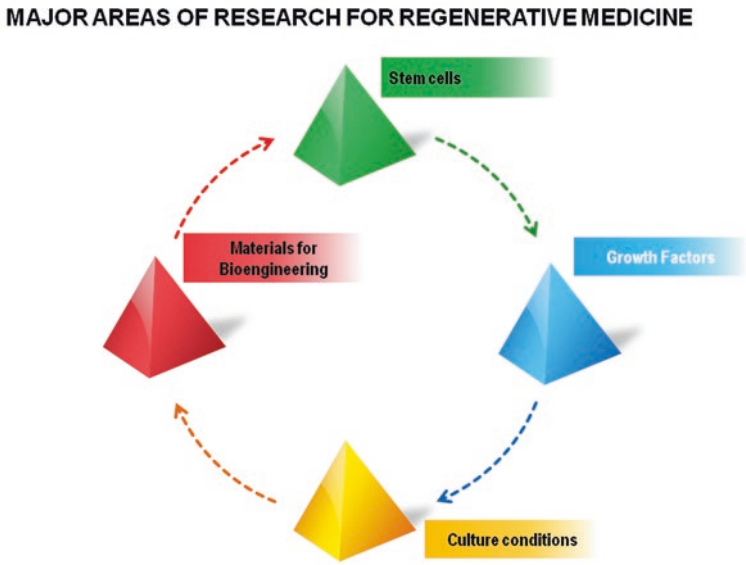

Fig. 1 Major areas of regenerative medicine and stem cell biology. Regenerative medicine is mainly focused on human stem/progenitor cells. The type of human stem/progenitor cells and the culture conditions (including the selection of growth factors) together with the appropriate bioengineered materials play important role in successful therapies

proteomics, cloning and tissue engineering. The extension of novel therapeutic areas, including organ generation with 3D structure, depends on scaffold engineering, material science and/or bioreactor technology [14, 43].

It is widely accepted that the central focus of regenerative medicine is the human cells. Along these lines, cell-based therapies fall into two broad categories of use:

(i) Cells for structural repair or replacement (e.g., cultured dermal fibroblasts as skin replacement or chondrocytes for repair of cartilage)
(ii) Cells for correction of a physiological or metabolic problem

Autologous cells are derived from the patient to be treated, whereas allogeneic cells are derived from a donor. Several recent studies described allogeneic cell therapies developed including cultured keratinocytes as dermal matrices for the repair of cutaneous wounds, hepatocytes for liver repair, pancreatic islets for diabetes and hematopoietic stem cells for bone marrow transplantation in leukemia and other types of cancers [14, 43, 44].

On the other hand, allogeneic cells are expected to elicit immune response from the host by the transient production of tissue stimulatory molecules. The use of allogeneic cells for short-term tissue restoration appears to be more complicated, with the risk for immunological rejection of donor cells. However, long-term repair of organ function is clearly the most complicated and problematic therapeutic application. Critical issues, such as the tissue structure and condition, the biological

function and the immunological component, must be taken under consideration for a successful cell-based therapy [14, 43, 44] (Fig. 1).

Understanding the nature of the problem that need treating, the role that the cell can play in solving the problem (i.e., engraftment and differentiation at the site of the injury or paracrine effect) and also the related molecular mechanisms are critical to develop a successful cell therapy [44]. Scientists in regenerative medicine have strived to understand the interaction of cells, extracellular matrices and biological factors in order to develop tissue-engineered products for repair and replacement of injured tissues.

However, there are several limitations related to the type of cells that can be isolated, the condition of the tissue, the patient's age, and others. To achieve this goal, extended in vitro assays and in vivo animal models are needed to recapitulate the molecular events that take place and understand the mechanisms underlying the interactions of cells, extracellular matrices, and biological factors [44] (Fig. 1).

Conclusion

To date, regenerative medicine introduces novel methods and strategies to replace or regenerate cells, tissue or organs in order to restore and establish normal function. These strategies mostly include cell-based therapies combined with the use of biomaterials and scaffolds. The characterization and the basic properties of stem/progenitor cell types are crucial in respect of their use in potential clinical applications. Most importantly, the type and the source of cells remain of central focus for the approaches adopted in cell-based therapies. A primary issue remains the choice between using patient's own cells or cells derived from allogenic donors.

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The Truth Behind Esophagus: The Stem Cells' Significance

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Introduction

The esophagus presents the unique feature of involving stem cells (SCs) in a wide spectrum of events even from its embryonic development to the complicated esophageal diseases, as well as the esophageal tumorigenesis. The scientific progress during years in the field of stem cells (SCs) was taking place in strong relationship to the evolution of our experience about esophageal pathophysiology. For instance, an observation about stem cells behavior could be a stimulus for a therapeutic implication in an esophageal disease or vice versa, and an expression of molecular markers in an esophageal disease could be extremely crucial for a discovery of a pathway in stem cells signaling. This strong association has been continued for years, and it seems that it will be maintained in the future too.

In this chapter, we will present all the recent data about the contribution of stem cells to the esophageal development and maintenance of esophageal homeostasis. In addition, we will demonstrate the regulatory effect of stem cells on the benign diseases of the esophagus as well as their role in esophageal tumorigenesis and message transportation between cancer stem cells. Finally, we will show several possible molecular therapeutic targets that are based on the SCs metabolic pathways as well as the new applications of SCs technology in creating tissue-engineered esophageal scaffolds that could replace natural esophagus due to a variety of reasons that lead to esophageal destruction.

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Stem Cells Are the Main Factors of Esophageal Homeostasis

The esophagus is derived from the anterior portion of the developmental intermediate foregut, which is a structure that also gives rise to other organs like the trachea, lung, and stomach. The separation of the esophagus from the trachea is ensured by Sox2, which is a key family member of SRY (sex-determining region Y)-related transcriptional factors and is essential for maintaining self-renewal and pluripotency in ESCs [56]. The absence of Sox2 expression has been associated with esophageal atresia and tracheoesophageal fistula [122]. In addition, except from its role in esophagus separation from the respiratory organs, Sox2 contributes to the esophageal basal progenitor cells proliferating and differentiating into squamous superficial epithelial cells in adult esophagus [33].

Nevertheless, a homolog of the tumor suppressor and transcription factor p53, p63 is the most potent regulator of the differentiation of simple columnar into squamous epithelium [17]. The esophageal epithelium in mouse models with negative expression of p63 remains columnar, exactly like the skin epithelium. Furthermore, a large portion of the esophageal epithelial cells with negative p63 expression is characterized by the presence of multi-cilia. It has been reported that ciliated epithelial cells are present in developing the esophagus, highlighting that epithelial differentiation is arrested when there is a mutation in p63 expression in esophageal cells.

Two transcriptional factors of the Krüppel-like factor (Klf) family also contribute to the maintenance of homeostasis in the adult esophageal epithelium. The expression of Klf5 seems to be restricted to the basal layer and regulates progenitor cells proliferation. If a transgenic overexpression of Klf5 is caused in the esophageal epithelium, a twofold increase in the proliferation rate would take place, without any other disturbance of the esophageal epithelium functionality [93]. On the contrary, Klf4 is expressed in the suprabasal cell layer of the esophageal epithelium and plays a crucial role in cell differentiation. Klf4 deficient mice present impaired differentiation and increased proliferation leading to dysplasia.

Another very important factor to the esophageal epithelium homeostasis is the Notch signaling pathway. In vitro organotypic cultures and in vivo mouse models have shown that Notch signaling through the transcriptional factor CSL is required for human esophageal epithelial differentiation, especially factors NOTCH1 and NOTCH3 [43]. The key role of Notch signaling has been also underlined by studies that have demonstrated a relationship between mutations in the Notch signaling pathway and ESCC. Upregulation of Notch pathway components (Dll3, Jag2, and Hes5) was observed in a mouse model that leads to increased esophageal precursor cell differentiation after chemical inducement of endoplasmic reticulum (ER) and following unfolded protein response (UPR) activation due to thapsigargin treatment [94]. Thapsigargin is a plant-derived inhibitor of cell proliferation through ER stress inducement that leads to increased cell differentiation and upregulation of different Notch signaling pathway components. In addition, UPR after increased ER stress

serves as a regulatory mechanism that forces progenitor cells with accumulated unfolded proteins to initiate differentiation.

The basal layer of the esophageal progenitor cells is not homogeneous. Mouse models demonstrate that the esophageal basal epithelial cells present a scaled potential of stemness depending on the expression of two SCs markers: α_6 integrin and transferrin receptor CD71. According to these models, there are three subpopulations of basal cells: one that presents α_6 integrin^{high} and CD71^{low} expression, which is a minor subpopulation of small and undifferentiated cells that are full of label-retaining cells and represent a putative esophageal stem cell population. On the other hand, basal cells that express both α_6 integrin^{high} and CD71^{high} levels are the majority of the esophageal basal cells and represent a transit-amplifying population as it is enriched of actively cycling cells. Finally, basal cells that present α_6 integrin^{low} and CD71^{high} leave the basal cell layer and differentiate [15].

However, there is an arguing statement against the heterogeneous hypothesis, which claims that the normal esophageal epithelium is generated by a single and homogeneous population of progenitor cells. More specifically, every basal cell possesses equal potential of self-renewal and differentiation into squamous cell. During periods that esophageal epithelium is guided by homeostatic mechanisms, cell production and cell loss are balanced as proliferating basal cells create equal proportion of dividing and nondividing cells. On the contrary, when an injury happens, basal cells that are adjacent to the site of injury generate more proliferating cells until the injury is repaired (Fig. 1) [24].

The distribution of esophageal epithelial cells into layers and functional groups does not seem to be of great importance, due to the proven remarkable plasticity for self-renewal that the esophageal epithelial cells present in ex vivo wounding response models and in vivo mouse models. Undoubtedly, proliferation and mitotic activity are higher in the interpapillary basal layer and lower superficially toward the tip of the papilla. On the other hand, the orientation of mitosis is random linearly through the basal layer, and the cell divisions are not restricted to specific cell compartments. The expression of epithelial and progenitor cell markers such as EpCAM and CD34 determines the accumulation of epithelial cells into distinct populations, but there is no difference in self-renewal ability depending on the presence of each cell as unique or into a population. In 3D organotypic cultures, all esophageal epithelial cells were capable of restoring the architecture of the tissue they came from, and the main factor of successful result was the number of cells plated in the culture rather than the cell type [4].

Our attempt to investigate the principles of esophageal stem cells has led to the development of mouse models in order to make research easier, but we have to keep always in mind that there is a number of obvious differences between mouse and human esophageal epithelia [16]. Firstly, human esophageal epithelium has more cell layers than mouse epithelium. Secondly, the basement membrane of the human esophagus is thrown into folds by submucosal projections, called papillae, just like the human skin. In addition, there are mucosal and submucosal glands in the human esophagus that are not observed in the mouse esophagus. Furthermore, the transition from the proliferating compartment to the differentiating compartment is more

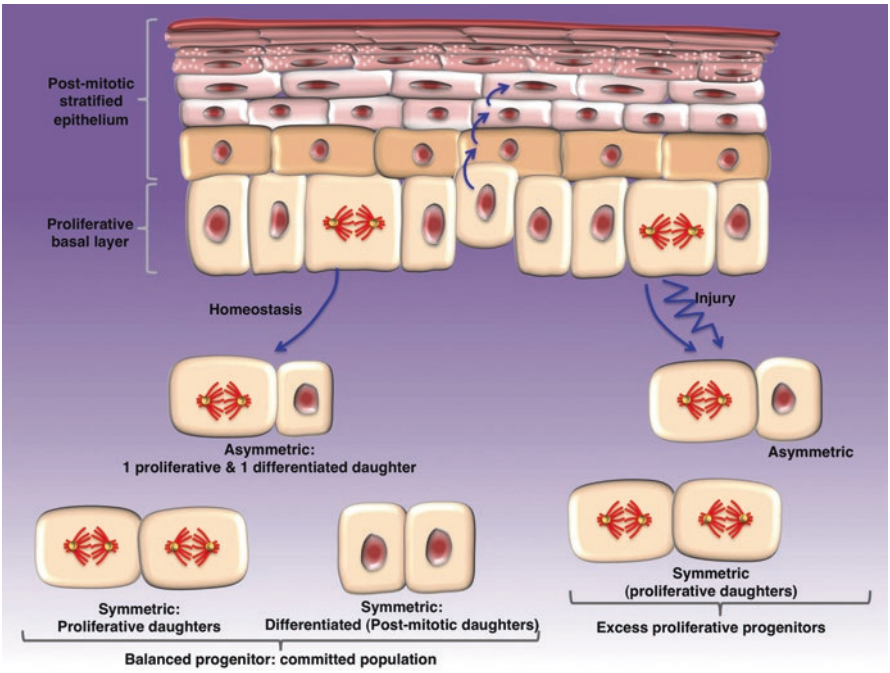

Fig. 1 The contribution of basal progenitors to epithelial self-renewal during homeostasis and in response to injury in the esophagus [5]

abrupt in the mouse esophageal epithelium than in human epithelium, in which mitoses are taking place five layers above the basement membrane. Finally, cell turnover in the human esophagus seems to be slower compared to mice. All these differences show that the distribution of the esophageal epithelial cells into the three layers of stem cells, transit-amplifying cells, and differentiating cells seems to be easier in the human esophagus than in mice or rats.

An extremely useful marker for distinguishing human esophageal cancer stem cells is the low-affinity neurotrophin receptor p75ntr, which is usually expressed in neural stem cells. Human esophageal epithelial cells with a high expression of that marker were found to present increased proliferative ability in vitro in comparison with those with low expression [80]. However, such measures need to be repeated due to the utilization of passage 2 cells rather than the use of freshly isolated esophageal cells, because in vitro cultivation influences cell surface markers.

SCs research in adult tissues revealed another quite interesting fact: the ESCs pluripotency transcriptional factor NANOG is selectively expressed in mouse stratified epithelia presenting a lineage-restricted mitogenic activity. More specifically, mouse NANOG is expressed in adult esophageal epithelium, where its promoter is hypomethylated [84]. Generally induced overexpression of NANOG in mouse models provokes hyperplasia especially in esophageal epithelium, accompanied by increased cell proliferation through the following mechanism: the exogenously

overexpressed NANOG activates the mitogenic pathways of the stratified epithelia via transcriptional factors such as Aurora A kinase (AURKA), and the endogenous NANOG binds to the AURKA promoter in the primary keratinocytes. Consequently, overexpression of NANOG or AURKA in mouse models causes increased proliferation and aneuploidy in esophageal basal cells. Finally, inactivation of NANOG in cell lines from ESSC results in decreased AURKA expression and diminished proliferation of both basal cells and keratinocytes; hence NANOG and AURKA are correlated with increased cancer cell proliferation in ESCC.

All the molecular SC mechanisms stated above conserve the goal of maintaining the homeostasis of the esophagus in embryonic and adult tissues. Sometimes though, the balanced self-renewal and proliferation of stem and progenitor cells that are required especially in quickly replenished tissues like the esophagus for achieving homeostasis are disrupted. A pathological condition that is associated with such a disruption is eosinophilic esophagitis (EoE), in which basal progenitor cells become hyperplastic due to proinflammatory stimulation. Once again, a stem cell mechanism seems to be responsible for the progenitor basal cells' irregular reaction to the inflammatory stimulation. Bone morphogenetic protein (BMP) signaling pathway is essential for epithelial morphogenesis in embryonic esophagus; however BMP signaling pathway seems to regulate tissue homeostasis and EoE development in the adult esophagus [37]. BMP signaling was activated in differentiated squamous cell epithelium, on the contrary to basal progenitor cells that express the BMP antagonist follistatin. Nevertheless, in mouse models BMP signaling was increased in basal progenitor cells and promoted squamous epithelial cells differentiation. In addition, BMP activation induced the production of intracellular ROS, initiating an NRF2-mediated oxidative response during the progenitor basal cell differentiation. On the other hand, both in human biopsies and in EoE mouse models, high levels of follistatin and disrupted BMP pathways led to reduced levels of differentiation. Consequently, BMP signaling pathway is responsible for basal cell differentiation into squamous epithelium, and EoE is related to a dysfunction of this mechanism which leads to decreased esophageal squamous differentiation with a consequent progenitor basal cell hyperplasia.

Except from intrinsic dysregulations that lead to esophageal dysfunction, there are several exogenous agents that could cause esophageal injury and activate a repair process by the esophageal epithelium. Severe caustic injury by alkali is a very common cause of esophageal injury, for the repair of which the intrinsic esophageal reaction sometimes is not enough. For that reason, ovine esophageal models have been developed that are utilized for testing the conditions under which viable autologous esophageal cells could be isolated in order to be used in tissue-engineering models for the replacement of the injured esophagus by caustic substances. It has been proven that an esophagus which has been exposed to low concentrations (2.5%) of NaOH would maintain a relatively large population of viable cells for tissue-engineering applications [65]. On the other hand, esophagi exposed to greater concentrations (15–25%) of NaOH could not provide tissue-engineering models with the required number of viable esophageal cells; thus alternative sources of esophageal cells should be searched, such as stem cells.

Even in less extensive injuries than caustic injuries, the esophageal epithelium presents a regenerative capacity which is based on its feature to maintain a balance between proliferation and differentiation. The progenitor basal cells proliferate, and then they migrate outward near luminal surface where they differentiate in squamous cells. An esophageal stem cell population, which is accumulated in the basal layer, is responsible for this process. This population maintains its capacity for self-renewal and epithelial reconstruction in both 3D organotypic culture models (in vitro) and in mouse models (in vivo). The esophageal stem cells both in vivo and in vitro give rise to undifferentiated and differentiated cells, uncovering the mechanism through which the adult esophagus faces injury insults and repairs itself [38].

Stem Cells in Esophageal Cancer

Esophageal cancer is the eighth most common cancer worldwide and the sixth most common cause of cancer death in the world. Signs and symptoms of esophageal cancer rarely present in early stages, so most of the time, this malignancy presents in advanced stages; thus it is related to low rates of survival (5-year survival 10–25%). In addition, the two main histological types are squamous cell carcinoma (ESCC) and adenocarcinoma (EAC). Interestingly, the incidence of the two types of esophageal cancer presents geographic patterns, with ESCC being the most common type worldwide presenting an incidence of 90%, but it mainly appears in Asian-belt, including Turkey, Iran, China, Japan, India, and Bangladesh. ESCC has been associated with tobacco and alcohol consumption, as well as diet traditions that include ingestion of spicy foods and hot beverages. On the other hand, EAC is the predominating type of esophageal cancer in the developed continents of the West, such as Europe and Northern America. EAC has been correlated with abnormal columnar metaplasia of the squamous cell esophageal epithelium and formation of Barrett's esophagus (BE) due to chronic gastroesophageal reflux disease (GERD), which is caused by the life trends of the Western societies such as obesity and maldietary habits [1].

During the recent years, the observations about esophageal tumor behavior in animal and human studies led to the development of the cancer stem cells (CSCs) hypothesis. Esophageal cancer is one of the most common death-related cancers worldwide. CSCs are considered to give esophageal cancer all the features that lead to greater mortality rates, such as tumor initiation, drug and radiation resistance, invasive growth, metastatic potential, and tumor relapse [70]. The next goal of the scientific society is to reveal specific markers in order to distinguish CSCs from non-CSCs. Esophageal CSCs derived from ESCC are related to increased β-catenin, Oct3/4, β1-integrin, miR-296, and miR-200c expression. In addition, aldehyde dehydrogenase-1 (ALDH1), Lgr5, and CD44 are useful for sorting esophageal CSCs [105]. Another very interesting observation is that the esophageal CSCs and the SCs of the normal embryonic developing esophagus follow the same trait of both upregulation and suppression of specific genes, so they express the same

molecular markers, making specific pharmaceutic targeting of esophageal CSCs extremely difficult [126]. Nevertheless, a fluorescent vector consisting of fluorescein ZsGreen fused to the carboxyl-terminal region of ornithine decarboxylase (cODC) has been used for targeting three chemotherapeutic drugs, AKT inhibitor XI, ERK inhibitor II, and JAK inhibitor I, which contribute as markers of esophageal CSCs [40]. Finally, except from the correlation of CSCs with the development of the two most common esophageal cancer types (EAC and ESCC), a less common type, small-cell esophageal cancer, seems to arise from a pluripotent esophageal progenitor cell [73].

CSCs development and consequent rise of esophageal carcinoma seems to initiate from a clonal region of paraneoplastic epithelium, a phenomenon called "field change." The quantitative analyses of scattered single esophageal epithelial progenitor cells expressing a mutation that inhibits the Notch signaling pathway, which is frequently inactivated in squamous cancers, demonstrate that cell divisions that produce two differentiated daughters are no longer present in mutant progenitors. In addition, mutant clones are maintained and become immortal, promoting the differentiation of neighboring wild-type cells, which are then lost from the tissue. As a result, the entire normal epithelium is replaced by mutant cells, in which Notch signaling has been disrupted and carrying p53 mutations has been established. Consequently, the phenomenon of "field change" is considered to be a result of imbalanced differentiation of individual esophageal progenitor cells [2]. Moreover, genetic lineage tracing has been used to quantify cell behavior during neoplastic transformation. It demonstrated that dividing esophageal tumor cells were characterized by an abnormality: more dividing than non-dividing daughters were produced in every division cycle. Furthermore, in invasive cancers induced by KRAS expression, a greater portion of the produced cells were dividing than nondividing, indicating that agents that determine proliferating cells' fate are the ideal targets for effective control of tumor growth [24]. The interpretation of the esophageal tumor growth could be achieved by bioinformatics and computational models, which have outlined the contribution of the ornithine metabolic pathway in the survival of chemotherapy-resistant CSCs, indicating possible targets for effective treatments against developing esophageal cancer [48].

It is well known that dietary habits are strongly associated with esophageal cancer. Alcohol consumption has been related to ESCC appearance. Nevertheless, the molecular events behind this association had never been clarified until the invention of the CSCs theory. More specifically, consumption and high concentration of ethanol in the squamous epithelial cells causes cell damage that usually leads to the cell death [59]. As a result, esophageal SCs are triggered to proliferate and differentiate in order to replace missed esophageal epithelial cells. The high rate of esophageal basal cells proliferation in combination with the carcinogenic effect of acetaldehyde, a liver-produced metabolite of ethanol, raises the possibility of a mutation to the proliferating esophageal progenitor cells and their transformation to esophageal CSCs, which give rise to invasive and usually fatal esophageal carcinomas [119]. In addition, another unclarified risk factor that seemed to be correlated with the development of esophageal cancer, although the exact mechanisms of that correlation

have not been specified yet, is the esophageal microbiota. The increase of esophageal adenocarcinoma during the last decades seems to be correlated with the radical treatments against *Helicobacter pylori*, because of the protective effect of *H. pylori* via IL-1b and TNF-a production against high levels of acid secretion and the antagonism against other pathogens that raised their population after *H. pylori* extinction. Moreover, specific bacteria, like *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, and *Spirochaetes*, have been correlated with esophagitis and Barrett's esophagus [118].

Following CSCs hypothesis described above, after CSCs formation at a specific tissue in the body, tumor dissemination takes part after insertion of CSCs from their tumor niche to the bloodstream. CSCs maintain the unique ability of epithelial-mesenchymal transformation. Mesenchymal cells are circulating in the bloodstream and when they disseminate to specific organs undergo transition again giving rise to primary tumors in a second tumor niche [14]. This theory of cancer generation is supported by observations that patients developed esophageal cancer after bone marrow-derived stem cells (BMSCs) transfusion [39]. In addition, abnormal esophageal alterations compatible with BE caused by providing a rat model with BMSCs and GERD products, bile and acid [53]. However, identification of the circulating CSCs in the bloodstream remains still a challenge, despite the fact that there is a hematopoietic growth factor, called stem cell factor (SCF), which is diagnostic for EAC presenting higher diagnostic sensitivity for EAC diagnosis than carcinoembryonic antigen (CEA), which is an ordinary esophageal tumor marker [62].

Esophageal cancer develops from CSCs that are located among basal cells and presents shorter telomeres than adjacent normal tissue, as well as chromosomal instability in the absence of histological inflammation [103]. This observation has been used to create immortalized epithelial models that simulate esophageal cancer. In such a model, in which immortalization of human esophageal epithelial cells was maintained by human papillomavirus type 16 and human telomerase reverse transcriptase (hTERT), cyclooxygenase-2 (COX-2) seemed to play a crucial role for the inducement and the conservation of the immortalized cells; thus it would be an ideal therapeutic target against esophageal carcinoma even in precancerous stage [124]. Furthermore, nestin, which is a member of the class VI family of intermediate filament proteins and was firstly identified as a protein expressed in progenitor cells of the central and peripheral nervous system, is another molecule that demonstrated an elevated expression in ESCC cell lines and an association with poor prognosis in ESCC patients, as well as a contribution to malignant proliferation and apoptosis of ESCC cell lines [123]. Finally, epithelial-mesenchymal transition (EMT), which has been implied in esophageal cancer morphogenesis as mentioned above, is regulated by TGF-β family molecules such as activin A that is strongly associated with colony formation, increased invasiveness, and cell migration of BE [106].

Several expression products and molecular biomarkers characterize the esophageal CSCs, however, without proven value as diagnostic markers. The most important is SOX2, which is a protein belonging to the family of high-mobility group transcription factors and is pivotal for early development and maintenance of undifferentiated ESCs [90]. Overexpression of SOX2 is responsible for development of ESCC through a complicated regulatory network of microRNAs, kinases, and

signaling molecules. In addition, high expression levels of SOX2 are associated with poor clinical prognosis of ESCC and increased proliferation rates of CSCs [56]. One possible pathway through which SOX2 promotes in vivo tumor growth of ESCC is activation of AKT/mammalian target of rapamycin complex 1 (mTORC1) signaling pathway, which enhances cell proliferation [28]. Furthermore, it has been shown that ESCC arises from esophageal stem/progenitor cells that are located in basal layer, and esophageal tumorigenesis driven by SOX 2 requires the interaction between SOX2 and microenvironment-activated STAT3 [55]. On the other hand, molecules that take part in SOX2-regulated oncogenic signaling pathways could be possible targets for pharmaceutic interventions against esophageal tumorigenesis. LSD1 (also known as KDM1, AOF2, or BHC110) which is a highly conserved flavin adenine dinucleotide (FAD)-dependent, lysine-specific demethylase that was initially found to specifically remove mono- and dimethyl groups from methylated histone H3 at lysine 4 (H3K4) to downregulate gene expression could serve as a selective epigenetic target for therapy in SOX2-expressing cancers [121]. Finally, except from the two most common types of esophageal cancer, SOX2 is highly expressed in small-cell esophageal cancer too. SOX2 overexpression in both small-cell esophageal cancer and esophageal embryogenesis highlights that esophageal small-cell carcinoma may arise from embryonic-like stem cells in the esophageal epithelium. Moreover, the two distinct differentiation patterns (neuroendocrine and glandular) of small-cell esophageal cancer is an indicator of the crucial role that SOX2 plays in the differentiation of pluripotent esophageal stem cells into esophageal small-cell carcinoma cells [36].

Notch signaling pathway which is responsible for multiple developmental activities, including stem cell survival, stem cell fate decision, and regulation of stem cell self-renewal by cross talk with other cell signaling pathways such as Wnt and Hedgehog, has been correlated with ESCC initiation, invasiveness, and metastatic potential. Moreover, Notch signaling has a fundamental role in controlling stem cell numbers through transcriptional activation of HEY (Hairy/enhancer of split related with YRPW motif) gene family members. Overexpression of specific products of Notch signaling pathway, such as HEY1 and HEY2, in ESCC seems to be associated with poor clinical prognosis, as well as increased progression and invasiveness of esophageal tumorigenesis [23]. In addition, Notch1 expression in clinical specimens was located in basal cells of esophageal epithelia and was associated with short survival intervals and high pathological grades of ESCC. On the other hand, in vitro expression of Notch1 in ESCC cell lines was correlated with increased cell aggressiveness and 5-FU drug resistance [54]. Wnt/β-catenin signaling pathway, which is parallel to Notch signaling pathway and has similar activity in maintenance of CSCs leading to poor clinical outcomes, has been associated with ESCC development. More specifically, microRNA-942 (miR-942), which is a crucial contributor to the Wnt/β-catenin signaling pathway, is overexpressed in ESCC leading to poor prognosis for the ESCC patients. Furthermore, miR-942 promotes esophageal tumor spheres formation, $CD90^{+}$ subpopulation cells development, and pluripotency markers expression. Another special role of miR-942 is upregulation of Wnt/β-catenin signaling activity via direct inhibition of sFRP4, GSK3β, and TLE1,

which are multiple-level negative regulators of the Wnt/β-catenin signaling cascade; thus miR-942 could be an ideal therapeutic target for future treatment attempts against esophageal cancer [26]. Additionally, WNT10A, which is another product of Wnt signaling pathway, is overexpressed in ESCC leading to poor clinical outcomes; promotes migration, invasion, and proliferation of transformed esophageal cells; induces a greater $CD44^{high}/CD24^{low}$ population, which are putative markers of cancer stem cells; and increases self-renewal capability of ESCC cells [57].

Aldehyde dehydrogenase-1A1 (ALDH1A1) is overexpressed in esophageal CSCs that initiate and develop ESCC and is associated with the pathological stage and clinical status of the ESCC patients; thus ALDH1A1 could serve as a biomarker for diagnostic and follow-up purposes in ESCC patients and as a prognostic factor too [108]. In addition, Oct4, which is a member of POU-domain transcription factors and is expressed normally by pluripotent cells of embryonic tissue and adult stem cells, seems to play an important role in identifying putative CSCs in esophageal tumor tissue, as well as determining response to treatment. Nevertheless, it has not been yet clarified if the Oct4-positive putative cancer stem cells exist in ESCC or the CSCs properties are acquired by tumor cells as a response to treatment given, resulting immediately in an uncontrolled cell proliferation and consequent treatment failure [109]. Moreover, H3K4 demethylase Jumonji/Arid1b (Jarid1b), which is an epigenetic factor that is required for continuous cell growth in melanoma, seemed to play an important role in maintaining CSCs in the esophagus; thus its continuous inhibition has been under investigation for providing a possible therapeutic option against esophageal and other squamous cell cancers [41]. Furthermore, CD44 and CD117 have been proven to have an important role in esophageal cancer progression; thus they could serve as reliable markers for undifferentiated malignant squamous cells of the esophagus and possible therapeutic targets [29, 89]. Finally, the expression of low-affinity neurotrophin receptor (p75NTR) in the infiltrative margin of ESCC indicates a crucial regulatory molecule of esophageal carcinogenesis and invasiveness with obvious therapeutic potential, the same as Hesca-2, a monoclonal antibody (mAb) IgM raised to the human embryonic stem cells (hESCs), which characterizes esophageal cancer as well [98].

Barrett's esophagus (BE) is the transformation of the original squamous esophageal epithelium into columnar epithelium, a process called intestinal metaplasia, which is considered to be a result of gastroesophageal reflux disease (GERD) and chronic injury caused by exposure to intestinal bile salts and gastric acid. There are three types of columnar epithelial cells metaplasia that substitute for squamous epithelium: (i) the intestinal type, which includes intestinal mucin (MUC2)-expressing goblet cells, as well as other intestinal cells, and is strongly associated with progression to EAC; (ii) the cardia type, which includes mucus cells; and (iii) the gastric fundic type, which includes mucus, parietal, and chief cells. The exact molecular events that lead from normal squamous esophageal epithelium to intestinal metaplasia (BE) and progression to EAC remain unclear. There are four theories about the orientation of a progenitor cell that gives rise to intestinal epithelium among squamous cells. Firstly, an esophageal squamous cell could be converted in an intestinal

columnar cell, a phenomenon called transdifferentiation. In addition, a native esophageal progenitor cell could diverge from its normal fate and differentiate into an intestinal columnar cell (esophageal progenitor cell transcommitment). Moreover, a circulating bone marrow-derived stem cell in the bloodstream could attach to the esophageal epithelium and differentiate into an intestinal columnar cell (circulating stem cell transcommitment). Finally, an adjacent columnar cell from gastroesophageal junction or gastric cardia could shift to replace a gap in esophageal squamous epithelium due to an injury and then undergoes intestinal differentiation (columnar progenitor cell transcommitment). There have been no indications yet about which theory is most possible to exist; however there are no indications that all four theories are not true [113].

Transdifferentiation is supported by the presence of multilayered epithelium (MLE) that contains both squamous and columnar esophageal cells. In addition, MLE in BE is characterized by a "transitional zone" of epithelial cells that demonstrate morphological features of both squamous and columnar epithelial cells, such as intercellular ridges, distinct microridges, microvilli, and bulging mucus. Nevertheless, microscopic assessment of normal gastroesophageal junction (GEJ) area did not demonstrate characteristics of transitional zone cells [97]. Moreover, the biphenotypic cell population of MLE is supported by the fact that esophageal basal epithelial cells of MLE express a combination of cytokeratin subtypes that are found in both squamous (CK4) and columnar (CK19) cells, and the stimulus of these MLE basal cells, after the intestinal transcription factor Cdx2 overexpression using CK14 promoter, causes the acquirement of both squamous and secretory features by MLE cells [8, 47]. However, the failure of complete transdifferentiation of a squamous epithelial cell into a columnar cell in vitro so far has raised some concerns against the theory of transdifferentiation. On the other hand, production of differentiated squamous epithelial cells that present several features of intestinal mucus producing cells resembling BE cells has been achieved, after the overexpression of the transcriptional factors HET-1A, EPC2, NES-B3T, and NES-B10T [35, 114]. Consequently, a pluripotent basal cell with preserved features of stemness seems to be required in order this formation of an intestinal goblet cell from a differentiated squamous cell to be achieved.

Transcommitment, which describes the genetic reprogramming of stem or progenitor cells in order to proliferate and differentiate into different cell types than they were initially programmed to do, seems to be a basic condition for developing BE intestinal metaplasia regardless the progenitor cells orientation. For example, BE epithelium could include Paneth cells, enteroendocrine cells, and goblet cells, which are usually diagnostic for BE [52]. BE development in patients that had undergone partial esophagectomy including GEJ and gastric cardia outlines that proximal shifting of progenitor cells from GEJ or gastric cardia to the main esophagus does not explain BE formation in every situation; however it seems that a reprogramming of the residual esophageal squamous or glandular progenitor cells takes place [60]. Furthermore, recurrence of BE development after ablation due to BE lesions demonstrates that esophageal epithelium is susceptible to environmental conditions that is exposed to, and as a result differentiation depends on them, as

normal reepithelialization with squamous epithelial cells happens after BE ablation when gastric acid levels are low in esophageal area, while development of BE lesions rises after previous ablation when gastric acid levels in the esophagus are high due to several reasons, such as non-compliance to acid-diminishing pharmaceutic therapy after ablation [9]. Finally, progenitor cells located in esophageal glands could give rise to multiple phenotypes, either squamous or columnar, due to transcommitment, as it has been observed that neo-squamous epithelium after ablation due to BE lesions shares the same mitochondrial DNA mutation with the underlying metaplastic epithelium of the submucosal esophageal glands that led to BE formation [74].

Passing from squamous fate to intestinal fate for an esophageal progenitor cell requires activation of transcription factors that would give the progenitor cell a columnar phenotype such us Sox9, as well as downregulation of the transcription factors that determined the squamous phenotype such as Sox2 and p63. In addition, the final differentiation into a goblet cell requires the expression of intestinal (Cdx1 and Cdx2) and mucus-related (Foxa2) transcription factors.

Sox9 is a member of Sox genes family and is expressed in intestinal crypts of GI tract as well as Paneth cells. In addition, Sox9 expression has been reported in esophageal embryogenesis together with CK8 and CK18, but when the epithelium matures to squamous, Sox9 expression gets lost. Sox9 has been described to express in 100% of BE specimens and in 85% of EAC specimens; however there was no expression in adjacent normal esophageal tissue [112]. Environmental conditions in the esophagus have been proven to play a crucial role in esophageal epithelium fate. As a result, Sox9 activation is caused by bile- and acid-stimulated Hedgehog ligand secretion by epithelial cells, which in turn induce BMP4 secretion by adjacent stromal cells. This stromal BMP4 acts back by increasing Sox9 expression [6]. In addition, the retroviral transduction of Sox9 in a mouse transplant culture system upregulated the expression of columnar CK8 and intestinal glycoprotein A33, as well as altered the esophageal epithelium architecture with inducement of one to two layers of cuboid or columnar-shaped epithelial cells. On the other hand, no alteration was observed neither in squamous epithelium architecture nor in gene expression of the transplant culture mouse model, after retroviral transduction of Cdx2, indicating the crucial role of Sox2 in BE development by altering the esophageal progenitor cells fate directing them toward intestinal phenotype [13].

Sox2 is another member of the Sox gene family that is expressed during esophageal embryogenesis and is responsible for maturing esophageal epithelium into its squamous phenotype [87]. Downregulation of Sox2 in mice leads to a thinner esophageal epithelium, characterized by mucus-secreting columnar cell, as well as decreased expression of p63 and CK14. Nevertheless, overexpression of Sox2 in mouse intestine caused loss of villi; appearance of p63 expressing basal cells, which are characteristic for esophagus and forestomach; and decreased attachment of Cdx2 to the promoters of its target genes [88]. Except from the role of squamous differentiation, Sox2 is responsible for the maintenance of stem cells, as its overexpression in several mouse models leads to esophageal basal cells hyperplasia [55]. Consequently, in the normal adult esophagus, Sox2 is expressed in the progenitor

basal cells of the stratified epithelium, while it is not expressed in MLE or in intestinal metaplasia of BE; thus its downregulation could be an important condition for reprogramming esophageal progenitor cells from which BE arises [11].

P63 is a member of the P53 transcription factors family and presents six isoforms. The key role of downregulated p63 in BE formation has been proven by studies in which mice null for p63 completely lack squamous esophageal epithelium and presents esophagi with simple columnar epithelium [17]. P63 presents escalated expression in esophageal epithelium depending on the different stages of esophageal dysplasia. More specifically, it presents absent to moderate expression in Barrett's esophagus and high expression in Barrett's esophagus with high-grade dysplasia and esophageal adenocarcinoma [34]. High expression of p63 has been observed in normal esophageal epithelium and esophageal squamous cell carcinomas too [27]. Despite the conflicting results of the studies mentioned above, it has been clarified that p63 is required for squamous differentiation, and BE without dysplasia is not likely to express P63, while adenocarcinomas may weakly express P63. In addition, combined exposure of esophageal squamous epithelium to bile salts and acid, like happening in patients suffering from GERD, diminishes the p63 expression in squamous cells leading to transcommitment of esophageal progenitor cells and consequent BE development [92].

However, esophageal intestinal metaplasia does not stop with the acquisition of columnar phenotype by esophageal epithelial cells. Cdx1 and Cdx2 are members of the caudal-related homeobox gene family and are expressed in the intestine, with Cdx1 expressing in the proliferative crypt compartment while Cdx2 in differentiated villus compartment [32]. The role of Cdx1 in BE development had been outlined after the observation that transgenic Cdx1 mice presented intestinal metaplasia of the gastric epithelium including all four cell types of the adult colon such as enterocytes, Paneth cells, goblet cells, and enteroendocrine cells [72]. Moreover, CDX1 mRNA has been found in Barrett's metaplastic tissue, but not in normal esophageal squamous tissue highlighting the ability of Cdx1 in reprogramming columnar progenitors into intestinal columnar cells [116]. Furthermore, increased expression of Cdx1 was observed in the metaplastic epithelium of a rat BE model, which was further induced after bile acid exposure of the esophageal epithelial cells, and promoted upregulation of Cdx2 expression as well, indicating the crucial role of Cdx1 in pathogenesis of BE after condition similar to GERD and its regulatory effect to the Cdx2 expression, establishing a positive feed-forward intestinalization loop [44].

Cdx2 has been involved in transcommitment of columnar progenitor cells in patients with GERD that present BE due to the observation that CDX2 expression has been found in 100% of biopsy specimens from nondysplastic and dysplastic Barrett's metaplasia and esophageal adenocarcinoma [31]. In addition, CDX2 expression has been found in inflamed esophageal squamous epithelium of GERD patients, but not in normal non-inflamed esophageal epithelium [83]. Moreover, human esophageal squamous epithelial cells from GERD patients with Barrett's esophagus differentially respond to acid and bile salt exposure by upregulating CDX2 when compared to human esophageal squamous epithelial cells from GERD

patients without Barrett's esophagus [71]. Finally, Cdx2 seems to be insufficient of stimulate a squamous cell transformation into intestinal cell, unless epigenetic alterations happen, while Cdx2 is able to promote intestinal metaplasia in columnar cells. However, Cdx2 remains a major transcriptional activator within the intestine; thus loss of its expression results in intestinal progenitor cell reprogramming into squamous cells [25, 66, 96].

Intestinal phenotype is characterized by mucus secretion, which in BE is provided by FOXA2 expression by intestinal columnar cells through presumed transcriptional regulation of MUC2 itself and of AGR2, which is required for proper processing of the MUC2 protein. Despite the fact that FOXA2 expression led to MUC2 protein expression, the cells did not acquire a full goblet cell phenotype [110]. It is possible that other factors may be required additionally to FOXA2 to induce a goblet cell phenotype. These other factors could include downregulation of SOX2 and P63, and similar to Noggin null mice, in which Bmp4 signaling is unopposed, Sox2 null or p63 null mouse embryos have esophagi with columnar epithelium containing goblet-like cells. In addition, Notch pathway modulation may also be required for the formation of goblet cells, as loss of Notch signaling in a surgical model of reflux esophagitis and Barrett's metaplasia led to almost a complete conversion of metaplastic epithelial cells to differentiated goblet cells [68].

Stem Cells in Novel Esophageal Therapeutic Attempts

The unique feature of the esophagus to include a functional population of pluripotent stem cells which regulate its homeostasis and are responsible for repairing possible injuries is usually the reason for several esophageal diseases that are related to stem cells pathophysiology; however stem cell molecular pathways could be possible therapeutic targets for such modalities. Recently, esophageal cancer has been correlated with CSCs, which seem to be responsible for resistance to chemotherapy and radiation; thus they are very attractive pharmacologic targets. CSCs express a variety of molecular markers such as CD44, CD133, and ALDH that contribute to drug resistance and give them features like quiescence, evasion of apoptosis, resistance to DNA damage, and expression of drug transporter pumps. In vitro clonogenic assays with sphere formation and in vivo studies in xenograft models demonstrate the stem-like self-renewal and differentiation capacities of CSCs. Consequently, future therapeutic trials should aim in the direction of exactly clarifying the mechanisms by which CSCs contribute to drug resistance in order to reveal specific molecular targets against esophageal CSCs [20].

Since irradiation has been inducted in the therapeutic protocols of the majority of the thoracic tumors, it has been demonstrated as one of the main factors that cause esophageal injury, commonly complicated with esophagitis. Nevertheless, the presence of subpopulations of esophageal progenitor cells that are characterized by in vitro ability of differentiation to multiple adherent lineages of cells gives the opportunity of using the isolated pluripotent esophageal cells in gene therapy

technology. There are ionizing irradiation mouse models, in which esophageal progenitor cells have been isolated either by the side population method or the serial preplate technique, and demonstrated repopulation in the irradiated esophagus of the recipient mouse [19]. More specifically, the side population cells differentiated to endothelin or vimentin positive colonies, while preplate cells formed colonies that were uni-lineage, bi-lineage, or tri-lineage for macrophage, endothelin, or vimentin positive colonies in vitro. On the other hand, there was no difference in the type of colonies that the two cell types formed in methylcellulose culture. As a result, the utilization of transgenes for the creation of soluble growth factors that would enhance repair process may facilitate innovating transplantation techniques for tissue regeneration after irradiation. Gene therapy with manganese superoxide dismutase plasmid liposome (MnSOD-PL) seems to be protective for esophageal side population cells against irradiation damage both in vivo and in vitro [77]. Another application of the stem cells technology that seems to contribute in healing after radioactive esophageal injury is dental pulp stem cell (DPSC) transplantation. DPSCs were cultured and transplanted into rats in which radioactive esophageal injury had been induced using radioactive I^{125}. In the injured esophagus, the labeled DPSCs were observed to co-localize with the SCs markers PCNA, CK14, CD71, and integrin α_6, which presented increased levels of expression too. After DPSCs transplantation, esophageal tissue presented an increase in epithelial thickness in combination with recovered esophageal functionality and diminished inflammation in the esophageal area [120].

Gastroesophageal reflux (GERD) is another common cause of chronic esophageal injury, which is managed pharmacologically in the majority of cases. However, pharmacologic management of GERD is restricted to the cure of symptoms and the complications, instead of facing the cause, which is the relaxation of the lower esophageal sphincter. As a result, there have been held a lot of studies on alternative invasive therapeutic options for GERD. Endoscopic injections of inert materials or cells have been attempted through the years with controversial results. Nevertheless, the injection of muscle precursor cells (MPCs) that were derived from expanded satellite cells isolated from skeletal muscle fibers, in the gastroesophageal junction presented promising results, offering both regenerative and functional action [22]. In addition, a full-thickness esophageal damage could be caused after swallowing of corrosive substances, with stricture formation presenting as a late complication, due to esophageal SCs destruction. The transplantation of MSCs in rats that had undergone caustic esophageal injury presented increased accumulation of MSCs at the site of injury, but there was no difference in healing between the transplanted and the control group histopathologically. However, new epithelial and muscle cells oriented by the transplanted MSCs were revealed. Consequently, transplantation of MSCs after caustic esophageal injury seems to be effective, but often injections seem to be required [42]. Moreover, esophageal damage has been studied in animal models of esophagogastric myotomy, in which autologous bone marrow mesenchymal stem cells (BM-MSCs) have been tested for their effectiveness to repair the lower esophageal sphincter (LES) after surgery. The results were interestingly promising as the autologous BM-MSCs improved muscle regeneration and

increased the contractile function of the damaged LES, without losing their position at the site of injury and without any phenotype alteration toward smooth or striated muscle cell [67].

Barrett's esophagus (BE) is the most common dysplasia that happens to the esophageal epithelium and the most common precursor lesion for esophageal cancer. During the last years, several efforts have been conducted for the investigation of new therapeutic alternatives instead of the radical surgical methods that have been applied over the years. Endoscopic ablation of BE foci with radiofrequency (RF) technology seems an effective alternative; however the system of ablation should achieve a balance between the radical excision of all the dysplastic cells from the esophageal epithelium, but not deeply enough to cause esophageal perforation or stricture formation. The HALO system seems to achieve the perfect balance using a balloon-based array of closely spaced electrodes to deliver radiofrequency energy to the esophageal mucosa, providing efficacy and safety at the same time [101]. Nevertheless, stem cells pathophysiology gave the chance for testing both the effectiveness of the RF ablation of BE lesions and the safety of the procedure. Enhanced AKT-mediated β-catenin phosphorylation, which is present in activated progenitor cells, is a characteristic of BE-associated carcinogenesis. Three months after RF ablation of BE lesions, an increased expression of AKT-mediated phosphorylated β-catenin was observed, while this increase was followed by a deep quiescence 6 months after RF ablation [49]. These findings reveal that 3 months after RF ablation, a repair process takes place in the neo-squamous esophageal epithelium.

Except from complicated gene therapies and molecular treatments, oral administration of agents that are based on the SCs principles could be proven effective in several esophageal diseases even cancer. An excellent example of this situation is the orally administered conditioned medium derived from mesenchymal stem cells after endoscopic submucosal dissection in the esophagus. The conditioned medium gel prevents esophageal stricture after the endoscopic procedure, diminishes the number of activated myofibroblasts, downsizes the fiber sickness, and restricts the inflammatory infiltration of neutrophils and macrophages at the site of excision [69]. For that reason, it could be used as a preventive agent of esophageal stricture after endoscopic procedures in the esophagus. However, orally administered agents are not used only for the prevention of mechanical injuries of the esophagus or benign diseases but even for cancer prevention. Several studies refer to the preventive role of aspirin against cancer. But, why aspirin could be so beneficial against a so complicated disease? The esophagus, and other tissues with increased concentration of SCs, gives the answer. Inflammatory stress which may be caused due to several reasons, different for each organ, provokes the SCs of each tissue to proliferate through prostaglandins and especially PGE2. Increased proliferative rates for a SC population lead to raised chance for mutations and so for CSCs creation. The anti-inflammatory capacity of aspirin against PGE2 is the key feature that makes it so useful against cancer, especially in tissues that contain squamous cell epithelium and high numbers of SCs, like the esophagus [58].

Apparently from human studies on SCs therapies or animal models based on human patterns, original animal studies have contributed to new therapeutic approaches against esophageal diseases. First of all, porcine 3D culture models have been developed that reproduce esophageal gland proliferation in vivo and provide laboratory technology with two different phenotypes of spheroids: one that expresses markers of squamous epithelium and one that expresses markers of columnar epithelium [111]. These models could allow the evaluation of the molecular factors that drive epithelialization toward the squamous or the columnar direction, as well as the generation of technically manufactured scaffold for different applications related to esophageal diseases. Furthermore, the unique feature of echinoderms to reconstruct both external appendages and internal organs has been studied during the last years and has uncovered plenty of secrets about SCs biology such as Notch signaling and expression of SCs markers (Piwi and Vasa) that are expressed in human tissues like the esophagus [91]. All this data will be the basis for the future therapies in organs (esophagus) that are molecularly similar to these organisms.

The complicated congenital diseases of the esophagus such as esophageal atresia or tracheoesophageal fistula and several benign esophageal diseases like caustic ingestion of toxic substances, esophageal cancer, and radical surgical operations of the adjacent thoracic or abdominal organs that involve the esophagus due to its anatomical complexity require replacement of whole or segment of the esophagus. So far, surgical connection of the two remaining esophageal segments and replacement of the missing segment with a transplant from an adjacent organ, like the stomach or large bowel, were the only therapeutic options after esophageal dissection [12]. Nevertheless, the raised morbidity rates after the surgical repairs in combination with the simple function of food and water transport from the pharynx to the stomach that the esophagus is responsible for raised the efforts of constructing a functional substitute based on the principles of tissue engineering, which includes tissue scaffolds, cell sources, and bioreactors (Fig. 2) [85]. Tissue engineering for the esophagus, as well as the rest of the tubular organs of the intestinal tube, utilizes somatic cells from human fibroblasts that are reprogrammed into induced pluripotent cells (iPS), which are able to differentiate into any type of cell of the three germ layers [107]. Autologous muscle cells, epithelial cells, or mesenchymal stem cells (MSCs) that are provided by this process undergo reproduction and then are implanted into artificial scaffolds that are constructed using biological materials. The enriched scaffolds are left to mature in a bioreactor toward the direction of the willing organ [85].

In the case of gastrointestinal organs, like the esophagus, scaffolds have to support proliferation, differentiation, and attachment of the iPS; thus both artificial materials and biological substances have been investigated for their properties to provide the ideal scaffold for esophageal tissue engineering [7]. A variety of materials such as polylactic acid (PLA), polyvinylidene fluoride (PVDF), polyglycolic acid (PGA), poly-dl-lactic acid (PLGA), poly-l-lactide-co-caprolactone (PLLC), polyvinylidene fluoride (PVDF), poly-caprolactone (PCL), and poly-l-lactic acid (PLLA) have been used for the construction of artificial scaffolds [18, 30, 63, 125]. Nevertheless, the use of these materials in scaffold constructing for tissue engineering

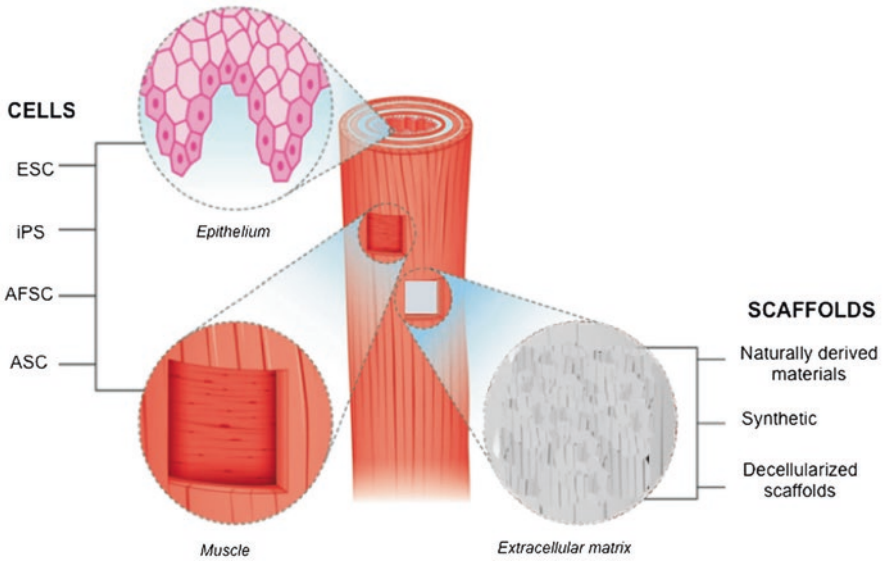

Fig. 2 Esophageal tissue engineering requires the combination of appropriate scaffolds and cells. Cells used for repopulation of the epithelial and muscular layers can be derived from ESC, iPS, AFSC, and ASC. ESC embryonic stem cells, iPS induced pluripotent stem cells, AFSC amniotic fluid stem cells, ASC adult stem cells [64]

has been correlated with anastomotic leakage and stricture formation after surgical operation. On the other hand, these limitations seem to be overtaken with the induction of acellular biological tissue scaffold in tissue-engineering technology. Acellular tissue scaffolds maintain the extracellular matrix (ECM) of the original tissue that they come from, presenting the advantage of improved cellular attachment on the scaffold [45, 46, 51]. In addition, they usually include vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF), which enhance the vascularization of an implanted transplant [61]. Acellular scaffolds include the esophageal submucosa, small intestinal submucosa, aortic acellular matrix, and aortic acellular matrix [21].

Except from a substrate where the "artificial esophagus" will develop, esophageal tissue-engineering models require a source of cells that would provide the developing esophagus with the appropriate number of functional cells in order to proliferate and differentiate into the specific esophageal epithelium. A possible source of cells for the developing esophagus could be provided by the adjacent esophageal epithelium, from which epithelial cells would migrate toward the developing organ [64, 82]. However, this process would be uncontrolled in terms of cell orientation that would be sparse and time intervals that would be undefined. Another possible technique that has been described is the transplantation of autologous buccal epithelial cells, which offer the advantage of the immunological similarity with the recipient tissue minimizing the risk for immune-mediated rejection, as well as

the capacity of inducing muscular regeneration at the site of implantation, with the latter being a controversial observation [86, 100]. Therefore, a specified cell source that would provide both epithelial esophageal cells that would protect the scaffold from caustic injuries and infections and specified muscle cells that would support the construction and help peristaltic function of the "artificial organ" is required. This cell source is provided by patient-derived iPS, for instance, bone marrow mesenchymal stem cells or adipose-derived stem cells, which differentiate into mesenchymal stem cells (MSCs), which maintain the ability of differentiating both in the direction of epithelial cells and in the direction of smooth muscle cells [76, 78, 81, 115]. Finally, the production of esophageal organoid units (EOU) after transplantation of murine-derived tissue-specific stem/progenitor cells in vitro in a degradable biological scaffold and after a few days, re-transplantation in vivo at the site of esophageal defect, with the formation of expanding spheres of proliferative basal cells on a neuromuscular network that demonstrated spontaneous peristalsis, gave another prospective in esophageal tissue engineering [95, 102].

After implantation of the cell source in the artificial or biological esophageal scaffold, two very important issues need to be overcome. Firstly, the new developing esophagus requires blood supply in order to maintain nutritional exchange for the proliferating iPS and differentiating epithelial cells. A possible approach is the implantation of the graft into the omentum or latissimus dorsi muscle before connecting it with the esophagus. Another option is to deliver angiogenic growth factors, such as fibroblast and platelet-derived growth factors, to the transplanted segment after implantation in the esophagus. Retention of VEGF in the protocol of an acellular scaffold after decellularization in combination with the proangiogenic properties of the scaffold enhances angiogenesis in a rodent tissue-engineering model [99]. In addition, gastrointestinal organs like the esophagus require peristaltic movement in order to be fully functional; thus a local neural network to initiate and maintain esophageal peristalsis is required. Intestinal organoids recombined with iPS-derived neural crest cells, which differentiated into neurons and glial cells, provided a neural network that was successfully integrated into intestinal smooth muscle and achieved a rhythmic wave movement [117].

Several animal models that apply the esophageal tissue-engineering expertise in the field have been developed. A full-thickness circumferential replacement of the esophagus of pigs has been attempted using synthetic polyurethane electrospun grafts seeded with autologous adipose-derived mesenchymal stem cells and a disposable bioreactor. After adipose tissue biopsy in order to provide adipose-derived mesenchymal stem cells, pigs underwent endoscopic circumferential resection of the mid-lower segment of the esophagus and replacement with the engineered scaffold. This model demonstrated gradual structural regrowth of endogenous esophageal tissue, including squamous esophageal mucosa, submucosa, and smooth muscle layers with blood vessel formation [50]. Furthermore, there has been a comparison between an acellular scaffold seeded with MSCs and an acellular scaffold alone after a 3 cm circumferential resection of the abdominal esophagus in a pig model. The comparative histological analysis presented a mature squamous epithelium covering the scaffold at 45th postoperative day for the MSCs group, while in

the control group, the mature esophageal epithelium was observed at 95th postoperative day. Moreover, desmin-positive cells were observed in the graft area in the MSCs group at 45th postoperative day, indicating muscle cell colonization, while in the control group, desmin-positive cells were never observed [10]. In addition, a dog model of 5 cm half circumference replacement of the esophagus with a small intestine scaffold seeded with BM-MSCs highlighted an increase in reepithelialization, revascularization, and muscular regeneration compared to the control group that included transplant of a small intestine scaffold alone [104].

Despite the fact that the findings of the in vitro experimental and animal models of esophageal tissue engineering are impressive, very few applications of tissue-engineered scaffolds have been conducted to humans. Esophageal endoscopic procedures that are the basic therapeutic option for early esophageal cancer in the stage of BE very often cause scar ulcers that lead to stricture formation, which is accompanied by annoying symptoms and requires often dilatations. Circumferential sleeve resection of the mucosa and placement of an ECM scaffold over the site of the resected tissue in five patients with high-grade esophageal dysplasia or BE demonstrated a successful prevention of intractable stricture, as well as complete maturation of squamous esophageal epithelium over the placed ECM scaffold 4 months after the operation [3]. Moreover, patch esophagoplasty with urinary bladder-ECM scaffolds in four patients presenting strictures due to surgery or past ingestion of a caustic substance outlined stricture avoidance and recovery of the oral intake with an obvious improvement of the patient's quality of life [75]. Finally, the application of cell sheets composed of the patients' oral mucosa (using a temperature-responsive culture dish) over post-ESD esophageal ulcers after endoscopic procedures due to esophageal carcinoma presented reduction of the reepithelialization period and stricture prevention of post-ESD stricture [79].

Conclusion

The principles of embryonic stem cells (ESCs) interaction have been utilized to explain the secrets of the development of the esophagus postnatally. In addition, the investigation of the metabolic pathways that regulate ESCs' differentiation into esophageal progenitor cells and finally into differentiated esophageal squamous epithelial cells established a matching between specific SCs markers and different steps of esophageal development. Consequently, specific molecules, such us mediators or receptors, have been correlated to specific cell features. Furthermore, SCs fundamentals have been applied in the explanation of the pathophysiology of several benign esophageal diseases such as eosinophilic esophagitis or esophageal achalasia.

Scientific progress about SCs has been widely used in the field of esophageal tumorigenesis and clinical features of esophageal malignancies. The raised morbidity and mortality rates of esophageal adenocarcinoma (EAC) and esophageal squamous cell cancer (ESCC) formed the necessity to reveal the behavioral patterns of

these tumors as well as the molecular factors that affect their clinical outcome. SCs played an important role to this as it is considered that esophageal cancer arises from a population of cancer stem cells (CSCs), a theory called the CSCs hypothesis. Moreover, the main precursor lesion of esophageal cancer, Barrett's esophagus (BE), causes a columnar metaplasia in the esophageal epithelium with an intestinal pattern instead of the ordinary squamous epithelium, due to progenitor cells' mutations and altered molecular profile.

A large amount of pediatric and adult esophageal diseases such as esophageal atresia, tracheoesophageal fistula, and post-endoscopic esophageal stricture require radical surgical treatment with high morbidity rates. Nevertheless, the development of SCs technology has opened new ways in the management of such modalities with the invention of tissue-engineered esophageal scaffolds that could replace circumferentially the damaged part of the natural esophagus. In addition, molecular therapies based on esophageal CSCs and progenitor cells markers have been developed, which target exclusively esophageal cancer cells without affecting other organs, ensuring greater quality of life for the patient with the highest effectiveness against the tumor.

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Pancreatic Diseases: The Role of Stem Cells

Konstantinos G. Apostolou

The Role of Stem Cells in Pancreatic Cancer

Introduction

Pancreatic cancer accounts for 277,000 new cases diagnosed each year in the world [1], among which approximately 55,000 occur in the United States [2]. Despite modest improvements in detection, which may have contributed to its rise in incidence, the 5-year overall survival (OS) rate only increased from 5% to 6% during the past three decades [3, 4]. While currently pancreatic cancer represents the fourth leading cause of cancer death in the United States (approximately 44,000 deaths annually), it is expected to become the second cause of cancer-related deaths in the United States in the next decade [2].

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer and continues to have one of the poorest prognoses of any malignancy [2, 5]. Smoking and overweight/obesity are among the known risk factors for the development of pancreatic cancer. However, despite the significant decrease in tobacco smoking since the 1990s, the prevalence of pancreatic cancer was not decreased, possibly due to the increase in the prevalence of obesity and type II diabetes mellitus [4, 6]. The dismal prognosis of pancreatic cancer is mostly due to the fact that no effective screening tests are available, as well as that its early stages are usually not associated with symptoms and if so, only non-specific symptoms do exist. Thus, the majority of patients are diagnosed suffering from a locally advanced or metastatic disease, which renders their prognosis worse. More than 50% of pancreatic cancers are identified in metastatic stage, where survival rates range from 7 to 11 months [7, 8], while in 30–40% of patients, the disease is localized but not

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surgically resectable, with an overall survival of 11–18 months [9, 10]. Patients with resected pancreatic cancer have poorer outcomes compared to other resected solid tumors, with the median survival after surgery and adjuvant therapy averaging 24 months [11–15].

Gemcitabine still represents the adjuvant therapy of choice for resected pancreatic cancer. However, since 2011, two combination regimens for metastatic disease have become the gold standard: 5-fluorouracil (5-FU)/leucovorin with irinotecan and oxaliplatin (FOLFIRINOX) [7] and nab-paclitaxel with gemcitabine [8]. With these approaches, response rates range between 23% and 31%, and overall survival is between 8.5 and 11 months. Given these modest improvements in survival rates, the target of recent clinical investigations has been the tumor microenvironment, the chemoresistant cancer stem cells, and the impaired drug delivery, as a result of the unique desmoplastic response that occurs in PDAC, thus necessitating the development of new treatments for patients with pancreatic cancer.

Cancer Stem Cells in Pancreatic Cancer

Originally identified in hematopoietic malignancies [16, 17], cancer stem cells (CSCs) have now been identified in a number of solid tumors [18–21]. CSCs are phenotypically distinct cells that are functionally defined by their ability to initiate tumor formation when implanted into immunocompromised mice; thus, they possess the capacity for self-renewal and differentiation [22].

In 2007, two groups of researchers isolated and identified pancreatic CSCs from human pancreatic cancer, using two different sets of cell surface markers [18, 23]. By using fluorescence-activated cell sorting (FACS) analysis, Li et al. isolated the $CD44^+/CD24^+/ESA^+$ pancreatic CSCs from pancreatic cancer tissue. These cells were found to harvest the stem cells properties of self-renewal, as well as the ability to produce differentiated progeny. In addition, they demonstrated that these cells had a 100-fold increased tumorigenic potential, when compared to marker-negative cancer cells [18]. Another study further examined these cell subpopulation pancreatic cancer tissues and reported that human pancreatic cancer tissues contained CSCs, which were identified by the expression of the CD133 [23]. One step further, the expression of the CD133 has been significantly associated with the clinical TNM stage, tumor differentiation, vascular endothelial growth factor C (VEGF-C) expression, lymph node metastasis, and a lower 5-year survival rate [24, 25]. However, CSCs do not represent a homogeneous clonal population of cells, as they undergo genetic evolution during the tumor's development and progression. Thus, subpopulations of CSCs, such as the $CD133^+$ $CXCR4^+$ [23] and $C\text{-}MET^+$ $CD44^+$ [26], respectively, bear distinct features and have been found both in primary tumors and in distant metastases.

Clinical Significance of Pancreatic Cancer Stem Cells

In recent years there is increasing evidence that CSCs, as also the pancreatic CSCs, are resistant to both chemotherapy and radiotherapy. In the clinical setting, the administered chemotherapy regimens may favor the depletion of the non-CSCs, while on the other hand, the remaining unaffected pancreatic CSCs are able to divide and repopulate the tumor with drug-resistant cancer cells. The underlying mechanisms of this phenomenon are multiple, with the most prominent being the high level of anti-apoptosis gene expression, the DNA repair mechanisms, as well as the drug efflux proteins [27–31].

The study published by Hong et al. demonstrated that an in vitro administered high dose of gemcitabine eliminated most of the pancreatic cancer cells. However, the cancer cell population was reconstituted via the proliferation of the $CD44^+$ CSCs, proving the resistance of CSCs to the administered gemcitabine [32]. These results were also evaluated in an in vivo setting, where $CD133^+/CXCR4^+$ CSCs were located in the invasive forms of pancreatic tumors. The removal of these cells from the pancreatic neoplasms resulted in a significant decrease in the metastatic potential of these neoplasms, suggesting that the $CD133^+/CXCR4^+$ CSCs harbor a higher invasive and metastatic phenotype, compared with the non-CSCs [23].

Apart from their resistance to both chemotherapy and radiotherapy, pancreatic CSCs were found to be associated with worse clinical outcomes. In the study published by Rasheed et al., the presence of aldehyde dehydrogenase (ALDH)-positive PDAC cells in resected surgical specimens was associated with worse survival, compared with patients with ALDH-negative tumors. Moreover, they reported that the expression of ALDH was greater in metastatic lesions than that in the primary site, suggesting a link between ALDH expression and disease progression [33]. In another study, the expression of the CD133 in resected PDAC specimens was significantly correlated with worse histologic tumor grade, lymphatic invasion, and lymph node metastasis. As a result, the 5-year survival rate of patients suffering from $CD133^+$ PDAC was significantly lower than the respective one of patients with CD133-negative tumors [25].

In addition to their crucial role in favoring tumor's chemo- and radiotherapy resistance, as well as their association with worse histologic types, lymph node infiltration, and lower survival rates, CSCs are also associated with a great potential to metastasize or favor tumor's metastatic potential. Herman et al. investigated the role of $CD133^+$ cells and found a distinct subpopulation of $CD133^+$ cells, which are CXCR4-positive, and reported that these cells were more metastatic than CXCR4-negative cells. One step further, they demonstrated that the elimination of $CD133^+$ $CXCR4^+$ cells significantly decreased the rate of metastases, without affecting the tumor-initiating process [23]. Maeda et al. reported that CD133 was not expressed in normal pancreatic ductal epithelium, while it was expressed at the circumference of the $cytokeratin^+$ cells, with its expression being correlated with the presence of lymph node infiltration. Moreover, another study demonstrated that a distinct subpopulation of $CD44^+$ CSCs, which expressed the c-MET, had a greater metastatic potential, compared to c-MET-negative cells, with the therapeutic targeting of

c-MET$^+$ cells resulting in an impaired function and metastatic potential of CSCs [26]. As a conclusion, the wide spectrum of stemlike gene expression profile of CSCs and the great frequency of phenotypic CSCs has definitely been associated with worse clinical outcomes and a greater metastatic potential, underlying the crucial role of CSCs in determining the progression of PDAC, as well as the metastatic and survival rates.

The Role of Stem Cells in the Treatment of Pancreatic Cancer

Despite the advances in understanding the biology of pancreatic cancer, which facilitated the development of new cancer treatments, major limitations still exist, including the short drug half-lives, the insufficient delivery to the tumor, the suboptimal specificity for malignant cells, the adverse side effects, and the deterioration of the quality of life of treated patients. Considering the aforementioned data as well as the marginal increase in survival rates during the past three decades, it becomes evident that new therapeutic strategies would be of clinical and survival benefit.

Cell-based anticancer therapy represents a novel strategy to target solid malignancies and has gathered growing interest in recent years, as it may improve tumor's therapeutic sensitivity and therefore therapeutic efficiency, by two mechanisms. First, it allows to target and elucidate critical molecular pathways, which are specific and essential to tumor growth [34], and secondly, it may enhance targeting specificity of cancer therapeutics to the desired site of action [35–37]. Mesenchymal stem cells (MSCs) represent a great candidate for the implementation of cell-based therapy against tumors, especially due to their tumortropic properties [38–46], and their therapeutic applications will be further discussed in the present chapter. Moreover, there are no published reports of any negative effects of transgene expression on MSCs proliferation capability, morphology as well as transformation properties [47–49].

Mesenchymal stem cells may be isolated from adipose tissue, bone marrow, umbilical cord, and peripheral blood. They are characterized by their innate ability to self-renew as well as differentiate into a variety of cell types [50]. However, MSCs differentiation capacity depends also on their tissue of origin, even if they are cultured in identical microenvironments [51]. The study published by Altaner demonstrated that MSCs remain plastic adherent under standard culture conditions; they express CD73, CD90, and CD105 markers; whereas they do not express hematopoietic lineage markers. Moreover, they may differentiate into adipogenic, osteogenic, and chondrogenic cellular lineages [52].

Several studies have demonstrated the innate characteristic of MSCs to home to sites of injury, inflammation, ischemia, as well as to tumors and metastases, including pancreatic carcinoma [53–55], which renders them as an attractive option for cell-based therapy of tumors. Despite the not fully elucidated mechanism of homing to the aforementioned sites, it is well documented that following the triggering tissue injury, they are recruited, then decelerated, and arrested within the blood vessels

and finally transmigrate across the endothelium to the injured tissue [50, 56]. Cytokines and chemokines, such as stromal-derived factor 1/CXC chemokine receptor 4 (SDF-1/CXCR4), stem cell factor/c-Kit (SCF/c-Kit), hepatocyte growth factor/c-MET (HGF/c-Met), vascular endothelial growth factor/vascular endothelial growth factor receptor (VEGF/VEGFR) [57, 58], monocyte chemotactic protein/CC chemokine receptor 2 (MCP/CCR2), and high-mobility group box protein 1/receptor for advanced glycation end-products (HMGB1/RAGE) [59–62]; adhesion molecules, β1- and β2-integrins, and L-selectin [60–62]; CC chemokine ligand 5 (CCL5/RANTES) [39, 63–65]; CC chemokine ligand 2 (CCL2) [39, 64, 65]; and vascular cell adhesion molecule 1 (VCAM) and cellular fibronectin ligands [37, 66, 67], all contribute to the arrest of MSCs within the vasculature of the tumor, followed by transmigration of MSCs across the endothelium into the tumor itself [39, 50, 56]. Furthermore, factors such as interleukin 8 (IL-8), neurotrophin-3, transforming growth factor β (TGF-β), interleukin 1β (IL-1β), tumor necrosis factor a (TNF-a), platelet-derived growth factor (PDGF), and epidermal growth factor (EGF) have also been shown to enhance MSCs tumor tropism capabilities [68, 69] (Fig. 1).

Mesenchymal stem cells may have a crucial role in cell-based therapy of tumors, by specifically targeting certain aspects of tumor's biology, either by introducing into the tumor genes, which interfere with specific molecular pathways or induce apoptosis, or by introducing specific genes, which contribute to the local activation of systemically administered prodrugs, avoiding thus the side effects, which are caused by the systemic administration of chemotherapy.

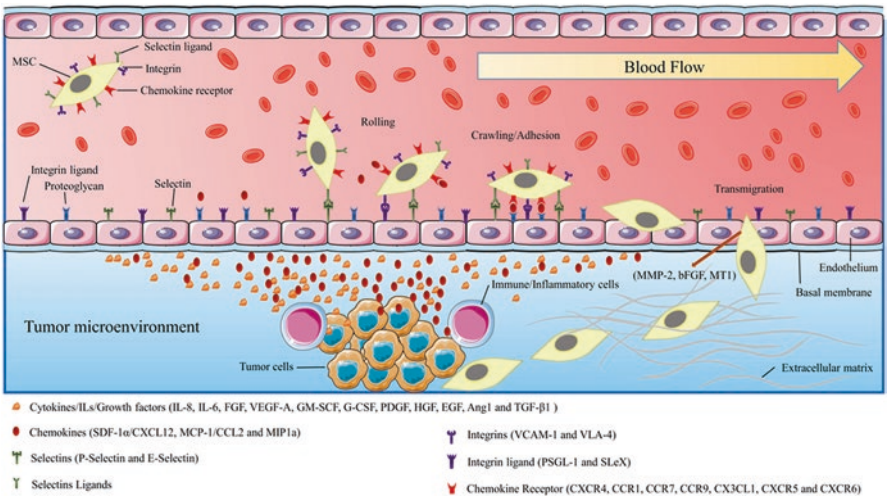

Fig 1 Schematic picture showing the mechanism of MSCs homing to sites of tissue injury. (Adapted by: Marofi et al. [151])

The Use of Genetically Engineered Mesenchymal Stem Cells to Induce Tumor Apoptosis

The use of MSCs as carriers of genes with specific antitumor activity has gained great popularity in recent years. The identity of the transgenes depends on the specific tumor properties and molecular pathways, while the expression of the introduced into the tumor transgenes may be regulated by signals of the tumor's microenvironment (Fig. 2). MSCs have been genetically engineered to express gene products with direct antitumor activity, such as the interferons (IFNs), as well as pro-apoptotic and anti-angiogenic agents [36, 52]. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and IFN-b have been demonstrated to bear antitumor activities. The therapeutic efficiency of the systemically administered TRAIL and IFN is limited by two factors, including their low bioavailability at the tumor's site, due to short protein half-life, and their dose-limiting side effects. Thus, genetically engineered MSCs to express the transgenes of TRAIL and/or IFNs may be used, to produce proteins with definite antitumor activity at the tumor's site, bypassing the aforementioned limiting factors of their systemic administration [70].

Considering the potency of TRAIL in specifically inducing apoptosis in many cancer cells, genetically engineered MSCs expressing TRAIL may be a possible mechanism to induce apoptosis in pancreatic cancer cells. However, it has been shown that pancreatic carcinoma cells carry an intrinsic resistance toward TRAIL, with the expression of the anti-apoptotic X-linked inhibitor of apoptosis protein (XIAP) being the ascendant mechanism [71]. More recent studies have demonstrated that the inhibition of XIAP, by using RNA interference, causes an enhanced TRAIL-induced apoptosis in pancreatic cancer cells, both in vitro and in vivo [72, 73]. One step further, the study published by Mohr et al. in a human pancreatic cancer mouse xenograft model demonstrated a decrease in tumor's growth rate, by genetically engineering human-derived MSCs (hMSCs) to express the soluble form

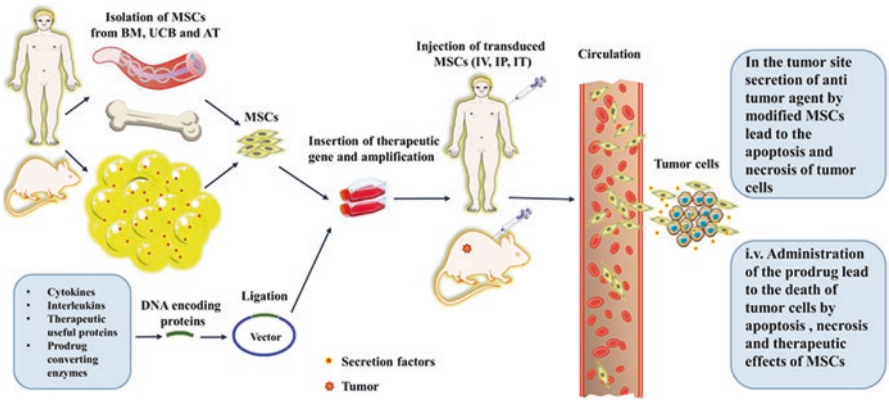

Fig 2 Schematic picture showing the procedures for the isolation, culture and gene transduction of MSCs, followed by their in vivo administration. (Adapted by: Marofi et al. [151])

of TRAIL (sTRAIL). Moreover, by combining XIAP inhibition and hMSCs expressing sTRAIL, they showed that the apoptotic activity was not only limited to slowing the tumor growth but also caused tumor's remission and inhibition of metastatic growth [70].

The intrinsic antitumor properties of MSCs have been observed in a number of studies, regarding lymphomas [74], hepatomas [74], breast cancer [75], as well as pancreatic carcinomas [76]. Considering the inflammatory nature and the extensive stromal compartment in pancreatic tumors, Kidd et al. investigated the role of MSCs intraperitoneal injection with regard to tumor growth, in a SCID mice model with orthotopically implanted human pancreatic carcinoma cells (PANC-1). The intraperitoneal injection was performed weekly for 3 weeks, using either MSCs or genetically modified MSCs to express the IFN-b transgene. They demonstrated that MSCs homed selectively to the sites of primary and metastatic pancreatic tumors and significantly inhibited tumor growth. One step further, the production of IFN-b within the tumor site, by the genetically modified MSCs, further suppressed tumor growth to a significant extent. However, this beneficial effect of IFN-b regarding the inhibition of tumor growth was not observed, when the genetically modified MSCs were administered in combination with an anti-inflammatory agent. Their results suggested that MSCs exhibit innate antitumor effects against PANC-1 cells and may serve as cellular vehicles for the specific expression of IFN-b in pancreatic tumors [76].

The Use of Genetically Engineered Mesenchymal Stem Cells in Gene-Directed Enzyme Prodrug Therapy

The concept of gene-directed enzyme prodrug therapy (GDEPT), also called suicide gene therapy (SGT), in cancer therapy, has been under investigation in recent years. By specifically targeting tumors, this approach consists of two steps: firstly, the use of genetically engineered MSCs as carriers of a specific gene that encodes a prodrug-activating protein and, secondly, the systematic administration of that particular prodrug that will be metabolized and activated into cytotoxic metabolites within the tumor [77]. Following the administration of the genetically engineered MSCs and their homing at the tumor's site, the inactive prodrug is systemically administered, causing the production of cytotoxic metabolites within the transduced MSCs, which will cause their apoptosis. However, in order to maximize the therapeutic potential of that treatment concept, the active cytotoxic metabolites should be able to diffuse also to the neighboring cancer cells, a phenomenon called "the bystander effect," which will lead to the cellular death of not only the transduced MSCs, but also of the neighboring tumor cells that do not express the transgene [78] (Fig. 3). In addition to that mechanism of cellular death, the dying tumor cells and MSCs will induce a host immune response, mediated by T cells, macrophages, and natural killer cells, as well as by increased levels of various cytokines, contributing to the drug-induced cellular apoptosis [79–82].

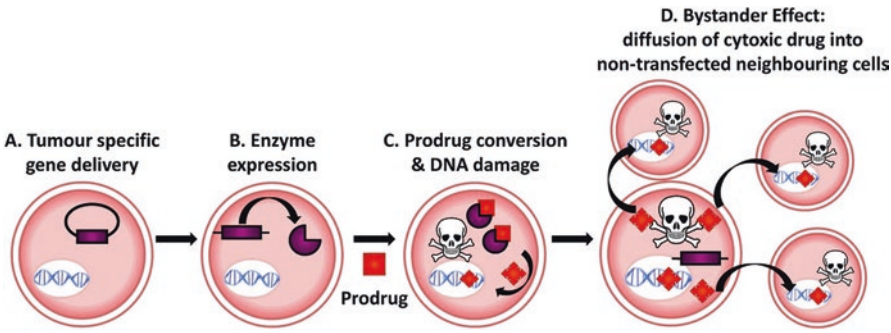

Fig 3 Schematic diagram depicting the principles of gene - directed enzyme prodrug therapy and the "bystander effect". (Adapted by: Williams et al. [152])

Following the transduction of MSCs with the transgene, it is essential to select the genetically modified MSCs, so as to obtain only the transduced cells that manage to highly express the transgene. This may be accomplished with the use of immunofluorescence or by using an enzymatic assay, which verifies that the transgene is actively expressed. However, the most commonly performed procedure is the fluorescence-activated cell sorting (FACS), which may determine the efficiency of transduction and at the same time may distinguish the transduced from the non-transduced MSCs.

Gene-directed enzyme prodrug therapy requires the cellular carriers of the transgene to manage to travel, then arrest within the blood vessels, and finally transmigrate across the endothelium to the site of the tumor. MSCs were found to be able to circulate in the bloodstream and then transmigrate to the site of the tumor, independently of their transduction with a lentiviral vector or the expression of a specific therapeutic protein [47]. This phenomenon was further facilitated by the immune-privileged properties of MSCs, arising from the low major histocompatibility complex (MHC) I expression and the absence of MHC II expression. However, it should be stated that the expression of the MHC I and II in undifferentiated MSCs may be proportionally increased with the increase in the differentiation grade of MSCs [83]. One step further, although the homing capabilities of MSCs render them as an attractive option for cell-based therapies, an additional specificity may be achieved by using tumor- or tumor stroma-specific promoters to drive the expression of the transgenes, once therapeutic MSCs have transmigrated to the targeted tumor [84–87].

Angiogenesis is essential for tumor initiation and growth. Several studies have demonstrated that this process depends mainly on the angiopoietins-TIE (ANGPT-TIE) system, which is necessary for the angiogenic switch in tumors [88]. Several agents targeting that particular pathway are being developed, with encouraging anti-tumor activity having been observed in early clinical studies [88, 89]. Genetically modified MSCs that express a therapeutic transgene under the control of TIE2 promoter upon reaching the tumor and in the presence of ANGPT2 ligand have been implicated in targeting the angiogenesis pathway, with the herpes simplex virus

thymidine kinase (HSV-tk) being the most commonly used transgene. Thus, the genetically modified MSCs that express the HSV-tk transgene under the control of the TIE2 promoter may express the transgenes products in a specific way to the targeted tumor, only after they are homed to the tumor and following the initiation of their differentiation process, in order to support the angiogenesis phenomenon. This mechanism of action, combined with the prodrug ganciclovir (GCV), has gained popularity as a gene-directed enzyme prodrug therapy of pancreatic cancer. In a syngeneic orthotopic mouse pancreatic carcinoma model, Conrad et al. demonstrated that the genetically modified murine MSCs expressing the HSV-tk under the control of the TIE2 promoter managed to successfully engraft into the growing tumor vasculature, followed by activation of the TIE2 promoter in that particular microenvironment, which finally resulted in a significant decrease in the volume of the pancreatic tumor, following the administration of the GCV as the prodrug [86].

As was previously described, CC chemokine ligand 5 (CCL5) represents, among other chemokines, an essential factor in the homing process of MSCs to sites of tissue injury, including tumors and their metastases [39, 63–65]. Taking advantage of that homing behavior of MSCs in response to CCL5, the study published by Zischek et al. used genetically modified MSCs to express the HSV-tk transgene under the control of the CCL5 promoter, in a mouse orthotopic model of pancreatic carcinoma, and demonstrated a selective expression of the transgene only within the tumor stroma microenvironment. Treatment with these genetically modified MSCs and the GCV as the prodrug resulted in a significant decrease both in the growth of the primary pancreatic tumor, and in the incidence of metastatic lesions [54].

The Role of Stem Cells in Acute and Chronic Pancreatitis

Introduction

Pancreatitis is an inflammatory process of the pancreatic gland and is characterized by the local activation and release of pancreatic enzymes, derived by the damaged exocrine cells of the gland. Clinically, it may manifest either as an acute inflammatory process, called acute pancreatitis, or as a progressive inflammatory process, called chronic pancreatitis.

Acute pancreatitis is one of the most common gastrointestinal disorders requiring acute hospitalization worldwide, with a reported annual incidence of 13–45 cases per 100,000 persons [90]. It is most commonly presented as an acute upper abdominal pain and therefore should be in the differential diagnosis work-up of any clinicians involved. In developed countries, the most common cause of acute pancreatitis is the gallstone disease, followed by alcohol abuse.

Acute pancreatitis is caused by the acute unregulated activation of trypsin within the pancreatic acinar cells, when the intracellular protective mechanisms preventing trypsinogen activation or reducing trypsin activity are overwhelmed. This

phenomenon leads to the lysis of the pancreatic tissue, followed by a subsequent local release of the activated proenzymes [91]. The inflammatory process is a major component in the development of acute pancreatitis, mediated by the local release of inflammatory cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α), whose production correlates with the severity of the acute pancreatitis in experimental models [92–94]. The localized pancreatic tissue damage is followed by a systematic inflammatory response syndrome (SIRS) and a possible subsequent organ failure, caused by the production and release in the bloodstream of inflammatory chemokines, such as the monocyte chemoattractant protein-1 (MCP-1) and fractalkine (FKN) [92, 95, 96].

Most of the acute pancreatitis cases are mild and self-limiting, needing only brief hospitalization. On the other hand, approximately 20% of patients develop a severe form of the disease, with local as well as extrapancreatic complications, a condition termed severe acute pancreatitis, characterized by extensive pancreatic necrosis alongside with pancreatic inflammation, and is accompanied by a high mortality rate, which is increased relatively to the increase in the severity grade of the disease and may reach 30% in the severe cases [97]. Several scoring systems have been used in order to predict patients at high risk for developing severe forms of the disease, such as the Ranson criteria [98, 99], the acute physiology and chronic health evaluation (APACHE II) score [100, 101], the sequential organ failure assessment (SOFA) score [102, 103], and the computed tomography (CT) severity index (CTSI) [104]. All the aforementioned scoring systems assess the severity of the acute pancreatitis episode, based on the assessment of the injury of the pancreatic gland as well as of the extrapancreatic organs. However, there exists a large variation between these scoring systems, in terms of predicting the severe forms of the disease [105, 106].

The treatment of acute pancreatitis depends mainly on the severity of the attack, as assessed by the aforementioned scoring systems. As a general rule, the first step should be the support of both the homeostasis and the functionality of multiple organs, including adequate fluid resuscitation, oxygen administration, and pain relief. In mild cases or predicted mild cases, which mostly are self-limiting, all these steps have been proven adequate, and oral feeding should be restarted as soon as the abdominal pain is decreasing and the inflammatory markers are improving [97]. On the other hand, the severe attacks of the disease require, besides the supportive care, a multidisciplinary approach, targeting in closely monitoring the vital signs of the patient, as well as assess the need for proper, either minimally invasive or surgical intervention, regarding the complications arising from the severe attack [97].

Chronic pancreatitis is a progressive inflammatory process of the pancreatic gland that causes damage of both the endocrine and exocrine components of the pancreas, therefore causing endocrine as well as exocrine pancreatic insufficiency, such as diabetes mellitus and steatorrhea. The most common causes of chronic pancreatitis are the alcohol abuse, the pancreatic duct obstruction as well as genetic mutations [107, 108]. The ongoing inflammation causes pancreatic fibrosis, atrophy of the acinar glands, and obstruction of the pancreatic duct [107, 108]. Given the progressive nature of the inflammatory process, the established pancreatic damage cannot be reversed. As a result, the treatment of chronic pancreatitis consists of

conservative methods, aiming to alleviate the abdominal pain and enhance body's endocrine and exocrine insufficiency status, by administering exogenous pharmacological agents.

As was previously mentioned, the innate characteristic of MSCs to home to sites of injury, inflammation, and ischemia renders them an attractive option for cell-based therapy. Various cytokines and chemokines [59–62], adhesion molecules [60–62], and ligands [37, 66, 67] contribute to the arrest of MSCs within the vasculature, followed by transmigration of MSCs across the endothelium into the inflammatory site [50, 56]. One step further, MSCs may decrease chronic inflammation and fibrosis via multiple mechanisms, including the downregulation of the expression of TGF-β1, which is a major regulator of chronic inflammation and fibrosis [109, 110]. In addition to that, MSCs may decrease the secretion of collagen, which is the major component of the extracellular matrix, thus decreasing the excessive secretion of collagen and its degradation during fibrosis [111, 112]. Considering these MSCs characteristics and due to the lack of effective targeted treatments for acute and chronic pancreatitis, new therapeutic approaches would be desirable, and MSCs may prove important contributors to these approaches.

Stem Cells in the Treatment of Acute Pancreatitis

Several studies have focused on the potential effect of MSCs administration on acute pancreatitis. All studies reported on experimental protocols, with the most commonly used MSCs being the bone marrow-derived mesenchymal stem cells (BM-MSCs), followed by the umbilical cord-derived MSCs (UC-MSCs), the adipose tissue-derived MSCs (ADSCs), and the fetal membrane MSCs (FM-MSCs). Given the self-limiting condition of the mild cases of acute pancreatitis and therefore their lack of pancreatic tissue damage, all published studies investigated the effect of MSCs administration in severe cases of acute pancreatitis.

Bone marrow MSCs (BM-MSCs) were, as reported earlier, the most commonly used MSCs [113–122], with the majority of studies reporting on the effect of their administration on severe attacks of acute pancreatitis and investigating their mechanism of action. The immunomodulatory effect of BM-MSCs was proposed by nearly all studies, as BM-MSCs were shown to downregulate the expression of several pro-inflammatory markers and cytokines, such as IL-1a, IL-6, IL-15, IL-17, TNF-a, TGF-β, nuclear transcription factor kappa B p65 (NF-κB p65), and nitric oxide synthase (NOS) [113, 118, 123]. One study showed that the administration of rat BM-MSCs increased the expression of anti-inflammatory cytokines, such as the IL-10 [117], with another study demonstrating that human clonal derived BM-MSCs suppressed the proliferation of T cells, whereas they increased the expression of Foxp3 regulatory T cells in the affected pancreatic gland [113]. The immunomodulatory effect of BM-MSCs was also supported by another one study, with pri-miR-9 BM-MSCs decreasing the levels of local and serum pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, myeloperoxidase (MPO), and CD68, alongside with

their effect in enhancing the regeneration of the affected pancreatic gland [122], as well as inducing an anti-apoptotic effect, via reducing the apoptosis of the pancreatic acinar cells, a finding also demonstrated in other studies [113, 122–124].

Apart from their immunomodulatory effects, other potential mechanisms of action of BM-MSCs are their antioxidant activities, by increasing the expression of glutathione peroxidase (GPx) and superoxide dismutase (SOD) [114, 120, 122]. Moreover, the administered BM-MSCs in severe cases of acute pancreatitis may enhance the neovascularization and angiogenesis phenomenon [115, 116], as well as increase the serum levels of MCP-1, which is a crucial chemokine in the pathogenesis of acute pancreatitis, at a significant extent [116]. Another finding suggests that the pancreatic gland damage may be further improved, by administering BM-MSCs together with granulocyte colony-stimulating factor (G-CSF), as apart from mobilizing the hematopoietic stem cells, G-CSF enhances the proliferation of the administered BM-MSCs, by binding to their G-CSF receptors [121, 125].

The therapeutic effect of BM-MSCs in acute pancreatitis is not only limited to the pancreatic gland, as BM-MSCs exert also their effects in other organs, which are frequently affected by the severe acute pancreatic episode. Lu et al. investigated the potential therapeutic effect of BM-MSCs on capillary endothelial barrier and water transportation, the impairment of which may lead to capillary leakage. They demonstrated that, apart from the induced pancreatic damage, the small intestinal capillary endothelial barrier is also impaired in the acute pancreatitis setting, accompanied by a significant reduction in the expression of aquaporin 1 (AQP1). Following treatment with BM-MSCs, the damage to the pancreatic tissue and the level of small intestinal capillary leakage were alleviated, whereas the reduction in the expression of AQP1 was reversed [126].

Three studies focused on the effect of UC-MSCs administration on severe attacks of acute pancreatitis [124, 127, 128]. The treatment of severe acute pancreatitis with UC-MSCs resulted in a decreased pancreatic gland damage, with the parameters of edema, inflammation, and necrosis being significantly improved. Moreover, all three studies confirmed the immunomodulatory effect of UC-MSCs, via reducing the serum levels of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α, and IFN-γ) and simultaneously by increasing the levels of anti-inflammatory cytokines (IL-4 and IL-10) [124, 127, 128]. Except for their immunomodulatory effect, UC-MSCs were shown to decrease the apoptotic rate of the pancreatic acinar cells [124], whereas in another study, UC-MSCs transfected with ANGPT1 demonstrated an enhanced immunomodulatory effect, alongside with the promotion of the angiogenesis phenomenon in the damaged pancreatic tissue [128]. Only the study published by Yang et al. investigated the possible effect of UC-MSCs administration on the mortality rate associated with the severe cases of acute pancreatitis and demonstrated that UC-MSCs caused a reduction in the mortality rate of severe forms of acute pancreatitis [127].

Apart from BM-MSCs and UC-MSCs, two studies reported on the mechanism of action of ADSCs and FM-MSCs in acute pancreatitis, respectively. Both ADSCs and FM-MSCs administration resulted in a decrease in the levels of pro-inflammatory cytokines (TNF-α, IFN-γ, IL-1β, and IL-6), with a simultaneous increase in the

levels of anti-inflammatory cytokines (IL-4 and IL-10), an immunomodulatory effect comparable with that of BM-MSCs and UC-MSCs [129, 130].

At this point, it should be emphasized that the tracking and homing properties of the administered MSCs within the damaged tissue is an essential step for determining their mechanism of action, since several studies have suggested that MSCs exert their function via a paracrine, dominantly immunomodulatory, effect, rather than by directly differentiating into pancreatic cells [131], as there is increasing evidence that the majority of the systemically administered MSCs are being trapped within the lung capillaries [113, 115, 116, 132, 133]. Regarding the acute pancreatitis injury model, only five studies [113, 117, 118, 121, 122] investigated the homing properties of the administered MSCs, with the BM-MSCs demonstrating definite homing properties to the damaged pancreatic gland in four studies [113, 118, 121, 122], whereas in the remaining study, the systemically administered BM-MSCs were passively trapped mainly in the lungs, as well as in other organs [117].

Stem Cells in the Treatment of Chronic Pancreatitis

A limited number of studies has investigated the potential effect of MSCs administration for the treatment of chronic pancreatitis [129, 134, 135]. In these studies, chronic pancreatitis was induced in rats, by intravenously administering dibutyltin dichloride [129, 135], followed by a systemic administration of MSCs, via either the penile vein [129] or the jugular vein [135], with the administration time point ranging from 4 h before the onset of chronic pancreatitis [134] till the 5th day following disease's onset [129, 135]. Various sources of MSCs were used, including BM-MSCs [134], UC-MSCs [135], and FM-MSCs [129]. Only two studies reported on the time of sacrifice, which ranged from 14 days [129] to 28 days after disease's onset [135].

Only one study investigated the tracking and homing properties of the MSCs and revealed that the administered UC-MSCs were successfully engrafted into the damaged pancreatic tissue [135]. All studies demonstrated a decrease both in the pancreatic damage and in the pancreatic fibrosis, following the administration of MSCs. The injection of UC-MSCs resulted in a reduced expression of cytokines, chemokines, and ligands, such as the VCAM-1, intercellular adhesion molecule 1 (ICAM-1), IL-6, TNF-α, and MCP-1 [135]. The immunomodulatory effects of MSCs were not confined to the aforementioned parameters, as the administered UC-MSCs, which were modified as for the expression of the inhibitor IκBαM, managed to inactivate the NF-kB factor, as well as reduce the levels of several pro-inflammatory cytokines, including IL-1, IL-6, IL-8, TNF-α, ICAM-1, and TGF-β1, and simultaneously increase the levels of anti-inflammatory cytokines, such as IL-10 [134]. In addition to their immunomodulatory properties, the administered BM-MSCs and UC-MSCs exerted an anti-apoptotic effect, by decreasing the apoptotic rate of the pancreatic acinar cells [134, 135].

Current Limitations of Stem Cell-Based Therapies

Cell-based therapy represents a very promising strategy for the treatment of various diseases, including cancer. However, there are certain prerequisites as well as efficacy and safety issues that should be carefully considered and require further investigation.

Stem cells for therapeutic applications need to meet the standards of Good Manufacturing Practice regulations, posing as important quality criteria, among others, the immunophenotype of the cells, the composition of the culture medium, and the risk for malignant transformation, as well as the aging and the immunosuppressive potential of the manufactured MSCs [136]. From one aspect, it is well known that MSCs derived from the adipose tissue may easily and repeatedly be isolated and then expanded in culture medium for a prolonged time, in contrast with the BM-MSCs which have to be used during early cell passages, in order to prevent any possible differentiation process [137]. Thus, fearing the potential malignant transformation of MSCs, a good practice suggests to avoid unnecessary manipulation and prolonged passaging of MSCs that will be used for therapeutic applications [138, 139].

Another issue regards the correct amount of MSCs that should be systemically administered, so as to home to the targeted tumor and exert their therapeutic effect. Several studies have focused on that issue, with some reports estimating the necessary amount of administered MSCs to be less than 10% of the targeted tumor mass [69, 140]. Considering that the targeted tumor responds and its mass is decreased as a result of the administered MSC-based therapy, it is a reasonable approach to administer the MSCs in a repeated fashion, in order to achieve tumor regression. This fact has been demonstrated in a metastatic renal cell carcinoma study, where repeated low dose injections of transduced MSCs exhibited stronger anti-metastatic effects, as compared to a single injection at a high dose [141]. However, the lack of evidence regarding that issue in other cancer protocols, including the pancreatic cancer, necessitates the conduction of studies which will shed more light on this topic.

The major concern regarding the use of MSCs for cell-based therapy is the potential risk for tumor and metastases formation, as there is evidence suggesting that, under some circumstances, MSCs are immunosuppressive and favor tumor growth [142–144]. Moreover, as with any therapy involving genetic manipulations, the malignant transformation of MSCs leading to the development of secondary tumors is also a concern, a phenomenon called “insertional mutagenesis,” which has been observed in a preclinical setting [145]. Several studies have demonstrated that the non-transfected MSCs may contribute to the growth of tumors [146–149], a mechanism which is probably mediated by the MSCs production of immunosuppressive agents, as well as by the effect of MSCs on tumor stroma and vascularization phenomenon [150]. Thus, as was previously mentioned, a good practice suggests to avoid unnecessary manipulation and prolonged passaging of MSCs that will be used for therapeutic applications [138, 139].

The genetic modification of MSCs and the transduction of the therapeutic gene may serve as a means of lowering the risk of either malignant transformation of MSCs or insertional mutagenesis, as the incorporated transgene enables efficient elimination of the transduced MSCs. Therefore, the validation of all the MSCs cell lines as for their transduction with the therapeutic-suicide gene is mandatory before their administration.

At this point, it should be emphasized that the majority of studies investigating the insertional mutagenesis phenomenon has been performed in rodent models, which have a relatively short life span, so that the true mutagenic risk cannot be estimated in the long-term. Therefore, the use of primate animal models, with a relatively longer life span, which may be administered greater amounts of MSCs, and possibly in a repeated fashion, will elucidate the true mutagenic risk of MSCs used for cell-based therapies.

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Stem Cell Therapy for Liver Diseases

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Stem Cell Categories for Liver Disease Therapy

There have been many reports that demonstrate the therapeutic potential of different categories of stem cells in liver diseases. These include embryonic stem cells; annex stem cells, obtained from placental and cordonal tissues; fetal liver stem cells; and induced pluripotent stem cells. Moreover adult stem cells, derived from liver as well as from extrahepatic tissues, such as bone marrow and adipose tissue, exhibit also the capacity to differentiate into hepatocyte-like cells and support liver regeneration (Fig. 1).

Embryonic Stem Cells: Human embryonic stem cells (hESCs) are totipotent cells that are isolated from the inner cell mass of blastocysts and have the ability of self-renewal and differentiation into specialized cell types, under appropriate cell culture conditions [1]. Several studies have shown that hESCs are able to successfully differentiate into hepatocyte-like cells (HLCs) in vitro, exhibiting similar properties of mature hepatocytes, such as urea secretion, glycogen storage, indocyanine green uptake and secretion, as well as cytochrome P450 expression [2–4]. Moreover, in vivo studies have revealed that the ESC-derived HLCs are able to engraft efficiently into mice and continue to retain their hepatic features, promoting proliferation of host hepatocytes and revascularization of injured liver tissues [4]. Additionally, Moriya et al. have shown that administration of ESC-derived HLCs into CCl_4-treated mice inhibited liver fibrosis without tumor formation [5]. More interestingly further analyses have shown that the ESC-derived HLCs contribute to liver repair not only by cell replacement but also by delivering trophic factors that support endogenous liver regeneration [1, 4, 5]. Thus, hESCs consist a valuable tool

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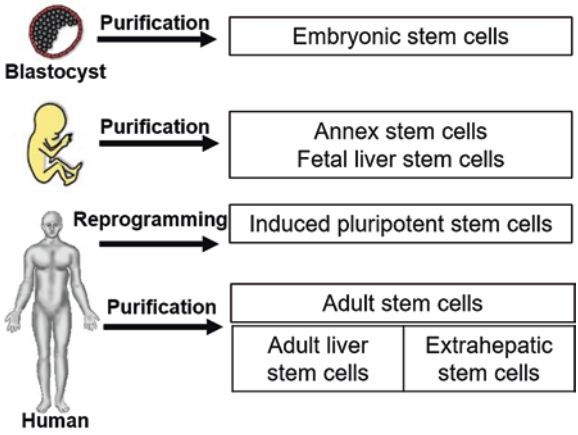

Fig. 1 Different stem cell categories in cell-based therapy for liver diseases. Pluripotent, totipotent, and multipotent stem cells can be purified or reprogrammed from embryonic, fetal, and adult human tissues

for studying the molecular basis of hepatocyte differentiation, evaluating liver toxicity in in vitro platforms [6], and providing the basis for cell therapy. Nonetheless, still exist limitations regarding the use of ESCs in cell therapies, such as immune incompatibility between donors and ESC transplant recipients [7], as well as ethical and legal concerns [8], which confine the progress of ESC research in the current therapeutic field.

Annex Stem Cells: This category includes cells derived from placental and cordonal tissues, such as umbilical cord, umbilical cord blood, placenta, and amniotic fluid. The advantage of annex stem cells compared to adult stem cells is that they exhibit higher proliferation potential that allows their extensive expansion in vitro and manipulation prior to cell therapies [9]. Moreover, these cells of fetal origin have a broad differentiation potential, since they are able to give rise to cells of all three germ layers and more importantly to hepatocyte-like cells (HLCs). Several studies have suggested protocols for the efficient differentiation of annex stem cells into functional HLCs that constitutively express hepatic lineage markers, albumin, a-fetoprotein, cytokeratin-19, and connexin-32 and are able to secrete urea, store glycogen, and exhibit CYP3A4 activity [10]. More interestingly, recent publications have demonstrated that umbilical cord blood-derived HLCs were able to promote liver regeneration and reduce mortality in NOD/SCID mice and rats with acute toxic liver damage [11, 12]. Moreover, placenta-derived stem cells exhibit immunomodulatory effects, and they have already demonstrated their therapeutic efficacy on animal models with liver diseases. More particularly, recent studies have categorized the repair mechanisms of placenta-derived stem cells in injured liver tissues as follows: (a) antifibrotic and anti-inflammatory action, (b) apoptosis and autophagy of damaged cells, (c) autophagy for recycling of cellular products, and (d) activation of hepatic cell proliferation [13]. Additionally, amniotic fluid-derived mesenchymal stem cells have been considered as a potential alternative treatment for terminal liver diseases, since they do not raise ethical issues, whereas they exhibit high proliferation and differentiation potential [14]. Most recent studies have revealed that

amniotic fluid-derived HLCs were able to induce liver repair in mouse models of acute hepatic failure [15], ameliorate liver fibrosis in mice [14], and promote improvement of fulminant hepatic failure in rats [16]. Overall, annex stem cells offer another advantage compared to adult stem cells in liver therapy: they do not form teratomas or teratocarcinomas in humans [17] that considers them as an alternative, more ethically acceptable source in the field of cell therapy.

Induced Pluripotent Stem Cells: Induced pluripotent stem cells exhibit similar characteristics to ESCs such as (a) unlimited in vitro self-renewal and (b) pluripotency, i.e., potential to differentiate into various cell lineages under appropriate conditions [18]. Due to these unique properties, iPSCs have been established as an important tool for modeling and investigating human diseases, as well as for drug screening. In more detail, the first report of iPSC generation refers to retroviral vectors that induce pluripotency in somatic cells, through the expression of four defined factors: (a) octamer-binding transcription factor 4, OCT4; (b) sex-determining region Y-box 2, SOX2; (c) Krüppel-like factor 4, KLF4; and (d) c-MYC [19]. However, this method exhibits drawbacks, such as spontaneous reactivation of the viral transgenes as well as their integration in the host genome that can lead to tumor formation [20]. Thus, current research has focused on the development of novel strategies for reprogramming iPSCs that include vectors which do not integrate into the host genome, like episomal, mini circle, adenoviral, and Sendai vectors [21–23]. Moreover, recent approaches for iPSCs' generation have established integration-free methods and DNA-/RNA-free strategies in order to improve reprogramming efficiency, renewing hopes for their use in regenerative medicine [24–27]. Another remarkable property of iPSCs is their differentiation potential into any cell type, depending on their origin [28], establishing them as valuable tools in the field of regenerative medicine for liver diseases [29]. More particularly, several studies have demonstrated that iPSCs of diverse origin are able to differentiate into HLCs, exhibiting similar properties to primary hepatocytes, such as albumin production, CYP activity, a-fetoprotein expression, urea secretion, and glycogen storage [30–32]. Various protocols have been developed for the differentiation of iPSCs toward the hepatic lineage which include the application of growth factors and small molecules that regulate epigenetic mechanisms and signaling pathways, without genetic alterations [31, 33]. Moreover, clinical applications of regenerative medicine require a large-scale production of hepatocytes. Thus, Yamashita T et al. have demonstrated a novel technology for a billion-scale production of homogenous and functional HLCs from human iPS cells, using a three-dimensional (3D) cell culture bioreactor, that can be utilized in the areas of drug screening and tissue engineering [34]. Regarding iPSC clinical applications in liver diseases, Asgari S et al. revealed that iPSC-derived HLCs improved a fibrotic mouse model after their transplantation [35], whereas other researchers demonstrated enhanced liver regeneration and reversed lethal fulminant hepatic failure, after iPSC-derived HLCs' administration [31, 36]. Furthermore, Takebe T et al. demonstrated a first report for the generation of vascularized and functional human liver from human iPSCs by transplantation of liver buds created in vitro [37], whereas a more recent study suggested a novel method of growing a liver bud through tissue connection, using a 3D bioprinter

[38]. Thus, iPSC technology provides opportunities for liver disease treatment, since these cells have the ability of self-renewal, differentiation, and generation of an unlimited amount of hepatocyte-like cells for transplantation. However, several issues need to be addressed before the use of iPSCs in cell therapy. One of the most important obstacles of iPSCs applications in liver transplantation is the identification of the optimal reprogramming method, performing clinically relevant methodologies. More particularly, the development of iPSC-derived HLCs requires novel differentiation protocols, since the monolayer culture of iPSCs is a time-consuming process, inappropriate for clinical applications [39]. The solution to these issues is the 3D culture of iPSCs that leads to high amount of metabolically functional hepatocytes [40], providing a new window in therapeutic applications for liver diseases. Recent studies have revealed that while undifferentiated autologous iPSCs lead to tumor formation in animal models, iPSC-derived progenitors formed functional tissues in vivo without any evidence of teratoma generation [41]. Thus, efficient differentiation protocols of iPSCs could block cells from entering a pluripotent state and preventing tumor formation. Overall, the production of iPSC-derived HLCs in a large scale with functional longevity is of high importance, whereas cost-efficient developmental procedures, long-term safety, and tolerability are valuable for clinical applications of iPSCs in liver diseases [39].

Extrahepatic Adult Stem Cells

Liver regeneration is an endogenous process characterized by the participation of mature hepatocytes and resident hepatic stem cell populations [42]. However, recent studies have suggested that populations of extrahepatic adult stem cells are also able to migrate into the liver and improve the endogenous regenerative potential [43]. Bone marrow is the main reservoir of adult stem cells, including endothelial progenitor cells, hematopoietic stem cells, and mesenchymal stem/stromal cells that are able to engraft to the injured liver tissue, modulate the endogenous repair mechanisms, and contribute to hepatic repopulation [44–46].

Endothelial Progenitor Cells (EPCs): EPCs are mobilized from bone marrow and incorporated into sites of vascular disorders, promoting neovascularization and tissue regeneration via secretion of growth factors that support endogenous repair mechanisms [47]. Studies have revealed that EPCs transplantation in rats with liver fibrosis could suppress activated hepatic stellate cells, increase matrix metalloproteinase activity, and regulate hepatocyte proliferation [48]. Moreover, liver sinusoidal endothelial cells (LSECs) are thought to promote tissue regeneration via their recruitment to injured rat livers, to exhibit increased HGF levels, and to regulate hepatocyte proliferation and organ recovery [49]. The beneficial role of EPCs in liver regeneration includes their effective transplantation into the hepatic central veins, formation of tubular structures along hepatic sinusoids, proliferation of hepatocytes, and production of several growth factors, such as hepatocyte growth factor, transforming growth factor-α, heparin-binding epidermal growth factor-like growth

factor, and vascular endothelial growth factor [50]. Most recent studies have revealed that bone marrow EPCs mobilization and differentiation into the injured liver tissue are regulated by the CXCL10/CXCR3 signaling [51].

Hematopoietic Stem Cells (HSCs): HSCs consist a cell population that is able to support liver healing process after tissue injury, via two major mechanisms: (a) transdifferentiation into hepatocytes and (b) genetic reprogramming of hepatocytes after cell fusion [52, 53]. First, Grompe et al. have demonstrated that HSCs could give rise to hepatocytes, whereas other research groups revealed that blood-system stem cells could be used clinically to generate hepatocytes and cholangiocytes for tissue regeneration [52, 54]. Further studies have proved the beneficial role of HSCs in ameliorating liver damage. More particularly, it has been shown that hepatic injury caused by extensive liver resection and partial hepatectomy could trigger the mobilization of hematopoietic stem cells able to differentiate into hepatocytes, promoting the liver recovery process [55, 56]. Moreover, the combination of portal vein embolization with CD133(+) BMSC administration substantially increased hepatic regeneration in patients with malignant liver lesions, whereas Thomas JA et al. observed in mouse models of liver fibrosis, paracrine signaling of exogenous unmanipulated BM cells to larger populations of endogenous cells, such as macrophages and neutrophils, that increased their reparatory effect. Finally, the beneficial role of HSCs could be a result of their paracrine effect that includes production of various cytokines and growth factors that induce the hepatic recruitment of endogenous macrophages and neutrophils in the tissue injury [57].

Mesenchymal Stem/Stromal Cells (MSCs): Mesenchymal stem cells have been described as potential candidates for cell therapy in liver diseases, since they exhibit easy accessibility, high proliferation rate, and multidirectional differentiation potential into cell types derived from mesoderm, ectoderm, and endoderm, such as hepatocyte-like cells (HLCs) [58, 59]. Bone marrow is the first described source of MSCs wherein they contribute to nonhematopoietic stromal cell renewal, involving adipocytes, chondrocytes, and osteocytes [60]. MSCs are positive for the mesenchymal markers CD73 (SH3), CD90, CD105 (SH2), CD44, CD29, CD51, CD106, CD166, and Stro-1; however they do not express the endothelial marker CD31, the hematopoietic marker CD45, as well as the CD11b, CD79a, CD19, and HLA-DR [61]. Several studies have demonstrated that MSCs have a dual positive effect in liver diseases: (a) transplanted MSCs are able to differentiate into functional HLCs in the injured tissue, and (b) MSCs secrete immunoregulatory factors that modulate the inflammatory progression of inflammation [62, 63]. In more detail, several protocols have suggested specific culture conditions that promote the differentiation of MSCs into functional HLCs. These cells express the hepatic lineage markers albumin, a-fetoprotein, cytokeratin-18, hepatocyte nuclear factor 4, connexin-32, and dipeptidyl peptidase IV [64–66]. According to hepatocytes, MSC-derived HLCs were able to store glycogen, produce urea, transport low-density lipoprotein (LDL), and express cytochrome P450 [64]. More interestingly, in vivo studies have revealed that transplanted MSCs into rodents with acute or chronic liver failure were able to transdifferentiate into HLCs, replacing the damaged hepatocytes in the injured area and improving liver regeneration [67, 68]. Further

research of Chamberlain et al. confirmed the transdifferentiating ability of MSCs into HLCs in larger animals, where intrahepatic injection of MSCs into preimmune fetal sheep resulted to widespread generation of hepatocytes [69]. Moreover, MSCs have few MHC-I, and they do not express on their surface the costimulatory molecules CD80, CD86, CD40, and MHC-II, failing to stimulate T-cell response [70]. Aggarwal et al. have reported that MSCs exhibit immunosuppressive effects via the secretion of prostaglandin E2 that leads to IL-10 secretion by dendritic cells, as well as through the decrease of tumor necrosis factor alpha (TNF-α), interferon-γ (IFN-γ), and IL-4, produced by TH-1 and TH-2 cells, respectively [71]. Subsequent studies have confirmed that the current immunosuppressive properties of MSCs could be beneficial for allogeneic transplantation in mouse models with acute and chronic liver injuries [72]. Overall, in vivo studies have demonstrated that the therapeutic mechanism of MSCs in liver failure includes promotion of hepatocyte proliferation [73], impairment of hepatic stellate cell apoptosis [74], antifibrotic effect [75], and reduction of inflammation [76].

Adipose Tissue Stromal Cells (ATSCs): Bone marrow has been considered as the main cell source of MSCs in the previous years; however it exhibits serious drawbacks. More particularly, harvest of bone marrow is a highly invasive procedure, whereas the proliferative and differentiation potential and the maximal life span of BM-MSCs decline with increasing age. Therefore, adipose tissue has been suggested as an alternative source of MSCs that can be obtained by a less invasive process and in larger quantities compared to bone marrow. Adipose tissue stromal cells (ATSCs) exhibit similar characteristics to BM-MSCs, such as the expression of MSC markers, like CD29, CD44, CD71, CD90, CD105/SH2, and SH3. Moreover, several studies have confirmed that ATSCs are able to differentiate into functional HLCs, induced by defined growth factors and chemicals, establishing them as an excellent source for liver-regenerative procedures [77]. Furthermore, researchers have demonstrated that systemically administration of ATSCs and HLCs derived from ATSCs led to cell engraftment and promotion of tissue repair in rodent models with acute hepatitis, possibly due to increased hepatocyte growth factor (HGF) levels in the site of injury [78, 79]. More recently, Sakai et al. published a Phase I clinical study of liver cirrhosis therapy using ATSCs that were administered via intrahepatic arterial transfusion into cirrhotic patients [80]. The results of the study revealed that 1 day after ATSCs transplantation, were detected increased levels of HGF and IL-6 in all patients, whereas serum albumin concentrations were improved, even 1 year after autologous cell transplantation, confirming liver regeneration and establishing ATSCs as powerful tools in liver therapy [80].

Liver-Derived Stem Cells

Fetal Liver Stem Cells: Fetal liver stem cells derived from hepatic endoderm during embryogenesis and are characterized as hepatoblasts, bipotent cells that express a-fetoprotein, able to give rise to hepatocytes or bile-duct epithelial cells [81, 82].

More particularly, hepatoblasts exhibit less immunogenicity, higher proliferation potential, and greater regenerative capacity compared to adult hepatocytes, considering them as a valuable tool for liver disease treatment [83]. The regenerative capacity of fetal liver stem cells has been confirmed in in vivo studies as well as in clinical trials [84–86]. Haridass D et al. have demonstrated that even cryopreserved fetal liver stem cells were able to engraft into the liver of athymic mice resulting in up to 10% repopulation and high expression of characteristic hepatic markers, including albumin, alpha1-antitrypsin, cytochrome P450, and alpha-glutathione S-transferase [87]. Moreover, a clinical study from Khan AA et al. has revealed that when human fetal liver-derived stem cells were transplanted into 25 patients with liver cirrhosis, it resulted to significant improvement of all clinical and biochemical parameters without adverse effects [85]. A more recent clinical study by Cardinale V et al. has suggested that transplantation of human fetal biliary tree stem cells via hepatic artery in patients with advanced cirrhosis led to biochemical and clinical improvement during the 12-month follow-up. More interestingly, the absence of signs of rejection and/or allergy correlated with minimal or null expression of HLA class I and II antigens both in hepatic and biliary tree stem cells from fetal liver [88, 89], confirming a safe and efficient therapeutic protocol for cirrhotic patients [23].

Adult Liver Stem Cells (LSCs): Several studies have identified activation and expansion of liver stem cells in patients with chronic liver injury or submassive hepatic necrosis, known as resident liver progenitor cells [90, 91]. Their role is to restore the liver parenchyma after extensive damage, by giving rise to hepatocytes and biliary epithelial cells [92, 93]. In 2006 Herrera et al. performed phenotypical characterization of progenitor cells in normal adult human liver, demonstrating that LSCs were able to express the mesenchymal stem cell markers CD29, CD73, CD44, and CD90 but not the hematopoietic stem cell markers CD34, CD45, CD117, and CD133. Moreover, LSCs were positive for vimentin, nestin, albumin, a-fetoprotein, and cytokeratins 8 and 18, whereas they did not express cytokeratin-19, CD117, and CD34, indicators of oval stem cells [94]. Additionally, LSCs, when cultured in the presence of hepatocyte growth factor, were able to differentiate into functional hepatocytes that express P450 and albumin and secrete urea [94]. Further in vivo studies have suggested that LSCs can contribute to liver parenchyma regeneration in severe-combined immunodeficient mice, as it has been histologically identified by the description of nodules, composed of small clusters of hepatocytes mixed with ductules [95]. Additional research has revealed that Wnt and Notch signaling pathways are responsible for the proliferation and differentiation of LSCs into hepatocytes or cholangiocytes in chronic liver diseases in mouse and human [96]. In more detail, Boulter L et al. demonstrated that in human diseased liver, Notch and Wnt signaling promote biliary and hepatocyte regeneration via the stimulated myofibroblasts and macrophages LSC niche, whereas the Wnt state is activated through engulfment of hepatocyte debris, influencing directly the LSCs. Additionally, Wnt3a expression promotes hepatocyte proliferation, providing a positive feedback in adult parenchymal regeneration [96].

However, liver-derived stem cells exhibit major drawback, comprising only 0.7% of the adult liver and hepatoblasts less than 0.1% of the fetal liver mass [39]. Thus, their isolation and expansion are restricted in small-scale applications, and their future use in clinical trials remains a great challenge.

Stem Cell-Treated Liver Diseases

Genetic Liver Diseases (GLDs): There is no permanent treatment for genetic liver diseases (GLDs), whereas liver transplantation has been suggested as a therapeutic option that is not affordable by most patients. Recent studies have proposed cell therapy as an alternative, since it can serve as a bridge to liver transplantation and can also support long-term correction of the metabolic deficiency [97]. GLDs include abnormalities in gene coding that result to expression of defective proteins which cause improper liver function [98]. GLDs are rare and include the following cases: a1-antitrypsin deficiency (a1-ATD), hereditary tyrosinemia type I (HT-1), Wilson disease, galactosemia, juvenile hemochromatosis, Crigler-Najjar type I syndromes, and familial hypercholesterolemia [99]. In GLDs, liver recovery can be mediated by use of BM-MSCs that are able to differentiate into hepatocytes and thus replace host hepatocytes carrying mutated genes. The first preclinical study for GLDs was performed by Markus Grompe's group in a case of HT-1 where transplantation of HSCs cured the genetic defect of HT-1 Fah-null mice [100]. Further studies revealed that donor-derived macrophages were fused with the recipient hepatocytes, resulting to gene alterations in the synthesis of hepatocyte-specific proteins [101]. In the case of "Crigler-Najjar" syndrome, BM-MSCs engrafted in the liver lobes of rats and differentiated into hepatic cells [102]. Moreover, significant progress in GLDs' treatment can be performed with the development of iPSC technology that can avoid the disadvantages of allogeneic transplantation. In more detail, this strategy can include (i) isolation of iPSCs from GLD patients, (ii) gene correction of isolated iPSCs, (iii) iPSCs differentiation into hepatocytes, and (iv) autologous, disease-free iPSC transplantation. Finally, a more recent study demonstrated that disease-corrected hepatocytes have been already successfully developed from a familial hypercholesterolemia patient's iPSCs in 4–5 months, holding great promise for the future treatment of GLDs [103].

Viral Hepatitis: Hepatitis B and C infections are widely prevalent among the general population, whereas the most effective treatment for HBV-associated end-stage liver disease is liver transplantation. However, the risk of HBV reinfection following transplantation may reach >80% [104]. Thus, the current treatment protocols suggest the combination of nucleoside analogues with hepatitis B immunoglobulin (HBIG) following liver transplantation that exhibit drawbacks, such as high cost and HBV resistance [105]. Therefore, a case report in 2003 by Chiba T et al. reported successful clearance of hepatitis B virus after allogeneic peripheral blood stem cell transplantation in a 38-year-old male patient with acute lymphocytic leukemia [106]. Moreover, BM-MSCs have been suggested as a novel tool to

prevent hepatitis B recurrence, following liver transplantation [105]. In more detail, the co-culture of BM-MSCs with hepatitis B (HBV)-infected lymphocytes in vitro led to inhibited proliferation of HBV-infected cells and decrease of HBV DNA levels, preventing immune responses to induce immune tolerance. This could happen via cytokine secretion from BM-MSCs, such as fibroblast growth factor (FGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF) that inhibit HBV replication, as well as IL-22, known to exert anti-inflammatory effects in HBV infection [107].

On the other hand, HCV consists a significant global public health problem, infecting 3% of the world population and leading to chronic liver diseases, such as steatosis, cirrhosis, and hepatocellular carcinoma. Despite that, it remains difficult to develop vaccines against HCV, whereas antiviral drugs have optimized the HCV therapeutic effect by increasing the virological response. However, the current vaccines exhibit serious drawbacks, such as virus resistance, concomitant adverse reactions, and high medical expenses [108]. Thus, a therapeutic strategy based on stem cells could be an alternative to HCV treatment regarding their paracrine effect, such as the secretion of exosomes and extracellular vesicles. A recent study has suggested that exosomes, derived from umbilical cord blood mesenchymal stem cell-derived exosomes (uMSC-Exo), could be potent anti-HCV agents since they were able to prevent viral replication and showed lower cytotoxicity compared to other antiviral agents [109]. In more detail, high-throughput miRNA sequencing of uMSC-Exo suggested that the antiviral process is mainly mediated by a series of miRNAs transported specifically through exosomes, illustrating a promising method for the development of anti-HCV therapy.

Parasitic Liver infections: Recently stem cells have been considered as a valuable, therapeutic tool for parasitic infections. More particularly, researchers demonstrated that culture supernatant derived from MSCs could inhibit activation and proliferation of macrophages induced by the soluble eggs of *S. japonicum*, leading to increase of survival rates and decrease of liver injury and fibrosis in infected mice with *S. japonicum* [110]. Moreover, stem cell therapy could offer a novel treatment for cerebral malaria, since current available antimalarial agents are insufficient to inhibit neurological disorders and cognitive impairment. Souza et al. reported that administration of BM-MSCs in an experimental model of cerebral malaria increased survival and reduced parasitemia and malaria pigment deposition in the spleen, liver, kidney, and lung, providing a new therapeutic strategy [111]. In addition, stem cell treatment could be effective in cases of Chagas disease, a neglected tropical illness caused by the parasite *Trypanosoma cruzi* that leads to cardiomyopathy [112]. Recent studies have shown that MSCs transplantation was effective in reducing inflammation, fibrosis, and right ventricular dilation in the hearts of chagasic mice [113]. Further clinical trials revealed that autologous bone marrow transplantation in 28 patients with heart failure due to Chagas disease led to significant improvement to the quality of life, as determined by the Minnesota Questionnaire and by NYHA class, suggesting an important therapeutic modality in the management of end-stage chagasic heart disease [114]. Finally, Hegab MH et al. reported in 2018 that BM-MSCs exhibited beneficial action on chronic diseased liver in *Schistosoma*

mansoni-infected mice. The MSCs' therapeutic effect was based on prevention of collagen deposition, reduction of collagen 1 gene expression, and regression of fibrosis in mice liver tissues, confirming the antifibrotic effect of MSCs in parasitic liver infections [115].

Autoimmune Hepatitis (AIH): It is a chronic, necroinflammatory liver disease, characterized by increased aminotransferase levels, hypergammaglobulinemia, and production of characteristic autoantibodies, such as primarily antinuclear antibodies (ANA), antismooth muscle antigen (SMA), antiliver kidney microsomal antibody (LKM), and antisoluble liver antigen/liver-pancreas (SLA/LP). The current therapy for the disease is based on corticosteroids, however not all the patients respond to the treatment, whereas those who do respond exhibit strong side effects or relapse after drug withdrawal. The emergence for a novel therapeutic strategy led to stem cell therapy, since MSCs display T-cell suppressive properties in vitro and in vivo. Thus Chen Yi et al. have demonstrated the beneficial effects of MSCs in an AIH mouse model and explored their therapeutic mechanism. In more detail, transplantation of BMSCs in an AIH experimental model led to recovery of the phenotype, as it was confirmed by immunohistochemical and biochemical analyses, suggesting as a potential mechanism the upregulation of PD-L1 and repression of IL-17 [116]. Additionally, a clinical trial has already begun in patients with AIH, based on their transplantation with umbilical cord blood mesenchymal stem cells (UCB-MSCs) combined with corticosteroids and azathioprine. The follow-up will be in 96 weeks in order to evaluate the safety and efficacy of UCB-MSCs transplantation in cases of AIH [117].

Nonalcoholic Fatty Liver Disease (NAFLD) and Nonalcoholic Steatohepatitis (NASH): NAFLD is one of the prominent liver diseases worldwide, including three stages: (i) steatosis that corresponds to lipid accumulation; (ii) nonalcoholic steatohepatitis (NASH), characterized by hepatocyte damage, leukocyte infiltration, and fibrosis; and (iii) cirrhosis [118]. There are several factors responsible for NAFLD progression, including accumulation of triglycerides into hepatocytes, hepatic inflammation, Kupffer cell activation, oxidative stress, and stimulation of hepatic cells that produce collagen type I, resulting in fibrosis and further cirrhosis [118]. Recently, stem cells have been considered as a promising tool for the development of therapeutic strategies for NAFLD and NASH. A very promising study from Ezquer M et al. has revealed that intravenous administration of BM-MSCs in obese mice with metabolic syndrome was able to prevent NASH. Their preclinical results demonstrated that BM-MSCs inhibited hepatomegaly, liver fibrosis, inflammation, and more interestingly slightly reverted steatosis secondary to obesity-induced metabolic syndrome [119]. In a more recent publication, compact bone MSCs were administered into NASH mice which resulted in reduction of weight loss, hepatic steatosis, hepatocyte ballooning, liver inflammation, and fibrosis [120]. According to these findings, the beneficial role of MSCs was based on their immunosuppressive effects and more particularly on the suppression of activation of $CD4^+$ IFN-γ^+ and $CD4^+$IL-6^+ lymphocytes [120]. Concluding, recently Lyall MJ et al. have developed an in vitro NAFLD model, based on ESC-derived HLCs, which will be useful

in the future for mechanistic and high-throughput therapeutic screening in NAFLD [121].

Alcoholic Liver Disease (ALD): Chronic alcohol consumption is a major cause of ALD, affecting a great number of people worldwide, with limited therapeutic options. Several studies have proved that stem cell therapy could offer great potential to these patients, revealing their contribution to liver repair [58, 68]. More particularly, transplanted BM-MSCs into mouse models with ALD are able to improve the phenotype through their transdifferentiation into HLCs and their fusion with native hepatic stem cells [101]. Further, ethanol toxicity or other chemical injuries lead to production of regenerative stimuli that drive homing and engraftment of BM-MSCs into the liver, such as SDF-1, IL-8, MMPs, HGF, and SCF [122]. Additionally, beyond transdifferentiation and cell fusion, BM-MSCs contribute to hepatocyte regeneration via secretion of microvesicles and exosomes that support communication between hepatic stem cells and hepatocytes in tissue injury. Thus, the microvesicles and exosomes may represent critical components that promote self-renewal and expansion of stem cells and hepatocytes and simultaneously activate proliferative/regenerative programs in hepatocytes by transferring specific nucleotides and proteins [123]. Lyra et al. performed the first randomized control trial with ALD patients who exhibited a significant improvement in their serum albumin, after BM-MSC transplantation [124]. Overall, stem cell-based transplantation consists a promising strategy in ALD cases; however the high death rate of posttransplanted stem cells is one of the major problems in clinical therapy. Thus, a very recent study has demonstrated that co-stimulation of LPAR1 and S1PR1/3 can enhance the engraftment of hMSCs in mouse models with ALD via involvement of downstream pathways, such as RAS/ERK and PI3K/Akt pathways, representing a safer strategy for enhancement of stem cell transplantation efficacy [125].

Liver Cirrhosis: The current disorder consists the major mortal cause of various progressive liver diseases, resulting in chronic liver failure with other complications, such as encephalopathy, spontaneous bacterial peritonitis, ascites, and esophageal varices [126]. Since the effective treatment is limited to liver transplantation that exhibits serious drawbacks, involving immunological rejection and the scarcity of donor sources, the current research interest is focused on stem cell-based therapy [127]. Several preclinical and clinical studies have demonstrated promising results for the use of stem cells in liver cirrhosis treatment [128, 129]. Despite the unsolved precise mechanisms, the results from these studies have confirmed the absence of posttransplantation complications and the decrease of hepatocellular carcinoma [58, 130]. Moreover, a clinical trial using human fetal liver-derived stem cells into 25 patients with cirrhosis revealed improvement of MELD scores [85], whereas clinical studies using HSCs have showed promising results, such as advancement of serum albumin levels and Child-Pugh score [131]. BM-MSCs have been considered as the most frequently used stem cell source in liver cirrhosis treatment. In more detail, different pilot studies have demonstrated the safety and short-term efficacy of BM-MSCs in cirrhotic patients, showing significant improvement of Child-Pugh and MELD scores [132]. Subsequent research revealed the antifibrotic activity of BM-MSCs in liver cirrhosis, whereas stem cell transplantation in 12 patients

resulted to decreased levels of transforming growth factor-β1, type 1 collagen, and α-smooth muscle actin, as well as histological improvement of hepatic fibrosis [133]. Apart from BM-MSCs, the efficacy of UCB-MSCs has also been evaluated in clinical trials for cirrhosis. In more detail, 45 patients received UCB-MSCs transfusion, whereas 1-year follow-up period demonstrated significant reduction in the volume of ascites, increase of serum albumin levels, and decrease of total serum bilirubin and sodium model for end-stage liver disease scores, confirming the liver function improvement [97]. Additionally, other stem cell sources have been considered as therapeutic candidates for liver cirrhosis, such as human fetal biliary tree stem or progenitor cells that were able to proliferate, differentiate, and repopulate a fibrotic rat liver [134]. More interestingly, during a subsequent clinical trial, two patients with advanced liver cirrhosis (Child-Pugh C) were transplanted with human fetal biliary tree stem/progenitor cells and observed through a 12-month follow-up, confirming stable biochemical and clinical improvement for 6–12 months [86]. Finally, ESCs or iPSCs could be ideal candidates for liver cirrhosis therapy, since studies have proposed that iPSCs are able to alleviate the inflammatory microenvironment, differentiate into functional hepatocytes, and restore liver tissue [36].

Paracrine Effect of Mesenchymal Stem/Stromal Cells in Liver Diseases

The paracrine effect of MSCs has been demonstrated to play an important role in liver repair and regeneration through downregulation of proinflammatory and fibrogenic activity and stimulation of hepatocyte proliferation (Fig. 2) [128, 135, 136]. In more detail, specific MSC-derived cytokines have been confirmed to improve liver injury, including IL-10 [71], interleukin-1 receptor antagonist [137], hepatocyte growth factor [138], vascular endothelial growth factor, insulin-like growth

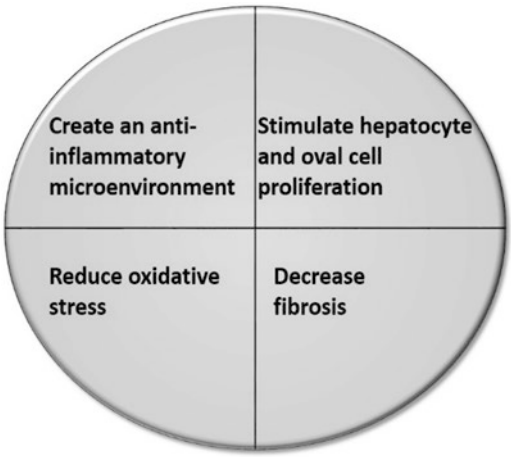

Fig. 2 Paracrine mechanisms of MSCs. The figure illustrates the different paracrine activities of MSCs in liver injury

factor-binding protein [139], tumor necrosis factor alpha [140], nerve growth factor, and matrix metalloproteinase-9 [75]. Parekkadan et al. have proposed that MSC-conditioned medium administration in an acute liver injury rat model resulted to phenotype improvement [140]. The authors suggested that MSC-secreted factors had an effect on immune cell migration to the liver, whereas a similar subsequent study revealed that the MSC paracrine effect led to decrease of IL-1β, TNF-α, and IL-6, increase of IL-10, lower lymphocyte infiltration in the liver, and reduced hepatocyte apoptosis [141]. In more detail, through secretion of PGE2, MSCs are able to reduce production of IFN-γ and IL-4 in Th1 and Th2 cells, promote proliferation of immunosuppressive CD4+CD25+forkhead box P3 (FoxP3)+ (Tregs), and inhibit cytotoxic CD8+T lymphocytes (CTLs) and natural killer (NK) cells [142, 143]. In another study, Zagoura et al. have demonstrated that administration of CM derived from AF-MSCs or hepatic progenitor-like cells (HPL) exhibited a significant therapeutic effect in mice with acute liver failure through secreted molecules, such as IL-10 [15]. The beneficial effect of MSCs' soluble factors was further examined in subsequent publications that revealed that MSC-CM could create an anti-inflammatory microenvironment in the liver tissue through the activation of M2 macrophages, main producers of IL-10 and C-C motif chemokine ligand 1 (CCL-1) cytokines [144]. More interestingly, researchers have shown that MSC-CM does not have only anti-inflammatory properties but exhibits profound inhibitory effects on hepatocellular death resulting in a 90% reduction of hepatocyte apoptosis and increased hepatocyte proliferation [145]. Additionally, several studies have demonstrated that MSC-CM therapy could have antifibrotic effect on mouse models with liver diseases through the expression of metalloproteinases 9 and 13 that degrade extracellular matrix [75], as well as through modulation of TGF-β signaling, attenuating infiltration of pro-fibrotic F4/80+ macrophages in the liver [144]. It should be mentioned that the paracrine effect of MSCs could be a result of their secreted extracellular vesicles (MSC-EVs) which include two basic types, (a) microvesicles (MSC-MVs) and (b) exosomes, that contain miRNA, mRNA, proteins from their cells of origin, and immunosuppressive or anti-inflammatory molecules [146]. Li et al. have demonstrated that administration of UCB-MSC-derived exosomes in animals with liver fibrosis reduced collagen deposition, decreased expression and production of TGF-β, and inhibited phosphorylation of Smad2, suggesting that alleviation of liver fibrosis could be a consequence of MSCs' paracrine effect [147]. Further studies revealed that MSC exosomes through the delivery of glutathione peroxidase 1 (GPX1) could reduce oxidative stress and apoptosis in mice with carbon tetrachloride-induced liver failure [148]. Moreover, MSC exosomes may have a superior safety profile, compared to the cells they derived from, since they do not replicate or cause microvascular embolism [149] and can be stored without losing their properties [150]. Thus, administration of MSC exosomes may represent an alternative therapeutic strategy for liver diseases by ameliorating oxidative stress and cell apoptosis.

Conclusion

The current chapter has attempted to draw out the exciting and dynamic area of stem cell-based therapies for liver diseases. Stem cells could offer an alternative to liver transplantation since they exhibit significant therapeutic benefit according to their positive effect in animal models and clinical trials. However, ethical, legal, and social issues of stem cells potential clinical applications need to be addressed in order to ensure their safety and efficacy in liver therapy.

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The Role of Stem Cells in Colorectal Cancer Carcinogenesis and Treatment

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Introduction

Carcinogenesis is a multistep process involving a series of genetic, epigenetic alterations of non-neoplastic cells with proliferative potential, which lead to the transformation of the cells, thereby driving the formation of highly progressive and malignant derivatives [1]. Cancer is an abnormal tissue, which constituted of heterogeneous population of cells differing in morphology, gene expressions, proliferative capacity and invasiveness [2]. This heterogeneity may occur as a result of hierarchically organising cancer cells with a subset of cells, called cancer stem cells (CSCs) at their top, which have the properties of stem cells (stemness; ability of self-renewal and multi-lineage differentiation) [3, 4]. These CSCs potentially contributed with the vital phenomena of cancer such as minimal residual diseases, resistance to therapies, cancer recurrence and ability to form metastases and thereby led to the poor outcome of patients with cancer [5]. In addition, CSCs could play roles in the predicting of biological aggressiveness of cancer due to its stemness through either asymmetric or symmetric division [6].

Colorectal cancer is the third leading cause of cancer-related death followed by lung and liver cancer worldwide, with an estimated 774, 000 deaths that occurred in the year 2015 [7]. According to the seminal discovery, the molecular pathogenesis of colorectal carcinogenesis encompasses multiple genetic and epigenetic alterations leading to adenoma-carcinoma progression [8]. In addition, studies provided

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profound insights into the nature of colorectal cancer and revealed extreme complexity of the diseases, including intertumoural and intratumoural heterogeneity [9, 10]. Thus, colorectal cancer consists of different genetic, epigenetic and functional heterogenic cancer cells, which favour the functional properties of tumour propagation and therapy resistance [11–13]. Moreover, identification of CSCs in colorectal cancer added a further layer of intratumoural heterogeneity by exposing the existing cancer cells with stemness [14–16]. However, recent studies suggested that CSCs are a dynamic population of cells rather than a distinct population, which continuously changed by a convergence of genetic, epigenetic and tumour microenvironmental factors [17, 18]. Considering this, the view of CSCs in colorectal cancer is facing profound transformation in parallel with a rapidly evolving concept of stemness in both cancer and non-neoplastic stem cells. Therefore, stemness is increasingly seen as a property of cell populations which is highly dependent on contextual factors not only cell-intrinsic features [19–22]. Despite its developing stage, the concept of targeting CSC possesses enormous potential for better therapies to the disease. In addition, the prognostic significance of CSCs profiles in patients with colorectal cancer has reinforced the existence of CSCs in colorectal cancer. The carcinogenesis of colorectal cancer is strongly associated with the presence of an altered stem cell pool. Therefore, exposing the mechanisms by which CSCs drive progression of colorectal cancer could provide better insights for clinicians to interfere with diseases and lead to improvement of treatment for patients with colorectal cancer. Given the importance of CSCs in colorectal cancer, this chapter focuses on the roles of CSCs in the progression as well as therapy resistance in colorectal cancer.

The Intestine and the Origin of Colorectal CSCs

Epithelial cells in the intestine have a lifetime of around 5 days and are continuously renewed by intestinal stem cells (ISCs) under the microenvironmental influence [23]. The components of this microenvironment include sub-epithelial stroma, adjacent epithelial cells, natural enteric flora and soluble epithelium-derived factors which construct a unique microenvironmental “niche” that directs genetically identical cell populations towards divergent behaviours [23, 24]. Thus, changes in niche components can lead to alterations and dysregulation of crypt behaviours, which in turn might involve in fostering malignancy.

The intestinal stem cells are located at the base of the mucosal crypt and undergo asymmetric division, giving rise to one identical daughter cell and one cell with the potential to differentiate into the intestinal cell, thereby maintaining tissue homeostasis and repair [1]. They are the prime suspects as the source of reliable of most, if not all, of colorectal cancers due to their pre-existing enhanced proliferative and self-renewal properties [25, 26]. This hyothesis is supported by the demonstration that deletions of adenomatous polyposis coli (APC) in intestinal stem cells (Leucine-rich repeat-containing G protein-coupled receptor 5 ($Lgr5^{+}$) rich promoted adenoma

formation in the small intestine in mice [27]. In addition, $Lgr5^{+}$ intestinal stem cells are 20-fold more efficient in forming adenomas in comparison to that of Lgr5-poor cells of similar origin. The adenomas are mostly monoclonal and shared an organisational resemblance to the original cells [28]. Moreover, deletion of APC in intestinal stem cells of the small intestine can efficiently produce tumours in mice [29, 30]. Therefore, it is assumed that intestinal stem cells are the ideal target source for neoplastic transform cells, which lead to adenomas and carcinoma through additional genetic and epigenetic changes in the intestine. It is unlikely intestinal stem cells alone are capable of inducing cancer progression.

The origin of colorectal CSCs remains elusive, and it is still a matter of active debate amongst scientist whether they derived from intestinal stem cells having stemness during cancer formation or they are the direct progeny (differentiated cells) of mutated cells [31]. The identification of stem cells in majority of normal tissues including colon crypts favours the hypothesis that non-neoplastic stem cells could be the possible target for cancer transforming genetic (e.g. mutations) and epigenetic (e.g. DNA promoter methylation, small RNA-mediated gene silencing, etc.) alterations and the origin of CSCs. Since intestinal stem cells and colorectal CSCs share many features such as longevity and self-renewal, intestinal stem cells might be the potential source of CSCs. In addition, CSCs could derive from cells outside a tumour. For example, bone marrow-derived mesenchymal stem cells can serve as CSC's ancestors [32]. Therefore, colorectal CSCs may arise from (i) intestinal non-neoplastic stem cells by accumulating genetic and epigenetic alterations, (ii) de-differentiation of cancer cells or (iii) cells outside the tumour (Fig. 1) [35, 36].

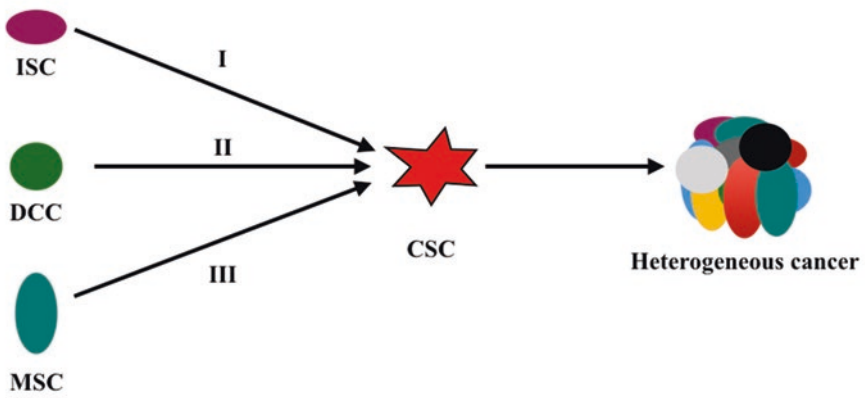

Fig. 1 Origin of cancer stem cells (CSCs) in colorectal carcinoma. Colorectal CSCs may arise from intestinal normal stem cells by accumulating genetic and epigenetic alterations (I), differentiated cancer cells by de-differentiation (II) and from cells outside the tumour such as bone marrow mesenchymal stem cell (III). The resulting CSCs can generate cancer cells of various phenotypic and genetic make-up, thereby producing heterogeneous cancer. ISC Intestinal stem cell, DCC Differentiated cancer cell, MSC Mesenchymal stem cell

Despite the origin, colorectal CSCs follow a trend of constant increase during cancer progression, generating increasingly competitive CSCs having more accumulating genetic and epigenetic alterations. During cancer initiation, competition occurs between CSCs and their non-neoplastic counterparts. In advanced cancer, CSC clones compete with each other, resulting in more aggressive clone emerging as the combined impacts of genetic, epigenetic alterations and environmental pressures, i.e. cancer therapy [33]. Moreover, in a highly competitive microenvironment of advanced cancer, cells may raise their survival chances by increased proliferation as well as other mechanisms. These mechanisms include increasing genetic and epigenetic drift and engaging in the mechanism of segregation, thereby creating metastatic CSCs [34].

Colorectal CSCs: Phenotypic and Functional Characterisation

Colorectal CSCs have been implicated in the carcinogenesis albeit their correct identification and isolation is challenging due to the complexity of their biology and unresolved technical issues [37]. Several in vitro assays including sphere formation, colony formation, cell surface markers and Hoechst dye efflux properties may be used to detect CSCs in various cancers. The gold standard for identification of CSCs is the capacity of tumour formation as a xenograft in immune-compromised mice in serial dilution. Many available methods such as functional assays, surface marker-based assays, etc. for detection and isolation of CSCs have potential pitfalls, thereby limiting their applications in detecting and profiling CSCs in patients with cancer [3].

Human colorectal CSCs were first identified and detected using CD133 (also called prominin-1) as cell surface marker [16, 17]. These CD133-expressing cells are capable of regenerating tumours in mice that resembled original cancer. However, the significance and applications of CD133 as a specific CSCs marker for colorectal cancer has subsequently disputed [38]. Later on, other surface and intracellular markers for colorectal CSCs have been identified (Table 1), which in turn described several phenotypes of CSCs in colorectal cancer. Nonetheless, the markers identified for CSCs are also expressed by intestinal stem cells, hence preventing their potential applications as therapeutic targets. In 2013, doublecortin-like kinase 1 (DCLK1) was identified as the CSCs specific marker as intestinal stem cells did not express the marker [39]. Intracellular DCLK1 efficiently can distinguish a tumour and normal stem cells in the intestine. However, its intracellular localisation further limits its use, as antibody targeting intracellular antigen is often non-specific.

Methods currently employed to identify colorectal cancer stem cells have various issues. Firstly, the sensitivity of markers may be affected by the instability of CSCs in colorectal cancers. For example, CSC populations that are LGR5 positive and LGR5 negative can interconvert followed by chemotherapy [33, 40]. Furthermore, cytokines released by tumour-associated cells can induce enhanced self-renewal

Table 1 Markers used to identify CSCs in colorectal cancer

Name of markers	Method used to identify markers	References
CD133	Xenotransplantation in immunodeficient mice	[17]
Lgr-5	Tumorigenicity assay; experimental metastasis assay	[108]
CD24	Colony formation assay; invasion assay; xenotransplantation in immunodeficient mice	[4]
CD29	Xenotransplantation in immunodeficient mice	[4]
ALDH-1	Xenotransplantation in immunodeficient mice	[109]
EpCAM	Immunohistochemistry; Western blot assay	[110]
CD44	Xenotransplantation in immunodeficient mice; colony formation assay	[4, 110]
CD166	Tumour growth in immunodeficient mice following xenograft; colony formation assay	[4]
CD26	Tumour formation and metastasis following xenotransplantation	[61]

and reprogramme trans-amplifying progenitors to CSCs [15, 41–43]. Thus, the number of CSC or proportion of cells expressing CSC markers may vary, depending on tumour stage, type and timing of therapy and many microenvironmental (e.g. oxygen, nutrients and extracellular matrix conditions) and individual factors. Therefore, in a dynamic state of cancer progression, CSCs vary in quantity and phenotype.

Molecular and functional characteristics of the CSCs could be used to identify CSCs in addition to the phenotypic features. Hyper-activated β-catenin pathway is the molecular standpoint of CSCs, which in turn confer the ability to generate tumours in serial dilution in mice [42]. The transcriptional DNA-binding protein inhibitors 1 and 3 (ID1 and ID3) regulate self-renewal properties of colorectal CSCs [44]. In addition, transcriptional regulator Polycomb complex protein (BMI-1) plays key roles in self-renewal of colorectal CSCs. Inhibition of BMI-1 induces loss of stemless and impaired tumour formation [18]. Finally, molecular tracking studies provided important insights into the functional properties of colorectal CSCs in an in vivo setting [45]. These studies revealed the existence of multiple types of colorectal CSCs with different roles in cancer initiation, progression, maintenance and metastasis formation.

Role of CSCs in the Pathogenesis of Colorectal Carcinoma

CSCs, which are also known as tumour-initiating cells, are heterogeneous and are highly tumorigenic. The colorectal CSCs rely on different pathways including the WNT pathway, the BMP pathway, the Notch pathway, etc. to maintain their stemness and to contribute tumour progression [2, 19]. CSCs mediate cancer pathogenesis by driving the fundamental processes, i.e. cell proliferation, growth,

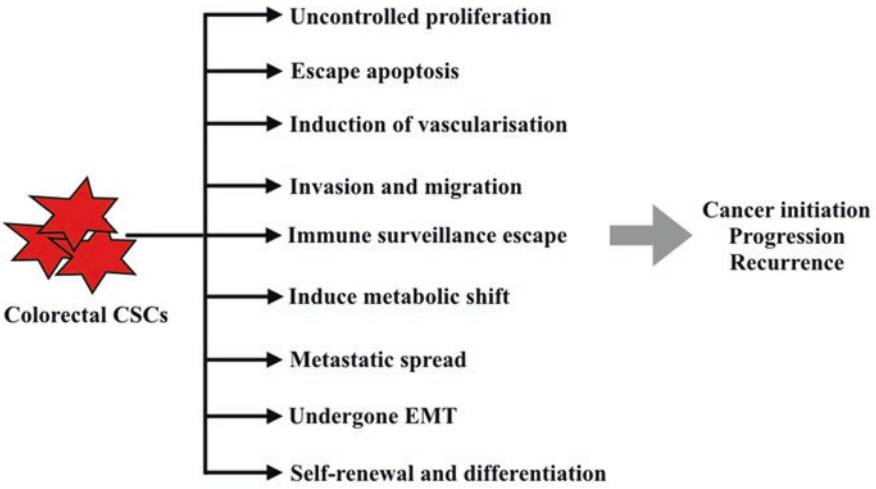

Fig. 2 Roles of cancer stem cells (CSCs) in pathogenesis of colorectal carcinoma. CSCs regulate different hallmarks such as uncontrolled proliferation, invasion, metastasis, escaping apoptosis and so on in various cancers including colorectal carcinoma (CRC). Alterations of these key processes lead to cancer initiation, progression, therapy resistance and thereby cancer recurrence

angiogenesis, invasion, metastatic dissemination and therapy resistance [2, 19]. The potential roles of colorectal CSCs in cancer progression are illustrated in Fig. 2.

CSCs in Initiation of Colorectal Carcinoma

Genetic and epigenetic alterations in adult stem cells and their progenitors or normal differentiated cells create CSCs, which disrupt the tightly controlled self-renewal and differentiation processes, thereby bypassing protective mechanisms in cells. This process leads to uncontrolled proliferation and escape of apoptosis of cells resulting in cancer [46, 47]. Dysregulation of key signalling pathways, including WNT/β-catenin, BMP and Notch pathways in colorectal CSCs, induces uncontrolled proliferation and self-renewal, resulting in intestinal polyp, adenoma and increased tumour formation in mice [33]. For example, activation of transcription factor GATA6 represses *BMP* gene expression which in turn activates WNT/β-catenin signalling, thereby promoting colonic CSCs expansion and self-renewal, resulting in neoplastic colon [48, 49]. In addition, colorectal CSCs are able to form large lumen-containing colonies consisting of three types of differentiated colonic epithelial cells in a three-dimensional culture system [48]. Purified single CSC from these colonies can reconstitute the lumen and can reproduce the tumours in immunodeficient mice [48]. This result indicated that a single CSC may expand and differentiate into a big tumour. Furthermore, multiple CSC subpopulation may arise

during tumour progression by the accumulation of additional epigenetic and genetic alterations and by microenvironmental influence. These new CSC populations may exhibit more aggressive growth potential, drive cancer progression and lead to the sustained malignancy.

CSCs in Vasculature of Colorectal Carcinoma

Tumour vascularisation includes angiogenesis (formation of new blood vessels) and lymphangiogenesis (formation of lymphatic vessels). They are critical for tumour growth, maturation and metastatic spread [50, 51]. For example, $CD133^+$ colorectal CSCs promote in vivo vessel formation and survival instead of original vessel ablation [52]. In addition, these $CD133^+$ colorectal CSCs are resistant to the anti-angiogenic therapies [52]. Therefore, colorectal CSCs induce tumour vessel formation in mice. CSCs increase vascular formation by enhanced secretion of a pro-angiogenic and lymphangiogenic factor and vascular endothelial growth factors (VEGFs) in a tumour, thus paving the way for the tumour to survive and advance using newly generated blood and lymphatic vessel [50, 51]. In addition, CSCs have the potential to induce angiogenesis and can produce angiogenic cells, whereas the differentiated non-CSCs are non-angiogenic [53, 54]. The CSCs in tumour microenvironment interact with the vascular niche and promote angiogenesis through the secretion of VEGF, stromal-derived factor 1 (SDF-1) and tumour microvesicles. The microvesicles act a cargo of pro-angiogenic mRNA and microRNA, which in turn confer the angiogenic phenotype to the normal human endothelial cells and stimulating their growth and vessel formation. Therefore, microvesicles in CSCs trigger the angiogenic switch and coordinates spread of cancer cells.

CSCs in Metastasis in Colorectal Carcinoma

Cancer metastasis is associated with approximately 90% of cancer-related mortality [55]. As compelling evidence suggested that CSCs are the only cells that can propagate cancer, it can be extrapolated that these cells can succeed in forming new tumours at distant sites [56, 57]. In the process, cells must be able to detach from the primary tumour, invade, access and survive in the circulation, disseminate at distant sites, transmigrate across the endothelial lining of the target tissue and form secondary tumours [58]. During these events epithelial to mesenchymal transition (EMT), a process in which polarised epithelial cells are converted to the motile mesenchymal cells, is very crucial and is involved in cancer cells dissemination and distant metastasis [59]. Interestingly, CSCs can interconvert themselves between epithelial to mesenchymal phenotype by acquiring EMT properties and can mediate the metastatic processes [60]. Moreover, with the acquisition of EMT, CSCs attain increased capacity for migration, resistance to apoptosis, enhanced production of extracellular

matrix-degrading enzymes and higher invasiveness, which in turn facilitate the metastatic spread of cancer.

Subpopulations of cells present in a primary tumour endowed with quiescence, resistant to therapies and extensive self-renewal capacity, i.e. possessing CSC phenotype, are responsible for metastases in colorectal cancer [18, 45]. For example, colorectal CSCs expressing CD26 were associated with the development of metastases in CRC [61]. In addition, CSCs in colorectal cancer expressing thrombopoietin receptor (CD110) and CUB-domain-containing protein 1 (CDCP1) are involved with metastasis to the liver and lung, respectively [62]. Moreover, it is evident that in colorectal cancer, CD44v6-expressing colorectal CSCs initiate the metastatic process [15]. Mechanistically, colorectal CSCs expressing CD44v6 display EMT phenotype and contributed to the enhancement of cell motility and invasiveness [15]. Thus, CSCs with EMT acquire the characteristics such as loss of the polarity, cell-cell adhesion and gain of migratory and invasive properties, which promote cancer metastasis, resulting in tumour progression.

CSC in Escaping Apoptosis in Colorectal Carcinoma

Cancer cell encounters a physiologically ubiquitous cellular programme: apoptosis aims to eliminate damaged or abnormal cells, during development. Thus, it is instrumental for cancer cells to acquire mechanisms to bypass programmed cell death [63]. CSCs have been shown to be critical for maintaining tumour growth and have been implicated in treatment resistance and tumour progression [2, 18]. Escaping apoptosis is one of the hallmarks of CSCs as they are highly resistant to undergo cell death including apoptosis in response to microenvironmental influence or cytotoxic insults [2, 18, 19]. CSCs adopt various strategies to override apoptosis including activating the anti-apoptotic machinery and inactivating the pro-apoptotic programmes [2, 19, 64]. For example, colorectal CSCs override apoptosis and survive the cytotoxic insults by inducing the expression of transcription factor, specificity protein 1 (Sp1) [65]. In addition, enriched CSCs isolated from colon cancer cells escaping apoptosis and engaged in enhanced proliferation by activation of epidermal growth factor (EGF) signalling pathway [66]. CSCs can also promote progression of various cancers including colorectal cancer by escaping immune surveillance, dysregulation of cellular metabolism and inducing genomic instability. For example, in regard to cellular metabolism, CSCs exhibited more glycolytic metabolism over oxidative phosphorylation, a phenomenon called “Warburg effect” when compared to that of non-CSCs cancer cells [67]. Altogether, CSCs in colorectal carcinogenesis can modulate key hallmarks of cancer, including uncontrolled proliferation, undergoing EMT and metastasis, escaping apoptotic death and increased vascularisation, thereby regulating cancer initiation and progression.

Roles of CSCs in Colorectal Carcinoma Therapy Resistance

CSCs are responsible for therapy resistance and cancer relapses due to their stemness [68–71]. The differentiated tumour cells undergo apoptotic death followed by chemotherapies, whereas the CSCs exhibited resistance to the process of apoptotic cell death [72]. In addition, the surviving CSCs fraction can re-establish the culture and contribute to cancer recurrence [72]. Moreover, CSCs had shown therapy bypass in an animal model of different cancers. The numbers of xenotransplanted CSCs increased in mice after exposed to chemotherapy [73, 74]. Furthermore, CSCs confer resistance to radiotherapy in colorectal cancer due to preferential activation of DNA damage checkpoints [70, 72, 75]. A number of studies demonstrated that colorectal CSCs displayed intrinsic properties of therapy resistance and associated with cancer regeneration and relapse after conventional therapy [43, 72, 73, 76]. The underlying mechanisms of therapy resistance of colorectal CSCs are:

Activation of Gene Signalling Pathways

Activation of signalling pathways such as Hedgehog, Notch, TGF-β, Wnt/β-catenin, SUMO, MET, etc. in colorectal CSCs has been implicated in the attribution of therapy resistance in colorectal cancer [66, 77–81]. Current conventional adjuvant treatment strategies to cancer are designed to target and eliminate all the differentiated cancer cells within a cancer. However, these treatments fail to detect and kill the CSCs which result in therapy escape/resistance and thereby cancer recurrence [81]. In principle, pharmacological inhibition of these pathways or their components increased the therapy sensitivity of CSCs. For example, exogenous overexpression of miR-140-5p (a regulator of Smad2 and the element of TGF-β signalling pathway) inhibited colorectal CSCs proliferation in vitro and nullified the tumour formation capacity in mice [78]. Inhibition of SUMO pathway via E1 and E3 SUMO inhibitors induces reduction of MMP14 and CD44 expression in colorectal CSCs, thereby functional loss of CSCs [77]. In addition to these pathways, other signalling cascades such as PI3K/Akt/mTOR, JAK/STAT and BMI1 also contribute to the therapy-resistant properties of CSCs.

Phenotypical Changes via Drug Resistance

Colorectal CSCs undergo multiple phenotypic changes, including overexpression of multidrug resistance (MDR) gene and G-glycoprotein transporter to acquire therapy resistance [82, 83]. For example, colon cancer cells expressing CD44 and ALHD1A2 (CSC markers) exhibited resistant to cytotoxic drugs by overexpressing G-glycoprotein transporters [83]. These cells also showed increased proliferation,

invasion and migration [83]. Overexpression of these proteins reduces or drains out the intracellular level of cytotoxic drugs from cells. Thus, CSCs can escape treatment by upregulation of drug transporters, which, in turn, lead to low or no drugs bioavailability inside the cells, resulting in the generation of therapy resistance.

Mechanisms Evade DNA Damage

Conventional radiotherapy and chemotherapeutic agents such as cisplatin, oxaliplatin (DNA cross-linkers), methotrexate (inhibitor of DNA synthesis), doxorubicin and daunorubicin (topoisomerase inhibitors) induce cancer cell death by damaging cellular DNA. Interestingly, CSCs can protect themselves by evading DNA damage caused by conventional therapeutics via multiple mechanisms, including (i) altering the cell cycle checkpoints, (ii) enhancing the capacity of DNA damage repair machinery and (iii) protection of DNA damage by an efficient scavenging of reactive oxygen species (ROS) [84]. For example, colorectal CSCs induced significant overexpression of DNA repair gene, *O(6)-methylguanine-DNA methyltransferase,* followed by DNA damage when compared to that of non-CSCs cells [85]. These cells also exhibited increased proliferation when compared to that of non-CSCs cells [85]. Moreover, CSCs from different cancers had shown resistance to genotoxic stress by low production of ROS and scavenging of already produced ROS after the therapy [86]. Activation of genes such as superoxide dismutase, glutathione peroxidase and catalase (responsible of ROS scavenging) in CSCs implied that CSCs escape DNA-damaging therapies by minimising the toxic insults of the treatments via scavenging ROS more efficiently [86]. Thus, high DNA damage repair activity and high free radical scavenging potentials aid in making colorectal CSCs resistant to cancer therapy.

Modification of Cell Cycle Phases

Current chemoradiotherapies to cancer target the highly proliferative S-phase cancer cells. As CSCs are slow growing and mostly quiescent, they survive off such treatment, whereas active proliferative cancer cells were eliminated. These quiescent, treatment-surviving CSCs re-enter the cell cycle and accelerate tumour regeneration through activation of cell growth and proliferative signalling pathways [82, 87]. For example, treatment of colon CSCs with 5-fluorouracil (5FU) induces a reversible quiescent G0 state. These quiescent cells overexpressed tyrosine kinase c-Yes that in turn confer the dormancy of colorectal CSCs [88]. In addition, colon CSCs enter the quiescent state via downregulating high-mobility group A1 proteins [89]. Therefore, designing and developing strategies targeting these quiescent and therapy-resistant CSC populations could improve the survival of patients with cancer.

Additionally, increased activation of detoxification enzyme aldehyde dehydrogenase, shifting the metabolisms, alterations of autophagy phenomenon, modulation of a tumour microenvironmental niche and impaired apoptotic pathways in CSCs of various cancers including colorectal cancer could contribute to their resistant to conventional treatment strategies.

Therapeutic Potential of CSCs in Colorectal Cancer

Current chemoradiotherapies only eliminate differentiated cancer cells but are insensitive to subpopulations of CSCs. This insensitivity can confer more complications to the patients during or after course of the disease by re-establishing cancers with more aggressive biological behaviour [90]. Thus, the development of therapeutic strategies targeting both differentiated cancer cells along quiescent CSCs has greater translational potential in a clinical setting for the better management of cancer. CSCs can be targeted by inhibiting self-renewal pathways, by interfering the components of anti-apoptotic or metabolic pathways, by activating differentiation pathways, by boosting anti-CSCs immunity or by acting on the protecting microenvironment [33].

Small molecules such as monoclonal antibodies or immunotoxins specific for the cell surface markers of CSCs have the potential to selectively eliminate the CSCs [90, 91]. Hence, the molecules targeting cell surface markers (e.g. CD133, CD44, CD26, CD29, EpCAM, etc.) could potentially eradicate CSCs and thereby increase sensitivity to therapies [14, 92–95]. For example, colorectal CSCs exhibited resistance to 5-fluorouracil and oxaliplatin by overproduction of cytokine IL-4 and escaped the apoptotic insults induced by the treatment [14, 96]. Importantly, these cells treated with 5-fluorouracil and oxaliplatin in conjunction with monoclonal antibodies to IL-4 as an adjuvant therapy remarkably augmented the antitumour activity of the treatments [14, 96]. Chemoresistant colon cancer (HT29 CD133+ and CD44+ fraction) cells showed increased expression of Type 1 insulin-like growth factor receptor (IGF-IR), and treatment of these cells with IGR-IR monoclonal antibody induced significant inhibition of tumour growth in a murine xenograft model [93]. Thus, targeting CSCs specific molecules can selectively eliminate CSCs and could improve the therapeutic outcome.

Treatment of patients with colorectal cancer by a monoclonal antibody against EpCAM (colon CSC marker) improved the cancer-free survival and prolonged the cancer remission in patients [97, 111]. Furthermore, treatment of colon cancer cells expressing CSC markers with antisense oligonucleotides, short hairpin RNA and/or natural products can target colorectal CSCs and inhibit tumour formation potential in vitro and in vivo [92, 94, 98]. Thus, development and application of strategies targeting cell surface markers of colon CSCs or their downstream signalling components in conjunction with conventional therapy have the potential to eradicate CSCs.

Inhibition of self-renewal pathways such as Notch, Wnt/β-catenin, TGF-β and Hedgehog by chemical intervention has increased the sensitivity of CSCs to chemotherapy [38]. For example, γ-secretase inhibitors inactivate Notch signalling and could be used to develop therapeutics for patients with colorectal cancer [99]. In colorectal cancer, Wnt/β-catenin pathways are constitutively activated; thereby inhibition of these pathways or its components is an important target for therapy development. Inactivation of this pathway or its components by small molecules or oligonucleotides caused inhibiting β-catenin accumulation and/or expression and disrupting its interaction with other components, thereby reducing colon cancer growth both in vitro and in vivo in mice [100, 101]. In addition, inhibition of Sonic Hedgehog by the treatment of cerulean, cyclopamine and itraconazole significantly induced apoptosis, decreased cell proliferation, inhibited spheres formation and reduced the expression of stemness factors in colon cancer cells [102]. Thus, the effective repression of CSC activities in colorectal cancer by targeting key signalling pathways has significant therapeutic implications in patients with colorectal cancer.

Additionally, colorectal CSCs can be targeted by interfering the tumour protective heterogeneous signals from fibroblasts, myofibroblasts, adipocytes, mesenchymal cells, infiltrating immune cells and endothelial cells [103, 104]. Treatment of CSCs with differentiating agents such as all-trans retinoic acid, hexamethylamine bisacetamide, dimethylsulfoxide, suberoylanilide hydroxamic acid, etc. has proven to induce cellular differentiation, resulting in effective CSC elimination by conventional therapies [105–107]. Furthermore, CSCs can be eliminated selectively in various cancers by adopting CSCs specific immunotherapy according to the CSCs associated tumour antigens. Thus, therapeutic options target CSCs via inhibiting self-renewal pathways or their components, interfering the components of anti-apoptotic or metabolic pathways by small molecules, inducing differentiation of CSCs by therapeutic agents, boosting anti-CSCs immunity by adoptive and/or cellular immunotherapy and disrupting the protecting microenvironment by therapeutic agents. These combined approaches against CSCs may improve the management of patients with cancers.

Concluding Remarks

The CSCs model for the pathogenesis of haematological and solid cancers continues to evolve, and accumulating information from the recent standpoint of cancer researches implied that cancer is driven by CSCs subpopulation. However, the current conventional chemoradiotherapies to cancer target the differentiated cancer cells; thereby CSCs may remain untouched, resulting in cancer recurrence. Thus, development of therapeutic strategies based on a combination of conventional therapies targeting non-CSCs along with CSCs specific pathways or their component has a higher potential to improve therapeutic outcome. However, the challenges remain

for specific identification of CSCs in different cancers including colorectal cancer as almost all markers of CSCs occur in normal intestinal stem cells. Therefore, specific identification of CSCs in colorectal cancer based on their surface markers could help in isolation as well as predicting of aggressive clinical behaviour, resistance to therapy, detection of cancer recurrence, survival predication and in the development of advanced cancer therapies. In addition, newly identified CSC markers in colorectal cancer in combination with the existing markers could help in therapy selection and in optimising the post-treatment surveillance of patients with colorectal cancer.

It is worth noting that a number of emerging therapeutic tools based on specific properties and functions of CSCs in colorectal cancer could be useful in a clinical setting and have the potential to improve clinical outcomes. The combination of CSCs targeted therapies in conjunction with other conventional anti-cancer therapies such as chemotherapy, radiation, molecular targeted therapy and immunotherapy, etc. may improve the management of patients with colorectal cancer. Thus, in-depth understanding of the biology, function, identification and clinical implications of CSCs in colorectal cancer is imperative to develop effective therapeutic modalities for the patients with colorectal cancer.

Conflict of Interest None

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The Role of Stem Cells in the Treatment of Anal Fistulas

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Introduction

Perianal Fistula

The (peri)anal fistula or fistula-in-ano is defined as an abnormal channel between the anal canal and the surrounding structures, usually the perianal skin. Idiopathic perianal fistulas are of cryptoglandular etiology, originating from infection of the anal glands [1, 2]. They represent a form of chronic anorectal sepsis as a result of an initial perianal abscess that had been previously spontaneously or surgically drained [3]. Clinically, they present with purulent drainage or periodical pain [1, 2]. The incidence of the disease is reported to be between 12 and 28 cases per 100.000 people with a greater incidence in men (12.3 per 100.000) than in women (5.6 per 100.000) [4].

The most common types of perianal fistulas are the idiopathic, cryptoglandular fistulas (about 90–95% of perianal fistulas), fistulas related to Crohn's disease (about 1.5% of perianal fistulas), and traumatic or iatrogenic fistulas (about 3.5% of perianal fistulas) [5]. A specific type is the rectovaginal fistula which when affecting women with Crohn's disease remains an entity very difficult to treat. There are also several other causes of perianal fistulas such as fungal infection, mycobacterial infection, and neoplasms [6]. A cryptoglandular anal fistula arises from infection of the Desfosses-Hermann glands; these are distributed alongside the circumference of

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the dentate line [7]. Perianal fistulas often complicate patients with Crohn's disease during the course of their lives. It has been estimated that 13–38% of patients with Crohn's disease may be affected especially those with colonic and anal involvement usually suffering of drainage, pain, and fecal incontinence [7, 8].

The most frequently used anatomical classifications are the Parks classification that classifies the fistulas according to their relationship to the anal sphincter and the AGA classification categorizing them as simple or complex (Tables 1 and 2) [9, 10].

Pelvic magnetic resonance imaging (MRI) is considered to be the gold standard for anal fistula imaging. T2-weighted sequences are essential in order to identify abscesses and fluid content into the fistula tract. The use of gadolinium could be useful to differentiate the tissues and the inflammatory masses [11, 12]. MRI is also used to monitor the results of therapy. Endoanal ultrasound (EUS) remains a reliable alternative [13, 14]. Examination under anesthesia (EUA) is essential, especially for patients with perianal Crohn's disease [15].

Clinical measurements have been established for the assessment of the activity of perianal disease. The most commonly used Perianal Disease Activity Index (PDAI) estimates the severity of the perianal disease and takes patients' quality of life into consideration [16]. The Anal Disease Activity Index evaluates symptoms, such as the pain, and the Fistula Drainage Assessment measures the disease activity according to whether the fistula is open or closed (no drainage despite compression) [16, 17].

Treatment modalities for perianal fistulas include various surgical interventions and, as far as it concerns Crohn's fistulas, immunosuppressive agents and anti-tumor necrosis factor (TNF) biologics, such as infliximab [18–20]. All therapeutic approaches are often accompanied by relapses [18–20]. The most commonly used antibiotics are metronidazole and ciprofloxacin. However, no significant positive effects have been proven from their use at Crohn's perianal fistulas [21]. As far as infliximab is concerned, it seems to be effective not only for inducing fistula closure but also for maintaining remission [18–20]. Immunomodulator agents such as tacrolimus, thiopurines, and cyclosporine could be used as adjuvant therapy to infliximab and surgical procedures in order to improve results [20]. The monoclonal antibodies vedolizumab and ustekinumab are promising novel drugs [20].

Table 1 Parks classification of anal fistulas

Superficial	Fistula tract not crossing any sphincter or anus muscular structure
Intersphincteric (20–45%)	Fistula tract between the internal and external sphincter anal muscles, in the intersphincteric space, opening near the anus
Transsphincteric (30–60%)	Fistula tract between the internal and external sphincter anal muscles crossing the external anal sphincter into the ischioanal fossa
Suprasphincteric (20%)	Fistula tract between the internal and external sphincter anal muscles, continuing over the top of the puborectal muscle, penetrating the levator anal muscles before reaching the skin and opening near the anus
Extrasphincteric (2–5%)	Fistula tract outside the external sphincter anal muscles crossing the perianal skin, through the ischiorectal fat and anus levator muscles, into the rectum

Table 2 American Gastroenterological Association (AGA) classification of anal fistulas

Simple	Complex
Low lying	High (high intersphincteric or transsphincteric, suprasphincteric, and extrasphincteric)
Only superficial tissues or distal part of the sphincters (low intersphincteric)	Multiple openings
Single opening	Local complications (pain, abscess, extension to nearby structures, such as vagina or bladder and rectal stricture)
Absent perianal complications (pain or fluctuation)	Proctitis
Continence preserved and unaffected by therapy	

The surgical techniques should target to the eradication of local sepsis induced by the fistula, the permanent closure of the fistula tract while maintaining fistula continence. Fistulotomy with or without sphincterotomy is suggested for low, non-Crohn's single tract anal fistulas with high success rates reaching almost 100%, although there are several reports of incontinence [22, 23]. Seton placement is preferred for high transsphincteric fistulas and chronic drainage, but its use is rather limited to eliminate the recurrence of the sepsis and not for the definite cure of the fistula [24, 25]. Ligation of the intersphincteric fistula tract (LIFT) procedure has arisen as an effective technique first described by Rojanasakul [26]. A meta-analysis of 2014 patients showed a success rate of 76.5%, 0% incontinence, and 5.5% post-operative complications [27]. Moreover, advancement flap is a sphincter preserving method, which is mainly used for complex, high fistulas. Healing rate is estimated to be 57–90% [28]. The high incontinence rates and the lack of long-term healing success rates have led to the development of other noninvasive techniques. Fibrin glue injections have a reported success rate of 30–60% to properly selected patients [28]. Fistula plug and fistula laser closure have not proven to have the expected benefit supported by the initial reports [28].

Stem cell transplantation is one of the most vigorously investigated and promising techniques for the management of difficult to heal anal fistulas, such as the ones related to Crohn's anal disease. Not only their anti-inflammatory function but also their regenerative effects when they are locally injected make them a promising treatment for the perianal fistulas [29, 30].

Stem Cell Properties

Stem cells are unspecialized cells that have two main characteristics. Firstly, they have the ability of renewing themselves long time after being inactivated. Secondly, under certain conditions, they can differentiate to certain tissue or organ cells with special functions such as a muscle cell, a red blood cell, or a brain cell or just remain

a stem cell. Two categories of stem cells have been described; the embryonic and the adult or somatic stem cells. The second population is divided to hematopoietic stem cells, from which all the types of blood cells are formed and to mesenchymal stem cells (MSCs), which are the stem cells mainly used in fistula healing as is analyzed bellow [31].

MSCs can be isolated from bone marrow, adipose tissue, muscles, and umbilical cord; however, the most commonly used in fistula healing are the MSCs derived from bone marrow and adipose tissue [31]. Bone marrow is suggested as the chief source of MSCs extraction due to their several advantages. More specifically they are easily isolated and replicable, and they have major immunological properties. The risk for immunological rejection is low, the co-existence in the host is prolonged, and their differentiation ability is maintained [32]. However, the highly invasive procedure that is needed in order to obtain them and the fact that their number is being diminished by aging have reduced their use as a therapeutic choice [33]. Thus, other sources of MSCs and especially the adipose tissue have gained more popularity. The advantage of adipose tissue-derived MSCs (ADSCs) is that their number is significantly higher than the bone marrow stem cells (BMSCs) and can be harvested by minimally invasive procedures through liposuction. Both adipose tissue- and bone marrow-derived MSCs from the same donor seem to have the same immunomodulatory characteristics [34].

There are two main types of stem cells transplants, the Stem cells that are derived from a matched related or unrelated healthy donor and are called allogeneic, and the stem cells come from the patient himself, they are called autologous. The main advantages of the allogeneic cells are that their consistency is standardized, they can be administrated immediately, and they can be used for many patients. Furthermore, allogeneic MSCs are characterized by low immunogenicity, since they lack major histocompatibility complex class II (MHC II) and have poor antigen-presenting features [35]. However, their cost is higher than the use of autologous stem cells. On the other hand, the consistency of autologous stem cells varies among the patients, and their administration demands days or weeks due to the need of in vitro expansion. However, their property of eliciting an immune response is nonexistent [35].

From a pathophysiological standpoint, MSCs have been specifically used for the treatment of perianal fistulas, because of three special functions: epithelialization, angiogenesis, and immunomodulation. MSCs lead to neovascularization due to the secretion of vascular endothelial growth factor (VEGF), insulin-like growth factor (ILGF), keratinocyte growth factor (KGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF), and other paracrine growth factors, resulting to accumulation of fibroblasts and macrophages to the damaged tissue. Hence, collagen production and angiogenesis are stimulated promoting tissue repair [36–39]. Transplanting MSCs are an optimal method for the tissue reconstruction, since, under certain circumstances, they can differentiate into endothelial cells [40, 41]. The principal role of MSCs is the immunomodulation, via their interaction with monocytes, T cells, B cells, and natural killer cells. When MSCs contact with the activated T cells, they induce the production of IL-6 and IL-10 leading to the inhibition of monocytes to dendritic cell maturation [42]. The latter leads to a decrease of

the production of CD80, CD86, CD40, IL-12, IFN-γ, and TNF-a [42]. Moreover, IL-10 is responsible for the stimulation of human leukocyte antigen G5 (HLA-G5) [43, 44]. HLA-G5 reduces effector T cell proliferation and increases the population of T-regulatory antigen-presenting cells (TREGCs). These cells suppress the proliferation of CD8(+) T lymphocytes and change the profile of CD4 cells by decreasing Th1 and Th17, which play a central role in the pro-inflammatory response enhancing Th2 and thus, the production of IL-4 and IL-10 [45, 46]. On the other hand, due to the high level of pro-inflammatory cytokines and especially IFN-γ into the fistula tract caused by the bacterial colonization, MSCs are stimulated to express indoleamine 2,3-dioxygenase (IDO), an enzyme that metabolizes tryptophan to kynurenine, which is an anti-inflammatory factor [47, 48]. Furthermore, MSCs alter the inflammatory (M1) phenotype of macrophages into anti-inflammatory (M2) [49]. IDO attains an antiproliferative and suppressive role for B cells and NK cells [42].

Techniques

Isolation of Stem Cells

Various techniques have been applied for the preparation of ADSCs and BMSCs before their introduction into the fistula tract. As for the ADSCs, the first step is liposuction. The technique has been well described by Borowski et al. [50, 51]. The procedure is carried out under local or general anesthesia, and then a manual liposuction is performed. In the beginning, two small bilateral flank incisions approximately 0.5 cm are made. Approximately 200 ml of a mixture containing 1000 ml of normal saline solution, 2 ml epinephrine 1:1000, 50 ml of 1% lidocaine, and 1500 U of hyaluronidase is injected in order to allow tumescence of fat with minimal blood loss. A hollow blunt-tipped cannula is moved rapidly back and forward into the anterior abdominal wall in order to disrupt the fatty tissue and to obtain approximately 300–400 ml of raw lipoaspirate. For isolation of allogenic stem cells, the technique remains almost the same, but the cells are extracted from a healthy donor instead.

According to Yong Lee et al., after the isolation, the adipose tissue is digested in phosphate-buffered saline containing 1% bovine serum albumin and 0.025% collagenase for 80 min at 37 °C with intermittent shaking [52]. The isolated ASCs are then cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1 ng/mL basic fibroblast growth factor (bFGF), in order to obtain the required number of ADSCs for the treatment of the perianal fistulas. After harvesting via trypsinization, cells are suspended in DMEM and packaged into vials containing $3X10^7$cells per milliliter at 10–20 °C before use [52]. As Cho et al. argued about, the minimum criteria for release are for cell viability percentage of 80% and, for purity, less than 1% of CD45-positive cells [53, 54]. Moreover, Yong Lee et al. certified the expression of stromal cell-associated markers such as CD10,

CD13, CD29, CD44, and CD90 and the lack of either hematopoietic stem cell-associated markers, CD34 and CD45, or bone marrow-derived stem cell-associated marker (STRO-1) [52]. Additionally they tested them for contamination with adventitious agents, mycoplasma, bacteria, fungi, and viruses.

A similar technique for isolation and expansion of allogenic ADSCs has been described by Portilla et al. [55]. Following liposuction of subdermal adipose tissue from the healthy donor, ADSCs were isolated by digesting the adipose tissue with type I collagenase and centrifugation. Resuspension and lysis of the cell pellet that was obtained were followed by centrifugation, which lead to the creation of a stromal vascular fraction. That was placed in cell culture containers in culture medium and antibiotics and incubated at 37 °C and 5% CO_2. One to 2 days later, the culture medium was removed to eliminate the nonattached cell fraction. ADSCs adhered to the plastic culture plates were expanded under in vitro conditions. Every 3–4 days, the culture medium was changed after reaching 90–95% confluence, and the cells were detached with trypsin/EDTA, collected, centrifuged, and expanded without antibiotics. They were then harvested and cryopreserved until use. Before the appointed administration date, sufficient cryopreserved vials were thawed to provide the required dose for administration. ADSCs were recovered from their cryopreserved state by plating and culturing (to confirm viability). On the day when the vials were filled and packaged, the cultures were washed with phosphate buffer solution and trypsin/EDTA. The ADSCs were immediately resuspended in Dulbecco's modified Eagle's medium and human albumin serum, in order to formulate the drug product. Before use the ADSCs were characterized in terms of their phenotypic profile, purity, potency, morphology, viability, and cell growth kinetics [55].

Borowski et al. have described an innovative technique called ALFA technique (enhanced lipofilling for fistula-in-ano); the technique uses autologous adipose tissue-derived regenerative cells (ADRC) and has the great advantage of not needing in vitro expansion of the stem cell fraction [51]. The technique includes the transfer of 50 mL lipoaspirate into the tissue collection chamber of the Celution 800/CRS system. There the harvested adipose tissue is washed in order to remove free blood and lipid and then digested with the enzyme reagent Celase 835/CRS stimulating the release of the stromal vascular fraction (SVF) of the lipoaspirate. After its concentration the SVF is centrifuged and washed to obtain the ADRCs. Regarding the estimation for the requirements of the injection, the latest are then taken out of the automated cycle of the Celution system and mixed with the supernatant adipose tissue fraction of the remaining lipoaspirate [51].

On the other hand, in order to obtain the crucial number of BMSCs that is needed for the final administration, a more complicated procedure has to be accomplished. More specifically, BM-MSCs are derived by iliac crest aspiration under local anesthesia, and their preparation, according to Ciccocioppo et al., is approximately the same as the procedures described above, with one exception: 10% fetal calf serum is substituted by 5% human platelet-rich plasma, 50 ml of which are acquired to form a healthy donor and utilized for the BMSC expansion. After the expansion the BMSCs have to be suspended in a solution of sodium chloride and human albumin

20% and satisfy the requirements mentioned previously, as far as their phenotype and the microbial contamination are concerned [56]. Exactly the same technique for the use of allogeneic BMSCs has been described by Molendjik et al. [57].

Administration

The first step for the injection procedure is to clean the external opening, as well as to core out or perform a thorough curettage of the fistula track, in order to remove the inflamed surrounding tissues. Then the internal opening is closed with absorbable stitches, in order to avoid fecal contamination [58, 59]. The fistula tract is filled with a combination of stem cells and fibrin glue injected with a dual syringe system using a 1 ml syringe and 24 gauge needle [58, 59]. The purpose of fibrin glue use is to reassure that stem cells are maintained into the injection area. Fibrin glue was reported to increase cell transplant survival and enhance the biological function of transplanted cells [58]. Fibrin glue can act as a scaffold to hold stem cells in place and fill large defects with ADSCs after coring out and curettage of the fistula tract. Supporters of this procedure have claimed that a maximum of 30% of the ADSCs must be mixed with fibrin glue for the injection [59]. Suspension of stem cells in fibrin sealant has not been followed in some of the published case series [57, 60]. The ADSCs are then injected in the submucosa around the internal opening and all along the fistula tract. Another proposed way of administration is to inject the cell suspension into the tract walls, placing half of the total cell dose in the intersphincteric tracts and those adjacent to the internal opening and the other half in the tract walls in the direction of the external opening [55]. At the ALPHA technique as described by Borowski et al., the ADRC solution is injected employing a crisscross lattice technique into the fistulae and surrounding tissue, in order to achieve maximal tissue density and filling of all adjacent tissue spaces, while the external opening is obliterated by tissue bulking injection around its orifice [51].

In general the total volume of ADSCs injected is based on preoperative MRI measurement of fistula tract diameter and length [55, 58]. Intraoperative measurements are also feasible with the aid of a fistula probe and a ruler [58]. According to Choi et al., fistulas less than 1 cm in diameter were injected with 1 mL ADSCs per cm of fistula length, while fistulas with a diameter between 1 and 2 cm were injected with 2 mL ADSCs per cm of fistula length [58]. Exploring the efficacy of allogeneic ADSCs for the treatment of perianal fistulas in Crohn's disease, Park et al. followed a similar protocol of fistula diameter-directed administration; when the diameter of the fistula was 1 cm or less, approximately 1×10^7 cells/cm length (group 1) or 3×10^7 cells/cm length (group 2) were injected, and when the diameter of the fistula was between 1 and 2 cm, the cells were administered in two doses [59]. According to several experts' opinion, ADSCs are preferred over BMSCs due to their quality and quantity. One liposuction treatment may obtain enough cells to make the first injection in raw form on the same day of the extraction, and the second injection

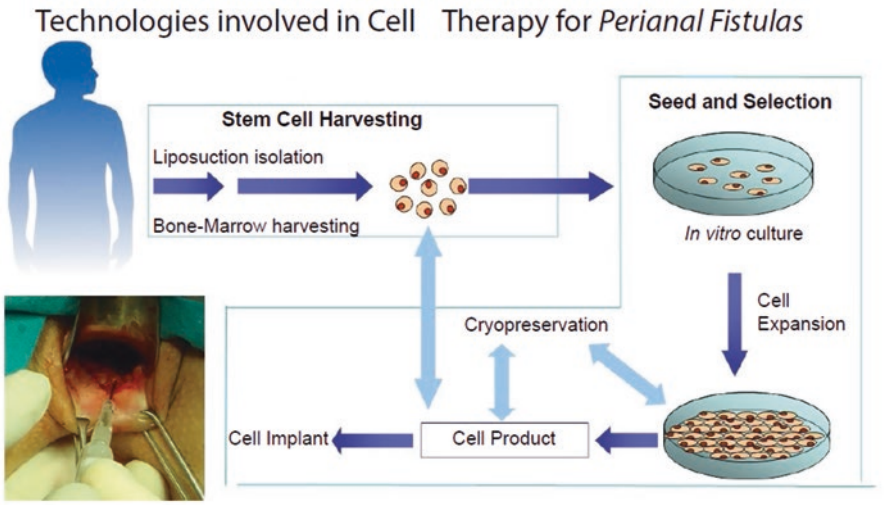

Fig. 1 Technologies involved in stem cell therapy for perianal fistulas. Cells can be harvested by isolation after liposuction or from bone marrow aspiration. Those cells can be injected in raw form after little manipulation. They can also be seeded in order to select specific MSCs based on their plastic adherent properties. Using "in vitro" culture technologies, MSCs can be expanded until they achieve the adequate quantity (millions). The final cell product can be cryopreserved or administered directly. (From Guadalajara et al. [61]. Reprinted with permission from "ClinMed International Library")

may use expanded ADSCs if needed; if not, they are cryopreserved [61]. MSCs technologies, treatment algorithms, and potentials are summarized in Fig. 1.

Results

The administration of stem cells was introduced as a novel treatment of perianal fistulas at the early onset of the twenty-first century. In 2005 García-Olmo et al. published a phase I clinical study with four patients with Crohn's disease who were treated with autologous ADSCs [62]. Among the eight inoculated fistulas that were followed up, six had their external opening covered with epithelium by the eighth postoperative week and were considered healed, while incomplete closure of the external opening with output decrease was achieved in the rest of the fistulas. Since no adverse events were observed, the authors concluded that their protocol was feasible and safe for the treatment of fistulas in Crohn's disease [62]. In their Spanish multicenter, randomized, active-controlled, open-label, add-on phase II clinical trial that is followed and conducted by the same group, 35 patients with idiopathic fistulas of cryptoglandular origin and 14 patients with Crohn's disease fistulas were assigned to either intralesional treatment with fibrin glue or fibrin glue plus 20×10^6 ADSCs [63]. Unlike their first study, which only used cells, in this study, cells were

co-administrated with fibrin glue. Fistula healing and quality of life were evaluated at 2 and 12 months. If healing had not been achieved at the 2nd month of follow-up, a second double dose of ADSCs with fibrin glue was administered. Patients treated with the combination regimen of ADSCs demonstrated significantly higher healing rates (71%) compared with the ones that were treated with fibrin glue only (16%), while the proportion of patients with healing was similar in Crohn's and non-Crohn's subgroups. Quality of life scores were also higher in patients who received ASCs than in those who received fibrin glue alone. At 1 year follow-up, the recurrence rate in patients treated with ADSCs was 17.6%. Again both treatments were not related to any serious adverse events with almost no risk of incontinence. The authors supported that the administration of expanded ADSCs ($20–60 \times 10^6$ ADSCs) in combination with fibrin glue was an effective and safe treatment for complex perianal fistulas achieving higher healing rates than fibrin glue alone [63]. In the retrospective analysis of this phase II results, that was published 3 years later, the same group found that only 7 of the 12 patients treated with ADSCs plus fibrin glue and who were included a follow-up of average 40 months remained free of recurrence and, actually irrespective of the MRI findings, which did not necessarily correlate with the clinical status of patients [64]. Long-term follow-up also reaffirmed the very good safety profile of the treatment. Nevertheless, a low proportion of the ADSC-treated patients with closure after the initial procedure remained free of recurrence after more than 3 years later [64].

A larger trial phase III multicenter, randomized, single-blind trial (FATT 1: Fistula Advanced Therapy Trial 1) by the same group separated 200 patients from 19 Spanish institutions into three groups with the first one receiving 20×10^6 ADSCs, the second one 20×10^6 ADSCs plus fibrin glue, and the third one only fibrin glue [65]. If the fistula had not healed at 12 weeks, a second dose (40×10^6 ADSCs in the first and second group) was administered. The outcomes at 12 weeks after therapy administration (healing rates: 26.56%, 38.33%, and 15.25%, respectively, for the three assigned treatments; $p = 0.01$) were comparable to the previous phase I and II studies, and significantly higher healing rates were reported in the groups receiving ADSCs versus fibrin glue alone. At 24–26 weeks (after a second dose if applied), the healing rates were still good (39.01%, 43.3%, and 37.3%), but there were no statistically significant differences between groups. Given that the risk of anal sphincter injury is almost negligible, these healing rates were still encouraging, but because healing at 24–26 weeks was the primary outcome measure, the higher efficacy of ADSCs in comparison with the control treatment was not finally established [65]. It has to be pointed out, though, that the results of the technique's pioneer center were definitely better than the rest of the study's participating institutions and healing rates actually differed significantly between groups, with the combination of ADSCs and fibrin glue achieving a healing rate of 83.33% versus 54.55% (ADSCs alone) and 18.18% (fibrin glue alone) at 24 and 26 weeks [65]. No serious adverse events were reported at this series either [65]. A point of importance for the aforementioned Spanish multicenter study was the fact that patients with inflammatory bowel diseases were excluded and, as a result, the study population was consisted of complex, conventionally untreated cryptoglandular fistulas.

Apart from the studies derived from Spain [62–66], two additional groups, one from the United Kingdom and one from Korea, have published results of small case series including non-Crohn's patients [50, 51, 58]. Initially, Borowski et al. reported the successful treatment of three consecutive patients with long-standing cryptoglandular anal fistula with a novel combination of mucosal advancement flap and ADRCs from the SVF obtained from a simple lipoaspiration procedure, using Celution technology [50]. There was no operative morbidity, and the one patient who had a colostomy for fecal diversion succeeded to have his bowel continuity restored. All fistulas remained healed at 2- to 3-year follow-up [50]. Later, the same group of researchers, using a similar technique in seven patients with complex cryptoglandular fistulas, succeeded and sustained healing at four of them at a follow-up of almost 4 years [51]. In a phase II Korean clinical trial, where 13 non-Crohn's patients were included, complete closure was achieved in 9 of them, 2 months after the injection of ADSCs, whereas a persistent response was maintained in 7 of them at 6 months reexamination [58]. Published series and results on MSCs local administration for cryptoglandular fistulas treatment are depicted on Table 3.

As expected most of the interest for the therapeutic effect of MSCs local application has been attracted in the context of the more difficult to treat and most resilient to pharmaceutical and conventional surgical interventions Crohn's disease-related anal fistulas [52–57, 59, 62–64]. In an Italian study focusing on Crohn's fistulas refractory to or unsuitable for currently, BMSCs were isolated and expanded ex vivo [56]. Sustained complete closure in seven cases and incomplete closure in the rest of the three fistula tracks with a parallel reduction of Crohn's disease and perianal disease activity indexes as well as rectal mucosal healing were induced by intrafistular BMSC injections without any adverse effects [56]. The percentage of mucosal and circulating regulatory T cells significantly increased during the treatment and remained stable until the end of follow-up. No case of persistence or recrudescence of draining fistulas was observed during the entire 12-month follow-up period [56]. That may be important since fistulizing Crohn's disease carries a high relapse rate despite the large therapeutic armamentarium.

Modestly encouraging results were obtained from a Spanish multicenter open-label, single-arm clinical trial, where allogenic stem cells were used to refractory Crohn's anal fistulas [55]. Allogenic MSCs are easily obtained from a healthy donor providing a product accessible to more patients and avoiding the need of collecting primary material from patients. Thus, an easily available treatment can be rapidly administered from a completely validated cell bank and provides an economically affordable therapy to large numbers of candidate patients. In this study, efficacy analysis revealed that almost 70% of the patients demonstrated reduction in the number of active fistulas, while one-third of the cases presented complete closure of all fistula tracts [55]. MRI scores of severity showed significant differences at 3 and 6 months postinjection [55]. On the other hand, when autologous ADSCs were injected at various concentrations based on fistula size, at a Korean phase I clinical trial, only 30% of patients achieved a sustained healing at 8 months [53]. In the phase II trial that is followed and conducted by the same group, the results were better, with healing rates reaching 80% at 2-year follow-up [54].

Table 3 Published series investigating the role of stem cells for the treatment of anal fistulas of cryptoglandular origin

Authors (year) Country of study	Type of study	Interventions (number of patients)	Healing rate (follow-up)	Recurrence rate (follow-up)	Adverse events
Garcıa-Olmo et al. (2009) [63] Spain	Phase II, open label, double arm, randomized	20×10^6 autologous ADSCs plus fibrin glue (17 patients) vs fibrin glue alone (18 patients); second dose of 40×10^6 ADSCs if no healing at 2 months	71% vs 17% (2 months)	17.6% (12 months)	15 nonserious; 4 serious (1 related to ADSCs: perianal abscess)
Herreros et al. (2012) [65]	Phase III, multicenter open label, double arm, randomized	Group A: 20×10^6 autologous ADSCs (64 patients) vs Group B: 20×10^6 ADSCs plus fibrin glue (60 patients) vs Group C: fibrin glue (59 patients)	Group A: 39.1% vs Group B: 43.3% vs Group C: 37.3% (6 months)	Group A: 0% vs Group B: 6.6% vs Group C: 0% (12 months)	Group A: 90.8 % vs Group B: 84.5% vs Group C: 85%
Spain			Group A: 57.1% vs Group B: 52.4% vs Group C: 37.3% (12 months)		Most nonserious; proctalgia (43.7%), abscess drainage (22.4%), pain (13.7%), perianal abscess (13.1%), pyrexia (9.3%), swelling (6.6%), pruritus (6.6%))
Borowski et al. (2012) [50] United Kingdom	Single arm	Autologous adipose tissue-derived regenerative cells (ADRC) (3 patients)	100% (12 months)	0% (12 months)	N/R
Garcia-Olmo et al. (2015) [66]	Single arm observational	Autologous ASDCs (7 patients)	71.5% (2 months)	N/R	None
Spain			57.1% (12 months)		

(continued)

Table 3 (continued)

Authors (year) Country of study	Type of study	Interventions (number of patients)	Healing rate (follow-up)	Recurrence rate (follow-up)	Adverse events
Borowski et al. (2015) [51] United Kingdom	Single arm observational	Autologous adipose tissue-derived regenerative cells (ADRC) (7 patients)	71.4% (6 months) 57,1% (46 months)	0.14% (10 months)	None
Choi et al. (2017) [58] Korea	Phase III, multicenter open label, double arm, randomized	Group 1: 10×10^6 autologus ADSCs (5 patients) vs Group 2: 10×10^6 autologus ADSCs (6 patients); second dose of twice the initial concentration administered if no healing at 2 months	Group 1: 60% vs Group 2: 75% Overall: 69.2% (2 months), 83.3% (6 months)	N/R	No grade 3 or 4 adverse events; postoperative pain (26.67%), fever (20%), chills (13.33%), anal hemorrhage (13.33%), anal pain (13.33%), perianal abscess (13.33%)

The largest and more meaningful, at least methodologically, study performed up to now is the multicenter phase III, double-blind, parallel-group, placebo-controlled, randomized trial by Panés et al. published in 2016 [67]. Two hundred and twelve patients were randomly assigned to either expanded allogeneic ADSCs single injection or placebo. The analysis of their results showed that for the difficult, unresponsive to other treatments complex Crohn's perianal fistulas, 50% of the patients treated with ADSCs injection alone or added on to current medical treatment achieved a combined remission at 6 months in contrast to the 34% of those who received the placebo injection [67]. This result was consistent across all statistical populations, although more patients in the ADSCs group than the placebo group had more than one fistula tract. The ADSCs treatment was well tolerated; the most commonly reported adverse events were proctalgia, anal abscess, and nasopharyngitis, while most of them were mild or moderate in intensity [67]. The authors pointed out that local ADSCs treatment added on to established treatments for Crohn's disease might open new therapeutic options for refractory perianal disease [67].

In 2017, Dietz et al. at a phase I clinical trial investigated the effect of autologous ADSCs transplantation with an absorbable matrix (Gore Bio-A Fistula Plug) on 12 patients with anal fistula associated with Crohn's disease [68]. The results of this study showed that 10 of 12 patients (83%) had complete clinical recovery at 6-month follow-up, and consequently, the safety and efficacy of ADSCs in treatment of anal fistula was once again emphasized [68]. This study along with the satisfactory results derived from the combination of ADSCs with fibrin glue may guide to conclude that MSCs scaffolding may be superior to their just direct injection. Therefore,

a scaffolding material, such as the fibrin glue or the fistula plug may be useful in keeping MSCs at the delivery site, leading to improved fistula healing rates (71% and 83%, respectively) compared to MSCs direct injection alone (50%). Published series and results on MSCs local administration for Crohn's perianal fistulas treatment are depicted on Table 4.

A recently published meta-analysis explored the effect of stem cells, either systemically or locally injected, on management of Crohn's perianal fistulas [69]. For the nine included studies that reported data on outcome after local MSCs injections, the random-effects pooled rate of fistula healing was 60% (95% CI: 44–75, $n = 203$), while the respective percentage after systemic stem cells treatment was 29% (95% CI: 3–85, 2 studies, $n = 24$) [69]. Patients treated with autologous MSCs, either local or systemic, had a pooled rate of fistula healing of 62% (95% CI: 44–77, 9 studies, $n = 113$) compared to 47% (95% CI: 33–61, 4 studies, $n = 138$) for patients who underwent allogeneic MSCs treatment [69]. Putting all current data together and aiming to better understand the potential befits of this emerging therapy, Lightner et al. performed a systematic review and meta-analysis of all phase I, II, and III clinical trials using MSCs to treat perianal Crohn's disease [70]. They sought to determine their safety, as long as their short-term (<6 months) and long-term (>6 months) efficacy. Among the 11 included studies, 7 were phase I, 3 were phase II, and 1 was a phase III randomized controlled trial (Table 4). Eight used autologous MSCs, and three used allogeneic cells. Nine studies used ADSCs and two used BMSCs. Six studies defined healing on the basis of a clinical end point of cessation of drainage and/or epithelization of the external opening, and five included MRI into the definition of fistula healing [70]. Healing rates ranged from 27% to 83%, with more than half of all study patients achieving healing at the primary end points of the studies. The most common adverse events were perianal pain and perianal abscess, but no significant differences were observed at both groups of interventions, and none were related to the MSC product. So, there were no significant increases in adverse events [OR = 1.07 (95% CI: 0.61–1.89); $p = 0.81$] when MSC and non-MSC cohorts were compared. MSCs were associated with improved healing when compared with control subjects at 6–24 weeks [OR = 3.06 (95% CI: 1.05–8.90]; $p = 0.04$) and 24–52 weeks [OR = 2.37 (95% CI:0.90–6.25); $p = 0.08$] [70]. According to this review, up to now, far more patients have been treated with allogeneic ($n = 269$) as compared with autologous ($n = 96$) MSCs, largely because of the ability to widely distribute across sites and patients without the need for a complex infrastructure to generate an autologous product for each patient [70]. Similarly, the use of ADSCs ($n = 334$) has far exceeded BMSCs ($n = 31$); this is likely related to the ease of adipose tissue harvest as compared with bone marrow harvest, rather than any suspected difference in efficacy [70].

MSCs may be a promising adjunct for treatment of rectovaginal fistulas. Actually, the first report on the use of stem cells in treatment of anal fistulas was on a successfully treated recurrent rectovaginal fistula in 2003 by Garcıa-Olmo et al. [71]. In this case report, autologous ADSCs of a Crohn's patient were used. The results of this case report indicated that 3 months after cell transplantation, the fistula was closed and no excretion of gas or stool from the vagina was seen. Apparently, the ADSCs

Table 4 Published series investigating the role of stem cells for the treatment of anal fistulas at patients with Crohn's disease

Authors (year) Country of study	Type of study	Interventions (number of patients)	Healing rate (follow-up)	Recurrence rate (follow-up)	Adverse events	Previous/concomitant treatments
García-Olmo et al. (2005) [62] Spain	Phase I, open label, single arm	10–30 × 10^6 autologous ADSCs (4 patients)	75% (2 months)	0% (2 months)	None	Unsuccessful treatment by conventional surgery at least twice
García-Olmo et al. (2009) [63] Spain	Phase II, open label, double arm, randomized	20 × 10^6 autologous ADSCs plus fibrin glue (17 patients) vs fibrin glue alone (18 patients); second dose of 40 × 10^6 ADSCs if no healing at 2 months	71% vs 14% (2 months)	17.6% (12 months) (including Crohn's and non-Crohn's fistulas)	15 nonserious, 4 serious (1 related to ADSCs: perianal abscess)	Previously: seton placement or conventional surgery (advancement flap or fistulectomy) (100%), infliximab (100%)
Ciccocioppo et al. 2011 [56] Italy	Open label, single arm	15–30 × 10^6 autologous BMSCs every 2 months until improvement or until ADSCs not available (2–5 injections) (10 patients)	67% (2 months) 100% (12 months)	0% (12 months)	None	Previously: fistulectomy (90%), immunosuppressors (80%),biological agents (70%)

de la Portilla et al. (2013) [55]	Phase I/II open label, single arm	20×10^6 allogeneic ADSCs; second injection of 40×10^6 if unhealed at 14 weeks (24 patients)	28% (3 months)	N/R	2 serious adverse events; fever (1 patient), perianal abscess (1 patient)	N/R
Spain			47% (6 months)			
Cho et al. (2013) [53]	Phase I, open label, single arm	10×10^6, 20×10^6, 40×10^6 autologous ADSCs based on the size of the fistula (up to 40×10^6 cells)	30% (2 months)	0% (8 months)	13 in 7 patients (70%); 3 in 2 patients (20%)	Previously: conventional surgery and/or setons (50%)
Korea		(10 patients)	30% (8 months)			Infliximab for new fistulas unrelated to the target fistula

(continued)

Table 4 (continued)

Authors (year) Country of study	Type of study	Interventions (number of patients)	Healing rate (follow-up)	Recurrence rate (follow-up)	Adverse events	Previous/concomitant treatments
Lee et al. (2013) [52]	Phase II, open label, single arm	30×10^6 or 60×10^6 autologous ADSCs per 1 cm of fistula length; second injection of 1.5 times more cells if fistula closure was not complete	82% (27/33 patients at 2 months)	11% (3/27 patients)	29 unrelated to ADSCs	No infliximab within 3 months
Korea		(43 patients)	88% (23/27 patients sustained at 12 months)	(9 months)		
Cho et al. (2015) [54]	Phase II, open label, single arm (long-term follow-up of Lee et al.'s study)	30×10^6 or 60×10^6 autologous ADSCs per 1 cm of fistula length; second injection of 1.5 times more cells if fistula closure was not complete	79.3% (12 months)	11.5% (12 months)	53 adverse events, all unrelated to ADSCs; abdominal pain (17.1%), eczema (9.8%), anal inflammation (7.3%), diarrhea (7.3%), (7.3%)	During follow-up: seton placement (7.3%), fistulotomy (2.4%), infliximab (19.5%)
Korea		(41 patients)	80.8% (24 months)	16.7% (24 months)		

Park et al. (2015) [59]	Multicenter, open-label, dose escalation pilot study	$10–30 \times 10^6$ allogeneic ADSCs	50% (8 months)	N/R	No grade 3 or 4 adverse events; postoperative pain (83.33%), perianal abscess (16.67%), site infection (16.67%), fever (16.67%), abdominal pain (16.67%) diarrhea(16.67%), numbness (16.67%), erythema (16.67%)	Previously: conventional surgery (66%), infliximab (50%)
Korea		(6 patients)				
Panés et al. (2016) [67]	Multicenter phase III, randomized	120×10^6 allogeneic ADSCs (107 patients) vs 24 mL saline solution (placebo) (105 patients)	50% (ADSCs) vs 34% (placebo) ($p = 0.024$) (6 months)	N/R	17% (ADSCs) vs 29% (placebo); anal abscess (6 vs 9 patients), proctalgia (5 vs 9 patients)	Previously: antibiotics (77%), immunosuppressants (88%), anti-TNF and seton placement (78%)
Europe, Israel						Concomitantly: antibiotics, immunosuppressants (15%), anti-TNF (35%), both (26%)
Molendijk (2015) [57]	Open label, 4 arms	10×10^6 allogeneic BMSCs (group 1, 5 patients) vs 30×10^6 allogeneic BMSCs (group 2, 5 patients) vs 90×10^6 allogeneic BMSCs (group 3, 5 patients) vs placebo (6 patients)	60%, 40%, 80% (group 1) vs 80%, 80%, 80% (group 2) vs 20%, 20%, 20% (group 3) vs 16.7% (placebo) (6 weeks, 3 months, 6 months)	N/R	Nonserious adverse events; anal abscess, 3 patients (BMSCs) vs 1 patient (placebo); abdominal pain, 5 patients (BMSCs); common cold, 8 patients (BMSCs) vs 2 patients (placebo)	Previously: anti-TNF, antibiotics, steroids, thiopurines, methotrexate, surgery
Netherlands						

(continued)

Table 4 (continued)

Authors (year) Country of study	Type of study	Interventions (number of patients)	Healing rate (follow-up)	Recurrence rate (follow-up)	Adverse events	Previous/concomitant treatments
Garcia-Olmo et al. (2015) [66]	Single arm observational	Autologous ASDCs (3 patients)	33% (2 months)	N/R	None	Previously: curettage (100%)
Spain			66% (12 months)			
Dietz et al. (2017) [68]	Phase I, open label, single arm	20×10^6 autologous ADSCs on a Gore bio A-plug (12 patients)	83% (6 months)	N/R	5 nonserious	Previously: incision and drainage (12%), seton placement (83%), fistulotomy (25%), infliximab and other immunomodulators (100%)
United States						

therapy was effective in this patient, and no ethical or safety problems arouse at that early stage [71]. Except for the few cases of rectovaginal fistulas that were included in the group of patients treated by the same group of experts later on, limited data may be extracted by small published case series [72]. Piejko et al. have recently reported the successful sealing of three rectovaginal fistulas and the subsequent restoration of intestinal continuity [60]. The aim of another clinical trial conducted in Spain by the pioneer group completed the first phase I and II studies was to determine the safety and feasibility of expanded ADSCs to treat Crohn's-related rectovaginal fistulas [72]. A phase I–II study was designed to treat ten female patients. Curettage was performed, and a vaginal or rectal flap was added according to surgeons' preference. The therapeutic protocol included intralesional injection of 20×10^6 ADSCs in the vaginal walls and fistula tract. Healing was evaluated at 3 months, and if the fistula had not healed, a second dose of 40×10^6 ADSCs was administered [72]. Serious adverse events were not observed. Five patients were excluded because biologic drugs were required to treat Crohn's disease flare-ups during follow-up [72]. Cytokine profiles and immunotoxicity assays showed no statistically significant alterations. Sixty percent of the non-excluded patients achieved a complete healing [72]. The authors concluded that ADSCs injection was safe and feasible, and the healing success rate underscored the promising results of this novel treatment alternative for rectovaginal fistulas [72].

Conclusions

Treatment of perianal fistulas, especially in the context of Crohn's disease, continues to remain a real surgical challenge due to the high rate of recurrence and the possibility of fecal incontinence after surgery. MSCs could be helpful in orchestrating and significantly promoting the process of complex fistulas healing due to their potent anti-inflammatory and immunomodulatory effects. MSCs may act by downregulating immune responses, by upregulating T-regulatory cells, by directly promoting tissue healing through tissue-specific differentiation, or remotely by secretion of growth factors and cytokines that promote angiogenesis and epithelial cell proliferation. A surge in clinical trials has been noted on the evaluation of safety and efficacy of MSCs for perianal fistula healing. MSCs have been proven to attain a satisfactory safety profile, and their local application in different concentrations and technical variations has offered decent results considering the challenging conditions surrounding this particular entity and the related interventions. Combination of MSCs with other available and well-tested surgical or medical treatments may potentiate healing results. Important practical issues regarding cell source, dosing, and frequency of administration and method of implantation still remain. Large clinical trials will enlighten controversies that have unavoidably arisen. Nevertheless, according to current data, it can be stated that MSCs may become a clinical reality for the treatment of complex perianal fistulas in the near future.

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Stem Cells in Inflammatory Bowel Disease: From Pathogenesis to Clinical Practice

Christos Zavos

Introduction

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is a chronic, relapsing, and remitting disease of the gastrointestinal (GI) tract, characterized by perpetual idiopathic intestinal inflammation, associated with uncontrolled innate and adaptive immunity. These disorders (CD and UC) have both distinct and overlapping pathologic and clinical characteristics.

IBD is more prevalent in the industrialized western countries; in one of the largest studies conducted in the United States (based on health insurance claims for nine million Americans), the prevalence of CD was 201 per 100,000 adult population, and the prevalence of UC was 238 per 100,000 adult population [1]. In Europe, the highest IBD prevalence values were reported in Norway (UC 505 per 100,000) and in Germany (CD 322 per 100,000) [2]. On the other hand, the incidence and prevalence of IBD appear to be lower in Asia and the Middle East; however, in some newly industrialized countries in Africa, Asia, and South America, the incidence of IBD has been rising [2–4]. These data suggest that the quality of life of a significant part of the population worldwide is profoundly affected.

Relegated to a "Cinderella status" behind many other chronic inflammatory and autoimmune diseases for over 40 years, a significant progress has recently been made with respect to the pathogenesis, diagnosis, and treatment of IBD. A series of factors have been named as culprits, such as genetic susceptibility, dysregulated immune responses, gut microbiome composition, and environmental factors in a susceptible host [5].

Nonsurgical IBD treatment initially included anti-inflammatory approaches, such as corticosteroids, 5-aminosalicylic acid formulas, and immunosuppressive agents, such as azathioprine and methotrexate. However, these medications would

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achieve a sustained remission only in a proportion of IBD patients, often accompanied by side effects, such as the toxicity and the decreased bone mineral density of corticosteroids and the cytopenia of azathioprine [6]. The development of biological agents with immunomodulatory activity has considerably enhanced our armamentarium of the available medications to treat IBD, and more new agents targeting excessive cytokines and immune responses in inflamed mucosa are currently tested in phase II and III clinical trials.

It is important to note, however, that concerns over the safety of a prolonged use of biological agents have been raised, as once they are introduced to an IBD patient, it is difficult to predict when they will be discontinued. Furthermore, in the cases of a failed response to the currently available biological therapies, surgery still remains an option, often offering a suboptimal solution especially in complicated CD, such as in the presence of fistulas [7]. Surgical approach in the latter cases includes fistulotomy, ligation therapy, and anal fistulectomy, which often come with a high recurrence rate. Failure of biological treatment must be attributed to the fact that the IBD pathogenesis is influenced by multiple factors and there are multiple cell types involved, thereby limiting the success of a therapeutic agent targeting a single mediator involved in gut inflammation.

Lately, stem cell (SC)-based therapy, mainly using hematopoietic SCs (HSCs) and mesenchymal SCs (MSCs), has been proposed as an alternative approach to biological agents for IBD treatment. The rapid advances occurred in MSC research, known to exhibit regenerative, paracrine, and immunoregulatory properties, have raised hope for their therapeutic potential in IBD in the near future [8]. This hope has been reinforced by the observation that the intestine, with its crypts and niches, allows the migration, engraftment, and differentiation of SCs [9]. In this chapter, the pathophysiology of GI SCs and all the latest literature concerning their use in IBD treatment are discussed, mainly focusing on a clinical perspective.

Pathophysiology of GI SCs

The intestinal crypts of Lieberkühn contain SCs that have the ability to self-replicate and give rise to all other epithelial cell lineages and also to intestinal immune cells. These properties make them essential since they maintain tissue homeostasis by regulating cell turnover, depending on the current demand; tissue-specific SCs are responsible for the maintenance of the epithelium throughout the GI tract [10]. The epithelium is renewed every 3–5 days by SCs residing in the base of each crypt.

Due to advances in our understanding of the cell populations involved in the pathogenic process and recent findings on the regenerative, trophic, and immunoregulatory potential of SCs, HSC and MSCs have been a field of enormous interest to develop new treatments for IBD. In the IBD-inflamed mucosa, impairment of the intestinal immune cell function and turnover has been demonstrated to play a pivotal role in the deregulated and extended inflammatory response.

Concerning HSCs, a high-dose immune ablation regimen might allow detrimental T lymphocytes to be eliminated, and, after HSC transplantation, hematopoiesis might generate naïve cells that can restore tolerance and reboot the immune system [11]. Trans-differentiation of HSCs into other cell types is yet to be elucidated, but there is evidence suggesting that HSCs can form endothelial precursors that could repair the intestine [12]. Indeed, HSCs have been shown to migrate directly to the injury site and to differentiate into the epithelial and immunomodulatory elements unique to the intestinal compartments, thereby restoring the inflamed intestinal mucosa [13].

Identified by Friedenstein et al. in bone marrow [14], MSC is a multipotent, mesoderm-derived cell type that can also be isolated from various other tissues, including adipose, muscle, umbilical cord blood, peripheral blood, liver, placenta, skin, amniotic fluid, breast milk, synovial membrane, and tooth root, while exhibiting immunomodulatory properties [15]. Like HSCs, MSCs also play a key role in coordinating events related to repair of the inflamed epithelium in IBD.

Specifically, it is well known that the repair process can usually restore normal intestinal architecture in UC patients, but this is difficult in CD patients due to excessive fibrosis leading to formation of strictures and obstructions. Fibrosis in CD is associated with mesenchymal cell persistence and hyperplasia, tissue disorganization, and collagen deposition. It is interesting to note that bone marrow-derived SCs have been suggested to repair fibrosis [16]. The mesenchymal cells (intestinal subepithelial myofibroblasts) regulate proliferation and differentiation of the epithelial cell basement membrane, as well as extracellular matrix metabolism [17].

MSCs possess numerous immunomodulatory effects both on the innate and the adaptive immune systems, both known to be affected in IBD (Table 1). MSCs have the ability to interact with many kinds of immune cells, including B cells, T cells, dendritic cells (DCs), natural killer (NK) cells, neutrophil, and macrophages [18]. The immunomodulatory effects of MSCs include the following: release of soluble factors (cytokines, chemokines, growth factors – such as transforming growth factor [TGF]-beta, insulin-like growth factor [IGF]-1, epidermal growth factor [EGF] – matrix metalloproteinase [MMP] and tissue inhibitors of metalloproteinases [TIMP], indoleamine 2,3-dioxygenase in humans, nitric oxide in mice, and others),

Table 1 Immunomodulatory effects of mesenchymal stem cells (MSCs) on innate and adaptive immune systems

Immune system	MSC effect
Innate	
Dendritic cells	Inhibit migration, activation, differentiation, maturation, and endocytosis
Natural killer cells	Inhibit migration, proliferation, differentiation, maturation, and activation
Macrophages	Activate macrophage polarization
Adaptive	
T cell	Inhibit survival, proliferation, differentiation, maturation, and activation and enhance T-cell recruitment
B cell	Inhibit proliferation, differentiation, maturation, chemotaxis, and activation

induction of cell cycle arrest in pro-inflammatory lymphocytes, and induction of T-cell apoptosis [19]. In the inflamed IBD mucosa, MMP-1, MMP-3, and MMP-9 are overexpressed compared to TIMP-1 and TIMP-2, mediated in part by TGF-beta1 which may also play a critical role in IBD tissue remodeling [20]. The MSCs accelerate the proliferation and migration of residual epithelial cells over denuded areas by releasing TGF-beta, EGF, basic fibroblast growth factor, and various inflammatory cytokines. MSCs also act by non-specifically modulating the immune response through their antigen-presenting abilities; suppressing the differentiation and maturation of DCs [21]; decreasing the level of inflammatory and Th1 cytokines and increasing the secretion of interleukin (IL)-10, thereby improving the clinical and histopathological severity of colitis; modulating the innate immune response; acting on resting NK cells; decreasing the respiratory burst and apoptosis of neutrophils [19]; and triggering the generation of Tregs and inducing antigen-dependent Treg proliferation [11]. Taken together, the immunomodulatory and tissue-healing effects of MSCs provide the basis for investigation of their therapeutic value in IBD.

Therapeutic Value of HSCs in IBD

In clinical practice, HSC transplantation was initially used to treat hematological malignancies (namely, leukemia and lymphoma). Subsequently, owing to experimental studies in animals and the clinical improvement experienced by patients with immune-mediated diseases subjected to HSC transplantation due to malignant conditions [22, 23], HSC transplantation has become increasingly used in selected patients with immune-mediated diseases refractory to conventional therapy. In IBD, current studies continue to focus on autologous HSC transplantation, intended to reset the immune system by de novo regeneration of T-cell repertoire, and repopulation of epithelial cells by bone marrow-derived cells to help patients achieve clinical and, potentially, endoscopic remission.

Autologous HSC transplantation consists of two phases: mobilization and conditioning. During the first phase, HSCs are mobilized from bone marrow to peripheral blood and collected by apheresis. After extraction of HSCs, some of the current protocols for HSC transplantations in autoimmune diseases include either ex vivo T-cell depletion with the aim of obtaining inoculums rich in CD34+ and poor in T cells or in vivo T-cell depletion by using intravenous rabbit antithymocyte globulin (rATG). In some cases, CD34+ population was selected ex vivo to avoid reinfusion of previously activated T cells; however, subsequent evolution of these patients was no better than in those who received nonselected products. The second phase involves the administration of chemotherapy to induce immune ablation and subsequent infusion of previously harvested cells.

We recently published our own experience on autologous HSC transplantation in a patient with moderate-to-severe CD, involving the jejunum, ileum, and colon, who had initially been treated with conventional treatment and later with surgery (right

colectomy/sigmoidectomy) [24]. Due to the lack of response to biological therapy (infliximab and adalimumab, available at that time) and a severe side effect, i.e., demyelinating disease of the optical nerve leading to a decrease in visual acuity in both eyes, biological treatment had to be discontinued. As the patient experienced a new severe relapse requiring partial colectomy and a jejuno-colonic fistula resection, and as all conventional treatment had failed, it was deemed that the patient was eligible for autologous HSC transplantation. The procedure was well-tolerated with no complications. Over a follow-up period of 31 months, the patient initially achieved and has maintained clinical, endoscopic, and histological remission without further treatment and with an excellent quality of life. Similar case reports or case series of refractory CD patients who successfully received autologous HSC transplantations have also been published from other countries [25–32].

As of September 2017, there had been a total of 172 transplant registrations of CD within the European Society for Blood and Marrow Transplantation (EBMT) registry, with 164 for autologous HSC transplantation [33]. A single-center cohort from Spain studied the effect of autologous HSC transplantation in 29 patients with CD, unresponsive to current available therapies [34]. Seventy percent of patients achieved drug-free clinical remission at 6 months of follow-up. The proportion of patients who remained in drug-free remission state at 5 years of follow-up was 15%. Interestingly, 80% of patients who relapsed were successfully managed with medical therapy.

Only one randomized controlled trial has been published to date, the Autologous Stem Cell International Crohn's disease (ASTIC) trial [35]. The endpoints of this study were to assess how common it was to achieve complete remission of disease and also whether it was the cyclophosphamide used in stem cell mobilization or the complete autologous HSC transplantation that was responsible for any benefit seen. To be eligible, patients had to have objective evidence of active disease and impaired quality of life despite having tried at least three immunosuppressive/biological treatments. The designated primary endpoint was the most stringent used in a clinical trial in CD: clinical remission (CD Activity Index, CDAI <150) for 3 months, off all immunosuppressive medication with no evidence of active disease on radiological or endoscopic assessment (by Simple Endoscopic Score for CD, SES-CD).

To cover the possibility that mobilization alone might have therapeutic benefit, all patients underwent mobilization with cyclophosphamide 4 g/m^2 and granulocyte colony-stimulating factor before randomization to autologous HSC transplantation or control treatment. Conditioning for the transplant was cyclophosphamide 200 mg/kg and rATG, and patients received unmanipulated grafts. All patients in either group could receive any additional treatment deemed necessary, but investigators were required to try to withdraw existing treatment to a standard protocol if disease activity allowed.

Of 132 patients submitted for evaluation by the trial steering committee, 48 went forward to stem cell mobilization, which was successful in 46. Following mobilization, there was a significant fall in CDAI at 6 weeks. Forty-five patients were randomized to autologous HSC transplantation (n = 23) or control (n = 22) treatment. Following autologous HSC transplantation, there was a further decline in CDAI at

1 year, whereas the initial improvement was not maintained in control patients, suggesting that mobilization cyclophosphamide alone is not sufficient for any benefit seen with autologous HSC transplantation.

Only two patients undergoing autologous HSC transplantation vs. one control achieved the ambitious primary endpoint in the ASTIC trial. Nevertheless, a number of patients improved on one or more of the component dimensions. Sixty-one percent of autologous HSC transplantation patients had been off all treatment for ≥3 months at 1-year follow-up in comparison with 23% of controls ($P < 0.01$), with remission CDAI values in 35% vs. 9% ($P = 0.053$) and no objective evidence of active disease on endoscopy and radiology in 35% vs. 9% ($P = 0.053$). Adverse events were significantly more common in the autologous HSC transplantation group within the first 100 days and included mostly infections. One patient died 20 days after starting conditioning and had evidence of sinusoidal obstructive syndrome at postmortem. However, it should not be assumed that this single trial provides the definitive answer regarding the benefit of autologous HSC transplantation in CD. In addition to the stringent primary endpoint, there are several further drawbacks to the ASTIC trial design. First, the doses of cyclophosphamide used are higher than current guidelines recommend, which may have added to the burden of toxicity reported [36, 37]. Second, all patients received 4 g/m^2 cyclophosphamide at mobilization prior to randomization; so even patients in the control group had significant cyclophosphamide exposure. Finally, patients were not treated with maintenance therapy after autologous HSC transplantation.

Patients randomized to the control group in ASTIC could undergo autologous HSC transplantation after the primary endpoint and underwent the same schedule of assessments over the subsequent year. A recent report of the combined cohort includes baseline assessments in 40 and 1-year outcome in 38 patients. There were significant improvements in clinical disease activity, quality of life, and endoscopic disease activity at 1 year with 43% patients being in steroid-free clinical remission and 50% having ileocolonic ulcer healing, of whom 26% had complete regression of all evidence of ileocolonic CD (SES-CD score of 0) [38].

Allogeneic marrow transplantation seems to be useful for the treatment of IBD patients with hematologic malignancies, such as acute leukemia, myelodysplastic syndrome, non-Hodgkin lymphoma, and multiple myeloma [39]. However, IBD is thought to increase the risk and the severity of graft-versus-host disease (GVHD) after allogeneic HSC transplantation, resulting in increased non-relapse mortality. Due to this high morbidity and mortality rate, allogeneic HSC transplantation has been reserved for monogenic diseases, like IL-10 deficiency, as it would correct the disease by building a new immune system in the host [40, 41]. However, in a limited number of IBD patients (7 with CD and 4 with UC), allogeneic HSC transplantation has been performed with promising results (10 of 11 patients free of disease with a median follow-up of 34 months) with no severe GVHD [42].

Taken together, despite the promising initial results, it is evident that autologous HSC transplantation does not achieve cure of CD and is associated with a heavy burden of serious adverse events, predominantly infections related to the immunosuppression required. However, in CD patients refractory to currently available

therapies, autologous HSC transplantation may still offer a significant benefit. Importantly, CD patients whose disease relapses after autologous HSC transplantation appear to respond to therapies which had previously failed. No trials have been published so far on HSC transplantation in UC patients.

Therapeutic Value of MSC in IBD

MSC-based therapies have quickly risen in prominence among immunology disease treatments in the past few years. It should be underscored however that MSCs represent a heterogeneous population and different subpopulations of cells are exhibiting a variety of functional potentials. To improve efficacy, robust priming of MSCs, to isolate and use those with enhanced immunosuppressive capabilities, is of basic importance. Furthermore, improving the targeting and engraftment of MSCs is of the ultimate importance for their potential use in cellular therapy and for the progression of MSC-based therapies to clinical practice because their efficiency of delivery into injury sites is quite low especially when delivered systemically. An extensively investigated approach is the regulation of the expression of cell surface antigens by forcing the expression of appropriate receptors to the desired site of injury.

As already mentioned in the Pathophysiology section of this chapter, MSCs can be isolated from several tissues, with differences in yield and in differentiation capacities. To date, the best-characterized MSC population used in IBD is the one found in bone marrow. Alternatively, umbilical cord- and adipose tissue-derived MSCs have been used, and all the relevant clinical studies will be reviewed later on this section. There have been 680 MSC-based clinical trials registered on the National Institutes of Health (NIH) Clinical Trial Databank (https://clinicaltrials.gov/) around the world as of July 2018. Of those, 22 clinical trials recruit CD patients, 3 clinical trials recruit UC patients, and 2 clinical trials recruit IBD patients (both CD and UC) for MSC (derived from bone marrow, adipose tissue, or umbilical cord tissue) transplantation (Table 2). More than 60% of trials are employing allogeneic MSCs, and in CD, more than 40% of the trials are evaluating intralesional injection into the fistula, which is the major and refractory complication of CD [43].

Clinical Data on Bone Marrow-Derived MSC

Published studies on bone marrow-derived MSC-based therapies show promising but rather inconclusive results concerning a definitive cure for IBD. Specifically, Molendijk et al. reported improved healing of refractory perianal fistulas using allogeneic bone marrow-derived MSCs; local injection of 3×10^7 allogeneic MSCs promoted the healing of perianal fistula [44].

Table 2 Clinical trials in inflammatory bowel diseases according to ClinicalTrials.gov

No.	NCT number	Status	Conditions	Interventions	Characteristics	Population	Country
1	NCT01874015	Recruiting	Crohn's disease	Biological: mesenchymal cell transplantation Biological: mesenchymal cell and fibroblast injection	Study type: interventional Phase: phase 1 Study design: Allocation: randomized Intervention model: parallel Assignment masking: single (investigator) Primary purpose: treatment Outcome measures: Fistula closure Crohn's disease activity index (CDAI)	Enrollment: 10 Age: 18–65 years (adult, older adult) Sex: all	Iran
2	NCT01157650	Completed	Crohn's disease	Other: autologous mesenchymal stem cells (MSC)	Study type: interventional Phase: Phase 1 Phase 2 Study design: Intervention model: single group assignment Masking: none (open label) Primary purpose: treatment Outcome measures: Security and tolerance Therapeutic effect	Enrollment: 15 Age: 18 years and older (adult, older adult) Sex: all	Spain
3	NCT00294112	Completed	Crohn's disease	Drug: Prochymal™ adult human MSC Drug: adult human MSC	Study type: interventional Phase: phase 2 Study design: Allocation: randomized Intervention model: parallel assignment Masking: none (open label) Primary purpose: treatment Outcome measures: CDAI Inflammatory bowel disease quality-of-life questionnaire Crohn's disease endoscopic index of severity Crohn's remission	Enrollment: 10 Age: 18–70 years (adult, older adult) Sex: all	USA

4	NCT02677350	Not yet recruiting	Crohn's disease Fistulizing Crohn's Disease Stem cells	Drug: allogeneic bone marrow-derived human MSC	Study type: interventional Phase: phase 1 Study design: Intervention model: single group assignment Masking: none (open label) Primary purpose: treatment Outcome measures: Evaluation of treatment-emergent adverse events from the bone marrow-derived allogeneic mesenchymal stem cell implant Clinically: to assess changes in CDAI, the perianal disease activity index (PDAI) Endoscopic assessment of rectum using a limited simplified endoscopic activity score for Crohn's disease (SES-CD) to evaluate extent and severity of ulcers Radiologic assessment using MRI Evaluate symptomatic patients Evaluate the effect of local treatment with allogeneic bmMSCs using the short inflammatory bowel disease questionnaire (sIBDQ) Evaluate the effect of local treatment with allogeneic bmMSCs using the Short Form (SF)-36 score C-reactive protein (CRP) Major adverse events	Enrollment: 20 Age: 18 years and older (adult, older adult) Sex: all	USA
5	NCT02445547	Completed	Crohn's disease	Other: Umbilical cord-MSCs by peripheral intravenous infusion Drug: received hormone maintenance therapy	Study type: interventional Phase: Phase 1 Phase 2 Study design: Allocation: randomized Intervention model: parallel assignment Masking: none (open label) Primary purpose: treatment Outcome measures: CDAI Harvey-Bradshaw index Corticosteroid dosage	Enrollment: 82 Age: 18–70 years (adult, older adult) Sex: all	China

(continued)

Table 2 (continued)

No.	NCT number	Status	Conditions	Interventions	Characteristics	Population	Country
6	NCT01144962	Completed	Crohn's disease fistula	Procedure: localization, curettage of the fistulous tract and closure of the internal opening without MSC injection Procedure: localization, curettage of the fistulous tract and closure of the internal opening with local MSC injection	Study type: interventional Phase: Phase 1 Phase 2 Study design: Allocation: randomized Intervention model: parallel assignment Masking: triple (participant, investigator, outcomes assessor) Primary purpose: treatment Outcome measures: Safety and efficacy (fistula closure) Clinical scores Endoscopic scores Quality of life CRP Safety	Enrollment: 21 Age: 18 years and older (adult, older adult) Sex: all	The Netherlands
7	NCT00482092	Active, not recruiting	Crohn's disease	Drug: adult human MSC Drug: placebo	Study type: interventional Phase: phase 3 Study design: Allocation: randomized Intervention model: parallel assignment Masking: quadruple (participant, care provider, investigator, outcomes assessor) Primary purpose: treatment Outcome measures: Disease remission (CDAI at or below 150) Disease improvement (reduction by at least 100 points in CDAI) Improvement in quality of life (IBDQ) Reduction in number of draining fistulas	Enrollment: 330 Age: 18–70 years (adult, older adult) Sex: all	USA

8	NCT00543374	Completed	Crohn's disease	Drug: placebo Drug: Prochymal adult human MSC	Study type: interventional Phase: phase 3 Study design: Allocation: randomized Intervention model: parallel assignment Masking: quadruple (participant, care provider, investigator, outcomes assessor) Primary purpose: treatment Outcome measures: Duration of clinical benefit (CDAI) Re-induction of clinical benefit (CDAI) Improvement in quality of life (Inflammatory Bowel Disease Quality of Life assessment IBDQ instrument)	Enrollment: 98 Age: 18–70 years (adult, older adult) Sex: all	USA
9	NCT01540292	Unknown status	Crohn's disease	Biological: MSC	Study type: interventional Phase: Phase 1 Phase 2 Study design: Intervention model: single group assignment Masking: none (open label) Primary purpose: treatment Outcome measures: Clinical response rate Clinical response Remission CDAI level CRP Fecal calprotectin levels Immune modulation investigation Incidence of infections	Enrollment: 20 Age: 18–75 years (adult, older adult) Sex: all	Belgium

(continued)

Table 2 (continued)

No.	NCT number	Status	Conditions	Interventions	Characteristics	Population	Country
10	NCT03183661	Enrolling by invitation	Crohn's disease	Biological: ALLOASC-CD	Study type: observational Study design: Observational model: case-only Time perspective: prospective Outcome measures: Number of participants with adverse events as a measure of safety and tolerability	Enrollment: 9 Age: 18–65 years (adult, older adult) Sex: all	Industry
11	NCT01541579	Active, not recruiting	Crohn's disease	Other: Cx601 Other: saline solution	Study type: interventional Phase: phase 3 Study design: Allocation: randomized Intervention model: parallel assignment Masking: double (participant, investigator) Primary purpose: treatment Outcome measures: Combine remission of perianal fistulizing Crohn's Efficacy assessment by week 24 Efficacy assessment by week 52 Efficacy assessment by week 104 Safety analysis throughout the study	Enrollment: 278 Age: 18 years and older (adult, older adult) Sex: all	Austria, Belgium, France
12	NCT01233960	Active, not recruiting	Crohn's disease	Drug: adult human MSC	Study type: interventional Phase: phase 3 Study design: Intervention model: single group assignment Masking: none (open label) Primary purpose: treatment Outcome measures: Disease remission Disease improvement Improvement in quality of life (IBDQ) Number of adverse events as a measure of safety Infusional toxicity as a measure of safety and tolerability	Enrollment: 120 Age: 18–70 years (adult, older adult) Sex: all	USA

13	NCT01510431	Completed	Crohn's disease	Drug: Prochymal (remestemcel-L)	Study type: interventional Phase: not applicable Study design: Intervention model: single group assignment Masking: none (open label) Primary purpose: treatment Outcome measures: CDAI	Enrollment: 11 Age: 18–70 years (adult, older adult) Sex: all	USA
14	NCT02580617	Recruiting	Crohn's disease	Biological: ALLOASC	Study type: interventional Phase: phase 1 Study design: Intervention model: single group assignment Masking: none (open label) Primary purpose: treatment Outcome measures: Safety (clinically measured abnormality of laboratory tests and adverse events) CDAI value The ratio of patients applicable to CDAI <150	Enrollment: 9 Age: 18–65 years (adult, older adult) Sex: all	Korea
15	NCT02403232	Recruiting	Crohn's disease perianal fistula	Procedure: Autologous adipose-derived stem cells (ASCs) injection Device: ASCs injection Device: closure of fistula tract	Study type: interventional Phase: phase 2 Study design: Allocation: nonrandomized Intervention model: single group assignment Masking: none (open label) Primary purpose: treatment Outcome measures: Healing fistula Morbidity Quality-of-life modification Relation between CDAI and fistula healing Mortality	Enrollment: 10 Age: 18 years and older (adult, older adult) Sex: all	Italy

(continued)

Table 2 (continued)

No.	NCT number	Status	Conditions	Interventions	Characteristics	Population	Country
16	NCT02000362	Recruiting	Crohn's disease	Biological: stem cells	Study type: interventional Phase: Phase 1 Phase 2 Study design: Intervention model: single group assignment Masking: none (open label) Primary purpose: treatment Outcome measures: Number of participants with adverse events, ratio of patients who is applicable to CDAI<150 The ratio of patients who reduce CDAI over 70 as contrasted with baseline value A variation of CRP value as contrasted with baseline A variation of MR enterographic score as contrasted with baseline A variation of fecal calprotectin as contrasted with baseline A variation of IBDQ score as contrasted with baseline A variation of SF-36 score as contrasted with baseline Reduction of the number of draining fistula All kinds of adverse effects which occur during the clinical study	Enrollment: 24 Age: 19–70 years (adult, older adult) Sex: all	Korea
17	NCT02926300	Recruiting	Crohn's disease	Biological: stem cells	Study type: observational Study design: Observational model: cohort Time perspective: prospective Outcome measures: All kinds of adverse events which occur during the clinical study Ratio of patients who is applicable to CDAI<150 Ratio of patients who reduce CDAI over 70 as contrasted with baseline (V7)	Enrollment: 24 Age: 19–70 years (adult, older adult) Sex: all	Korea

18	NCT01314092	Terminated	Complex perianal fistula	Biological: autologous cultured ASC (low-dose group) Biological: autologous cultured ASC (high-dose group)	Study type: interventional Phase: phase 2 Study design: Allocation: randomized Intervention model: parallel assignment Masking: single (participant) Primary purpose: treatment Outcome measures: Number of patients with complete closure of fistula (week 8) Grade of investigator's satisfaction Number of patients with closed fistula Photo of target fistula Number of patients with adverse events	Enrollment: 15 Age: 18 years and older (adult, older adult) Sex: all	Korea
19	NCT03220243	Active, not recruiting	Crohn's disease Fistula vagina	Drug: MSC-AFP (fistula plug coated with autologous MSCs)	Study type: interventional Phase: phase 1 Study design: Intervention model: single group assignment Masking: none (open label) Primary purpose: treatment Outcome measures: Number of participants with treatment-related adverse events (safety and toxicity) Number of participants with response to the treatment regarding potential cessation of drainage from their fistula	Enrollment: 15 Age: 18–65 years (adult, older adult) Sex: female	USA
20	NCT01623453	Terminated	Perianal fistula Primary; complex	Biological: autologous ASC (low-dose group) Biological: autologous ASC (high-dose group)	Study type: observational Study design: Time perspective: prospective Outcome measures: Number of patients with sustained efficacy of complete closure of fistula Number of patients with sustained efficacy of closure of fistula Evaluation of fecal incontinence score Grade of investigator's satisfaction Number of patients with adverse events as a measure of systemic tolerance and physical examinations	Enrollment: 6 Age: 18 years and older (adult, older adult) Sex: all	Korea

(continued)

Table 2 (continued)

No.	NCT number	Status	Conditions	Interventions	Characteristics	Population	Country
21	NCT01915927	Active, not recruiting	Perianal Crohn's disease	Drug: MSC-AFP	Study type: interventional Phase: phase 1 Study design: Intervention model: single group assignment Masking: none (open label) Primary purpose: treatment Outcome measures: To determine the safety and toxicity of using autologous MSC-coated fistula plug in patients with fistulizing Crohn's disease To assess in preliminary fashion the response of fistula healing induced by the GORE plug containing MSC	Enrollment: 20 Age: 18–65 years (adult, older adult) Sex: all	USA
22	NCT03449069	Recruiting	Crohn's disease Fistula-in-ano	Drug: MSC-AFP	Study type: interventional Phase: phase 1 Study design: Intervention model: single group assignment Masking: none (open label) Primary purpose: treatment Outcome measures: Number of participants with treatment-related adverse events (safety and toxicity) Number of participants with response to the treatment regarding cessation of drainage from the treated fistula Number of participants with radiographic response to the treatment regarding the treated fistula	Enrollment: 5 Age: 12–17 years (Child) Sex: all	USA

23	NCT01914887	Unknown status	Ulcerative colitis	Drug: allogeneic adipose tissue-derived mesenchymal stem cells	Study type: interventional Phase: Phase 1 Phase 2 Study design: Intervention model: single group assignment Masking: none (open label) Primary purpose: treatment Outcome measures: Safety Efficacy: change from baseline in modified Truelove-Witts score Efficacy: change from baseline in quality-of-life index, inflammatory bowel disease questionnaire (IBDQ-32) Efficacy: change from baseline in Mayo endoscopic index Change from baseline in CRP Change from baseline in fecal calprotectin	Enrollment: 8 Age: 18 years and older (adult, older adult) Sex: all	Spain
24	NCT01221428	Unknown status	Ulcerative colitis Mesenchymal stem cells Umbilical cord	Biological: umbilical cord MSCs	Study type: interventional Phase: Phase 1 Phase 2 Study design: Allocation: nonrandomized Intervention model: single group assignment Masking: none (open label) Primary purpose: treatment Outcome measures: The result of enteroscopy and pathological report The clinical symptom (including stomachache, abdominal distention, bloody purulent stool)	Enrollment: 50 Age: 18–70 years (adult, older adult) Sex: all	China

(continued)

Table 2 (continued)

No.	NCT number	Status	Conditions	Interventions	Characteristics	Population	Country
25	NCT02442037	Unknown status	Ulcerative colitis	Biological: Umbilical cord MSC group Other: control group (normal saline)	Study type: interventional Phase: Phase 1 Phase 2 Study design: Allocation: randomized Intervention model: parallel assignment Masking: single (participant) Primary purpose: treatment Outcome measures: Safety will be determined by the assessment of major adverse events Clinical response (CDAI points) Endoscopic improvement is assessed by Ulcerative Colitis Endoscopic Index of Severity score (UCEIS) Level of CRP	Enrollment: 30 Age: 18–65 years (adult, older adult) Sex: all	China
26	NCT03115749	Not yet recruiting	Inflammatory bowel diseases	Other: intestinal biopsies during colonoscopy Other: biopsies taken on surgical specimen	Study type: interventional Phase: not applicable Study design: Allocation: nonrandomized Intervention model: parallel assignment Masking: none (open label) Primary purpose: basic science Outcome measures: Proliferation and differentiation of intestinal MSCs in IBD patients Comparison of MSCs between IBD patients and control patients	Enrollment: 60 Age: 18–75 years (adult, older adult) Sex: all	France

27	NCT03299413	Recruiting	Inflammatory bowel diseases	Biological: Wharton jelly MSCs	Study type: interventional Phase: Phase 1 Phase 2 Study design: Intervention model: single group assignment Masking: none (open label) Primary purpose: treatment Outcome measures: Incidence of treatment-emergent adverse events [safety and tolerability] Evaluation of the efficacy of the injected cells (change from baseline in partial Mayo score)	Enrollment: 20 Age: 18–75 years (adult, older adult) Sex: all	Jordan

Duijvestein et al. used autologous bone marrow-derived MSCs a phase I study of luminal refractory CD. Conventional treatments and antitumor necrosis factor (anti-TNF) therapy had previously failed to all ten patients who participated in the study. Although three of the ten patients showed clinical response (CDAI decrease ≥70 from baseline) 6 weeks posttreatment, another three patients required surgery due to disease worsening. However, the authors concluded that administration of autologous bone marrow-derived MSCs appears to be safe and feasible in the treatment of refractory CD [45].

Ciccocioppo et al. evaluated the effect of bone marrow-derived MSCs on fistulizing CD with promising results [46]. Ten of 12 consecutive outpatients (two refused) received intrafistular MSC injections scheduled every 4-week intervals, with a median of 2×10^7 cells each time, and were monitored by MRI, surgical, and endoscopic evaluation for 12 months afterwards. MSC expansion was successful in all cases; 70% of the patients got sustained complete closure, and 30% got incomplete closure of fistula tracks with a parallel reduction of CD and perianal disease activity indexes ($P < 0.01$ for both), and rectal mucosal healing was induced after treatment without any serious adverse effects.

Forbes et al. reported a phase II study using allogeneic bone marrow-derived MSCs for luminal CD refractory to biologic therapy. They administered 2×10^6 cells/kg weekly for 4 weeks and found that allogeneic MSCs reduced the CDAI and CD endoscopic index of severity scores in patients with luminal CD refractory to biologic therapy. Improvement was noticed in 12 patients, remission in 8, and endoscopic improvement in 7, respectively [47].

In another phase III, prospective, randomized, placebo-controlled study of Prochymal® (allogeneic bone marrow-derived MSCs) in CD, 270 CD patients were enrolled with active moderate-to-severe disease who had previously failed treatment with steroids, immunosuppressants, and anti-TNF agents. The patients received 2 infusions of 600 million cells, or 1200 million cells, or placebo. After 28 days, the patients were evaluated for disease remission and clinical response [48]. The results were encouraging, and in 2007 Prochymal® received orphan drug designation from the Food and Drug Administration and the European Medicines Agency, but, until July 2018, only for the treatment of GVHD. However, there are efforts made to expand its indications for the potential enhancement of bone marrow transplants in cancer patients, for the prevention of GVHD, and for the treatment of CD [49].

Clinical Data on Umbilical Cord-Derived MSC

There are few published studies on umbilical cord-derived MSC in IBD (1 in UC and 2 in CD patients). Hu et al. reported a phase I/II study for severe UC using umbilical cord-derived allogeneic MSCs by combination injection through the peripheral blood and superior mesenteric artery with a 7-day interval. They

confirmed the safety of MSCs and alleviation of diffuse and deep ulcer formation and severe inflammatory mucosa by MSCs [50].

Zhang et al. performed a prospective open-label trial on the efficacy and safety of systemic infusion of umbilical cord-derived MSCs in patients with steroid-dependent CD [51]. In this study, 82 patients with steroid-dependent CD were randomized, and 42 patients assigned to the MSC infusion group received umbilical cord-derived MSCs via peripheral intravenous infusion of 1×10^6 cells/kg once a week, for 4 weeks. At 12 months after treatment, the CDAI, Harvey-Bradshaw index, and corticosteroid requirement had decreased by 62.5 ± 23.2, 3.4 ± 1.2, and 4.2 ± 0.84 mg/day, respectively, in the umbilical cord-derived MSC group, while they decreased by 23.6 ± 12.4, 1.2 ± 0.58, and 1.2 ± 0.35 mg/day, respectively, in the control group ($P < 0.01$, $P < 0.05$, and $P < 0.05$, umbilical cord-derived MSC vs. control, respectively). However, most patients still received the steroid treatment after 12 months. This means this stem cell therapy does not solely work compared with other therapies like anti-TNF agents. In addition, they did not confirm histological improvement of the inflamed lamina propria to show direct evidence of MSC's action [52].

Mayer et al. reported that umbilical cord-derived MSCs appeared to be safe and well-tolerated in subjects with treatment-resistant CD in their phase I trial [53]. All six subjects who received two infusions of 2×10^8 cells (low dose) achieved a clinical response. In six patients in the high-dose group (two infusions of 8×10^8 cells), two patients achieved response because of more severe disease activity. The most adverse events were mild to moderate in severity and included headache, nausea, fever, and infusion site reactions.

Clinical Data on Adipose Tissue-Derived MSC

As there are no sufficient numbers of bone marrow-derived MSCs in adults and isolation and expansion of these cells requires weeks before transplantation, the use of these MSCs is limited. Adipose tissue-derived MSCs have been also reported to retain promising potential for ulceration healing mainly in perianal disease. What's more, adipose tissue-derived MSCs can be isolated from liposuction aspirates and can overcome the defect of bone marrow-derived MSCs. Thus, more researchers focus their attention on the use of adipose tissue-derived MSCs in autologous or allogeneic transplantation of refractory CD [54].

Garcia-Olmo et al. were the first to report a clinical trial using autologous adipose tissue-derived MSCs obtained from lipoaspirates for direct injection in patients with perianal CD [55]. In their phase I study, they followed eight fistulas inoculated with using autologous adipose tissue-derived MSCs, of which six (75%) healed and two did not (25%) [55]. As the procedure appeared to be feasible and safe, a multicenter, phase II trial followed to assess the effectiveness and safety of adipose tissue-derived MSCs in combination with fibrin glue in the treatment of complex perianal fistulas [56]. Fistula healing was observed in 17 (71%) of 24 patients who

received adipose tissue-derived MSCs in addition to fibrin glue compared with 3 (12%) of 25 patients (erroneously reported as 4 patients) who received fibrin glue alone ($P < 0.001$). A retrospective study of this phase II trial was conducted by the same group to extend the follow-up period for a mean of 3–4 years [57]. Of 12 patients treated with autologous adipose tissue-derived MSCs plus fibrin glue included in the retrospective follow-up in the complete closure group, only 7 remained free of recurrence. However, a major weakness of this retrospective long-term follow-up study was that only 13 patients (of the 25 included in the phase II study) originally treated with fibrin glue alone were included, mainly because their fistulas failed to close during treatment and they opted for surgical procedures in their reference hospital. The authors admitted that many of those patients were disappointed that they had not received active treatment.

Cho et al. conducted a phase I, dose-escalation study assessing adipose tissue-derived MSCs feasibility and safety in ten patients with more than one fistula [58]. The first group of three patients was treated with 1×10^7 adipose tissue-derived MSCs/mL; all of them showed partial closure at week 8. A second group of four patients was treated with 2×10^7 adipose tissue-derived MSCs/mL, 2 of whom showed complete healing. Finally, a third group of three patients received 4×10^7 adipose tissue-derived MSCs/mL, of whom only one patient showed complete healing at week 8. All three patients with complete healing at week 8 showed a sustained effect without recurrence 8 months after injection. No grade 3 or 4 severity adverse events and no adverse events related to the study drug were observed.

The same group of researchers subsequently conducted a phase II trial to evaluate the efficacy and safety of adipose tissue-derived MSCs in a statistically significant number of patients suffering from Crohn's fistulas using the adipose tissue-derived MSCs dose determined by their previous phase I study [58, 59]. According to the modified per protocol analysis, 82% (27/33) of patients showed complete fistula healing at week 8 in contrast with 64.3% (27/42) in the intention-to-treat (ITT) analysis. In addition, three patients exhibited recurrence during the next year of follow-up. The long-term outcome after 2 years was reported by a subsequent study; of note, complete healing was observed in 80.6% (21/26) of patients in the modified per protocol analysis and 75% (27/36) of patients in the modified ITT analysis [60].

In an initial Letter to the Editor, Wainstein et al. reported their preliminary results of a prospective, observational study that included 9 refractory CD patients with complex anal fistulas, treated with 100–120 million adipose tissue-derived MSCs mixed with platelet-rich plasma (PRP) to improve the results of a surgical repair of cryptoglandular fistulas [61]. The purpose of this protocol was to offer patients with perianal CD a combined treatment with adipose tissue-derived MSCs, PRP, and surgical repair and assess the midterm outcomes. At 4 months of follow-up, four patients had completely healed, and one showed partial healing, and at 12 months of follow-up, all five patients were completely healed. No complications related to adipose tissue-derived MSC/PRP treatment were observed. The long-term outcome after 2–3 years was reported by a subsequent study of the same group of researchers.

Surprisingly, at the end of the follow-up period, 10/11 (91%) fistulas were completely healed and 1/11 (9%) was partially healed in the 9 patients included [62].

In a phase III randomized, double-blind, parallel-group, placebo-controlled study by Panés et al. [63], expanded allogeneic adipose-derived MSCs (Cx601) were used to treat adult CD patients and treatment-refractory, draining complex perianal fistulas, at 49 hospitals in 7 European countries and Israel. The primary endpoint was the combined remission at week 24 (clinical assessment of closure of external openings combined with MRI). Results showed that 24 weeks after the treatment, a significantly greater proportion of patients treated with Cx601 versus placebo achieved combined remission in the ITT (53 of 107 [50%] vs. 36 of 105 [34%]; $P = 0.024$) and modified ITT populations (53 of 103 [51%] vs. 36 of 101 [36%]; $P = 0.021$). Eighteen (17%) of the 103 patients in the Cx601 group versus 30 (29%) of the 103 in the placebo group experienced treatment-related adverse events, the most common of which were anal abscess (6 in the Cx601 group vs. 9 in the placebo group) and proctalgia (5 vs. 9). This challenging clinical trial showed effective and well-tolerated new treatment options for patients with Crohn's disease and complex perianal fistulas. On March 23, 2018, the European Commission approved Alofisel® (darvadstrocel), previously Cx601, for the treatment of complex perianal fistulas in adult patients with nonactive/mildly active luminal CD, when fistulas have shown an inadequate response to at least one conventional or biologic therapy. In the press release, it is stated that Alofisel® should be used after conditioning of fistula. This marks the first allogeneic MSC therapy to receive central marketing authorization approval in Europe [64].

Safety of SC Therapeutic Approaches

Several potential risks should be taken into account before the clinical use of SCs for IBD treatment, such as cell immunogenicity, culture media safety, risk of ectopic tissue formation, and in vitro cell transformation during expansion [49]. Other dominant SC transplantation-associated complications include infection and GVHD, especially for HSC transplantation [65].

With respect to immunogenicity, the majority of the clinical trials on MSC-based therapies in IBD patients have reported a decreased immunogenicity [49]. Because the expansion of several types of SCs is based on the presence of fetal calf serum, the risk of zoonoses transmission and possibly immune reactions in the host as a result of bovine proteins cannot be ruled out. For this reason, animal-free additives, such as platelet lysate/PRP and growth factors, have been alternatively used.

Although the formation of mesenchymal tissues at ectopic sites in vivo has not been reported in clinical trials, however, a long-term follow-up of patients treated with MSCs is required to monitor this risk. Homing and tissue integration might be the possible mechanisms for not forming ectopic tissues.

There are still limited and conflicting data concerning the potential risk of malignant transformation [66, 67]. Nevertheless, a phenotypic, functional, and genetic

assay, although known to have limited sensitivity, should be routinely performed on MSCs before in vivo use, in particular for patient-derived MSCs [49].

As for infection, bacteremia and viremia were found after autologous HSC transplantation in CD patients, with the latter being the most frequent, severe, and life-threatening adverse event (specifically, cytomegalovirus and Epstein-Barr virus). Therefore, it is advisable to prevent complications by conducting drainage of perianal disease beforehand, and by implementing strict hygienic contact measures, and adequate antibiotic prophylaxis schedules [68]. In a recent study that evaluated the feasibility and toxicity of autologous HSC transplantation for the treatment of a series of patients with refractory CD, after improving prophylactic measures, including prophylactic regimens in both mobilization and conditioning transplantation periods, no new multidrug-resistant Gram-negative microorganisms were isolated [69]. It is also important to note that antibiotic policies should be adopted according to the isolates at each center.

rATG reaction can be more pronounced in CD patients undergoing HSC transplantation compared with the patients undergoing transplants for hematological malignancies, possibly due to a weaker immune response in patients with hematological malignancies who underwent prior chemotherapy regimens or due to the higher burden of immune cells in patients with CD. This reaction can be minimized using higher doses of corticosteroids (500 mg) before each rATG infusion [69].

Finally, acute GVHD is a common and unfortunate cause of morbidity and mortality in patients undergoing allogeneic HSC transplantation, affecting the skin, liver, and GI tract [70]. Symptoms of acute GVHD typically include diarrhea and also vomiting, abdominal pain, and anorexia. Diarrhea in GVHD is secretory and usually voluminous; bleeding and ulceration of the mucosa is also common and a poor prognostic factor. The histology in GVHD is characterized by crypt apoptosis, glandular atrophy, and flattening of the surface epithelium. Differential diagnosis includes IBD symptoms. However, glandular architectural distortion, diagnostic of IBD, is not a typical characteristic of GVHD. Both IBD and GVHD involve a disruption and injury of the intestinal epithelium. Administration of corticosteroids is standard first-line therapy for acute GVHD but leads to remission in less than half of patients. Second-line therapy is variable, depending on the experience of the clinician and availability of therapies. ATG, pentostatin, mycophenolate mofetil, and other monoclonal antibodies (e.g., rituximab, etanercept) have been used to treat steroid-refractory GVHD. Patients with acute GVHD are immunosuppressed and already at high risk of mortality from infection and organ damage from GVHD, but these biological therapies may increase the risk of infection. Therefore, autologous HSC transplantation and MSC-based therapies are recommended to avoid GVHD.

Concluding Remarks

The currently available clinical data indicate that HSC- and MSC-based therapeutic approaches for IBD are very promising. Autologous HSC transplantation is a feasible therapeutic approach for selected patients with refractory CD but should be performed in highly experienced centers using a multidisciplinary approach (gastroenterologists, hematologists, radiologists, infectologists, and surgeons). Allogeneic HSC transplantation should not be recommended due to high morbidity and mortality, as it increases the risk of acute GVHD. To avoid this risk, Alofisel® (darvadstrocel), the only approved allogeneic MSC therapy for the treatment of complex perianal fistulas in adult refractory CD patients, should be preferred. The basic challenges however still remain concerning the determination of the best SC type and administration route and also the optimal cell dose needed at the lesions to guarantee safe and effective therapy.

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Paneth Cell Physiology and Pathophysiology in Inflammatory Bowel Disease

Billy R. Ballard and Amosy E. M'Koma

Introduction

Paneth cells (PCs) are cells that provide host defense against microbes in the gut mucosa and were first described by Joseph Paneth, a physiologist from Vienna, Austria (October 6, 1857–January 4, 1890) [1]. Paneth cells are unique epithelial cells responsible for secreting the antimicrobial peptides (AMPs) known as human

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Billy R. Ballard – Critical reading of the manuscript and evaluation of the validity of the content.

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alpha-defensin 5 (*DEFA5* also known as HD5) and human alpha-defensin 6 (*DEFA6* also known as HD6) as well as enzymes including lysozyme and phospholipase A2 that act to keep the intestinal crypts sterile [2–7]. They play a major role in defensive mechanisms against a broad range of intestinal microbes and in the regulation of host immunity [8]. Paneth cells derive from fast-cycling crypt base columnar cells, which are involved in crypt regeneration; they differentiate while migrating toward the crypt base, from which they are eventually phagocytosed [9, 10]. Paneth cells are mainly located within the ileum (Fig. 1), but they may occur in sporadic numbers in the proximal colon (caecum to transverse colon), and have been reported more distally only in pathological conditions [11].

Paneth cells are termed "metaplastic" when seen in areas in which they are "ectopic" or out of place and is in not normally found locations: Paneth cells in the distal colon (descending colon, sigmoid, and rectum) are always metaplastic. In pathological states, an increase in PC numbers – known as PC hyperplasia – may occur in the proximal colon [11]. Paneth cell metaplasia (PCM) has often been described in idiopathic inflammatory bowel disease (IBD), both ulcerative colitis (UC) and Crohn's disease (CD). It is thought to be a sign of a long colitis history: it correlates with disease duration, and it has been attributed to the effects of repair and regeneration [11–13]. Guidelines for reporting gastrointestinal biopsies published by the British Society of Gastroenterology in 1997 [14, 15] concurred that PCM was an indicator of chronic epithelial cell damage, though it was not included in the data set for IBD reporting as its diagnostic value was unclear. PCM may be present in other pathological states – in neonates, PC numbers are increased in the regenerating bowel following necrotizing enterocolitis [15] but is not seen in self-limiting infectious colitides [16, 17].

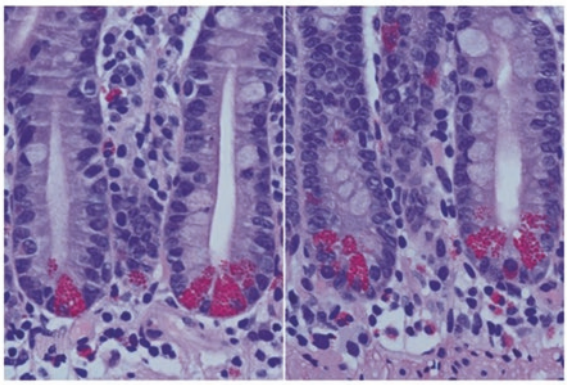

Fig. 1 Normal small intestinal crypts with basal orientated Paneth cells. (**a**) Paneth cells are characterized by their apical located granules (arrows). Between Paneth cells undifferentiated progenitor cells are found. In the normal *Lamina propria mucosae*, a mixed population of immune cells and stroma-resident cells is found; (**b**) occasionally, Paneth cells at the bottom of small intestinal crypts are mixed up with enteroendocrine cells (arrow). They are characterized by basal located granules. In the upper part of the crypt, a mitotic figure is shown. (Adapted with permission, Ref. [19])

There have been few studies of PCM in pediatric groups. Inflammatory bowel disease may present differently in this age population: new data of ulcerative colitis (UC) in children show a different pattern of inflammation from that seen in adults [18]. In this *e*-book chapter, we describe the distribution of PCs in symptomatic children without significant gastrointestinal pathology, compare the findings with newly diagnosed IBD, and evaluate the relation between PCM and histological features of chronic disease.

Core Focus

Paneth cells physiologically locate in ileal crypts [19]. Wnt signaling promotes their differentiation and abundance of events to relocate into crypts, whereas Notch activities act in opposition to PC maturation. The cells essentially promote to assists crypt morphogenesis and intestinal homeostasis, making the microbiome by excreting antimicrobial peptides, like defensing 5 and defensing 6, and crypt fission. In distinct intestinal disorders, there is increasing molecular evidence that PC disorders and malfunction are strongly involved in the etiopathogenesis physiology of several intestinal diseases including ileal Crohn's disease [19], Crohn's colitis [20], and necrotizing enterocolitis [21].

Paneth Cells

Paneth cells are an important part of the IBD differentiation because they are the only producer of DEFA5 and DEFA6 in the ileum. In normal conditions PCs should not be present in the colon [22]. Interestingly, there are multiple publications that show a decrease in the levels of PCs in the ileum in patients with CD [23–26]. However, the reverse is true in the case of Crohn's colitis (CC). There is evidence proving ectopic metaplasia of PCs in patients suffering from CC as reported the abundance of PCs in the colon with Crohn's [20]. Further, in animal studies, pharmacological inactivation of the $^{\gamma}$-secretase, an activator enzyme involved in the activation of the Notch, has been elucidated. In the experimental treatment of wild-type black-6 mice with iminostilbene, also known as dibenzazepine (DBZ), demonstrated significant increase in the secretory of differential cells located through ileal crypt. Subsequent to DBZ treatment, ileal crypts are lined by orthotopic Paneth cells and transdifferentiated cells with morphological features mixed from PCs and mucus-retaining cells (Fig. 2). The alteration in secretory differentiation is termed secretary metaplasis [19].

The action of dysregulation of PCs in both ileal CD and colonic CC has very important mechanistic implications because it leads to dysregulation of the secretion of DEFA5/ HD5, DEFA6/HD5, and antimicrobial peptides (AMPs) which are vital in the checks and balances regulation of gut microbes and homeostasis [27]. In

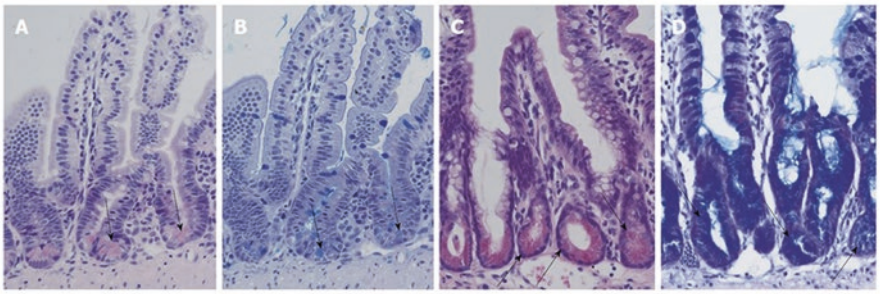

Fig. 2 Differentiation of Paneth cells and Notch inhibition. Normal ileal mouse mucosa with normal crypts and Paneth cells: HE staining (**a**) and alcian-PAS staining (**b**). Arrows indicate Paneth cells. Ileal mouse tissues after treatment with dibenzazepine show an increase in secretory cells in the crypts with differentiation of Paneth cell-like epithelia (arrows): HE staining (**c**) and alcian-PAS staining (**d**). (Adapted with permission, Ref. [19])

the ileum, these AMPs are critical to maintaining the sterile environment required for healthy small intestinal function [28–31]. A reduction in the "normal" levels of PCs, and therefore AMPs, during an episode of IBD, will therefore lead to dysbiosis (a microbial imbalance or maladaptation) of gut flora [32]. Now that we are aware that there is metaplasia of PCs in the large bowel in CC which is significantly increase when compared with UC, there is an interest in elucidating the repercussion of high levels of crypt PCs on the large bowel flora.

Human Alpha-Defensin 5

Human alpha-defensin 5 (DEFA5 also known as HD5) is a human protein that is encoded by the DEFA5 gene and is highly expressed in secretory granules of crypt Paneth cells (PCs) of the ileum [33]. Crypt PCs are normally not found in normal colon epithelium [34]. However, metaplastic PCs are abundantly observed in CC colectomy specimens and occasionally in patient with UC [20].

Human alpha-defensin 5 is a 32-residue cysteine-rich host defense peptide which exerts broad-spectrum antimicrobial activity and contributes to innate immunity in the human gut and other organ systems [33]. Human α-defensin 5 is classified as an antimicrobial peptide (AMP), along with other defensins and small immune proteins responsible for killing bacteria in the gut [29–31].

Defensins are aberrantly regulated in IBD, but there is no clear or known mechanism for their involvement in the IBD etiopathogenesis process [20, 35]. In addition, the literature varies on which form of IBD shows an increased expression of DEFA5, and some publications suggest that levels may fluctuate depending on location within the colon and severity of disease and some suggests that AMPs studies vary broadly based on the background. This may explain some of the conflicts

within the published literature and highlight the importance of using human tissues and cell lines to study defensins and IBDs.

In addition to be a potential diagnostic to differentiate UC from CC, there is interest in studying DEFA5 in IBD because of the importance of regulation of the immune system and gut flora in these diseases. Human α-defensin 5 is an important immune system protein that has been shown to regulate pro-inflammatory cytokine production in host cells, which may have an important function in inflammatory diseases. If we are able to determine the function of DEFA5 in IBD, we may gain some valuable insight that can be applied to diagnosis, prognosis, and biologic treatment of these diseases.

Human alpha-defensin 5 may be important in the development of dysbiosis. Dysbiosis of the gut microbiome has been associated with behavioral deficits via the interconnected network known as the microbiota-gut-brain axis. Given the growing number of clinical advances, luminal DEFA5/HD5 has been considered the most crucial factor to impact the gut microbiome composition and therefore provides a promising target for the precise control of IBD, behavior, and mental health.

Ectopic Colonic Paneth Cell Metaplasia

In different intestinal disorders, the normal physiology of Paneth cells is disturbed and becomes dysfunctional because of true injuries from such intestinal disorders, where differentiation is necessary to compensate cellular stress or misdirected mucosal differentiation and/or tissue homeostasis [19]. The important histomorphological findings include loss, transdifferentiation, or metaplasia of PCs.

Metaplasia is defined as the occurrence of differentiated cells in a histomorphological location, where they are physically and physiologically not found, such as in the colon. Paneth cell metaplasia is observed throughout the GI tract but frequently manifests in the stomach and is associated with different intestinal injuries, Fig. 3.

Paneth cell loss is a hallmark of special types of ileal Crohn's disease [19]. The reverse is true for colonic Crohn's disease; instead ectopic metaplastic PCs appear [20]. There is some contradictory phenomenology that PC loss, formation, and transformation could be due to reprogrammed stem cells [36]. Presently, it is well established that the molecular framework underlying the process of regular PC differentiation and maturation depends on Wnt activity [37]. In contrast to the crypt physiology, the role of Wnt signaling in the development of PC metaplasia is poorly elucidated. However, there is some understanding that Wnt activity is a driving force in metaplasia [38]. In an experimental study with the constitutive expression of a β-catenin-Lef1 fusion protein under control of a lung-endoderm-specific promoter from the surfactant protein C gene, transgenic lungs included cells expressing marker genes strongly characteristic of intestinal PCs [38]. The data strongly supports the view that hyperactive Wnt signaling could be crucial in stem cell lineage commitment and the generation of intestinal metaplasia. Lysozyme was used in the lung study, where as a marker protein of mature PCs, increased Wnt activity was

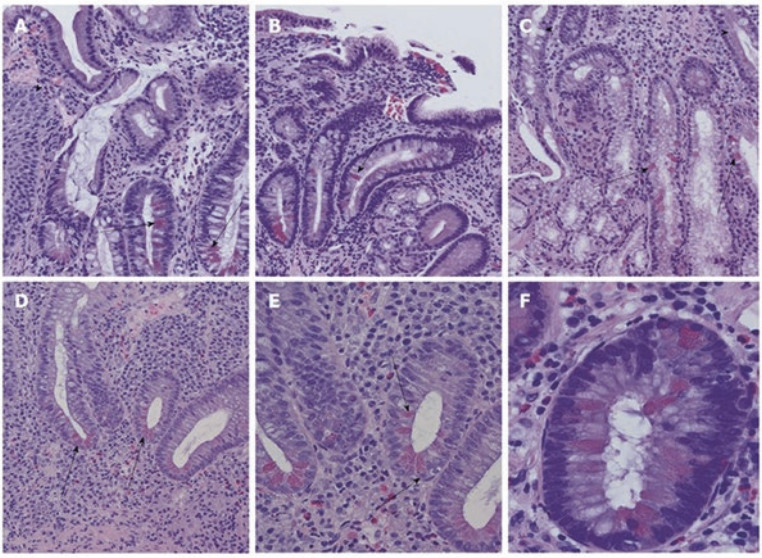

Fig. 3 Examples of Paneth cell metaplasia throughout the intestinal tract. (**a**) Paneth cell metaplasia (arrows) in Barrett's mucosa. Squamous epithelia of the esophagus are marked with an arrowhead; (**b**) chronic atrophic gastritis with Paneth cell metaplasia (arrow); (**c**) Paneth cell metaplasia (arrows) of Brunner's gland. In the upper part, small intestinal mucosa and secretory ducts are shown (arrowheads); (**d**) colon mucosa in ulcerative colitis with disturbed crypt architecture, increased numbers of stroma infiltrating inflammatory cells, and Paneth cell metaplasia (arrowheads); (**e**) higher magnification of ulcerative colitis-associated Paneth cell metaplasia (arrows) in colon mucosa as demonstrated in (**d**); (**f**) Paneth cell metaplasia (arrows) in tubular adenoma of the colon with low-grade dysplasia. (Adapted with permission, Ref. [19])

associated with diminished lysozyme expression in metaplastic cells [38]. This intriguing discovery is highly suggestive that Wnt activity is a critical prerequisite for PC metaplasia, but additional factors and supportive signaling mechanisms are essential. The auxiliary components necessary for PC differentiation and maturation probably differ between tissues and may require experimental validations. For example, inflammation seems to induce PC metaplasia/hyperplasia in the colon [20, 23], but not in the ileum [23]. The intestinal microbiome is suggested as an important variable in promoting signaling activities and the expression level of several intestinal pathways and regulates phenomena including inflammation and cellular differentiation.

Paneth cell source is from crypt base columnar cells involved in crypt regeneration which differentiate while migrating toward the crypt base from which they are eventually phagocytized [9, 10]. Paneth cells are abundantly located in the ileum; in adults they sporadically appear in the cecum to transverse colon but have been reported more distally only in pathological states [11, 20]. Paneth cells are supposedly not found in normal colon [34]. When PCs are found ectopically in areas they are not supposed to be (such as distal colon – descending, sigmoid, and rectum),

they are termed “metaplastic” PCs [20, 39]. In pathological conditions, an increase in PC abundancy – known as PCs “hyperplasia” – may be seen in the proximal colon [11]. Paneth cell metaplasia (PCM) has most recently been described in idiopathic IBD especially in Crohn’s colitis (CC) [20]. It is thought to indicate sign of a long colitis history, as correlates with disease duration and has been translated attributed to the effects of repair and regeneration [11–13]. The British Society of Gastroenterology which published the guidelines for reporting gastrointestinal (GI) biopsies concurred that PCM was an indicator of chronic epithelial cell damage [14], but it was inconclusive in the data set for IBD diagnostics until recently when both DEFA5 and PCs were abundantly detected in Crohn’s colitis when compared to ulcerative colitis of surgical pathology colectomy samples [20]. However, we believe that DEFA5 will serve as a better diagnostic candidate biomarker than PCs for Crohn’s colitis and indeterminate colitis that are eventual Crohn’s colitis [20]. We describe, in this paper, the abundance distribution of PCs in CC compared to UC and evaluate the relationship between PCM and diagnostic features of CC.

Paneth Cell Functionality

Paneth cells are found throughout the small intestine and the appendix at the base of the intestinal glands [40, 41]. The PC numbers demonstrate an ascending trend with highest numbers toward the distal end of the small intestine. Like the other epithelial cell lineages in the ileum, PCs originate at the stem cell region near the bottom of the gland [42]. However, unlike the other epithelial cell types, PCs migrate downward from the stem cell region and settle just adjacent to it [42]. This close relationship to the stem cell region is thought to suggest that PCs are vital in defending the gland stem cells from microbial damage [42], although their function is not entirely elucidated [40, 41]. Intriguingly, among the four aforementioned intestinal cell lineages, the PCs live the longest, 18–23 days.

As described earlier, these cells synthesize and secrete substantial quantities of AMPs and proteins. More recent studies have demonstrated that these antimicrobial molecules are key mediators of host-microbe interactions, including homeostatic balance with colonizing microbiota and innate immune protection from gastrointestinal pathogens. Perhaps more intriguing, PCs secrete factors that help sustain and modulate the mucosal stem and progenitor cells that cohabitate in the crypts and rejuvenate the ileal epithelium [30].

Ileal crypts house stem cells that serve to constantly refill epithelial cells that die and are lost from the villi. Protection of these stem cells is essential for long-term maintenance of the epithelium, and the location of PCs adjacent to stem cells suggests that they play a critical physiological role in defending epithelial cell renewal.

Paneth cells sense pathogens via MyD88-dependent toll-like receptor (TLR) activation which then triggers antimicrobial action [42, 43]. These AMPs are hydrophobic and are positively charged domains that can interact with phospholipids in cell membranes. This composition allows defensins to insert into membranes, where

they interact with one another to form pores that disrupt membrane function, resulting to cell lysis. Due to the higher concentration of negatively charged phospholipids in bacterial than vertebrate cell membranes, defensins favorably bind to and disrupt bacterial cells, leaving the cells they are functioning to protect [44].

Paneth cells are stimulated to secrete defensins when exposed to both Gram-positive and Gram-negative bacteria (or such bacterial products as lipopolysaccharide, muramyl dipeptide, and lipid A). In addition to defensins, PCs secrete lysozyme, tumor necrosis factor-alpha, and phospholipase A2. Lysozyme and phospholipase A2 both have clear antimicrobial functionality. This battery of secretory molecules gives PCs a potent arsenal against a broad spectrum of agents, including bacteria, fungi, and even some encapsulated viruses.

Paneth Cells and Disinfectant Activities

In the intestine, overabundance of autochthonous long-term colonizers microbes and allochthonous transient microorganisms is found [19]. One important function of indigenous commensal microbiota is host defense through the promotion of the mucosal innate and/or adaptive/ acquired immune system and killing of pathological microbes [45]. Another function is the nutrition of the host, because the microorganisms symbiotically have the capacity to ferment components of the diet. The synthesized short-chain fatty acids (SCFAs), vitamins, and amino acids are mandatory in maintenance of host homeostasis physiology and energy balance. SCFAs act as signaling molecules and highly specialized free fatty acid receptors (FFARs) exist in the gut epithelium. FFARs contribute to the chemosensory ileal system and differ in their molecular structure, ligand specificity, and functionality [46]. The available data indicate that SCFAs induce glucagon-like peptide-1 release from intestinal cells. The molecular link is important to display the integral role of the gut microbiome in the regulation host's energy homeostasis. Metabolic disorders and obesity are associated with changes in the intestinal microbiota [47]. In the intestine, an integrated complex molecular system is established to sustain microbiome-host homeostasis and to shape the composition of microbes colonizing the gut. For this reason, PCs sufficiently express and secrete different types of AMPs which are essentially important host defense substances in the symbiotic communication between host and microbiome.

The secretory capacity of PCs is reflected by the cytoarchitecture with apical clustering of large secretory granules. Ultrastructurally, the endoplasmic reticulum (ER) is hyperplastic and associated with a well-organized Golgi infrastructural network. Upon prosecretory stimuli like bacterially derived toll-like receptor ligands, the cytosolic calcium content enhances via KCa3.1, a calcium-activated potassium channel, and leads to granule secretion [44, 48, 49]. Defensins are the major AMP family involved in intramolecular desulfated bonds [41, 50]. In addition to alpha-defensins, PC granules contain lysozyme as another potent host defense molecule. The biosynthesis of active alpha-defensins depends on proteolytic processing. In

murine PCs, matrix metalloprotease 7 (MMP7) is found cleaving defensin precursor molecules to active alpha-defensins [51, 52]. Disturbed proteolytic activity as found in MMP7 knockout mice is associated with a thwarted gut microbiome and an enhanced distribution of bacterial pathogens [53].

In comparison to mice, trypsin is found in human PCs as an activating protease for prodefensins and the antimicrobial protein Reg3A [54]. The DEFA5 and (DEFA6) are essential in host protection from gut pathogens. Using a DEFA5 transgenic mouse model with physiological defensin levels, animals were resistant to enteric *Salmonella* infections due to reduced viability of bacteria, diminished bacterial translocation, and increased survival after lethal *Salmonella* infections [55]. In the same standard, evidence of a direct role of alpha-defensins in regulating and shaping the ileal microbiome was shown [53]. In the intestine of DEFA5 transgenic animals, the number of indigenous commensals was changed with an increase in *Bacteroides*, a reduced in *Firmicutes*, and the loss of segmented filamentous bacteria (SFB), designated *Candidatus arthromitus*, accompanied by an absence of Th17 cell differentiation. SFB are host-specific gut symbionts belonging to the *Clostridiaceae* displaying differentiation of filaments that interact intimately with the lining mucosal epithelia. Secreted proteins are expressed by SFB including different ADP-ribosyltransferases and a myosin cross-reactive antigen, all involved in modifying the host response and postnatal maturation of the luminal immune system [56, 57]. Recently, an SFB-host cell co-culturing system was established producing viable infectious particles, which can colonize intestinal mucosa with the initiation of an immune response [58]. Regional difference in the expression and secretion of PC AMPs along the intestinal tract are well balanced by the colonizing activity of SFB with the establishment and shaping the intestinal microbiome [59]. The DEFA6 secreted by human PCs acts differently to DEFA5. The DEFA6 is antibacterial by the configuration of self-assembled peptide nanonets and formation of nanofibrils [60, 61]. Targeted bacteria surrounded by the fibrils and nanonets are unable to invade the gut mucosa. The DEFA6 self-assembling function is usually based on the presence of histidine-27, which forms a salt bridge essential for multimerizing the peptide. The vital PC produced DEFA5 and DEFA6 can be further classified by their specific functions. The antimicrobial DEFA5 activity with disruption of bacterial membranes is called “HARPOON” activity, whereas DEFA6 represents so named “NET FORMING” activities [62]. In brief, DEFA5 acts antimicrobially and exhibits lectin-like properties, whereas DEFA6 entangles microbes and protects host cell invasion [28, 33].

Further to defensins, PCs secrete other AMPs including lysozyme, secretory phospholipase A2 (sPLA2), RegIII, angiogenin 4, and cathelicidins [29–31]. Among the AMPs, RegIII proteins are critically important in antibacterial defense, and murine RegIIIγ shares 65% identity with human RegIIIα. RegIII proteins belong to the family of C-type lectin regenerating islet-derived proteins and bind glycan chains of peptidoglycans on the cell wall of Gram-positive bacteria. In contrast to other C-type lectin AMPs as mannose-binding lectin, the complement recruitment domains are not constantly expressed in RegIII suggesting a direct anti-bactericidal function [63].

The AMP LL-37 belongs to the cathelicidin family and acts in a similar way as DEFA5 by puncturing holes in microbial membranes. LL-37 mediates antimicrobial activities in addition to immunological functions via various cellular receptors [64]. LL-37 is an inducible AMP, because LL-37 expression increases in the presence of SCFAs. Consequently, the protein is predominantly found in the transit-amplifying zone in colonic crypts, when compared with small intestinal mucosa [65]. In quantitative terms, the ratio of DEFA5 to DEFA6 is about 3:1, whereas DEFA5 expression levels are higher by a factor of up to 100 than those of lysozyme and sPLA2 [25].

Paneth cells are secretory cells with the excretion of a plethora of molecules including several types of AMPs [28–31]. The secretory function depends on adapted ER, which is determined by the behavior and smoothed by autophagy. Disruption of autophagy by the deletion of the unfolded protein response transcription factor X-box binding protein-1 results in ER stress, PC impairment, and spontaneous inflammation resembling special variants of CD [66]. The experimental data show that lipids and lipid metabolizing enzymes are involved in the signaling cascade for exocytosis of granules from PCs [67]. Expression profiling revealed a panel of target genes and related proteins including lipoprotein lipase, apolipoprotein A-IV, stearoyl-CoA desaturase-1, adiponectin, and leptin. Mapped pathways include PPAR signaling, statin pathway, adipocytokine signaling, and polyunsaturated fatty acid biosynthesis. It has been speculated that the lipid-associated exocytosis is an additive mechanism to the chemosensory system of FFARs and enteroendocrine cells to perform synergisms between the intestinal microbiome and host metabolism.

The release of AMPs from PCs into the gut mucus and lumen is initiated by stimulation of pattern recognition receptors (PRRs) located on intestinal surfaces. The class of PRRs includes toll-like receptors (TLRs) activated by lipopolysaccharide and nucleotide-binding oligomerization domain-containing molecules (NODs), which recognize muramyl dipeptide.

Independently of PPRs, bacterial cell wall glycolipids can stimulate the release of defensins by PCs. The myeloid differentiation primary response gene (MyD88) acts as an important cytoplasmic TLR limiting bacterial penetration into the intestinal mucosa [25].

In short, Paneth cells are necessary for the development of a mucus layer enriched with AMPs resting on the epithelia. The makeup of AMPs contrasts along the intestine, implicating various functions in communication with the gut microbiome.

Paneth Cells and Gastrointestinal Disorders

The characteristic histomorphological feature of intestinal mucosa is as a result of high numbers of *Lamina propria mucosae* infiltrating leukocytes with different types of specialized lymphocytes, plasma cells, mast cells, monocytes, and eosinophils. The even correspondence of the immune cells with the gut microbiome via

the luminal epithelium is necessary for intestinal homeostasis. Subsequently, an inflammatory response with a further increase in leukocytes is very common in most gastrointestinal diseases and is established in the most of gastrointestinal diseases with remarkable differences in the infiltrating leukocytes quantitatively as well as qualitatively. The differences of leukocytes are helpful to classify the basal disease using morphological, immunohistochemical, and functional techniques. In addition to resting PCs, infiltrating leukocytes are an important transient source for AMPs. They are aided by metaplastic PCs, available in the colon and stomach. In intestinal inflammation, sPLA2, which is physiologically not found in the colon, is expressed by metaplastic PCs [68, 69]. There are experimental data showing a direct role of the microbiome in controlling the defensin secretion. Using *NOD2* knockout mice in co-housing experiments, the wild-type microbiome was able to regulate defensing secretion to physiological level [70]. The inflammation-related expression profile of AMPs includes some characteristics showing a defined gastrointestinal disease [71, 72]. For example, β-defensin types 2, 3, and 4 are increased in UC but not in ileal and/or colonic CD [73]. In concise, *NOD* is highly expressed in ileal PCs that essentially contribute to the regulation of ileal microbiota through the secretion of AMPs [27].

As outlined above, PCs are the main source of AMPs and act in stabilizing the stem cell zone and are a site of origin for intestinal inflammation. This point of view is of high relevance concerning the pathogenesis of IBD, "The Colitides." A fundamental feature of ileal CD is a reduced expression of DEFA5 and DEFA6, and a reverse is true for UC. A fundamental feature of colonic CD is an increased expression of DEFA5 and DEFA6 [20]. The finding is sometimes paralleled by a reduced number of PCs but may be also a result of an injured microbiome [24, 25, 74].

An in-detail analysis of PCs in CD revealed subgroups of the disease characterized by unique molecular, morphological, and clinical features. The clinical phenotype of ileal CD is mostly associated with PC injuries. The first evidence from loss-of-function mutations was in *NOD2* (SNP 13), a gene that is strongly expressed by PCs [27, 75–77]. Subsequently, the molecular mechanisms underlying several injuries of PCs were reported and linked to ileal CD. One important finding was low Wnt signaling activity in ileal CD with diminished TCF-4 and reduced secretion of defensins [26, 78]. Another milestone was the identification of defective granule exocytosis from PCs with diminished levels of defensins due to abnormal autophagy in homozygosity for the risk allele autophagy-related 16-like 1 [67, 79]. The molecular characterization of disturbed autophagy and unresolved ER stress due to genetically and environmentally controlled dysfunction of unfolded protein response (UPR) was cornerstone in understanding the underlying mechanisms for the possible genesis of ileal CD [66, 67, 80].

Further to PCs, enterocytes are an additional source to secret AMPs including Reg proteins, defensins, and cathelicidins [28–31]. This is of high relevance to physiologically reimburse the perinatal period prior to the inception of PCs and to pathologically restore PC disorders [81].

Paneth Cells and Morphogenesis

Crypt developmental fission, the division of a single crypt into two daughters, is fundamental in intestinal tissue expansion and morphogenesis but is also found in tumorigenesis driving the clonal expansion of mutant adenomatous crypts [82–85]. A specific cellular arrangement in the intestinal stem cell niche is observed that controls crypt fission. In a recent model, PCs and CBCs (Lgr5+) are both importantly involved in crypt morphogenesis [86]. The findings summarize the data from intestinal organoids, where PCs are essentially involved in crypt budding [87, 88] and as mice lacking intestinal PCs, which are able to sufficiently repair crypt injuries [34, 89]. The morphogenic activities of PCs depend on Wnt signaling and redundant sources contribute [22, 90].

Important for the crypt fission model is the observation that Paneth cells adhere to their substrate more strongly than other crypt cells. In view with this strong evidence is given that so-called "mislocalized" PCs change the symmetry of fission in determining the site of fission [86].

In summary, Paneth cells are important secretory cells in the small intestinal mucosa. They are found as metaplastic cells in several other gastrointestinal tract locations. Important secretory products of PCs belong to the AMPs. They are essential for the symbiotic dialogue between the microbiome and the host. Besides to PC functions in controlling the microbiome, the cells are functionally and structurally inculpated in forming the stem cell zone of ileal crypts and entangled in morphogenesis of the crypt-villus axis (CVA). In the last decade, molecular mechanisms of different PC injuries have been identified and correlated with gastrointestinal PC disorders [19]. Experimental data indicate that ileal Crohn's disease and necrotizing enterocolitis are strongly associated with pathological PCs. The important role of PCs in gastrointestinal physiology and pathophysiology firmly supports the view that these cells are the guardian of small intestinal crypts.

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