Advances in Experimental Medicine and Biology 1237
Cell Biology and Translational Medicine

Kursad Turksen

Cell Biology and Translational Medicine, Volume 7

Stem Cells and Therapy: Emerging Approaches

Advances in Experimental Medicine and Biology

Cell Biology and Translational Medicine

Volume 1237

Subseries Editor
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Editor

Cell Biology and Translational Medicine, Volume 7

Stem Cells and Therapy: Emerging Approaches

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ISSN 0065-2598 ISSN 2214-8019 (electronic)
Advances in Experimental Medicine and Biology
ISSN 2522-090X ISSN 2522-0918 (electronic)
Cell Biology and Translational Medicine
ISBN 978-3-030-37844-8 ISBN 978-3-030-37845-5 (eBook)
https://doi.org/10.1007/978-3-030-37845-5

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Preface

In this next volume of Cell Biology and Translational Medicine series, we continue to explore the potential utility of stem cells in regenerative medicine. Chapters in this volume cover several crucial aspects of tissue and organ regeneration and restoration of function in clinical settings.

I remain very grateful to Gonzalo Cordova, Associate Editor of the series, and acknowledge his continuous support.

I would also like to acknowledge and thank Sara Germans-Huisman, Assistant Editor, for her outstanding efforts in helping to get this volume to the production stages.

A special thanks goes to Rathika Ramkumar for her outstanding efforts in the production of this volume.

Finally, sincere thanks to the contributors not only for their support of the series but also for their insights and efforts to capture both advances and remaining obstacles in their areas of research. I trust readers will find their contributions as interesting and helpful as I have.

Ottawa, ON, Canada Kursad Turksen

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Adv Exp Med Biol - Cell Biology and Translational Medicine (2019) 7: 1–16
https://doi.org/10.1007/5584_2019_430
© The Author(s) 2019, corrected publication 2021
Published online: 30 August 2019

Application of iPSC to Modelling of Respiratory Diseases

Ben A. Calvert and Amy L. Ryan (Firth)

Abstract

Respiratory disease is one of the leading causes of morbidity and mortality world-wide with an increasing incidence as the aged population prevails. Many lung diseases are treated for symptomatic relief, with no cure available, indicating a critical need for novel therapeutic strategies. Such advances are hampered by a lack of understanding of how human lung pathologies initiate and progress. Research on human lung disease relies on the isolation of primary cells from explanted lungs or the use of immortalized cells, both are limited in their capacity to represent the genomic and phenotypic variability among the population. In an era where we are progressing toward precision medicine the use of patient specific induced pluripotent cells (iPSC) to generate models, where sufficient primary cells and tissues are scarce, has increased our capacity to understand human lung pathophysiology. Directed differentiation of iPSC toward lung presented the initial challenge to overcome in generating iPSC-derived lung epithelial cells. Since then major advances have been made in defining protocols to specify and isolate specific lung lineages, with the generation of airway spheroids and multi cellular organoids now possible. This technological advance has opened up our capacity for human lung research and prospects for autologous cell therapy. This chapter will focus on the application of iPSC to studying human lung disease.

Keywords

Differentiation · Human models · iPSC · Lung disease · NKX2.1 · Stem cell

Abbreviations

ADA-SCID	adenosine deaminase deficiency-related severe combined immunodeficiency
AFE	anteriorization of the ventral foregut endoderm
ALI	air-liquid interface

The original version of this chapter was revised: This chapter was previously published non-open access which has now been changed to open access under a CC BY 4.0 license and the copyright holder updated to 'The Author(s)'. The correction to this chapter is available at https://doi.org/10.1007/978-3-030-37845-5_442

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APS	anterior primitive streak
BMP	bone morphogenetic protein
CDX2	caudal type homeobox 2
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane regulator
COPD	chronic obstructive pulmonary disease
CXCR4	C-X-C chemokine receptor type 4
DE	definitive endoderm
DMD	Duchenne's Muscular Dystrophy
DMH-1	dorsomorphin homolog 1
ESC	embryonic stem cells
FACS	flow activated cell sorting
FGF-2	fibroblast growth factor 2
FOXA2	forkhead box A2
GATA	GATA binding protein
GD	Gaucher disease
HBEC	human bronchial epithelial cells
HD	Huntington disease
IPF	idiopathic pulmonary fibrosis
iPSC	induced pluripotent stem cells
ITGA6 or CD49f	integrin alpha 6
JDM	juvenile-onset, type 1 diabetes mellitus
KLF4	Kruppel like factor 4
KRT5	cytokeratin 5
LP	lung endodermal progenitor cells
mRNAs	messenger ribonucleic acid
NGFR	nerve growth factor receptor
OCT4/POU5F1	POU domain, class 5, transcription factor 1
PAX6	Paired box gene 6
PAX8	Paired box gene 8
PCD	primary ciliary dyskinesia
PD	Parkinsons disease
PDX1	pancreatic and duodenal homeobox 1
RA	Retinoic acid
RNA	ribonucleic acid
SBDS	Shwachman-Bodian-Diamond syndrome
Shh	Sonic hedgehog
SOX2	sex determining region Y 2
SPC	surfactant protein C
TEER	trans-epithelial electrical resistance
TGFβ	transforming growth factor beta
TP63	tumor protein p63
TTF1	thyroid transcription factor 1
Wnt	wingless INP pathway

1 Introduction

Respiratory disease is currently the third leading cause of morbidity and mortality worldwide (Lozano et al. 2012). It is also the leading cause of hospitalisations in developed countries (Hubbard 2006), placing huge individual and socioeconomic burdens on healthcare systems. Respiratory diseases encompass a wide range of disorders extending from more common diseases such as chronic obstructive pulmonary disease (COPD) and asthma to rare genetic disorders including cystic fibrosis (CF) and primary ciliary dyskinesia (PCD). Whilst each individual respiratory disorder possesses its own aetiology and pathophysiology, they often share many disease relevant commonalities, such as abnormal inflammation, increased susceptibility to infection and dysfunctional or damaged epithelia. Currently, many respiratory diseases are symptomatically managed with no effective treatment. Our understanding of disease initiation and progression is hindered through lack of robust *in vitro* models that closely reflect the disease phenotype as it occurs in humans for investigative research and drug screening. Many therapeutic "hits" discovered in mouse models do not translate successfully into humans leading to a high failure rate of lung therapeutics in clinical trials (Barnes et al. 2015). In this review, we evaluate the use of induced pluripotent stem cells (iPSC) for respiratory research and their potential for therapeutic applications in respiratory disease.

2 Induced Pluripotency

The discovery that fully differentiated/mature somatic cells can have pluripotency induced by Yamanaka et al. in 2006, ushered in a new era of genetic and cell biology research (Takahashi and Yamanaka 2006). This work identified that a minimal cocktail of 4 transcription factors, Oct4, Sox2, Klf4 and c-Myc, in combination with specific culture conditions was sufficient to

reprogram terminally differentiated cells back into a state of pluripotency, akin to that of embryonic stem cells (ESCs) found in the inner cell mass of the blastocyst (Takahashi et al. 2007a, b; Okita et al. 2007) (Fig. 1). These cells acquired an infinite capacity for self-replication and differentiation into cells and tissues from all germ layers, including endodermal lung progenitors. As iPSC are generated by isolating cells from somatic tissues, they circumnavigate the ethical issues surrounding the use of ESCs (Murugan 2009). iPSC have revolutionized our capacity to carry out research in relevant human cells providing an exceptional tool for disease modelling, as well as possessing a huge potential for regenerative therapy.

Yamanaka and colleagues originally generated iPSC by transducing mouse fibroblasts with Oct4, Sox2, Klf4 and c-Myc transcription factors via pMX based retroviral vectors. Since then, other methods and factors have been utilized to successfully induce pluripotency in a wide range of somatic and germ line cells, these are summarized in Table 1. Initially, lentivirus became favoured over retroviruses due to its capability of infecting post-mitotic cells as well as dividing cells (Yamashita and Emerman 2006). Other virus types are also used, such as adenovirus and Sendai virus, favoured for their non-integrating nature (Zhou and Freed 2009), helping to maintain host genomic integrity with the original viral RNA diluted with each cell division (Fusaki et al. 2009). The most recent shift in technology is

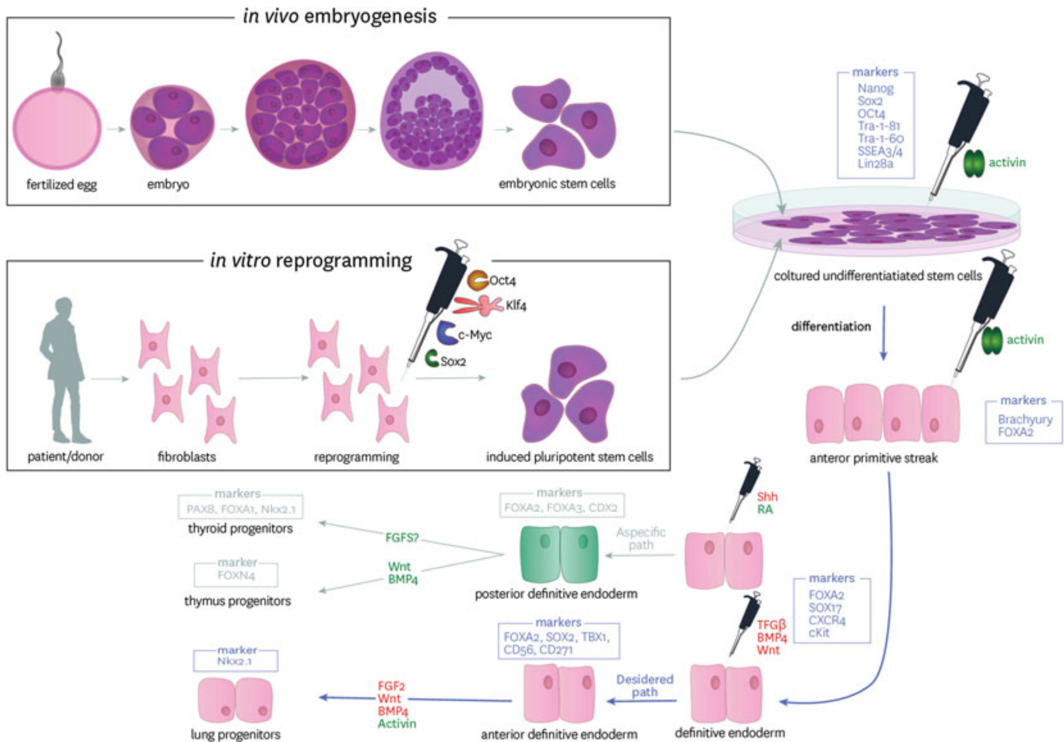

Fig. 1 Pluripotent cell differentiation toward primordial lung progenitor cells. Pluripotent stem cells are isolated and expanded in vitro from the inner cell mass of the blastocyst (Embryonic Stem Cells or ESC) or from reprogramming of somatic cells from individuals (induced pluripotent stem cells or iPSC). Following a stepwize differentiation protocol mimicking the key steps in embryogenesis, cells are differentiated through FOXA2, SOX17 expressing definitive endoderm to anterior foregut endoderm and then NKX2.1 expressing primordial lung progenitors. The pipette symbol inidcates the cytokines and growth factors applied at each stage. The boxed genes represent key genes expressed at each stage. The red text indicates signalling that must be repressed and green text that must be activated

Table 1 Methods for reprogramming somatic cells to iPSC

Method	Vector	Genomic integration	Advantages	Disadvantages	References
Viral	Lentivirus, retrovirus	Integrating	High efficiency stable expression can be inducible	Tendency for insertional mutagenesis	(Takahashi et al. (2007b), Yu et al. (2007), Ohnuki et al. (2014), Carey et al. (2009), Hotta et al. (2009), Sommer et al. (2009) and Liao et al. (2008)
Viral	Sendai, adenovirus	Non-integrating	High efficiency Non-integrating	Tendency to carry host genome	Zhou and Freed (2009), Fusaki et al. (2009), Fujie et al. (2014) and Suzuki et al. (2008)
Non-viral	Episomal vectors	Non-integrating	Virus free Single transfection	Lower efficiency	Okita et al. (2011), Yu et al. (2009) and Hu and Slukvin (2013)
Non-viral	PiggyBac transposon	Non-integrating	Evidence for more rapid reprogramming	Labour intensive and relatively low efficiency Inefficient excision	Yusa et al. (2009) and Stadtfeld and Hochedlinger (2009)
Non-viral	Mini-circle vectors	Non-integrating	Virus free. Higher efficiency of transfection	Longer ectopic expression	Narsinh et al. (2011) and Jia et al. (2010)
Non-viral	Plasmid	Non-integrating	Virus free	Low efficiency Multiple rounds of transfection	Kim et al. (2016), Dowey et al. (2012), Karow et al. (2011), Si-Tayeb et al. (2010) and Okita et al. (2010)
Non-viral	Protein	Non-integrating	No genetic material, direct protein delivery	Very slow reprogramming kinetics, very low efficiency	Tammam et al. (2016), Nemes et al. (2014), Thier et al. (2012) and Thier et al. (2010)

toward the use of non-viral methods of reprogramming including mRNAs (Warren et al. 2010), episomal plasmids (Okita et al. 2008), recombinant proteins (Zhou et al. 2009; Kim et al. 2009) using the four original Yamanaka factors. Other transcription factors have also found to be useful in the generation of iPSC. Many of these relate to the superfamilies of the transcription factors identified by Yamanaka, such as Oct3, Sox1 and Klf2 (Yu et al. 2007).

3 iPSC and Their Capacity for Disease Modelling

iPSCs have evolved rapidly as a technology, enabling the effective modelling of human disease, complimenting the more typical approaches using animal models and immortalised cell lines. Each model system has its own benefits and limitations (summarized in Table 2). While animal models of lung disease have substantially contributed to our knowledge of fundamental lung biology there has been little success in the translation of findings into the clinic for human use (Ma et al. 2018). Animal model studies of human diseases are often limited in the pathogenic aspects of the disease that they accurately recapitulate; for example, bleomycin instillation of animal models is often used to generate *in vivo* models of idiopathic pulmonary fibrosis, however does not accurately represent the onset or propagation of the disease. While informing us of some aspects, these models do not always replicate the complete aetiology and pathogenesis of the disease being studied. Primary isolated human cells are difficult to expand in culture without losing their phenotype with passage (Schiller and Bittner 1995). Further, human tissue availability can be limited and most often acquired post-mortem.

Table 2 Possible iPSC derived models for lung disease

Model	Species	Model usage	Benefits	Limitations	References
Organoid	Human	Lung structural development	Multiple cell types, spatially organized 3D system	Unsuitable for specific pathway analysis. No air interface	Dye et al. (2015), Wilkinson et al. (2018) and Chen et al. (2017)
Air liquid Interface	Human mouse	Epithelial barrier formation and function	Physiologically relevant air interfacing system, high throughput potential, TEER measurement	No presence of mesenchymal niche cells	Firth et al. (2014), Wong et al. (2012) and Hawkins et al. (2017)
Transplant	Human mouse	Cell engraftment and in vivo regeneration	Study engraftment potential of cell-based therapy, In vivo niche	Long-term human studies lacking, immune suppression	Shafa et al. (2018) and Okuyama et al. (2019)
Spheroid	Human mouse	Cellular and structural modelling, functional assays	Suitable for stringent pathway analysis, functional swelling	No air interface, usually lacks niche cells	Konishi et al. (2016), Gotoh et al. (2014), Dye et al. (2015) and Jacob et al. (2017)

TEER Trans Epithelial Electrical Resistance

This leads to a finite number of cells available for research from a limited patient population, which can result in limited use in high-throughput and drug screening research. Also, research into primary post-mitotic cells, such as that of neurones (Frade and Ovejero-Benito 2015), are restricted to the number of cells that can be initially isolated. This also limits the study of disease propagation and onset to what is typically an advanced disease state.

iPSCs provide an alternative and complimentary research tool that can overcome several limitations of animal models, primary and immortalized human cells. Restrictions of using primary and immortalized cell lines are surmounted due to their indefinite clonal expansion when maintained under specific culture conditions with the capacity for differentiation into multiple cell types comprising the human body (Kogut et al. 2014; Firth et al. 2015; Menon et al. 2015; Ward and Gilad 2019; Hnatiuk and Mercola 2019; Meijer et al. 2019; Fyfe 2019; Fiorotto et al. 2019; Hoshina et al. 2018; Mucci et al. 2018; Tan et al. 2018) including cells within the respiratory system. iPSC, therefore, have the potential to provide a seemingly unlimited source of patient/disease specific cells. This opens up multiple new options for research and the prospect for autologous cell therapy (Ebert et al. 2012). This chapter will focus on their application to studying human lung disease.

Genetic disorders are a prime example of where iPSC benefit over conventional *in vitro* disease modelling. Genetic diseases are often rare and have multiple subtypes, such as those seen in CF (Marson et al. 2016). Whilst the specific mutations of these subtypes are documented, access to patient specific material is limited, severely hindering studies of disease pathology. Instead, self-renewing iPSC can have the genetic mutation induced via state of the art gene editing technology, such as clustered regularly interspaced palindromic repeat (CRISPR)/Cas9 (Qi et al. 2013; Haurwitz et al. 2010; Wang et al. 2013; Cong et al. 2013). A concerted effort over the past decade has seen the evolution of protocols to differentiate iPSC to cells of the respiratory epithelium (Firth et al. 2014; Wong et al. 2012; Green et al. 2011; Cheng et al. 2012; Hawkins et al. 2017). This technology now enables rare genetic disorders to be modelled in a relevant and human cellular system. Several disease states have successfully been induced in iPSC including adenosine deaminase deficiency-related severe combined immunodeficiency (ADA-SCID), Shwachman-Bodian-Diamond syndrome (SBDS), Gaucher disease (GD) type III, Duchenne's Muscular Dystrophy (DMD),

Parkinson disease (PD), Huntington disease (HD), juvenile-onset, type 1 diabetes mellitus (JDM) (Park et al. 2008). The concept here is to develop an iPSC line and induce a disease phenotype in the cells by knocking out/in certain genes, or challenging the cells with factors that may onset disease. The field of neuroscience has particularly benefited from the use of iPSC (Wang and Doering 2012), as obtaining primary neuronal tissue is particularly challenging. Such diseases include Alzheimer's disease (Kondo et al. 2013), Huntington's disease (Kaye and Finkbeiner 2013) and schizophrenia (Brennand et al. 2011). The connexion to this paradigm is that iPSC derived from patient populations with rare genetic disorders, can be gene corrected through use of the same gene editing technology. While providing isogenic controls for *in vitro* evaluation this additionally opens up the potential for autologous cell based therapies reducing the need for immunosuppression and the likelihood of tissue rejection. In the respiratory field, proof-of–principle studies have demonstrated the correction of cystic fibrosis transmembrane regulator (CFTR) in CF patient derived iPSC, which were subsequently differentiated into functional epithelial cells (Firth et al. 2015; Crane et al. 2015). Providing a basis for novel iPSC based therapies for CF patients in the future.

4 Specification of Primordial Lung Progenitors from iPSC

Directed differentiation of iPSC toward lung endoderm presents its own set of challenges, which the field has made substantial progress toward elucidating over the past decade (Firth et al. 2014; Hannan et al. 2015; Wong and Rossant 2013). The lungs are a sophisticated organ system comprising of complex structures and over 40 different cell types; they include a complex vasculature, sympathetic and parasympathetic neuronal innovation, structural support and a specialized respiratory epithelium. To add to this complexity, the structure of the airways changes to meet its functional requirements along the proximal-distal axis. Similar to the gut, the respiratory system also contains a natural homeostatic microbiota, which can drastically alter during times of disease and stress (Dang and Marsland 2019; Man et al. 2017) and is an internal organ exposed to the exterior environment increasing the potential for epigenetic modification (Sakurada 2010; Hagood 2014). These features must all be considered when creating an *in vitro* model of respiratory disease and reflected in the advantages and limitations of any given model system. iPSC have the potential to investigate mechanisms of human lung development providing insights into the differentiation pathways from stem cell to fully differentiated tissues. In addition, they provide an opportunity to reverse a disease phenotype and investigate mechanisms of disease onset.

The first methods describing directed differentiation of the respiratory epithelium from iPSC focused primarily on specification of the lung endoderm (Cheng et al. 2012; Kadzik and Morrisey 2012; Longmire et al. 2012a). Subsequently, three pioneering papers were published differentiating cells to more mature cells in the respiratory epithelium (Wong et al. 2012; Kadzik and Morrisey 2012; Firth et al. 2014). These studies all strived to mimic lung embryonic development in a dish pushing cells through mesendoderm, to definitive endoderm (DE) followed by anteriorization of the ventral foregut endoderm (AFE) to primordial NKX2.1 expressing lung endodermal progenitor cells (LP). These cells have the capacity for differentiation into cells akin to that for the mature human lung including club, goblet, multiciliated, basal, alveolar and neuroendocrine cells.

Understanding lung development is critical to efficiently driving pluripotent cells to generate the cells and structures comprising the human lung. There is still a sparsity of specific knowledge of human lung development and much of our information is gained from transgenic mouse models tracing lineage specification (Bellusci et al. 1997a, b; Weaver et al. 1999, 2003; Okubo and Hogan 2004; Rawlins et al. 2009a, b). Lung organogenesis begins in the embryonic period with independent outpouchings of the ventral wall in the primitive foregut endoderm that

elongate and branch into the surrounding mesenchyme. The respiratory mesenchyme is crucial in many developmental and homeostatic processes within the lung. It provides key signalling ligands to promote the development of lung structures, including alveolargenesis, airway branching and the vasculature. The mesenchyme is the primary source of transforming growth factor beta (TGFβ) in the developing lung (McCulley et al. 2015; White et al. 2006). It is an integral component for natural development and TGFβ knockout studies demonstrate impaired lung development (Sanford et al. 1997). The mesenchyme also provides the primary source of Wnt signalling, key for branching morphogenesis of airway epithelium (Miller et al. 2012) (Fig. 1).

DE gives rise to lungs, thyroid, pancreas, liver and intestines and is specified from the anterior primitive streak (APS), induced from pluripotent cells through strong activation of nodal and canonical wnt signaling, which are synergistically activated during gastrulation. This is mimicked in culture using Activin A and Wnt3a or Wnt agonist CHIR99021 (Kubo et al. 2004). The APS can be pushed to DE through persistent activation of nodal signalling and inhibition of bone morphogenetic protein (BMP), using DMH-1, to suppress mesoderm derivation (Green et al. 2011; Ogaki et al. 2013). Specification of DE from the mesendoderm has been optimized and results in a high efficiency of DE cells from iPSC (Mfopou et al. 2010). In embryonic development Wnt signalling plays an important role in many cellular functions, including differentiation and proliferation, *in Vitro* Wnt3A signalling is used to skew away from SOX2 expressing ectoderm and promote endodermal differentiation. DE also expresses cell surfaces markers CXCR4, a chemokine receptor important in cellular proliferation and cKit that can purify the DE through FACS sorting (Wong et al. 2010; Wang et al. 2012) (Fig. 2).

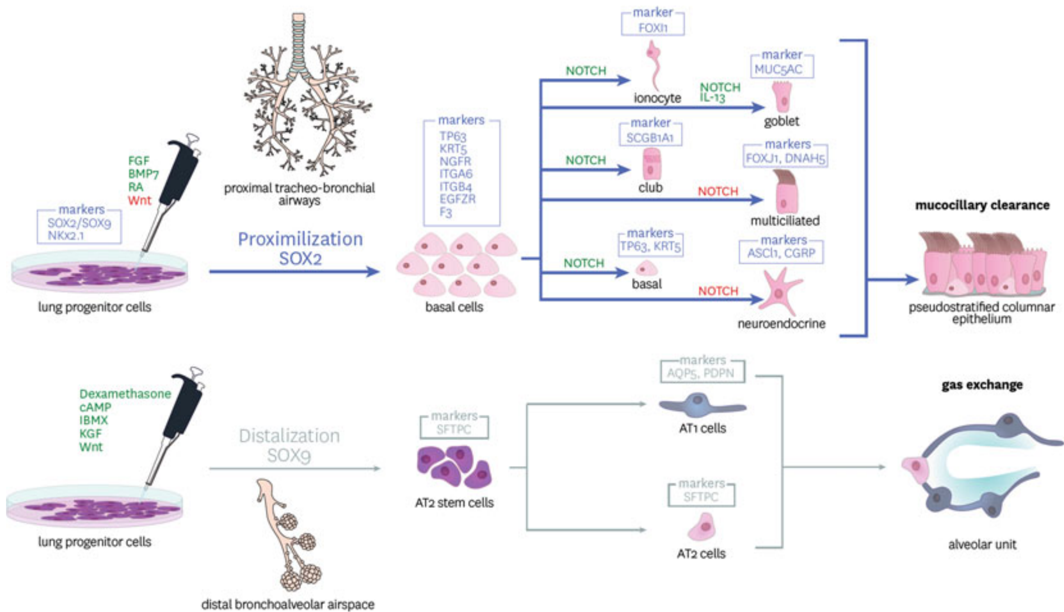

Fig. 2 Differentiation of primordial lung progenitors towards proximal and distal lung fate. iPSC derived and purified lung progenitor cells expressing NKx2.1 can be directed toward proximal and distal fates through activation (green) and inhibition (red) of signalling pathways including those driven by FGFs, BMPs and wnts. The markers of the specific lineages are indicated in boxes above the cell types. Alveolar Type II (ATII) cells are the progenitor cells giving rise to mature ATII and ATI cells responsive for the functional alveolar unit for gas exchange. Sox2 expressing proximal basal cells are able to differentiate and give rise to all cells of the mature conducting airways including secretory, basal and multiciliated cells responsible for mucociliary clearance

Anterioriziation of the DE to generate the foregut, identified through SOX2 and FOXA2 expressing cells does not appear to critically depend on Activin A/TGF-β-signalling. Inhibition of TGFβ is known to assist in driving AFE (Green et al. 2011) and an inhibition of Wnt- and BMP-signalling is critical in optimizing this transition. FOXA2 is an essential transcription factor for lung development, FOXA2−/− mice do not develop lungs (Wan et al. 2004; Aubin et al. 1997). Retinoic acid (RA), commonly used in lung differentiation protocols has dual effects and can either posteriorize or dorsalize the foregut creating PDX1-positive pancreatic duodenal cells. Its use in *in vitro* differentiation protocols is, therefore, not entirely clear. A number of studies have demonstrated the importance of RA, influencing micro RNAs, however, short pulses of RA can also maintain the stemness of iPSC through inhibition of the canonical Wnt pathway, essential for differentiation (De Angelis et al. 2018). In combination with Wnt/β-catenin, RA can act synergistically with FGF-2 and BMP-4 to generate CDX2-positive posterior endoderm further complicating the methods applied to iPSC differentiation (Davenport et al. 2016).

The successful generation of a primordial lung progenitor cell is accredited to the induction of lung transcription factor NKX2.1 (also known as TTF1) (Longmire et al. 2012a; Lazzaro et al. 1991). The function of NKX2.1 is not entirely understood. In mouse models, it is important in the development of respiratory tissue as well as other thoracic structures (Minoo et al. 1999; Minoo 2000). Purification of iPSC derived NKX2.1 primordial lung progenitor cells initially proved inefficient and purification was limited through lack of a suitable surface antigen. A recent study has shown that these NKX2.1 cells can be selected using a CD47-high, CD26-low surface marker expression profile (Hawkins et al. 2017). Alternatively, Carboxypeptidase M is also expressed in these cells and can be used to purify a similar population of lung progenitors (Konishi et al. 2016; Gotoh et al. 2014). While NKX2.1 defines specification of lung progenitor cells, it also has notable expression in the brain and thyroid tissues (Lee et al. 2001; Rossi et al. 1995; Acebron et al. 1995). Although the pathways that distinguish between these organ systems are not well-characterised, lineages can be identified though co-expression of PAX8 (thyroid, (Rossi et al. 1995) and PAX6 (forebrain, (Corbin et al. 2003; Takahashi and Osumi 2002).

Fibroblast Growth Factor (FGF) signalling is integral in defining lung endoderm and inducing NKX2.1 expression (Rankin and Zorn 2014; Dailey et al. 2005; Xian et al. 2005). In addition, sonic hedgehog (Shh) and transcriptional programs of the forkhead (Fox), and GATA-family members, are involved in specification of the lung from the AFE (Hogan 1999; Whitsett 1998). Differentiation towards lung progenitors can be directed away from specification of thyroid progenitors through controlled FGF2 expression. Studies have demonstrated that high concentrations of FGF activate Shh expression to generate NKX2–1 expressing lung progenitors and thyroid (Rankin and Zorn 2014; Serra et al. 2017; Kurmann et al. 2015; Longmire et al. 2012b). Dye et al. demonstrated that suppression of FGF activity whilst maintaining Shh signalling allowed for a more specific differentiation to lung primed NKX2.1 expressing cells (Dye et al. 2015). Sequential inhibition of TGFβ signalling, followed by subsequent activation of FGF and BMP4 signalling pathways can support further differentiation to lung epithelium (Longmire et al. 2012a).

5 Proximal and Distal Fate of Lung Progenitors

Lung buds arise from the lateral part of the foregut prior to forming the trachea and recent data suggests that the progenitors at the leading tip of these lung buds differ in humans and mice and can specify both the proximal and distal regions of the lung (Miller et al. 2018; Danopoulos et al. 2018; Nikolic et al. 2017). To study these fate decisions in humans a three-dimensional organoid system has been established to culture fetal lung bud tips (Miller et al. 2018; Danopoulos

et al. 2018; Nikolic et al. 2017). Cells at the leading tip of these buds express NKx2.1, SOX2 and SOX9 in humans; this contrasts with the cells in the same region of the mouse which either co-express NKX2.1 and SOX2 or SOX9 (Miller et al. 2018). A similar population of cells has been observed in iPSC derived lung progenitors from humans (Miller et al. 2018).

Detailed analysis of the regulation of proximal and distal fate decisions has been extensively studied in mice using lineage tracing models (Rawlins et al. 2009b; Barkauskas et al. 2013; Rock et al. 2009). In humans, we rely on the development of robust *in vitro* models. Both fetal and iPSC derived lung bud organoids will differentiate when exposed to FGF7, CHIR and Retinoic Acid, generating cells akin to those in the human airways (Miller et al. 2018). By utilizing more sophisticated scaffold materials, tubular airway-like structures can also be replicated *in vitro*, resembling that of the canalicular development in lung embryogenesis (Dye et al. 2016). Additional factors to consider when developing more complex models, is the importance of the mesenchymal supporting cells in controlling the fate of lung progenitors towards a proximal or distal epithelial phenotypes. Signals between the mesenchyme and epithelium are critical in lung development, and supplying the exogenous growth factors to an *in vitro* system may be in sufficient to allow us to fully appreciate the signals responsible for proximal and distal human lung fate decisions (El Agha et al. 2014).

6 Tracheo-Bronchial Differentiation and Disease Models

Specification of distal and proximal lung cells requires precise spatiotemporal regulation of Wnt, Notch and FGF signaling pathways. The proximal airways, comprising of tracheal and bronchial cartilaginous airways, are populated by basal cells, as the predominant progenitor cell, in addition to club and goblet secretory cells and multiciliated cells as the primary functional epithelium. SOX2 expression delineates the proximal airways from their distal counterparts that continue to selectively express SOX9 (Danopoulos et al. 2018). As discussed above, bud tip progenitors co-express both SOX2 and SOX9 during psuedoglandular phase of human embryogenetic development, however, will become determined before development reaches the canalicular stages, controlled by signals received within their proximity microenvironment (Danopoulos et al. 2018).

Currently there are no published studies specifically focusing on the specification and expansion of an iPSC-derived basal cell. Proximal airway basal cells are currently identified by the expression of cytokeratin 5 (KRT5), TP63, nerve growth factor receptor (NGFR) and integrin alpha 6 (ITGA6 or CD49f) (Daniely et al. 2004). During development, it appears that SMAD signaling plays a role in the lineage differentiation pushing away from lung progenitor stem cells. TGFβ and BMP4 mediated SMAD signaling has, however, been demonstrated to promote the differentiation from bud tips to a basal cell like phenotype, using an organoid based *in vitro* system (Dye et al. 2015). At this stage, SMAD inhibition promotes the maintenance of the basal cell phenotype and further differentiation beyond a precursor cell (Mou et al. 2016).

Another key component for differentiation to lung basal cell epithelium is NOTCH signaling (Rock et al. 2011). Activation of Notch signaling pathways is critical in embryonic development and plays various roles in a more developed system. In the basal cell, NOTCH signaling is involved in its further differentiation to a mature epithelial subtype. Maturation of airways cells is most commonly achieved at an “air-liquid interface” or ALI, a platform arguably more complex than *in vitro* systems for most other organ systems (de Jong et al. 1994). In this system, human bronchial epithelial cells (HBEC) are seeded to transwell inserts, allowed to grow to confluence generating an epithelial barrier with tight junctions. Once sufficient trans-epithelial electrical resistance (TEER) is generated, the apical media is removed generating an apical air interface. Over a 28-day period the cells undergo process of polarization, pseudo stratification and

maturation comprising predominantly of basal, secretory (goblet and club cells) and multiciliated cells. Inhibition of NOTCH promotes a basal cell to ciliated cell transition, whist continued activation of NOTCH pathways promote a secretory fate (Rock et al. 2011). Successful differentiation of ALI cultures from iPSC was demonstrated in some of the first protocols published (Wong and Rossant 2013; Firth et al. 2014). Since than several other laboratories have generated pseudostratified epithelium reflecting that of the primary airway cell differentiation in vitro (Konishi et al. 2016; McCauley et al. 2017; Huang et al. 2015).

7 Distal Lung and Alveolar Differentiation and Disease Models

Cells expressing NKX2–1 stand as the distinct lung progenitor that may differentiate into any lung cellular phenotype. Lung patterning during embryogenesis requires determination to distinguish the saccular generation of the distal alveolar spaces. *In vitro* generation of more distally aligned airway cells can be controlled via Wnt signalling. It had been demonstrated that high Wnt activation could generate alveolar progenitors whilst conversely supressed Wnt signalling generated more proximal cell types (McCauley et al. 2017). This is primarily achieved by culturing the cells in the presence of a potent GSK3β inhibitor known as CHIR99021. Inhibiting the ability of this enzyme to activate Wnt and its downstream machinery. The result is the robust generation of distal/alveolar epithelial cells (Jacob et al. 2017).

Alveolar epithelium is comprised of two major subtypes; alveolar type 1 (AT1) & alveolar type 2 (AT2) cells. In short, AT1 cells provide the structural basis of the alveolar spaces and primary function for gaseous exchange, whilst AT2 cells are primarily secretory and provide a supporting role to the AT1 cells. However, there is distinct multifunctional heterogeneity within these cell types. This was eloquently demonstrated utilising a surfactant protein C (SPC) (AT2 specific marker) reporter line, whereby phenotypic profiling multiple subtypes within this specific cell population (Lee et al. 2013). Interestingly, mutations in SPC are known to cause interstitial lung disease, and have been modelled utilising iPSC derived AT2 cells (Jacob et al. 2017). The AT2 cells derived from iPSCs are found to be NKX2.1 and closely resemble that of the foetal lung AT2 cells, based on a genetic profiling. This model is now being utilised to study the influence of 173 T SPC mutation and effectively model interstitial lung disease *in vitro*. Other distally aligned respiratory diseases have also been modelled *in* vitro utilising iPSC-based techniques. These include IPF models where 3-D organoids have been able to replicate disease characteristics including accumulation of extracellular matrix and mesenchymal cells, suggesting the potential for modelling fibrotic lung disease *in vitro* (Wilkinson et al. 2017; Strikoudis et al. 2019).

The development of lung progenitor cells is limited by our understanding of the phenotype and function of their primary counterpart. To date no direct comparison has been made between iPSC-derived cells and *in vitro* cultured primary cells. With substantial profiling of cells in progress through programs such as LungMAP (https://lungmap.net), it is hoped that a more extensive profile of basal cells and potential sub populations of progenitor cells will lead to increased options of specific cellular surface markers for specific identification and isolation of the definitive stem cells.

8 iPSC and their Capacity for Tissue Regeneration

Many degenerative disorders, such as COPD, do not have effective disease modifying treatments. In theory, iPSC could be generated from each patient diseases, differentiated to the relevant stem/progenitor cell and engrafted back into the patient's diseased and damaged lung. Furthermore, genetic disorders, such CF and primary ciliary dyskinesia, could be corrected using state-of-the-art gene editing technologies prior to

engraftment. Published data has demonstrated the successful use of iPSC-derived cells and their regenerative therapeutic potential in a multitude of disorders using animal models and *in vitro* based techniques. These include models of liver injury (Liu et al. 2011), muscular related disorders (Kazuki et al. 2010; Tan et al. 2012), blood/immunological disorders (Suzuki et al. 2013), cardiovascular disease (Shiba et al. 2016) and spinal cord injury (Nori et al. 2011), among others (Li et al. 2017). In the clinic, patient specific, iPSC-derived retinal epithelial cells have successfully been transplanted back into patients with macular degeneration, marking the first attempt of iPSC to treat a patient population (Mandai et al. 2017). Unfortunately, the reality of this is infinitely more challenging and complex for the lung. The lung comprises of over 40 different functional cell types forming airways, vasculature, cartilage, immune system, sympathetic and parasympathetic neural tissues, glands and supportive parenchyma. In the case of lung disease, it unlikely that one cell type is affected in isolation and more reasonable to think of changes more globally with specific microenvironments adapting to maximize protection and function of the lung for gas exchange. Long-term replacement of cells will likely require access to the relevant cellular niche for long-term reconstitution and adaptation of the niche to reflect that of a non-diseased lung to sustain a "healthy" engrafted cell and derivatives. Progress in the field of lung regeneration has been substantial but we now need to start thinking toward more complex models, which more closely recapitulate the *in vivo* cellular niche. This will require, at minimum, collaborative efforts between biologists, bioengineers and novel translational imaging techniques.

9 The Future of iPSC for Respiratory Disease

iPSC present a novel, human and patient specific avenue for research and therapeutic advancement. iPSC have enabled the generation of human and disease-related models to be created in vitro providing an unprecedented access to human biology. Like any model system, they are not without their limitations and should be used alongside other available systems suitable for completely addressing the specific experimental question at hand. For the lung, exposure of the cells to the environment, a constant for the millieu of cells in the airways, is not yet considered in this model system. Furthermore, epigenetic changes causative of disease are likely wiped during the reprogramming process loosing these markers as disease phenotypes. However, by utilising iPSC, a seemingly limitless source of cells is available and representative of the parent genetic profile enabling both human and patient specific cellular models to be developed. Further, the use of iPSC allows for robust investigations into the developmental pathways involved in a human cell-based system that would otherwise be challenging. In iPSC, individual genes can be manipulated and evaluated side-by-side with their isogenic counterparts enabling precise effects of specific genes to be evaluated. As such, iPSC provide an incredibly valuable model system as we progress to an era of personalized medicine.

10 Concluding Remarks

Substantial progress has been made since Yamanaka's discovery of induced pluripotency in humans in 2007. From initially identifying an iPSC inducing minimal cocktail of transcription factors, to sucessful use of iPSC as a therapeutic tool, highlights the speed at which this technlogy has evolved. Although initial derivation of relevant respiratory cells from iPSC lagged behing other organ systems, we still have a plethora of methods available to study respiratory disease in a biologically relevant cell type, overcoming the shortfall of current model systems. Progress is currently limited by our fundamental lack of understanding of the mechanisms controlling human lung development, the precise identity and function of human lung cell types and the genetic and epigenetic control of human lung fate. As our capacity to model human lung disease

evolves, so will our understanding of the pathogenesis of human lung disease. iPSC models remain an exciting prospect.

Acknowledgements Thank you to Sergio Bianco in the USC Department of Stem Cell and Regenerative Medicine for production of the artwork for the schematic diagrams in figures 1 and 2.

Funding A.L.R. is funded by the Hastings Foundation, Cystic Fibrosis Foundation Therapeutics (CFFT, Firth15XX0, Firth17XX0) and NIH/NHLBI, R01 HL139828.

References

Acebron A, Aza-Blanc P, Rossi DL, Lamas L, Santisteban P (1995) Congenital human thyroglobulin defect due to low expression of the thyroid-specific transcription factor TTF-1. J Clin Invest 96(2):781–785

Aubin J, Lemieux M, Tremblay M, Berard J, Jeannotte L (1997) Early postnatal lethality in Hoxa-5 mutant mice is attributable to respiratory tract defects. Dev Biol 192 (2):432–445

Barkauskas CE et al (2013) Type 2 alveolar cells are stem cells in adult lung. J Clin Invest 123(7):3025–3036

Barnes PJ et al (2015) Barriers to new drug development in respiratory disease. Eur Respir J 45(5):1197–1207

Bellusci S et al (1997a) Involvement of Sonic hedgehog (Shh) in mouse embryonic lung growth and morphogenesis. Development 124(1):53–63

Bellusci S, Grindley J, Emoto H, Itoh N, Hogan BL (1997b) Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung. Development 124(23):4867–4878

Brennand KJ et al (2011) Modelling schizophrenia using human induced pluripotent stem cells. Nature 473 (7346):221–225

Carey BW et al (2009) Reprogramming of murine and human somatic cells using a single polycistronic vector. Proc Natl Acad Sci U S A 106(1):157–162

Chen YW et al (2017) A three-dimensional model of human lung development and disease from pluripotent stem cells. Nat Cell Biol 19(5):542–549

Cheng X et al (2012) Self-renewing endodermal progenitor lines generated from human pluripotent stem cells. Cell Stem Cell 10(4):371–384

Cong L et al (2013) Multiplex genome engineering using CRISPR/Cas systems. Science (New York, NY) 339 (6121):819–823

Corbin JG, Rutlin M, Gaiano N, Fishell G (2003) Combinatorial function of the homeodomain proteins Nkx2.1 and Gsh2 in ventral telencephalic patterning. Development 130(20):4895–4906

Crane AM et al (2015) Targeted correction and restored function of the CFTR gene in cystic fibrosis induced pluripotent stem cells. Stem Cell Rep 4(4):569–577

Dailey L, Ambrosetti D, Mansukhani A, Basilico C (2005) Mechanisms underlying differential responses to FGF signaling. Cytokine Growth Factor Rev 16 (2):233–247

Dang AT, Marsland BJ (2019) Microbes, metabolites, and the gut-lung axis. Mucosal Immunol 12(4):843–850

Daniely Y et al (2004) Critical role of p63 in the development of a normal esophageal and tracheobronchial epithelium. Am J Phys Cell Physiol 287(1):C171–C181

Danopoulos S et al (2018) Human lung branching morphogenesis is orchestrated by the spatiotemporal distribution of ACTA2, SOX2, and SOX9. Am J Physiol Lung Cell Mol Physiol 314(1):L144–L149

Davenport C, Diekmann U, Budde I, Detering N, Naujok O (2016) Anterior-posterior patterning of definitive endoderm generated from human embryonic stem cells depends on the differential signaling of retinoic acid, Wnt-, and BMP-signaling. Stem Cells 34 (11):2635–2647

De Angelis MT, Parrotta EI, Santamaria G, Cuda G (2018) Short-term retinoic acid treatment sustains pluripotency and suppresses differentiation of human induced pluripotent stem cells. Cell Death Dis 9(1):6

de Jong PM et al (1994) Ciliogenesis in human bronchial epithelial cells cultured at the air-liquid interface. Am J Respir Cell Mol Biol 10(3):271–277

Dowey SN, Huang X, Chou BK, Ye Z, Cheng L (2012) Generation of integration-free human induced pluripotent stem cells from postnatal blood mononuclear cells by plasmid vector expression. Nat Protoc 7 (11):2013–2021

Dye BR et al (2015) In vitro generation of human pluripotent stem cell derived lung organoids. elife 4:e05098

Dye BR et al (2016) A bioengineered niche promotes in vivo engraftment and maturation of pluripotent stem cell derived human lung organoids. elife 5:pii: e19732

Ebert AD, Liang P, Wu JC (2012) Induced pluripotent stem cells as a disease modeling and drug screening platform. J Cardiovasc Pharmacol 60(4):408–416

El Agha E et al (2014) Fgf10-positive cells represent a progenitor cell population during lung development and postnatally. Development 141(2):296–306

Fiorotto R et al (2019) Liver diseases in the dish: iPSC and organoids as a new approach to modeling liver diseases. Biochim Biophys Acta Mol basis Dis 1865 (5):920–928

Firth AL et al (2014) Generation of multiciliated cells in functional airway epithelia from human induced pluripotent stem cells. Proc Natl Acad Sci U S A 111(17): E1723–E1730

Firth AL et al (2015) Functional gene correction for cystic fibrosis in lung epithelial cells generated from patient iPSCs. Cell Rep 12(9):1385–1390

Frade JM, Ovejero-Benito MC (2015) Neuronal cell cycle: the neuron itself and its circumstances. Cell Cycle 14 (5):712–720

Fujie Y et al (2014) New type of Sendai virus vector provides transgene-free iPS cells derived from chimpanzee blood. PLoS One 9(12):e113052

Fusaki N, Ban H, Nishiyama A, Saeki K, Hasegawa M (2009) Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. Proc Jpn Acad Ser B Phys Biol Sci 85 (8):348–362

Fyfe I (2019) Mutation-specific amyloid-beta processing in iPSC-derived neurons. Nat Rev Neurol 15(6):310

Gotoh S et al (2014) Generation of alveolar epithelial spheroids via isolated progenitor cells from human pluripotent stem cells. Stem Cell Rep 3(3):394–403

Green MD et al (2011) Generation of anterior foregut endoderm from human embryonic and induced pluripotent stem cells. Nat Biotechnol 29(3):267–272

Hagood JS (2014) Beyond the genome: epigenetic mechanisms in lung remodeling. Physiology (Bethesda) 29(3):177–185

Hannan NR, Sampaziotis F, Segeritz CP, Hanley NA, Vallier L (2015) Generation of distal airway epithelium from multipotent human foregut stem cells. Stem Cells Dev 24(14):1680–1690

Haurwitz RE, Jinek M, Wiedenheft B, Zhou K, Doudna JA (2010) Sequence- and structure-specific RNA processing by a CRISPR endonuclease. Science (New York, NY) 329(5997):1355–1358

Hawkins F et al (2017) Prospective isolation of NKX2-1-expressing human lung progenitors derived from pluripotent stem cells. J Clin Invest 127(6):2277–2294

Hnatiuk A, Mercola M (2019) Stars in the night sky: iPSC-cardiomyocytes return the patient context to drug screening. Cell Stem Cell 24(4):506–507

Hogan BL (1999) Morphogenesis. Cell 96(2):225–233

Hoshina A et al (2018) Development of new method to enrich human iPSC-derived renal progenitors using cell surface markers. Sci Rep 8(1):6375

Hotta A et al (2009) Isolation of human iPS cells using EOS lentiviral vectors to select for pluripotency. Nat Methods 6(5):370–376

Hu K, Slukvin I (2013) Generation of transgene-free iPSC lines from human normal and neoplastic blood cells using episomal vectors. Methods Mol Biol 997:163–176

Huang SX et al (2015) The in vitro generation of lung and airway progenitor cells from human pluripotent stem cells. Nat Protoc 10(3):413–425

Hubbard R (2006) The burden of lung disease. Thorax 61 (7):557–558

Jacob A et al (2017) Differentiation of human pluripotent stem cells into functional lung alveolar epithelial cells. Cell Stem Cell 21(4):472–488. e410

Jia F et al (2010) A nonviral minicircle vector for deriving human iPS cells. Nat Methods 7(3):197–199

Kadzik RS, Morrisey EE (2012) Directing lung endoderm differentiation in pluripotent stem cells. Cell Stem Cell 10(4):355–361

Karow M et al (2011) Site-specific recombinase strategy to create induced pluripotent stem cells efficiently with plasmid DNA. Stem Cells 29(11):1696–1704

Kaye JA, Finkbeiner S (2013) Modeling Huntington's disease with induced pluripotent stem cells. Mol Cell Neurosci 56:50–64

Kazuki Y et al (2010) Complete genetic correction of iPS cells from Duchenne muscular dystrophy. Mol Ther 18 (2):386–393

Kim D et al (2009) Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. Cell Stem Cell 4(6):472–476

Kim JS, Choi HW, Hong YJ, Do JT (2016) Generation of partially reprogrammed cells and fully reprogrammed iPS cells by plasmid transfection. Methods Mol Biol 1357:85–95

Kogut I, Roop DR, Bilousova G (2014) Differentiation of human induced pluripotent stem cells into a keratinocyte lineage. Methods Mol Biol 1195:1–12

Kondo T et al (2013) Modeling Alzheimer's disease with iPSCs reveals stress phenotypes associated with intracellular Abeta and differential drug responsiveness. Cell Stem Cell 12(4):487–496

Konishi S et al (2016) Directed induction of functional multi-ciliated cells in proximal airway epithelial spheroids from human pluripotent stem cells. Stem Cell Rep 6(1):18–25

Kubo A et al (2004) Development of definitive endoderm from embryonic stem cells in culture. Development 131(7):1651–1662

Kurmann AA et al (2015) Regeneration of thyroid function by transplantation of differentiated pluripotent stem cells. Cell Stem Cell 17(5):527–542

Lazzaro D, Price M, de Felice M, Di Lauro R (1991) The transcription factor TTF-1 is expressed at the onset of thyroid and lung morphogenesis and in restricted regions of the foetal brain. Development 113 (4):1093–1104

Lee BJ et al (2001) TTF-1, a homeodomain gene required for diencephalic morphogenesis, is postnatally expressed in the neuroendocrine brain in a developmentally regulated and cell-specific fashion. Mol Cell Neurosci 17(1):107–126

Lee JH et al (2013) Surfactant protein-C chromatin-bound green fluorescence protein reporter mice reveal heterogeneity of surfactant protein C-expressing lung cells. Am J Respir Cell Mol Biol 48(3):288–298

Li Y-C, Zhu K, Young T-H (2017) Induced pluripotent stem cells, form in vitro tissue engineering to in vivo allogeneic transplantation. J Thorac Dis 9(3):455–459

Liao J et al (2008) Enhanced efficiency of generating induced pluripotent stem (iPS) cells from human somatic cells by a combination of six transcription factors. Cell Res 18(5):600–603

Liu H, Kim Y, Sharkis S, Marchionni L, Jang Y-Y (2011) In vivo liver regeneration potential of human induced pluripotent stem cells from diverse origins. Sci Transl Med 3(82):82ra39–82ra39

Longmire TA et al (2012a) Efficient derivation of purified lung and thyroid progenitors from embryonic stem cells. Cell Stem Cell 10(4):398–411

Longmire TA, Ikonomou L, Kotton DN (2012b) Mouse ESC differentiation to Nkx2.1+ lung and thyroid progenitors. Bio Protoc 2(22):pii: e295

Lozano R et al (2012) Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the global burden of disease study 2010. Lancet (London, England) 380 (9859):2095–2128

Ma Q et al (2018) Regeneration of functional alveoli by adult human SOX9(+) airway basal cell transplantation. Protein Cell 9(3):267–282

Man WH, de Steenhuijsen Piters WA, Bogaert D (2017) The microbiota of the respiratory tract: gatekeeper to respiratory health. Nat Rev Microbiol 15 (5):259–270

Mandai M et al (2017) Autologous induced stem-cell–derived retinal cells for macular degeneration. N Engl J Med 376(11):1038–1046

Marson FAL, Bertuzzo CS, Ribeiro JD (2016) Classification of CFTR mutation classes. Lancet Respir Med 4 (8):e37–e38

McCauley KB et al (2017) Efficient derivation of functional human airway epithelium from pluripotent stem cells via temporal regulation of Wnt signaling. Cell Stem Cell 20(6):844–857. e846

McCulley D, Wienhold M, Sun X (2015) The pulmonary mesenchyme directs lung development. Curr Opin Genet Dev 32:98–105

Meijer M et al (2019) A single-cell model for synaptic transmission and plasticity in human iPSC-derived neurons. Cell Rep 27(7):2199–2211. e2196

Menon T et al (2015) Lymphoid regeneration from gene-corrected SCID-X1 subject-derived iPSCs. Cell Stem Cell 16(4):367–372

Mfopou JK, Chen B, Sui L, Sermon K, Bouwens L (2010) Recent advances and prospects in the differentiation of pancreatic cells from human embryonic stem cells. Diabetes 59(9):2094–2101

Miller MF et al (2012) Wnt ligands signal in a cooperative manner to promote foregut organogenesis. Proc Natl Acad Sci U S A 109(38):15348–15353

Miller AJ et al (2018) In vitro induction and in vivo engraftment of lung bud tip progenitor cells derived from human pluripotent stem cells. Stem Cell Rep 10 (1):101–119

Minoo P (2000) Transcriptional regulation of lung development: emergence of specificity. Respir Res 1 (2):109–115

Minoo P, Su G, Drum H, Bringas P, Kimura S (1999) Defects in tracheoesophageal and lung morphogenesis in Nkx2.1(−/−) mouse embryos. Dev Biol 209 (1):60–71

Mou H et al (2016) Dual SMAD signaling inhibition enables long-term expansion of diverse epithelial basal cells. Cell Stem Cell 19(2):217–231

Mucci A et al (2018) iPSC-derived macrophages effectively treat pulmonary alveolar Proteinosis in Csf2rb-deficient mice. Stem Cell Rep 11(3):696–710

Murugan V (2009) Embryonic stem cell research: a decade of debate from bush to Obama. Yale J Biol Med 82 (3):101–103

Narsinh KH et al (2011) Generation of adult human induced pluripotent stem cells using nonviral minicircle DNA vectors. Nat Protoc 6(1):78–88

Nemes C et al (2014) Generation of mouse induced pluripotent stem cells by protein transduction. Tissue Eng Part C Methods 20(5):383–392

Nikolic MZ et al (2017) Human embryonic lung epithelial tips are multipotent progenitors that can be expanded in vitro as long-term self-renewing organoids. elife 6: pii: e26575

Nori S et al (2011) Grafted human-induced pluripotent stem-cell-derived neurospheres promote motor functional recovery after spinal cord injury in mice. Proc Natl Acad Sci U S A 108(40):16825–16830

Ogaki S, Shiraki N, Kume K, Kume S (2013) Wnt and Notch signals guide embryonic stem cell differentiation into the intestinal lineages. Stem Cells 31 (6):1086–1096

Ohnuki M et al (2014) Dynamic regulation of human endogenous retroviruses mediates factor-induced reprogramming and differentiation potential. Proc Natl Acad Sci U S A 111(34):12426–12431

Okita K, Ichisaka T, Yamanaka S (2007) Generation of germline-competent induced pluripotent stem cells. Nature 448(7151):313–317

Okita K, Nakagawa M, Hyenjong H, Ichisaka T, Yamanaka S (2008) Generation of mouse induced pluripotent stem cells without viral vectors. Science (New York, NY) 322(5903):949–953

Okita K, Hong H, Takahashi K, Yamanaka S (2010) Generation of mouse-induced pluripotent stem cells with plasmid vectors. Nat Protoc 5(3):418–428

Okita K et al (2011) A more efficient method to generate integration-free human iPS cells. Nat Methods 8 (5):409–412

Okubo T, Hogan BL (2004) Hyperactive Wnt signaling changes the developmental potential of embryonic lung endoderm. J Biol 3(3):11

Okuyama H et al (2019) Transplantation of multiciliated airway cells derived from human iPS cells using an

artificial tracheal patch into rat trachea. J Tissue Eng Regen Med 13(6):1019–1030
Park IH et al (2008) Disease-specific induced pluripotent stem cells. Cell 134(5):877–886
Qi LS et al (2013) Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell 152(5):1173–1183
Rankin SA, Zorn AM (2014) Gene regulatory networks governing lung specification. J Cell Biochem 115 (8):1343–1350
Rawlins EL, Clark CP, Xue Y, Hogan BL (2009a) The Id2 + distal tip lung epithelium contains individual multipotent embryonic progenitor cells. Development 136(22):3741–3745
Rawlins EL et al (2009b) The role of Scgb1a1+ Clara cells in the long-term maintenance and repair of lung airway, but not alveolar, epithelium. Cell Stem Cell 4 (6):525–534
Rock JR et al (2009) Basal cells as stem cells of the mouse trachea and human airway epithelium. Proc Natl Acad Sci U S A 106(31):12771–12775
Rock JR et al (2011) Notch-dependent differentiation of adult airway basal stem cells. Cell Stem Cell 8 (6):639–648
Rossi DL, Acebron A, Santisteban P (1995) Function of the homeo and paired domain proteins TTF-1 and Pax-8 in thyroid cell proliferation. J Biol Chem 270 (39):23139–23142
Sakurada K (2010) Environmental epigenetic modifications and reprogramming-recalcitrant genes. Stem Cell Res 4(3):157–164
Sanford LP et al (1997) TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. Development 124(13):2659–2670
Schiller JH, Bittner G (1995) Loss of the tumorigenic phenotype with in vitro, but not in vivo, passaging of a novel series of human bronchial epithelial cell lines: possible role of an alpha 5/beta 1-integrin-fibronectin interaction. Cancer Res 55(24):6215–6221
Serra M et al (2017) Pluripotent stem cell differentiation reveals distinct developmental pathways regulating lung- versus thyroid-lineage specification. Development 144(21):3879–3893
Shafa M et al (2018) Human induced pluripotent stem cell-derived lung progenitor and alveolar epithelial cells attenuate hyperoxia-induced lung injury. Cytotherapy 20(1):108–125
Shiba Y et al (2016) Allogeneic transplantation of iPS cell-derived cardiomyocytes regenerates primate hearts. Nature 538(7625):388–391
Si-Tayeb K et al (2010) Generation of human induced pluripotent stem cells by simple transient transfection of plasmid DNA encoding reprogramming factors. BMC Dev Biol 10:81
Sommer CA et al (2009) Induced pluripotent stem cell generation using a single lentiviral stem cell cassette. Stem Cells 27(3):543–549
Stadtfeld M, Hochedlinger K (2009) Without a trace? PiggyBac-ing toward pluripotency. Nat Methods 6 (5):329–330
Strikoudis A et al (2019) Modeling of fibrotic lung disease using 3D organoids derived from human pluripotent stem cells. Cell Rep 27(12):3709–3723. e3705
Suzuki K et al (2008) Highly efficient transient gene expression and gene targeting in primate embryonic stem cells with helper-dependent adenoviral vectors. Proc Natl Acad Sci U S A 105(37):13781–13786
Suzuki N et al (2013) Generation of engraftable hematopoietic stem cells from induced pluripotent stem cells by way of teratoma formation. Mol Ther 21(7):1424–1431
Takahashi M, Osumi N (2002) Pax6 regulates specification of ventral neurone subtypes in the hindbrain by establishing progenitor domains. Development 129 (6):1327–1338
Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4):663–676
Takahashi K, Okita K, Nakagawa M, Yamanaka S (2007a) Induction of pluripotent stem cells from fibroblast cultures. Nat Protoc 2(12):3081–3089
Takahashi K et al (2007b) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131(5):861–872
Tammam S et al (2016) Nuclear delivery of recombinant OCT4 by chitosan nanoparticles for transgene-free generation of protein-induced pluripotent stem cells. Oncotarget 7(25):37728–37739
Tan Q, Lui PP, Rui YF, Wong YM (2012) Comparison of potentials of stem cells isolated from tendon and bone marrow for musculoskeletal tissue engineering. Tissue Eng A 18(7–8):840–851
Tan YT et al (2018) Respecifying human iPSC-derived blood cells into highly engraftable hematopoietic stem and progenitor cells with a single factor. Proc Natl Acad Sci U S A 115(9):2180–2185
Thier M, Munst B, Edenhofer F (2010) Exploring refined conditions for reprogramming cells by recombinant Oct4 protein. Int J Dev Biol 54(11–12):1713–1721
Thier M, Munst B, Mielke S, Edenhofer F (2012) Cellular reprogramming employing recombinant sox2 protein. Stem Cells Int 2012:549846
Wan H et al (2004) Foxa2 is required for transition to air breathing at birth. Proc Natl Acad Sci U S A 101 (40):14449–14454
Wang H, Doering LC (2012) Induced pluripotent stem cells to model and treat neurogenetic disorders. Neural Plast 2012:346053
Wang P et al (2012) A molecular signature for purified definitive endoderm guides differentiation and isolation of endoderm from mouse and human embryonic stem cells. Stem Cells Dev 21(12):2273–2287
Wang H et al (2013) One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell 153(4):910–918

Ward MC, Gilad Y (2019) A generally conserved response to hypoxia in iPSC-derived cardiomyocytes from humans and chimpanzees. elife 8:pii: e42374

Warren L et al (2010) Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. Cell Stem Cell 7(5):618–630

Weaver M, Yingling JM, Dunn NR, Bellusci S, Hogan BL (1999) Bmp signaling regulates proximal-distal differentiation of endoderm in mouse lung development. Development 126(18):4005–4015

Weaver M, Batts L, Hogan BL (2003) Tissue interactions pattern the mesenchyme of the embryonic mouse lung. Dev Biol 258(1):169–184

White AC et al (2006) FGF9 and SHH signaling coordinate lung growth and development through regulation of distinct mesenchymal domains. Development 133 (8):1507–1517

Whitsett J (1998) A lungful of transcription factors. Nat Genet 20(1):7–8

Wilkinson DC et al (2017) Development of a three-dimensional bioengineering technology to generate lung tissue for personalized disease modeling. Stem Cells Transl Med 6(2):622–633

Wilkinson DC et al (2018) Development of a three-dimensional bioengineering technology to generate lung tissue for personalized disease modeling. Curr Protoc Stem Cell Biol 46(1):e56

Wong AP, Rossant J (2013) Generation of lung epithelium from pluripotent stem cells. Curr Pathobiol Rep 1 (2):137–145

Wong JC et al (2010) Definitive endoderm derived from human embryonic stem cells highly express the integrin receptors alphaV and beta5. Cell Adhes Migr 4(1):39–45

Wong AP et al (2012) Directed differentiation of human pluripotent stem cells into mature airway epithelia expressing functional CFTR protein. Nat Biotechnol 30(9):876–882

Xian W, Schwertfeger KL, Vargo-Gogola T, Rosen JM (2005) Pleiotropic effects of FGFR1 on cell proliferation, survival, and migration in a 3D mammary epithelial cell model. J Cell Biol 171(4):663–673

Yamashita M, Emerman M (2006) Retroviral infection of non-dividing cells: old and new perspectives. Virology 344(1):88–93

Yu J et al (2007) Induced pluripotent stem cell lines derived from human somatic cells. Science (New York, NY) 318(5858):1917–1920

Yu J et al (2009) Human induced pluripotent stem cells free of vector and transgene sequences. Science (New York, NY) 324(5928):797–801

Yusa K, Rad R, Takeda J, Bradley A (2009) Generation of transgene-free induced pluripotent mouse stem cells by the piggyBac transposon. Nat Methods 6 (5):363–369

Zhou W, Freed CR (2009) Adenoviral gene delivery can reprogram human fibroblasts to induced pluripotent stem cells. Stem Cells 27(11):2667–2674

Zhou H et al (2009) Generation of induced pluripotent stem cells using recombinant proteins. Cell Stem Cell 4(5):381–384

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Adv Exp Med Biol - Cell Biology and Translational Medicine (2019) 7: 17–28
https://doi.org/10.1007/5584_2019_439
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Published online: 15 November 2019

Gene Editing in Human Pluripotent Stem Cells: Recent Advances for Clinical Therapies

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Abstract

The identification of human embryonic stem cells and reprogramming technology to obtain induced pluripotent stem cells from adult somatic cells have provided unique opportunity to create human disease models, gene editing strategies and cell therapy options.

Development of pluripotent stem cells from somatic cells and genomic manipulation tools enabled to use site specific nucleases in the cell therapy research. Identification of efficient gene manipulation, safe differentiation and use will provide a novel strategy to treat many diseases in the near future. Current available registered clinical trials clearly indicate the need for pluripotent stem cell and gene therapy treatment options. Although gene editing based pluripotent stem cell research is a popular field for research worldwide, improvement of clinical approaches for treatment still remains to be investigated. In this review, we summarized the current situation of gene editing based pluripotent cell therapy developments and applications in clinics.

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Keywords

Cell therapy · Clinical trial · Gene therapy · Pluripotent stem cell · Regenerative medicine

Abbreviations

AAV	Adeno-associated virus
AAVS1	AAV integration site 1
Cas9	CRISPR-associated system
CCR5	CC chemokine receptor 5
CD4	Cluster of differentiation 4
CMV	Cytomegalovirus
CRISPR	Clustered regularly interspaced short palindromic repeat
CRX	Cone-rod homeobox
DNA	Deoxyribonucleic acid
DSB	Double-strand break
ESC	Embryonic stem cell
FIH	First-In-Human
GBA	Glucosylceramidase Beta
GCase	Glucocerebrosidase
GFP	Green fluorescent protein
HDR	Homology-directed repair
hES	Human embryonic stem
hESC	Human ESC
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HR	Homologous recombination
HSC	Hematopoietic stem cell
iPS	Induced pluripotent stem

iPSC	Induced pluripotent stem cell
MELAS	Mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes
MiPSC	Mitochondrial disease patient-specific induced pluripotent stem cell
mtDNA	Mitochondrial DNA
MTS	Mitochondrial targeting sequence
NHEJ	Non-homologous end-joining
PITX3	Pituitary homeobox 3
PSC	Pluripotent stem cell
RNA	Ribonucleic acid
RPE	Retinal pigment epithelium
sgRNA	Single guide RNA
SHANK3	SH3 and multiple ankyrin repeat domains 3
SSN	Site-specific nuclease
TALE	Tal effector protein
TALEN	Transcription activator-like effector nuclease
ZF	Zinc finger
ZFN	Zinc-finger nuclease

1 Introduction

Gene editing of pluripotent stem cells is a milestone in stem cell research in terms of drug discovery, disease modelling and regenerative medicine. Upon discovery of induced pluripotent stem cells (IPSCs) in 2006 (Takahashi and Yamanaka 2006), problems associated with adult and embryonic stem cells (ESCs) have been resolved for potential future cell replacement therapies. IPSCs and human ESCs (hESCs) have similar biological properties and have brought an enthusiasm to basic research and regenerative medicine. Although several limitations exist in the therapeutic usage of pluripotent stem cells, genomic technology coincides with stem cell technology and has brought new insight to pluripotent stem cell research. This is due to the need for adequate, efficient and reliable cell therapy sources for tissue regeneration and disease treatment. Owing to recent advances in site-specific nuclease (SSN)-mediated gene manipulation techniques, pluripotent stem cells might be able to use in clinical studies in the future. The most popular and widely used SSNs are zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated system (Cas9) (Chang et al. 2018). Site-specific double-strand breaks (DSBs) are introduced into the genomic DNA and repaired by non-homologous end-joining (NHEJ) or homology-directed repair (HDR) which results in insertion or deletion in the targeted locus based on engineered design (Hendriks et al. 2016). Combination of SSN-mediated gene manipulation and pluripotent stem cell research enable modelling of developmental pathways and diseases, modulation of cell fate decision and differentiation, correction of genetic abnormalities for certain disorders and avoiding immunogenicity problems. Promising trend of SSN based gene editing and pluripotent stem cells can be adapted to clinics for cell based therapies in the near future. Although there are limited number of ongoing clinical research all around the world, challenges and problems related to efficient genetic engineering of pluripotent stem cells still remain to be encountered for further human use. In this paper, recent techniques especially SSNs in pluripotent stem cells and current clinical trials, are reviewed. Most recent situation of the pluripotent stem cell and gene editing in clinical trials were reviewed and analyzed in detail.

2 Human Pluripotent Stem Cells

Human pluripotent stem cells are divided into two categories: human embryonic stem (hES) cells and induced pluripotent stem cells (iPSCs). hES cells are accepted as gold standard for pluripotent cells and the discovery of hES cells is considered as a milestone for basic research and regenerative medicine (Thomson et al. 1998). The story of

pluripotent stem cell based cellular therapy has started by this research when James Thomson isolated human embryonic stem cells (hESCs) from the internal cell mass of developing embryos (Thomson et al. 1998). On August 9, 2001, US President George W. Bush adopted a federal policy that limits the use of federal funds in hESCs. In 2009, US President Barack Obama eased restrictions on the NIH related to the funding and addition of hESC lines to the NIH registry (Wolinsky 2009). In 2012, Shinya Yamanaka and Sir John Gurdon were awarded for the Nobel Prize which discovered that “mature cells are reprogrammable to become pluripotent”. Introduction of transcription factors (Oct4, Sox2, KLF4, and c-Myc) to create iPSCs by Yamanaka is a key step for disease modelling, cellular therapy and regenerative medicine applications (Takahashi and Yamanaka 2006; Takahashi et al. 2007). iPSCs have additional advantages over ESCs as they can produce patient-specific pluripotent stem cells that are autologous, non-immunogenic and can provide easier disease models and patient specific cellular therapy (Kimbrel and Lanza 2015). iPSCs might provide an opportunity for cell replacement therapy without the need for immunosuppressant, as autologous transplantation of genetically identical cells, tissues and organs prevents immunological rejection (Trounson and Dewitt 2016). Recent developments in iPSCs cell technology in combination with gene editing techniques have emerged as a new hope for the treatment of many diseases such as neurodegenerative diseases (Liu et al. 2013), cardiovascular diseases (Doppler et al. 2013), liver diseases (Yanagida et al. 2013; Liu et al. 2009) and many others. All these findings are very promising for future therapies and will lead to open new scientific fields for gene editing based clinical therapy approaches. Although clinical trials are currently developing for iPSC based cell therapies, it will take a lot of effort and time to administer basic research to clinics successfully. Cellular therapies based on iPSCs and genetic editing, although innovative, are known as complex therapeutic concepts that requires more detailed research.

3 Gene Therapy

Gene therapy is a subject of therapeutic potential in the medical field. All studies conducted by research communities, pharmaceutical companies and clinical communities focus on the discovery, research and clinical monitoring of therapeutic gene construct The first human gene therapy clinical trials was performed by Rosenberg et al. at the Bethesda National Cancer Institute, Department of Cancer Therapy in 1989. These researchers used retrovirus to introduce the bacterial gene encoding neomycin resistance into human tumor-leaking lymphocytes. Subsequently, the modified lymphocytes were re-injected into patients with metastatic melanoma. Thanks to this study, the applicability of retroviral gene transduction to human gene therapy, the treatment of serious hereditary diseases without treatment has provided hope (Rosenberg et al. 1990). After this trial, an increasing number of studies have been started on the application of gene therapy in clinical trials. Genomics, transcriptomics, interactions and functional profile studies of therapeutic targets are developed and comprehensive database is created (Li et al. 2018).

Dr. Takahashi and the research team conducted the first-in-human (FIH) clinical trial using induced pluripotent stem cells (iPSCs) in 2014 and open up the way for these cell therapy studies (Takashima et al. 2018). This is an iPSC-FIH trial in which a patient was transplanted with a retinal pigment epithelium (RPE) cell layer. Genome regulation in IPS cells is a powerful tool that allows researchers to investigate human genome studies on this subject and can broaden the possibilities of gene therapy for the treatment of innate diseases. Modification of a specific gene is called gene targeting. Gene targeting allows scientist to have control over the cellular genome by deleting the gene in the studies or replacing a foreign gene with a gene of interest. It is used in viral vector-mediated approaches as well as DNA-based vectors to take advantage of high transduction yields. Adenovirus as a double-stranded DNA virus (Hotta and Yamanaka 2015)

or Adeno-associated virus (AAV) vectors can be used to introduce a variety of specific mutations to homologous chromosomal regions (Khan et al. 2010) and can be used in research and therapy. The advantage of using Toxicity and immunogenicity are disadvantages of these type of viral vectors and needs to be overcome (Wold and Toth 2013). It has led to the development of synthetic vectors for safety concerns as therapeutic DNA. In the simplest non-viral gene delivery system, "naked" DNA uses plasmid DNA (Ginn et al. 2018).

DNA vectors can be used to reprogram somatic cells to produce induced pluripotent stem cells (iPSC). Jia et al. created a polycystic minicircle connected to 2A for reprogramming factors Lin28, Oct4, Sox2 and Nanog. In this study, iPSC, reprogrammed with minicircle DNA, produced embryo bodies in culture and teratomas in immunocompromised mice. They found that the yield of minicircle was higher than that of plasmid DNA. Non-viral methods for generating iPS cells using excision of reprogramming factors using plasmids, Cre/LoxP or piggyBAC transposition have been reported. Efficient derivation of iPS cells without foreign or chemical elements is absolutely essential (Jia et al. 2010). Minicircles have been used as in vitro and in vivo targets in certain areas, including lung epithelial cells containing enhanced GFP (eGFP), firefly luciferase (Luc), or DNAH5, which encode an external dynein arm protein in primary ciliary dyskinesia. The minicircles carrying these genes exhibited higher levels of gene expression compared to plasmids (Munye et al. 2016). Multiple recombinase systems are used to produce minicircles (Gaspar et al. 2015). Plasmid contaminants in minicircle preparations may be as high as 10% of the total yield, but this is a problem because it is above 1.5% allowed by health institutions. Several methods are being developed to increase the purity of minicircle (Hou et al. 2015). Daneshvar et al. also created iPSCs from umbilical cord mesenchymal stem cells using minicircles without the need for a feeder cell layer with these four reprogramming factors (Jia et al. 2010; Hou et al. 2015).

In the early 1980s, targeted gene studies by homologous recombination (HR) in cultured mammalian cells were performed (Folger et al. 1982). Using the host DNA repair pathway, an endogenous genomic region may be replaced by the external sequence when supported by a targeting vector via HR. A double-stranded break (DSB) in genomic DNA is a form of DNA damage that requires rapid repair of cells to preserve the integrity of the genome and the information it encodes. Repair of DSBs is achieved by non-homologous end joining (NHEJ), HR, and microhomology mediated end joining (Hotta and Yamanaka 2015). The lack of an ideal vector remains a major obstacle in the treatment of human diseases. In the last few years, the use of non-viral vectors has increased significantly (Ramamoorth and Narvekar 2015).

Induced pluripotent stem cell (iPSC) based disease modeling and cell replacement therapy approach has proven to be very effective in biomedical research and personalized regenerative medicine with the resolution of new pathological mechanisms of a large number of monogenic diseases in recent years (Doss and Sachinidis 2019) and identification of gene editing strategies in detail.

3.1 Zinc Finger Nucleases (ZFNs)

Zinc fingers (ZFs) are naturally found eukaryotic proteins that are dependent on zinc ion as a structural cofactor to function (Shimberg et al. 2018). ZFs consist of 30 amino acid residues with repeats of four cysteine and/or histidine within their sequence (Hamed and Arya 2016). In the field of genome engineering, Zinc Finger Nucleases (ZFNs) have been widely used as synthetic nucleases to target and edit any specific region found within the genome since 2003 (Perez-Pinera et al. 2012). ZFNs are comprised of two domains including the Cys2-His2 zinc finger protein domain which are series of DNA-binding motifs and a catalytic domain known as FokI nuclease which is a natural type

IIS restriction enzyme that can cleave the DNA within or in the proximity of its recognition sequence (Carroll 2011; Pingoud and Jeltsch 2001). Each Cys2-His2 zinc finger domain recognizes 3–4 adjacent nucleotide bases, and the linkage of two or three zinc finger motifs allow binding up to 18 base pairs of DNA (Carroll 2011). The restriction enzyme, FokI, must form dimers in order for it to apply its nuclease activity and cleave the DNA. Therefore, two sets of ZFNs are constructed and directed on opposite ends of the target site to allow dimerization of FokI and thus the cleavage of DNA in the specific region (Fig. 1).

In many organisms including humans, ZFNs have been successfully utilized as a tool for inducing DNA double strand break and specifically editing targeted genes in the genome (Carroll 2011; Urnov et al. 2010). Manipulating the genome using ZFN technology has been shown to be successful with high efficiency in hESCs and hiPSCs (Hockemeyer et al. 2009; Zou et al. 2009). In a study done in 2014, ZFNs have been shown to be a powerful tool for introducing and expressing transgenes in hESCs at specific regions by avoiding gene silencing during hESCs differentiation (Tiyaboonchai et al. 2014). CD43-GFP construct has been targeted with ZFN technology in hESCs to a locus in the human genome called AAVS1 to allow stable gene expression. Afterwards, the recombined hESCs have been differentiated into hematopoietic cells. Furthermore, it has been suggested that the introduction of CD43-GFP reporter construct in hESCs using ZFNs is a successful gene editing tool for the expression of transgenes (Tiyaboonchai et al. 2014). Brigham J. Hartley and his colleagues have established a protocol to target Pituitary homeobox 3 (PITX3) locus in hESCs by introducing ZFNs along with a PITX3-EGFP-specific DNA donor vector to generate a PITX3 reporter cell line. This reporter cell line has been suggested to allow tracking and isolating the cells of interest following differentiation of hESCs for use in studies including *in vitro* Parkinson's disease modeling or stem cell therapy (Hartley et al. 2014). In 2015, Jia Liu and his colleagues have improved specific protocols for implementing ZFN-based genome editing in hESCs. Furthermore, they have shown that ZFNs can be efficiently expressed and purified within 6 days and used for stimulating genomic modifications in hESCs within 2 days (Chandrasekaran et al. 2017). In 2016, Joseph Collin and his colleagues have used ZFNs to

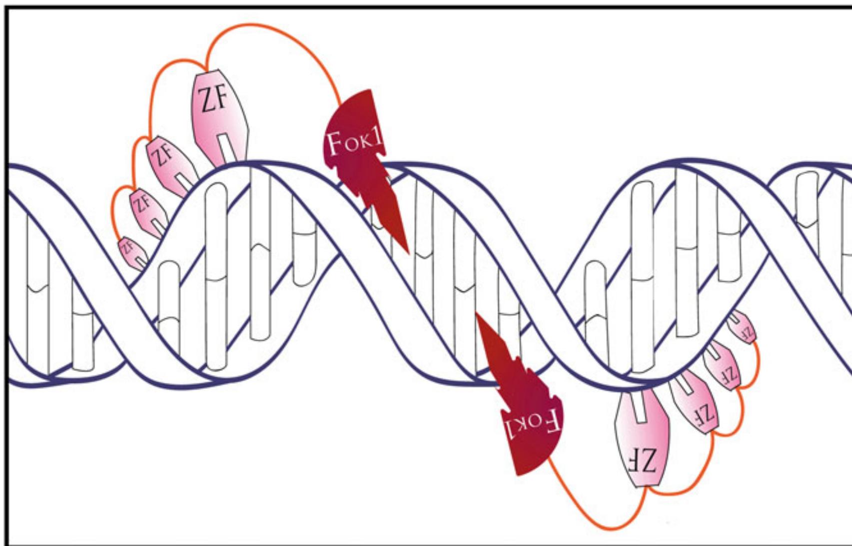

Fig. 1 The schematic representation of zinc finger nucleases. Four contiguous zinc finger proteins bind to the targeted sequence in the human genome and the dimerized nuclease, FokI, cuts the DNA in its recognition site

create CRX-GFP labeled hESCs to study the development of retina and detect photoreceptor precursors at different stages of differentiation (Collin et al. 2016). In a study published in 2017, scientists have demonstrated that stable long-term molecular imaging in hESCs can be achieved by using ZFN-mediated genome editing technology (Wolfs et al. 2017). A. Kathuria and his co-workers have also engineered hESCs with ZFNs in 2017 to knockout SHANK3 which is a gene associated with Autism Spectrum Disorder (Kathuria et al. 2018). In another recent study, ZFNs have been used for constructing a homozygous GBA gene deletion in a hESC line to generate loss of function in a lysosomal enzyme known as beta-glucocerebrosidase (GCase). Furthermore, this ZFN engineered hESC line has been suggested to be a promising *in vitro* model for studying GCase function in human disorders such as Gaucher's and Parkinson's disease (Gundner et al. 2017). Recently in 2018, ZFNs have been used for expressing fluorescently tagged endocytic markers in hESCs to measure clathrin-dependent endocytosis quantitatively during differentiation (Dambournet et al. 2018).

In the clinical aspect of genome editing, ZFNs have been firstly taken part in the clinical trials on patients with human immunodeficiency virus (HIV) (18). In this study, a gene responsible for encoding a coreceptor for HIV known as CCR5 has been genetically edited and made dysfunctional in CD4 T cells by using ZFNs. Consequently, the infusion of these modified cells has successfully demonstrated the potential clinical use of ZFN based genome editing in the treatment of HIV (Tebas et al. 2014). This clinical study has not only indicated ZFNs to be a therapeutic approach for HIV but also shown to be a promising gene-editing tool in hESCs for the treatment of other human disorders in the future.

3.2 Transcription Activator-Like Effector Nucleases (TALENs)

Similar to ZNFs, transcription activator-like effector nucleases (TALENs) are formed by the cooperation of DNA binding domain and nuclease (Fok1) domain (Singh et al. 2015). DNA binding domain is constituted by tandem repeats originated from TAL effector proteins (TALEs) which are naturally found in *Xanthomonas* bacteria (Boch and Bonas 2010; Tran et al. 2018). Double strand DNA breaks are generated by Fok1 nuclease at desired location (Mahata and Biswas 2017). This strategy is based on design of specific nucleases that are fused to DNA binding domains which have the ability to bind to desired DNA sequence. These specialized and targeted nucleases are used to generate DSBs on specific DNA sites followed by repair via different mechanisms which lead to DNA sequence adjustments at the cleavage site. TALENs have received extensive attention because of simple design and high efficiency compared to other gene editing strategies. The targeted DNA sequence is scanned through nucleotide by nucleotide and cleaved by TALENs (Singh et al. 2015). (Fig. 2).

In 2011, TALENs were tested in stem cell research for the first time by Hockemeyer et al. (2011). This study evaluated the efficacy of TALENs in human embryonic stem cells and iPSCs by the insertion of a particular gene modification for comparing the efficiency and precision of TALENs with ZFNs in PPP1R12C (the AAVS1 locus), OCT4, PITX3 genes. The results indicated that TALENs and ZNFs showed similar efficiency, however, the application and design process of TALENs were more suitable for *in vitro* studies. Because TALENs have short application time and easy design process compared to ZNFs (Hockemeyer et al. 2011). In another study, Sakuma et al. modified the TALEN proteins to increase the nuclease activity and used mammalian promoters CMV (cytomegalovirus) and T7 promoters for vector design (Sakuma et al. 2013). Same research showed that the entire gene editing protocol in iPS cells requires only one week by using TALENs indicating the time advantage (Sakuma et al. 2013). Besides, TALENs were regarded as more specific towards the target sequence compared to ZNF nucleases because of its specific DNA binding domain to targeted DNA region (Boch 2011). Furthermore, gene editing with ZNFs were

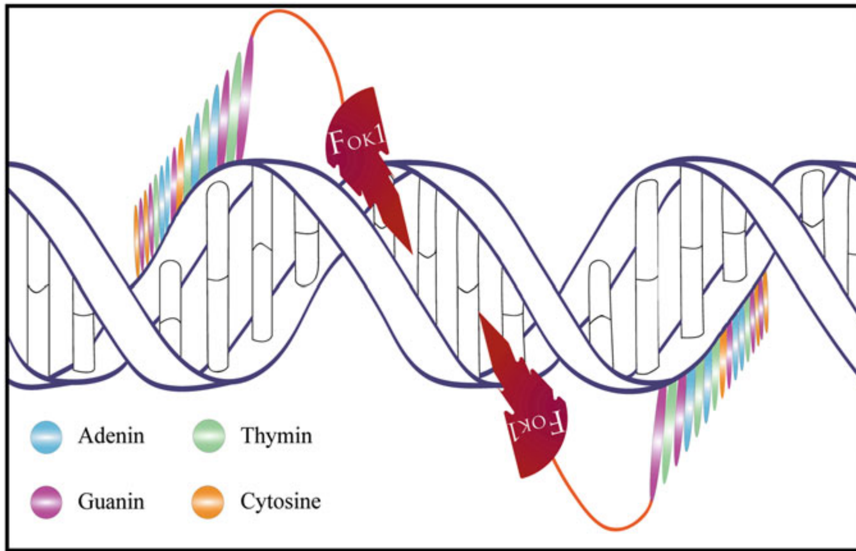

Fig. 2 Illustration of TALEN endonucleases. TAL effector DNA binding domains that has 15–20 TALE subunits and the endonuclease Fok1. Each monomer of TALE binds to specific nucleotide and dimerized Fok1 nucleases cut the double strand of DNA

observed to be more cytotoxic than TALENs since TALE binding domain allows for more efficient DNA cleavage with the help of reliable recognition and results in efficient editing without toxicity (Mussolino et al. 2011).

Additionally, a study in zebrafish embryo proved that TALE nucleases activity had shown no toxicity, unlikely to ZNFs, suggesting the difference between off-target binding of two nucleases types (Sander et al. 2011). Predictably, off-target nuclease activity causes cell death, therefore shows toxicity due to untoward double strand DNA breaks.

TALENs were reported to be very effective in repairing the mitochondrial genome of iPS cells. In 2017, Yahata et al. used MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) patients (carrying m.13513G > A mutation) derived iPS cells for gene editing with TALENs. Mitochondrial genome mutation related diseases mostly occur due to the increased ratio of mutant mitochondrial DNA mtDNA in cells (Yahata et al. 2017). Yahata et al. aimed to reduce the ratio of mutant mtDNA in iPS cells by using TALENs. A significant decrease in the ratio of mutant mtDNA was reported after insertion of mitochondrial targeting sequence (MTS) to TALEN encoding plasmid. Moreover, in 2018 another study on m.3243A > G mutant MELAS patients' mtDNA also supported the findings of Yahata et al. (2018) as the crucial role of TALENs in reduction in mutant mtDNA had been highlighted once again. This group generated mitochondrial disease patient-specific induced pluripotent stem cells (MiPSCs) that have m.3243A > G mutation and they successfully decrease the mutation in MiPSCs. Although there is not an available on-going clinical trial about the function of TALENs in pluripotent stem cells, there are three different clinical trials that focus on gene editing approach, using TALENs. These studies comprise acute myeloid leukemia and in human papillomavirus-related malignant neoplasm. A trail on acute myeloid leukemia has been carried out to investigate whether UCART123 (Car-T cell generated with TALENs gene editing method) is safe and efficient since June 16, 2017 (**ClinicalTrials.gov Identifier: NCT03190278**). Another trail conducted by Jingchu University of Technology, aimed to identify the safety and efficacy of TALEN-HPV E6/E7 and CRISPR/Cas9-

HPV E6/E7 in treating Human papillomavirus (HPV) Persistency and HPV-related Cervical Intraepithelial Neoplasia (**ClinicalTrials.gov Identifier: NCT03057912**). In the third trail, researches evaluate safety and efficacy of T27 and T512 in treating HPV Persistency and HPV16-positive cervical intraepithelial neoplasia (**ClinicalTrials.gov Identifier: NCT03226470**). *In vitro* studies about TALENs in pluripotent stem cells might highlight the gaps in gene editing research leading to identification of TALENs in clinical trials. Ongoing trials might be an initial point for further future clinical research on TALEN based gene editing of pluripotent stem cells and their use in regenerative medicine.

3.3 CRISP-Cas9 System

Genetic manipulation of pluripotent stem cells holds a great promise for DNA level therapeutic approaches to genetic disorders. Targeted genome modification techniques with the site-specific nucleases, ZFNs (Urnov et al. 2010; Miller et al. 2007), TALENs (Hockemeyer et al. 2011; Wood et al. 2011) and clustered regularly interspaced short palindromic repeats (CRISPR) CRISPR-associated nuclease 9 (Cas9) (Sander and Joung 2014; Hsu et al. 2014) have brought a new breath to genetic engineering for stem cell therapy. CRISPR-Cas system gained a wide usage thanks to its simplified design and enhanced efficiency of gene editing to generate genetic alterations in cells compared to other site-specific nucleases. Engineering only a single guide RNA (sgRNA), as a sole determinant for DNA-binding specificity, made this technique highly favorable for scientists (Doudna and Charpentier 2014).

CRISPR-Cas systems originate from adaptive immune system of bacteria and archaea, and RNA-guided nucleases acts as a guardian defending the host against foreign genetic elements (Marraffini and Sontheimer 2010). Among the 6 main types of CRISPR-Cas systems identified so far from a wide range of hosts, type II based on Cas9 effectors is the best characterized system for genome editing (Barrangou and Horvath 2017). CRISPR-Cas9 system performs the gene editing at a target locus by generating double strand breaks using Cas9 nuclease directed by a sgRNA (Ran et al. 2013). Double strand breaks stimulate DNA repair mechanisms, NHEJ DNA repair and HDR, within the cell (Cubbon et al. 2018; Komor et al. 2016) (Fig. 3).

Disease modelling with pluripotent stem cells mediated by CRISPR-Cas9 involves incorporation of the disease-related mutations to the genome of pluripotent stem cells and its elimination from the sick pluripotent cells (Shi et al. 2017). To make pluripotent stem cells more applicable for clinical therapies, abundance of CRISP-Cas9 mediated therapies were performed up to date. Chia-Wei et al. used iPSCs of a human severe combined immunodeficiency patient and targeted JAK3 mutation with CRISPR-Cas9 to restore T cell development (Chang et al. 2015). Chao Li et al. also used iPSCs of the patient having a sickle cell disease and corrected the mutation by CRISPR-Cas9 delivery (Li et al. 2016). Another disease model, Cystic Fibrosis, was also dealt by iPSCs generation from a Cystic Fibrosis patient. Amy L. Firth et al. corrected the deletion of F508 at CFTR gene by CRISP-mediated targeting of the correct sequence to the CFTR locus (Firth et al. 2015). Chul-Yong Park also took advantage of CRISPR-Cas9 nucleases to treat inversion of F8 gene of iPSCs from a Hemophilia A patient. Accordingly, when the corrected iPSCs functioning as wild type were differentiated to endothelial cells, F8 gene was expressed correctly and so the functional blood coagulating factor VIII expression was achieved (Park et al. 2015). Successful corrections of the disorders using CRISPR-Cas9 in vitro prepared the ground for in vivo clinical applications. The first promising in vivo genetic disease treatment using CRISPR-Cas9 therapy was achieved at mice with Duchenne muscular dystrophy disease. The disease-causing gene was removed by CRISPR-Cas9 to let the muscle cells express the protein (Bengtsson et al. 2017). There are also several on-going human clinical researches utilizing CRISP-Cas9 technology, such as transplantation of CRISPR-Cas9-mediated CCR5

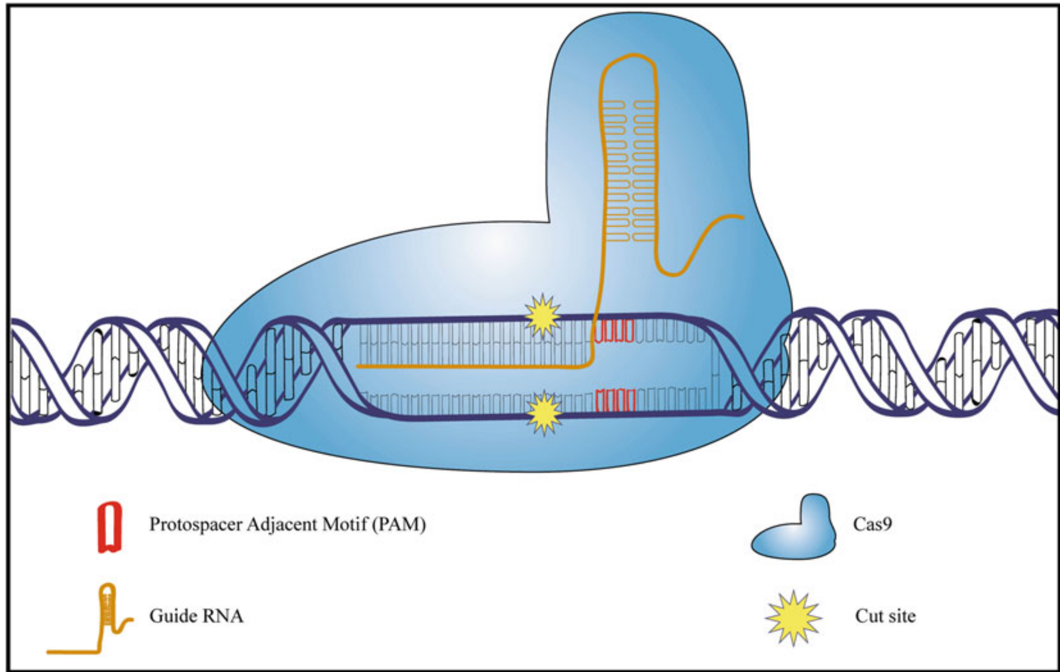

Fig. 3 Illustration of Crispr/Cas 9 system. The system consist of Cas 9 endonuclease and single-quided RNA (sg RNA) molecule. Cas 9 nuclease sites cleave both strands within the protopacer-associated motif (PAM) target sequence

gene disruption of CD34+ hematopoietic stem cells (HSCs) from HIV-1 infected patient to treat the disease (**ClinicalTrials.gov Identifier: NCT03164135**).

The applications of CRISPR-Cas9-based treatments show quite a potential for its clinical use. It is not far the use of CRISPR-Cas9 therapies in clinics for human including genetic disease treatment, cancer treatment and embryo treatment. However, there are still concerns about the safety and some challenges, including effective and safe delivery of the system to the cells of the patients and minimizing off-target cuts that must be overcome to make CRISP-Cas9 technology reach to its full potential.

4 Conclusion

Microbiological mechanisms have always been providing an enormous wealth to develop biotechnological tools for scientific researches. By taking advantage of these mechanisms, scientists have developed a number of gene editing technologies over the last decades.

Ever since the discovery of stem cells, researchers have been trying to utilize it for therapeutic approaches. Considering the pluripotency and self-renewal potentials, pluripotent stem cells became the major candidate for these studies. However, ethical concerns and lack of source were the biggest obstacles for the researcher. Thanks to the generation of iPSCs, as one of the major breakthroughs in science, stem cell-based therapy found its potential use in regenerative therapy. Nevertheless, the ultimate aim for the therapeutic use of stem cells could be reached by merging gene editing technologies with the potential of stem cells. Targeted genetic manipulations of pluripotent stem cells opened a new platform for treatment of genetic diseases and development of therapeutic agents. Ever since then increasing clinical trials also resulted in success and cured the patients with genetic diseases. According to the U.S. National Institute of Health web page of Clinical Trials, U.S. takes the lead in the world for gene therapy, stem cell

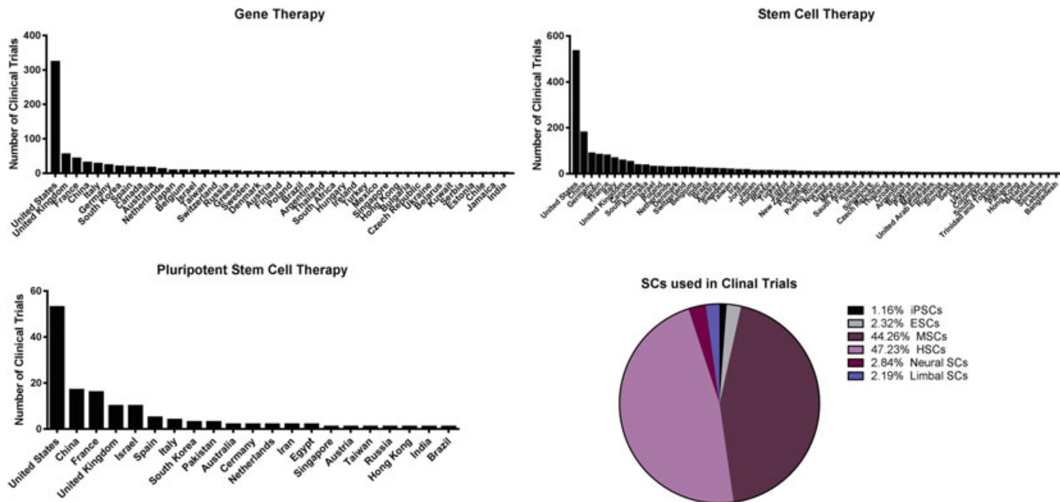

Fig. 4 Gene and Stem cell therapy clinical trials registered on ClinicalTrials.gov website

therapy and pluripotent stem cell therapy in the clinical trials. While China follows U.S. in stem cell therapy and pluripotent stem cell therapy, U.K. ranks second in clinical gene therapy applications. Following them, France takes the third place for gene therapy and pluripotent stem cell therapy, whereas Germany comes third in stem cell therapy clinical trials (Fig. 4). Hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) comprise almost 99% of the total stem cell usage in clinical trials indicating the lack of pluripotent stem cell based therapeutic approaches (Fig. 4). iPSCs is restricted to a small portion (1.16%) of application in the clinical trials according to the recent data (https://clinicaltrials.gov). This ratio is expected to be extended in the future by the administration of gene editing technology to stem cell based therapies. Although the success rate of clinical trials, these therapeutic developments are still far from being used in clinics. This powerful technology still needs to be advanced to provide maximum benefit with minimum risk.

References

Barrangou R, Horvath P (2017) A decade of discovery: CRISPR functions and applications. Nat Microbiol 2:17092

Bengtsson NE, Hall JK, Odom GL, Phelps MP, Andrus CR, Hawkins RD et al (2017) Muscle-specific CRISPR/Cas9 dystrophin gene editing ameliorates pathophysiology in a mouse model for duchenne muscular dystrophy. Nat Commun 8:14454

Boch J (2011) Tales of genome targeting. Nat Biotechnol 29(2):135–136

Boch J, Bonas U (2010) Xanthomonas AvrBs3 family-type III effectors: discovery and function. Annu Rev Phytopathol 48:419–436

Carroll D (2011) Genome engineering with zinc-finger nucleases. Genetics 188(4):773–782

Chandrasekaran AP, Song M, Ramakrishna S (2017) Genome editing: a robust technology for human stem cells. Cell Mol Life Sci 74(18):3335–3346

Chang CW, Lai YS, Westin E, Khodadadi-Jamayran A, Pawlik KM, Lamb LS Jr et al (2015) Modeling human severe combined immunodeficiency and correction by CRISPR/Cas9-enhanced gene targeting. Cell Rep 12(10):1668–1677

Chang CY, Ting HC, Su HL, Jeng JR (2018) Combining induced pluripotent stem cells and genome editing technologies for clinical applications. Cell Transplant 27(3):379–392

Collin J, Mellough CB, Dorgau B, Przyborski S, Moreno-Gimeno I, Lako M (2016) Using zinc finger nuclease technology to generate CRX-reporter human embryonic stem cells as a tool to identify and study the emergence of photoreceptors precursors during pluripotent stem cell differentiation. Stem Cells 34(2):311–321

Cubbon A, Ivancic-Bace I, Bolt EL (2018) CRISPR-Cas immunity, DNA repair and genome stability. Biosci Rep 38(5):BSR20180457

Dambournet D, Sochacki KA, Cheng AT, Akamatsu M, Taraska JW, Hockemeyer D et al (2018) Genome-edited human stem cells expressing fluorescently labeled endocytic markers allow quantitative analysis

of clathrin-mediated endocytosis during differentiation. J Cell Biol 217(9):3301–3311
Doppler SA, Deutsch MA, Lange R, Krane M (2013) Cardiac regeneration: current therapies-future concepts. J Thorac Dis 5(5):683–697
Doss MX, Sachinidis A (2019) Current challenges of iPSC-based disease modeling and therapeutic implications. Cell 8(5):pii: E403
Doudna JA, Charpentier E (2014) Genome editing. The new frontier of genome engineering with CRISPR-CAS9. Science 346(6213):1258096
Firth AL, Menon T, Parker GS, Qualls SJ, Lewis BM, Ke E et al (2015) Functional gene correction for cystic fibrosis in lung epithelial cells generated from patient iPSCs. Cell Rep 12(9):1385–1390
Folger KR, Wong EA, Wahl G, Capecchi MR (1982) Patterns of integration of DNA microinjected into cultured mammalian cells: evidence for homologous recombination between injected plasmid DNA molecules. Mol Cell Biol 2(11):1372–1387
Gaspar V, De Melo-Diogo D, Costa E, Moreira A, Queiroz J, Pichon C et al (2015) Minicircle DNA vectors for gene therapy: advances and applications. Expert Opin Biol Ther 15(3):353–379
Ginn SL, Amaya AK, Alexander IE, Edelstein M, Abedi MR (2018) Gene therapy clinical trials worldwide to 2017: an update. J Gene Med 20(5):E3015
Gundner AL, Meyer CA, Aigner S, Christensen K, Patsch C, Jagasia R et al (2017) Generation of a homozygous GBA deletion human embryonic stem cell line. Stem Cell Res 23:122–126
Hamed MY, Arya G (2016) Zinc finger protein binding to DNA: an energy perspective using molecular dynamics simulation and free energy calculations on mutants of both zinc finger domains and their specific DNA bases. J Biomol Struct Dyn 34(5):919–934
Hartley BJ, Fabb SA, Finnin BA, Haynes JM, Pouton CW (2014) Zinc-finger nuclease enhanced gene targeting in human embryonic stem cells. J Vis Exp 90(51764):1–7
Hendriks WT, Warren CR, Cowan CA (2016) Genome editing in human pluripotent stem cells: approaches, pitfalls, and solutions. Cell Stem Cell 18(1):53–65
Hockemeyer D, Soldner F, Beard C, Gao Q, Mitalipova M, Dekelver RC et al (2009) Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. Nat Biotechnol 27(9):851–857
Hockemeyer D, Wang H, Kiani S, Lai CS, Gao Q, Cassady JP et al (2011) Genetic engineering of human pluripotent cells using TALE nucleases. Nat Biotechnol 29(8):731–734
Hotta A, Yamanaka S (2015) From genomics to gene therapy: induced pluripotent stem cells meet genome editing. Annu Rev Genet 49:47–70
Hou XH, Guo XY, Chen Y, He CY, Chen ZY (2015) Increasing the minicircle DNA purity using an enhanced triplex DNA technology to eliminate DNA contaminants. Mol Ther Methods Clin Dev 1:14062
Hsu PD, Lander ES, Zhang F (2014) Development and applications of CRISPR-Cas9 for genome engineering. Cell 157(6):1262–1278
Jia F, Wilson KD, Sun N, Gupta DM, Huang M, Li Z et al (2010) A nonviral minicircle vector for deriving human iPS cells. Nat Methods 7(3):197–199
Kathuria A, Nowosiad P, Jagasia R, Aigner S, Taylor RD, Andreae LC et al (2018) Stem cell-derived neurons from autistic individuals with SHANK3 mutation show morphogenetic abnormalities during early development. Mol Psychiatry 23(3):735–746
Khan IF, Hirata RK, Wang PR, Li Y, Kho J, Nelson A et al (2010) Engineering of human pluripotent stem cells by AAV-mediated gene targeting. Mol Ther 18 (6):1192–1199
Kimbrel EA, Lanza R (2015) Current status of pluripotent stem cells: moving the first therapies to the clinic. Nat Rev Drug Discov 14(10):681–692
Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR (2016) Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature 533(7603):420–424
Li C, Ding L, Sun CW, Wu LC, Zhou D, Pawlik KM et al (2016) Novel HDAd/EBV reprogramming vector and highly efficient Ad/CRISPR-Cas sickle cell disease gene correction. Sci Rep 6:30422
Li YH, Yu CY, Li XX, Zhang P, Tang J, Yang Q et al (2018) Therapeutic target database update 2018: enriched resource for facilitating bench-to-clinic research of targeted therapeutics. Nucleic Acids Res 46(D1):D1121–D11D7
Liu T, Wang Y, Tai G, Zhang S (2009) Could co-transplantation of iPS cells derived hepatocytes and MSCs cure end-stage liver disease? Cell Biol Int 33(11):1180–1183
Liu Y, Liu H, Sauvey C, Yao L, Zarnowska ED, Zhang SC (2013) Directed differentiation of forebrain GABA interneurons from human pluripotent stem cells. Nat Protoc 8(9):1670–1679
Mahata B, Biswas K (2017) Generation of stable knockout mammalian cells by TALEN-mediated locus-specific gene editing. Methods Mol Biol 1498:107–120
Marraffini LA, Sontheimer EJ (2010) CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea. Nat Rev Genet 11(3):181–190
Miller JC, Holmes MC, Wang J, Guschin DY, Lee YL, Rupniewski I et al (2007) An improved zinc-finger nuclease architecture for highly specific genome editing. Nat Biotechnol 25(7):778–785
Munye MM, Tagalakis AD, Barnes JL, Brown RE, Mcanulty RJ, Howe SJ et al (2016) Minicircle dna provides enhanced and prolonged transgene expression following airway gene transfer. Sci Rep 6:23125
Mussolino C, Morbitzer R, Lutge F, Dannemann N, Lahaye T, Cathomen T (2011) A novel TALE nuclease scaffold enables high genome editing activity in combination with low toxicity. Nucleic Acids Res 39 (21):9283–9293

Park C, Kim DH, Son JS, Sung JJ, Lee J, Bae S et al (2015) Functional correction of large factor VIII gene chromosomal inversions in Hemophilia A patient-derived iPSCs using CRISPR-Cas9. Cell Stem Cell 17 (2):213–220

Perez-Pinera P, Ousterout DG, Gersbach CA (2012) Advances in targeted genome editing. Curr Opin Chem Biol 16(3–4):268–277

Pingoud A, Jeltsch A (2001) Structure and function of type II restriction endonucleases. Nucleic Acids Res 29 (18):3705–3727

Ramamoorth M, Narvekar A (2015) Non viral vectors in gene therapy- an overview. J Clin Diagn Res 9(1): Ge01–Ge06

Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F (2013) Genome engineering using the CRISPR-CAS9 system. Nat Protoc 8(11):2281–2308

Rosenberg SA, Aebersold P, Cornetta K, Kasid A, Morgan RA, Moen R et al (1990) Gene transfer into humans — immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. N Engl J Med 323 (9):570–578

Sakuma T, Hosoi S, Woltjen K, Suzuki K, Kashiwagi K, Wada H et al (2013) Efficient TALEN construction and evaluation methods for human cell and animal applications. Genes Cells 18(4):315–326

Sander JD, Joung JK (2014) CRISPR-Cas systems for editing, regulating and targeting genomes. Nat Biotechnol 32(4):347–355

Sander JD, Cade L, Khayter C, Reyon D, Peterson RT, Joung JK et al (2011) Targeted gene disruption in somatic zebrafish cells using engineered TALENs. Nat Biotechnol 29(8):697–698

Shi Y, Inoue H, Wu JC, Yamanaka S (2017) Induced pluripotent stem cell technology: a decade of progress. Nat Rev Drug Discov 16(2):115–130

Shimberg GD, Pritts JD, Michel SLJ (2018) Chapter 4: Iron–sulfur clusters in zinc finger proteins. In: David SS (ed) Methods in enzymology, vol 599. Academic, Cambridge, MA, USA pp 101–137

Singh AM, Adjan Steffey VV, Yeshi T, Allison DW (2015) Gene editing in human pluripotent stem cells: choosing the correct path. J Stem Cell Regen Biol 1 (1):1–5

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4):663–676

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K et al (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131(5):861–872

Takashima K, Inoue Y, Tashiro S, Muto K (2018) Lessons for reviewing clinical trials using induced pluripotent stem cells: examining the case of a first-in-human trial for age-related macular degeneration. Regen Med 13 (2):123–128

Tebas P, Stein D, Tang WW, Frank I, Wang SQ, Lee G et al (2014) Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. N Engl J Med 370(10):901–910

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS et al (1998) Embryonic stem cell lines derived from human blastocysts. Science 282 (5391):1145–1147

Tiyaboonchai A, Mac H, Shamsedeen R, Mills JA, Kishore S, French DL et al (2014) Utilization of the AAVS1 safe harbor locus for hematopoietic specific transgene expression and gene knockdown in human ES cells. Stem Cell Res 12(3):630–637

Tran T, Doucoure H, Hutin M, Jaimes Nino LM, Szurek B, Cunnac S et al (2018) Efficient enrichment cloning of TAL effector genes from xanthomonas. Methods 5:1027–1032

Trounson A, Dewitt ND (2016) Pluripotent stem cells progressing to the clinic. Nat Rev Mol Cell Biol 17 (3):194–200

Urnov FD, Rebar EJ, Holmes MC, Zhang HS, Gregory PD (2010) Genome editing with engineered zinc finger nucleases. Nat Rev Genet 11(9):636–646

Wold WS, Toth K (2013) Adenovirus vectors for gene therapy, vaccination and cancer gene therapy. Curr Gene Ther 13(6):421–433

Wolfs E, Holvoet B, Ordovas L, Breuls N, Helsen N, Schonberger M et al (2017) Molecular imaging of human embryonic stem cells stably expressing human PET reporter genes after zinc finger nuclease-mediated genome editing. J Nucl Med 58(10):1659–1665

Wolinsky H (2009) The Pendulum Swung. President Barack Obama removes restrictions on stem-cell research, but are expectations now too high? EMBO Rep 10(5):436–439

Wood AJ, Lo TW, Zeitler B, Pickle CS, Ralston EJ, Lee AH et al (2011) Targeted genome editing across species using ZFNs and TALENs. Science 333(6040):307

Yahata N, Matsumoto Y, Omi M, Yamamoto N, Hata R (2017) TALEN-mediated shift of mitochondrial DNA heteroplasmy in MELAS-iPSCs with m.13513G>A mutation. Sci Rep 7(1):15557

Yanagida A, Ito K, Chikada H, Nakauchi H, Kamiya A (2013) An in vitro expansion system for generation of human iPS cell-derived hepatic progenitor-like cells exhibiting a bipotent differentiation potential. PLoS One 8(7):E67541

Yang Y, Wu H, Kang X, Liang Y, Lan T, Li T et al (2018) Targeted elimination of mutant mitochondrial DNA in MELAS-iPSCs by mitoTALENs. Protein Cell 9 (3):283–297

Zou J, Maeder ML, Mali P, Pruett-Miller SM, Thibodeau-Beganny S, Chou BK et al (2009) Gene targeting of a disease-related gene in human induced pluripotent stem and embryonic stem cells. Cell Stem Cell 5 (1):97–110

Adv Exp Med Biol - Cell Biology and Translational Medicine (2019) 7: 29–36
https://doi.org/10.1007/5584_2019_421
© Springer Nature Switzerland AG 2019
Published online: 4 December 2019

Vascular Wall as Source of Stem Cells Able to Differentiate into Endothelial Cells

Roberto Tamma, Simona Ruggieri, Tiziana Annese, and Domenico Ribatti

Abstract

The traditional view of the vascular biology is changed by the discovery of vascular progenitor cells in bone marrow or peripheral blood Further complexity is due to the findings that the vessel walls harbor progenitor and stem cells, called vascular wall-resident vascular stem cells (VW-VSCs), able to differentiate to mature vascular wall cells. These immature stem/progenitor cell populations and multipotent mesenchymal lineage participate in postnatal neovascularization and vascular wall remodeling. Further studies are necessary to deepen the knowledge on characterization and biology of VW-VSCs, in particular of endothelial progenitor cells (EPCs) in order to improve their use in clinical settings for regenerative approaches.

Keywords

Angiogenesis · Endothelial cells · Stem cells · Vascular wall · Vasculogenesis

Abbreviation

AC133	CD133
Adv	adventitial
BM	bone marrow
CD	cluster differentiation
c-kit	proto-oncogene c-Kit
ECFCs	endothelial colony-forming cells
EPCs	endothelial progenitor cells
FOXF-1	forkhead box F-1
HSCs	hematopoietic stem cells
KLF4	Krüppel-like transcription factors
PECAM-1	platelet/endothelial cell adhesion molecule 1
Sca-1	stem cells antigen-1
shh	Sonic Hedgehog
SMCs	smooth muscle cells
TGF-β	transforming growth factor beta
VEGFR-2	vascular endothelial growth factor-2
VW-EPCs	vascular wall-endothelial progenitor cells
VW-HPCs	vascular wall- hematopoietic progenitor cells
VWMSCs	vascular wall-mesenchymal cells
VW-PCs	vascular wall-progenitor cells
VW-VSCs	vascular wall-resident vascular stem cells
Wnt	Wingless-related integration

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1 Background

Stem cells have attracted the attention of many biomedical science researchers due to their possible use in various therapeutic approaches and in regenerative medicine. Their characteristic features are the self-renewal and the ability in differentiating into almost all human cell types, a process known as potency, crucial for lifelong maintenance of organ function. Self-renewal is the process by which stem cells generate undifferentiated daughter cells in order to preserve stem cell populations (reservoir) in different tissues (Ahmed 2009).

Stem cells are divided in embryonic and adults stem cells. Embryonic stem cells reside the inner cell mass of pre-implantation embryos and have the ability to form all three embryonic germ layers (Evans and Kaufman 1981). They are classified in totipotent if they differentiate in all the human cells including embryo annexes and they are present in zygote and early blastomeres, pluripotent if they differentiate in all the adult human cells located in inner cell mass of blastocysts (days 4–14 after oocyte fertilization), and generate all cell types excluding extra embryonic trophoblast lineage, multipotent and unipotent if they differentiate respectively in many or one lineage of adult human cells (Evans and Kaufman 1981; Smith 2001).

There are two general strategies by which stem cells generate differentiated progeny. In the first one, a stem cell gives rise to one stem daughter and one daughter that undergoes differentiation through an asymmetric cell division. The second one implicates all the regulative mechanisms through which stem cell gives rise to daughter cells that have the potential to maintain the stem features or committed to progenitors. These last mays differentiate along different pathways depending on the combination of extrinsic factors to which they are exposed into a specific niche microenvironment. This mechanism regards most mammalian self-renewing tissues (Altman and Das 1965; Ambler and Maatta 2009).

2 Stem Cell Niche and Endothelial Progenitor Cells

Stem cell resides in a dynamic and specialized microenvironment defined as niche, source of many stimuli and playing a critical role in differentiation potential and maintenance of stem cells in a quiescent state. The stem cell niche corresponds to a particular location in a tissue where stem cells can reside for an indefinite period of time and produce progeny of self-renewing cells (Ohlstein et al. 2004). Usually a proof of the existence of a niche is the re-acquiring and maintaining the introduced stem cells after a previous emptying (Ohlstein et al. 2004). Adult stem cell niches are scattered in the adult and are represented by hematopoietic stem cell (HSC) niche, hair follicle stem cell niche, intestinal crypt niche, muscle satellite cell niche, neural stem cell niche. HSCs are not spread throughout the body but are organized in niches localized in the bone marrow (BM) cavity including the endosteal and the vascular niches. Both are fundamental source of instructive signals that maintain and regulate the activity of HSCs throughout life. Moreover, numerous chemical mediators and the extracellular matrix play an important role in the regulation of HSC fate (Tamma and Ribatti 2017). BM blood vessels not only separate the hematopoietic compartment from the peripheral circulation but are able to regulate hematopoiesis as well as stem cell mobilization and homing. BM is also involved in the process of vasculogenesis/angiogenesis by a complex signaling network towards endothelial and endothelial progenitor cell (EPCs) residing into the vascular niche.

The discovery of vascular progenitor cells in BM or peripheral blood (PB) have modified the traditional views of vascular biology. In fact, until more than two decade ago it was believed that new vessel formation in the adult was only provided by sprouting of vessels from pre-existing blood vessels (Folkman et al. 1989;

Risau et al. 1988). Later, it was postulated that endothelial BM derived EPCs isolated from peripheral blood as CD34, vascular endothelial growth factor receptor-2 (VEGFR-2), or AC133 antigen-positive cells may be incorporate into foci of neovascularization (Asahara et al. 1997).

In addition, it was found that the vessel walls harbor progenitor and stem cells, called vascular wall-resident vascular stem cells (VW-VSCs), able to differentiate to mature vascular wall cells (Fig. 1). These cells include subtypes of progenitors such as VW-EPCs (vascular wall endothelial progenitor cells), VW-SMPCs (vascular wall smooth muscle progenitor cells) and VWMSCs (vascular wall mesenchymal cells). These immature stem/progenitor cell populations and multipotent mesenchymal lineage participate in postnatal neovascularization and vascular wall remodeling (Ingram et al. 2005; Psaltis et al. 2011).

Although endothelial cell turnover is thought to be related to the recruitment of EPCs from remote sites including BM and non-BM adult tissues, including skeletal muscle (Majka et al. 2003), adipose tissue (Grenier et al. 2007), spleen (Werner et al. 2003), liver, intestine (Aicher et al. 2007) and myocardium (Bearzi et al. 2009), EPCs have been isolated from the intimal endothelial layer (Ingram et al. 2005) and vessel wall (Wabik and Jones 2015). Not all the VW-EPCs seems to be enrolled during angiogenesis. In adult rat aorta endothelial cells maintained residual rate of replication related to the endothelium turnover and are organized in clusters of cells localized in focal areas (Schwartz and Benditt 1977). After 2–3 weeks of renal hypertension, the rate of replication rises tenfold respect to the normal value in order to cover partially the expanded luminal surface of the dilated vessel (Schwartz and Benditt 1977).

The characterization of the EPCs is complicated because they share some markers with the HSCs including c-kit, CD133, Sca-1, VE-cadherin, VEGFR-2 and CD105 (endoglin) (Kopp et al. 2006; Rafii and Lyden 2003). The existence of a specialized cells forming the hemogenic endothelium, able to generate hematopoietic progenitors has been demonstrated (Boisset et al. 2010; Jaffredo et al. 1998). Cytometry and immunohistochemistry revealed

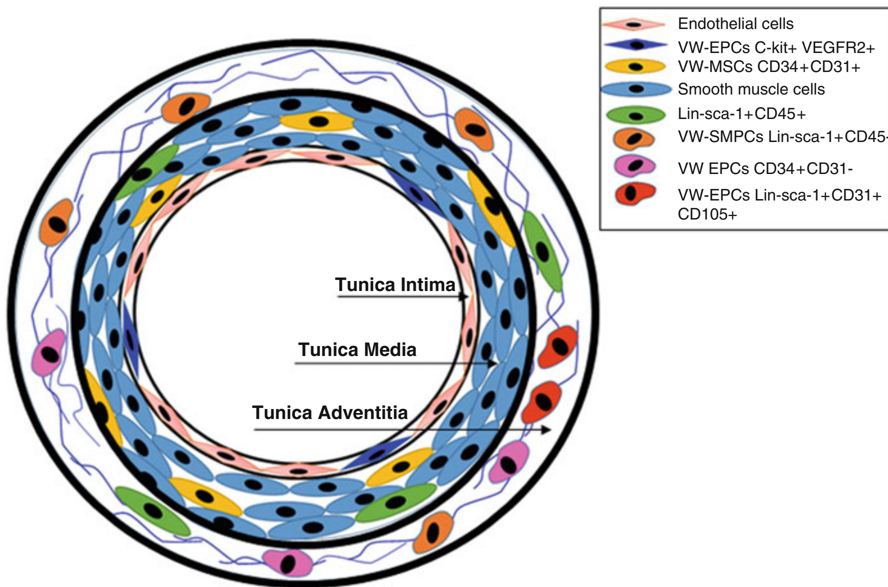

Fig. 1 Vessel wall distribution of different types of progenitor cells (VW-PCs) in postnatal vascular mural layers of arteries and veins

that $CD45^+$ $CD34^-$ cells are present in the adult arterial wall suggesting the potential existence of vascular wall-resident HPCs (VW-HPCs) (Klein et al. 2011). The coexistence of HPCs and EPCs is not surprising because they share a common embryonic precursor. Initially, it was suggested that some blood cells differentiate directly from endothelial cells (Sabin 1920; Tavian et al. 2005). Later, it was proposed that a bi-potent mesoderm progenitor cell named 'hemangioblast' (Choi et al. 1998; Ribatti 2008) generates both endothelial and blood cells that form the rudimentary circulatory system. The study of the nature of EPCs remains controversial, since monocytic-myeloid cells can transiently express cell surface markers typically found also on endothelial cells. This cell population may mimic an endothelial phenotype but do not have the other characteristics used in defining an EPC, such as the ability of a single cell to give rise to a proliferative colony of cells. Many studies have attempted to identify cell surface markers to be considered as unique to EPCs in order to distinguish them from mature adult endothelial cells as well as from myeloid-monocytic cells, but these attempts have met little success (Zhang et al. 2014). In the effort to solve this problem, Yoder (2012) proposed to redefine EPCs through a clonal analysis instead of cell surface antigen expression. They identified a very rare population of highly proliferative colony forming endothelial cells in preparations of cultured peripheral blood mononuclear cells, which were indeed able to give rise to clonal colonies and referred to this population as "endothelial colony-forming cells" (ECFCs). Although ECFCs behave like true progenitor cells and do not express myeloid or monocytic markers, it is unclear whether they play any significant role in physiological vascular repair and angiogenesis in the adult due to their rarity.

Human coronary arteries contain vascular niches composed of clusters of cells expressing c-kit and VEGFR-2. These cells are located in the intima, media, and adventitia, and are connected with endothelial cells, smooth muscle cells (SMCs), and fibroblasts by connexin 43 and N-cadherin (Bearzi et al. 2009). The intima-derived progenitor cells express endothelial cell surface markers such as CD31, CD141, CD105, CD146, CD144, von Willebrand factor, VEGFR-2 and displayed clonogenic potential similar to umbilical cord EPCs. Lin et al. (2011) demonstrated that leukemia inhibitory factor combined with VEGF can maintain progenitor phenotype of EPCs and reduce EPCs differentiation by upregulating Krüppel-like transcription factors (KLF4). Moreover, it has been demonstrated that KLF4 is upregulated by shear stress (Clark et al. 2011), and modulates e nitric oxide syntase (eNOS) (Shen et al. 2009; Yamawaki et al. 2010) and VE-cadherin expression (Cowan et al. 2010), inhibiting SMC maturation (Yamaguchi et al. 2011). KLF4 also regulates the choice of differentiation pathway of these cells through β-catenin activation and is regulated by the canonical Wnt pathway activator lithium chloride (Campagnolo et al. 2015).

As regards CD105, it is a transmembrane glycoprotein expressed on endothelial cells that functions as a co-receptor for several ligands of the transforming growth factor beta (TGF-β) family (Barbara et al. 1999). CD105 is also a cell adhesion molecule in mature and progenitor endothelial cells interacting with vascular mural cells, and is considered marker of angiogenesis (Rossi et al. 2019). Moreover, it has been attributed a role for CD105 in both hemangioblast specification and hematopoietic commitment (Perlingeiro 2007).

Observations resulting from adventitia derived cells suggest that the cell population residing within a particular mural domain and expressing Sonic Hedgehog (shh) is to be considered as stem cell antigen-1 $(Sca\text{-}1)^+$ vascular progenitor cells and named Adv Sca1 (Majesky et al. 2012). These cells are thought to constitute a source of circulating EPCs. Experimental evidences showed that endothelial cells of vein grafts are derived from circulating progenitor cells, of which one-third are derived from BM progenitor cells and the other from the vascular derived progenitors (Hu et al. 2002). It has been established that adventitial cells of the aorta not only contribute to the formation of the tunica adventitia but also serve as a reservoir for vascular progenitor cells. Zheng et al. (2009) identified

a population of Sca1$^+$ cells in adult mouse pulmonary vessels that contained a rare subset of endothelial colony-forming cells (c-kit$^+$Lin-CD31$^+$CD105$^+$) showing long-term self-renewal capacity, a functional feature of adult stem and progenitor cells. The same cell population were observed in the capillaries, arteries, and veins of different organs (lung, liver, kidney, and subcutaneous tissues), as well as in new-forming vessels in subcutaneous Matrigel plugs and in murine and human melanoma cells and breast cancer bioptic samples. A stem cell population CD31$^+$ was identified in the intima of preexisting blood vessels, showing the ability of produce large number of endothelial cells and when transplanted into ischemic lesions, restore blood flow and reconstitute *de novo* long-term surviving blood vessels (Naito et al. 2012).

Ohtani et al. (2011) have emphasized the importance of epigenetic regulation in the endothelial cell population differentiation. They showed that endothelial commitment in subpopulation of pro-angiogenic CD34$^+$ progenitor cells could be repressed by histone modifications of the endothelial NO synthase promoter, partly reversible. A population of CD34$^+$/CD31$^-$ cells located in intermediate zone between media and adventitial layers of human adult vascular wall may be considered as EPCs able to originate new vessels *in vitro*, named vasculogenic zone (Zengin et al. 2006).

Recent studies have identified PW1 as new marker of vascular associated progenitor cells (Malinverno et al. 2017). PW1 is a parentally imprinted gene that encodes a large zincfinger protein that functions both as a regulator of cell stress and as a transcription factor (Thiaville et al. 2013). PW1 is expressed in adult stem cells found in gut, skin, bone, skeletal muscle, and central nervous system (Besson et al. 2011). PW1$^+$ cells express comparable levels of most endothelial cell markers, including VE-cadherin, PECAM 1 and claudin 5. Although PW1$^+$ cells also co-express mesenchymal markers both *in vitro* and *in vivo*, it is unbelieved that these cells belong to hemogenic lineage because this specific markers are expressed at very low levels; moreover, PW1$^+$ cells do not form any hemopoietic colonies *in vitro* (Malinverno et al. 2017).

Sturtzel et al. (2018) in a recent study have identified forkhead box F1 (FOXF1) as the most preferentially expressed transcription factor in resident endothelial progenitors when compared to mature endothelial cells (Sturtzel et al. 2018). The FOX family of transcription factors is generally involved in the determination of cell lineage and organ specificities and in particular in formation of embryonic vasculature by regulating VEGFR genes (Ren et al. 2014). Its ability in the regulation of the progenitor status of endothelial progenitors and in regulation of their sprouting capabilities is associated to the modulation of Notch2 receptors and Notch 2 expression. Moreover, FOXF1 augments VEGFR-2 expression and upregulates ephrinB2, downregulating EphB4 (Sawamiphak et al. 2010).

EPCs have attracted significant scientific attention because their prospective for translational studies, gene therapy and vascular regeneration. Although the origin is until unclear it is believed that the sources of these cells the source on these cells is the BM (Asahara et al. 1997), the tissue vascular niches (Tura et al. 2013) and vascular resident endothelial cells (Corey et al. 2016; Mondor et al. 2016). EPCs have been used for autologous cell therapy in several pre-clinical models of ischemic vascular diseases (Wang et al. 2013; Wei et al. 2007). These cells incorporate in the ischemic tissue improving it functional recovery. Moreover, their use ischemic stroke models and traumatic brain injury suggests that EPCs could constitute a promising treatment by promoting angiogenesis by a direct involvement in new blood vessels formation, or stimulating endogenous neovascularization (Huang et al. 2013; Moubarik et al. 2011; Zhang et al. 2013).

Another application of isolated EPC regards their use as cellular vehicle for gene therapy. In this context, advantages of these cells include the possibility of isolation from peripheral blood and expansion, genomic stability in cell culture and easy genetic manipulation. Promising results have been obtained in hemophilia A (Matsui et al. 2007), Von Willebrand Desease (De Meyer et al.

2006) anemia (Lin et al. 2011) and cancer (Chong et al. 2016; Ruggeri et al. 2018).

In cancer, genetically engineered EPCs have been used to vehicle inhibitors of angiogenesis or other suicide genes directly into the tumor endothelium indicating circulating EPCs as potential candidates for tumor-specific delivery of cancer gene therapy (Dudek et al. 2007; Wei et al. 2007). In this context, the systemic delivery of EPCs expressing VEGFR-2 and/or angiostatin-endostatin fusion protein inhibit tumor growth and increased survival in mice cancer models (Bodempudi et al. 2010; Wei et al. 2007).

Future studies are necessary to deepen the knowledge on characterization and biology of EPCs in order to develop the use of EPCs in clinical settings for regenerative approaches. Two drawbacks are represented by the fact that EPCs cannot be successfully isolated from peripheral blood of all individuals because their low number as circulating cells, and the use of culture methods not containing media or supplement derived from the animals.

References

Ahmed S (2009) The culture of neural stem cells. J Cell Biochem 106:1–6

Aicher A et al (2007) Nonbone marrow-derived circulating progenitor cells contribute to postnatal neovascularization following tissue ischemia. Circ Res 100:581–589

Altman J, Das GD (1965) Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol 124:319–335

Ambler CA, Maatta A (2009) Epidermal stem cells: location, potential and contribution to cancer. J Pathol 217:206–216

Asahara T et al (1997) Isolation of putative progenitor endothelial cells for angiogenesis. Science 275:964–967

Barbara NP et al (1999) Endoglin is an accessory protein that interacts with the signaling receptor complex of multiple members of the transforming growth factor-beta superfamily. J Biol Chem 274:584–594

Bearzi C et al (2009) Identification of a coronary vascular progenitor cell in the human heart. Proc Natl Acad Sci U S A 106:15885–15890

Besson V et al (2011) PW1 gene/paternally expressed gene 3 (PW1/Peg3) identifies multiple adult stem and progenitor cell populations. Proc Natl Acad Sci U S A 108:11470–11475

Bodempudi V et al (2010) Blood outgrowth endothelial cell-based systemic delivery of antiangiogenic gene therapy for solid tumors. Cancer Gene Ther 17:855–863

Boisset JC et al (2010) In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. Nature 464:116–120

Campagnolo P et al (2015) c-Kit+ progenitors generate vascular cells for tissue-engineered grafts through modulation of the Wnt/Klf4 pathway. Biomaterials 60:53–61

Choi K et al (1998) A common precursor for hematopoietic and endothelial cells. Development 125:725–732

Chong MS et al (2016) Concise review: endothelial progenitor cells in regenerative medicine: applications and challenges. Stem Cells Transl Med 5:530–538

Clark PR et al (2011) MEK5 is activated by shear stress, activates ERK5 and induces KLF4 to modulate TNF responses in human dermal microvascular endothelial cells. Microcirculation 18:102–117

Corey DM et al (2016) Dynamic patterns of clonal evolution in tumor vasculature underlie alterations in lymphocyte-endothelial recognition to foster tumor immune escape. Cancer Res 76:1348–1353

Cowan CE et al (2010) Kruppel-like factor-4 transcriptionally regulates VE-cadherin expression and endothelial barrier function. Circ Res 107:959–966

De Meyer SF et al (2006) Phenotypic correction of von Willebrand disease type 3 blood-derived endothelial cells with lentiviral vectors expressing von Willebrand factor. Blood 107:4728–4736

Dudek AZ et al (2007) Systemic inhibition of tumour angiogenesis by endothelial cell-based gene therapy. Br J Cancer 97:513–522

Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292:154–156

Folkman J et al (1989) Induction of angiogenesis during the transition from hyperplasia to neoplasia. Nature 339:58–61

Grenier G et al (2007) Resident endothelial precursors in muscle, adipose, and dermis contribute to postnatal vasculogenesis. Stem Cells 25:3101–3110

Hu Y et al (2002) Both donor and recipient origins of smooth muscle cells in vein graft atherosclerotic lesions. Circ Res 91:e13–e20

Huang XT et al (2013) Intracerebroventricular transplantation of ex vivo expanded endothelial colony-forming cells restores blood-brain barrier integrity and promotes angiogenesis of mice with traumatic brain injury. J Neurotrauma 30:2080–2088

Ingram DA et al (2005) Vessel wall-derived endothelial cells rapidly proliferate because they contain a complete hierarchy of endothelial progenitor cells. Blood 105:2783–2786

Jaffredo T et al (1998) Intraaortic hemopoietic cells are derived from endothelial cells during ontogeny. Development 125:4575–4583

Klein D et al (2011) Vascular wall-resident CD44+ multipotent stem cells give rise to pericytes and smooth muscle cells and contribute to new vessel maturation. PLoS One 6:e20540

Kopp HG et al (2006) Contribution of endothelial progenitors and proangiogenic hematopoietic cells to vascularization of tumor and ischemic tissue. Curr Opin Hematol 13:175–181

Lin IRZ et al (2011) Induction of erythropoiesis using human vascular networks genetically engineered for controlled erythropoietin release. Blood 118:5420–5248

Majesky MW et al (2012) The adventitia: a progenitor cell niche for the vessel wall. Cells Tissues Organs 195:73–81

Majka SM et al (2003) Distinct progenitor populations in skeletal muscle are bone marrow derived and exhibit different cell fates during vascular regeneration. J Clin Invest 111:71–79

Malinverno M et al (2017) Peg3/PW1 is a marker of a subset of vessel associated endothelial progenitors. Stem Cells 35:1328–1340

Matsui H et al (2007) Ex vivo gene therapy for hemophilia A that enhances safe delivery and sustained in vivo factor VIII expression from lentivirally engineered endothelial progenitors. Stem Cells 25:2660–2669

Mondor I et al (2016) Clonal proliferation and stochastic pruning orchestrate lymph node vasculature remodeling. Immunity 45:877–888

Moubarik C et al (2011) Transplanted late outgrowth endothelial progenitor cells as cell therapy product for stroke. Stem Cell Rev 7:208–220

Naito H et al (2012) Identification and characterization of a resident vascular stem/progenitor cell population in preexisting blood vessels. EMBO J 31:842–855

Ohlstein B et al (2004) The stem cell niche: theme and variations. Curr Opin Cell Biol 16:693–699

Ohtani K et al (2011) Epigenetic regulation of endothelial lineage committed genes in pro-angiogenic hematopoietic and endothelial progenitor cells. Circ Res 109:1219–1229

Perlingeiro RC (2007) Endoglin is required for hemangioblast and early hematopoietic development. Development 134:3041–3048

Psaltis PJ et al (2011) Resident vascular progenitor cells—diverse origins, phenotype, and function. J Cardiovasc Transl Res 4:161–176

Rafii S, Lyden D (2003) Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. Nat Med 9:702–712

Ren X et al (2014) FOXF1 transcription factor is required for formation of embryonic vasculature by regulating VEGF signaling in endothelial cells. Circ Res 115:709–720

Ribatti D (2008) Hemangioblast does exist. Leuk Res 32:850–854

Risau W et al (1988) Vasculogenesis and angiogenesis in embryonic-stem-cell-derived embryoid bodies. Development 102:471–478

Rossi E et al (2019) Endoglin as an adhesion molecule in mature and progenitor endothelial cells: a function beyond TGF-beta. Front Med (Lausanne) 6:10

Ruggeri A et al (2018) Endothelial and circulating progenitor cells in Hematological diseases and allogeneic hematopoietic stem cell transplantation. Curr Med Chem 25:4535–4544

Sabin FR (1920) Studies on the origin of blood vessels and of red corpuscles as seen in the living blastoderm of chicks during the second day of incubation. Contrib Embryol 9:213–262

Sawamiphak S et al (2010) Ephrin-B2 regulates VEGFR2 function in developmental and tumour angiogenesis. Nature 465:487–491

Schwartz SM, Benditt EP (1977) Aortic endothelial cell replication. I. Effects of age and hypertension in the rat. Circ Res 41:248–255

Shen B et al (2009) Kruppel-like factor 4 is a novel mediator of Kallistatin in inhibiting endothelial inflammation via increased endothelial nitric-oxide synthase expression. J Biol Chem 284:35471–35478

Smith AG (2001) Embryo-derived stem cells: of mice and men. Annu Rev Cell Dev Biol 17:435–462

Sturtzel C et al (2018) FOXF1 mediates endothelial progenitor functions and regulates vascular sprouting. Front Bioeng Biotechnol 6:76

Tamma R, Ribatti D (2017) Bone niches, hematopoietic stem cells, and vessel formation. Int J Mol Sci 18:151

Tavian M et al (2005) The vascular wall as a source of stem cells. Ann N Y Acad Sci 1044:41–50

Thiaville MM et al (2013) DNA-binding motif and target genes of the imprinted transcription factor PEG3. Gene 512:314–320

Tura O et al (2013) Late outgrowth endothelial cells resemble mature endothelial cells and are not derived from bone marrow. Stem Cells 31:338–348

Wabik A, Jones PH (2015) Switching roles: the functional plasticity of adult tissue stem cells. EMBO J 34:1164–1179

Wang JW et al (2013) Analysis of the storage and secretion of von Willebrand factor in blood outgrowth endothelial cells derived from patients with von Willebrand disease. Blood 121:2762–2772

Wei J et al (2007) Targeted release of oncolytic measles virus by blood outgrowth endothelial cells in situ inhibits orthotopic gliomas. Gene Ther 14:1573–1586

Werner N et al (2003) Intravenous transfusion of endothelial progenitor cells reduces neointima formation after vascular injury. Circ Res 93:e17–e24

Yamaguchi S et al (2011) The role of microRNA-145 in human embryonic stem cell differentiation into vascular cells. Atherosclerosis 219:468–474

Yamawaki H et al (2010) Omentin, a novel adipokine, induces vasodilation in rat isolated blood vessels. Biochem Biophys Res Commun 393:668–672

Yoder MC (2012) Human endothelial progenitor cells. Cold Spring Harb Perspect Med 2:a006692
Zengin E et al (2006) Vascular wall resident progenitor cells: a source for postnatal vasculogenesis. Development 133:1543–1551
Zhang Y et al (2013) Transplantation of expanded endothelial colony-forming cells improved outcomes of traumatic brain injury in a mouse model. J Surg Res 185:441–449
Zhang M et al (2014) Endothelial progenitor cells and vascular repair. Curr Opin Hematol 21:224–228
Zheng F et al (2009) The primary study of CD90(+)CD34(−)and Sca-1(+) stem cells mobilized by EPO plus G-CSF in mice. Int J Stem Cells 2:129–134

Adv Exp Med Biol - Cell Biology and Translational Medicine (2019) 7: 37–47
https://doi.org/10.1007/5584_2019_427
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Published online: 30 August 2019

Physiological and Therapeutic Roles of Neuropeptide Y on Biological Functions

Pravin Shende and Drashti Desai

Abstract

Neuropeptide Y (NPY), an amino acid, used for various physiological processes for management and treatment of various ailments related to central nervous system, cardiovascular system, respiratory system, gastro-intestinal system and endocrinal system. In nasal mucosa, high concentrations of NPY are stored with noradrenaline in sympathetic nerve fibers. NPY Y_1 receptor mediates nitric oxide levels and reduction in blood flow in nasal mucosa of the human. NPY plays a role in dietary consumption via various factors like signaling the CNS for a prerequisite of energy in hypothalamus by mediating appetite and shows orexigenic effect. NPY emerges as a natural ligand of G-protein coupled receptors which activates the Y-receptors (Y_1–Y_6). But applications of NPY are limited due to shows the cost inefficiency and stability issues in the formulation design and development. In this review, authors present the findings on various therapeutic applications of NPY on different organ systems. Moreover, its role in food intake, sexual behavior, blood pressure, etc. by inhibiting calcium and activating potassium channels. The combination therapies of drugs with neuropeptide Y and its receptors will show new targets for treating diseases. Further evaluation and detection of NPY needs to be investigated for animal models of various diseases like retinal degeneration and immune mechanisms.

Keywords

Anxiety · Brain · Depression · Obesity · Stress

Abbreviations

6-OHDA	6-hydroxydopamine
BBB	Blood-Brain barrier
BDNF-TrkB	Brain-derived Neurotrophic Factor-Tropomyosin receptor kinase B
cAMP	Cyclic adenosine monophosphate
CNS	Central Nervous System
CSF	Cerebrospinal fluid
CVS	Central Vascular System
EEG	Electroencephalogram
MALDI	Matrix Assisted Laser Desorption/Ionization
NPY	Neuropeptide Y
PET	Positron emission tomography
PTSD	post-traumatic stress disorder
PYY	Peptide YY
RIA	Radioimmunoassay
SC	Stem cell

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1 Introduction

A large number of peptides like insulin, oxytocin, melittin, etc. are present in the human body which act as hormones by sequencing with amino acids to function as a biological messenger. Peptides play a major role in the human body for maintaince of homeostasis, whereas polypeptides show repeating sequence of amino acids. The first polypeptide was isolated from the pancreas of avian and then further investigated to develop the antibodies for the pancreatic polypeptide but immunoreaction was observed in the brain because of the presence of peptide structure. Pancreatic polypeptides isolated from the porcine brain were called as peptide YY but showed immunoreactivity, furthermore succeed in the preparation of neuropeptide Y (NPY) (Fig. 1), i.e. polypeptide of 36 amino acid starts and ends with tyrosine moiety mostly present in the various regions of brain.

NPY is also found in non-sympathetic neurons in some organs like salivary glands, gastrointestinal tract, thyroid gland, urogenital system, pancreas, heart and respiratory system. Many physiological conditions such as learning, cognition, depression, stress, etc. are also linked with NPY. Moreover, NPY causes vasoconstriction of vascular smooth muscles, post-junction noradrenaline-induced vasoconstriction elevation and inhibition of pre-junction nor-adrenaline release. Non-amidated NPY shows no pre-junctional and post-junctional effects but represents the significance of biological activity possessed by the C-terminal carboxamide group for the activation of protein kinase. (Decressac and Barker 2012; Dumont et al. 1992; Cox 2007; Colmers and El Bahh 2003; Botelho and Cavadas 2015; Beck 2006; Baraban 2004; Mittapalli and Roberts 2014; Horsnell and Baldock 2016).

1.1 Synthesis of Fragments ($NPY_{2–36}$ and $NPY_{3–36}$)

A large quantity of NPY is present in the central nervous system (CNS) belongs to the pancreatic polypeptides and peptide YY family, using a precursor of 97- amino acid derives NPY. The synthetic procedure for preparation of $NPY_{3–36}$ are shown in Fig. 2. Pre-Pro-NPY, 28-residue peptide precursor, detaches to a yield Pre-NPY in endoplasmic reticulum to produce mature NPY. It uses splitting prohormone convertase to form $NPY_{1–39}$ which is further reduced to $NPY_{1–37}$ by the carboxypeptidase-like enzyme to yield aminated $NPY_{1–36}$ from the mature NPY sequence. The degradation of active NPY by specific enzymes like a removal of N-terminal tyrosine by aminopeptidase P and Tyr-Pro dipeptide by dipeptidyl peptidase IV produces $NPY_{2–36}$ and $NPY_{3–36}$, respectively. $NPY_{18–36}$ have not shown any agonist activity by inhibiting the NPY effect but it eliminates the cardiac adenylate cyclase activity. (Balasubramaniam 1997, 2002; Malva et al. 2012; Pedrazzini et al. 2003)

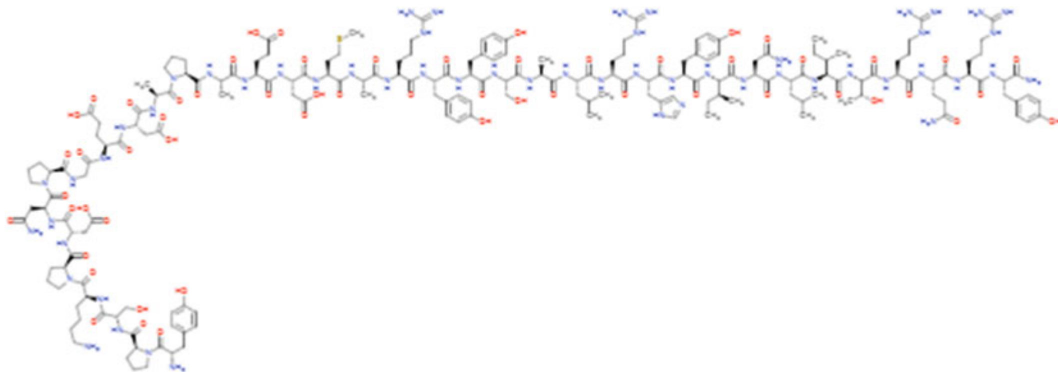

Fig. 1 Structure of NPY

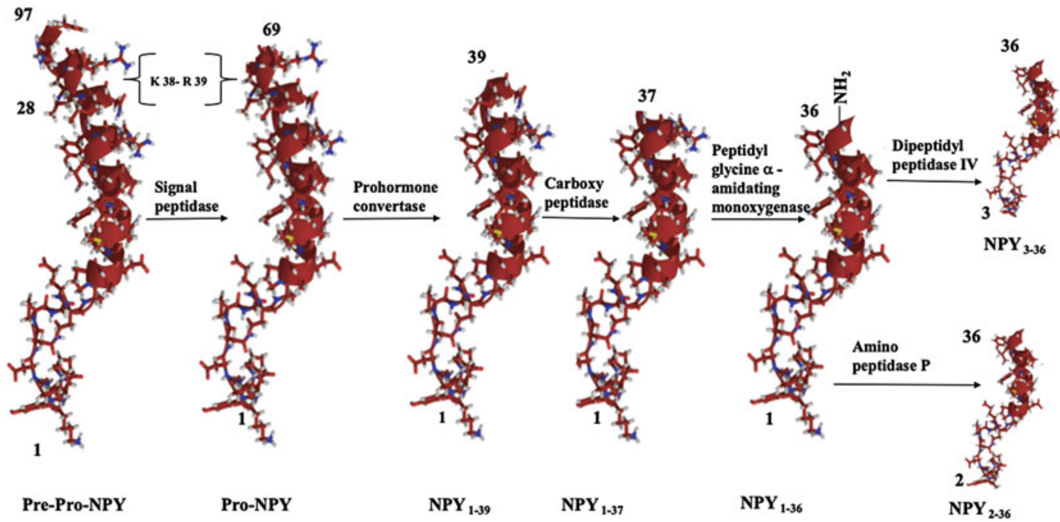

Fig. 2 Synthesis of NPY_{3-36}

For the targeted drug delivery system, mainly peptide-drug conjugates emerge as a promising carrier due to their high selectivity and desirable particle size.

1.2 Types of NPY Receptors

NPY emerges as a natural ligand of G-protein coupled receptors which activates the Y-receptors (Y_1–Y_6). Glutamatergic synaptic transmission is selectively inhibited whereas glutamate release is suppressed by NPY. It can further be sub-classified into Y_1 (Y_1, Y_4, Y_6, Y_8), Y_2 (Y_2, Y_7) and Y_5. NPY and peptide YY bind with Y_1, Y_2 and Y_5 receptors for various therapeutic actions like analgesic, hypertensive, antidepressant, etc. (Santos-Carvalho et al. 2015; Farzi et al. 2015; Reichmann and Holzer 2016).

Receptor Y_1 is located in CNS, postrema, blood vessels and amygdala, hippocampus, thalamus, heart, kidney, GIT and post-synaptic areas. Some Y_1 agonists are [Phe^7, Pro^{34}] NPY, [Leu^{31}, Pro^{34}] NPY, [Pro^{34}] NPY and antagonist are N-terminally trimmed NPY analogues like GR231118, 1,229 U91 INPI-Phenylalanine-RLRY, BMS-193885, SR120819, 1,3-disubstituted benzodiazepines dihydropyridine like substances, BIBO3304 and BIBP3226. It plays an important role in various functions by regulating feeding behaviour, immunoreactivity, vasoconstriction, anxiolytic, alcohol consumption regulation, antidepressant, neuroprotection, analgesia, hypertension, sedation and antiepileptic action. (Reichmann and Holzer 2016; Silva et al. 2002; Munglani et al. 1996; Martin et al. 2015)

Receptor Y_2 located at a pre-synaptic region in the brain mainly in frontal cortex, lateral septum, lateral hypothalamus, hippocampus, intestine, thalamus, postrema and certain blood vessels, human astrocytoma cell line, amygdala, brainstem, vascular smooth muscle cells, intestine, liver, spleen and adipose tissue. Some Y_1 agonists are cyclic analogs of NPY like Ac-Ac-[Lys^{28}, Glu^{32}] NPY_{25-36}, TASP-V, NPY $_{3-36}$, PYY_{3-36} and antagonist are T_4-[NPY_{33-36}], JNJ-5207787 and BIIE0246. Activities like immunoreactivity, autoreceptor, appetite regulator, inhibition of neurotransmitter release, anxiolytic, presynaptic autoreceptors modulating endogenous NPY release, antiepileptic, neuroprotector, antinociceptive, circadian rhythm regulation, anorexia, bone formation, alcohol and drugs dependence and cancer are demonstrated by Y_2. (Martin et al. 2015; Duarte-Neves et al. 2016)

Receptor Y_3 is located in post-synaptic region, in adrenal medulla in human and tractus solitaries, cardiac membranes and bovine chromaffin cells in the rat. The agonist and antagonist of Y_3 are not reported yet, but reduces vasomotor tone and releases catecholamine inhibitor and also affects cardiovascular activities. (Silva et al. 2002; Munglani et al. 1996)

Receptor Y_4 presents in brain regions like medial postrema post-synaptic and preoptic involving hypothalamus, hippocampus, thyroid gland, heart, skeletal muscle, prostate, stomach, small intestine, colon, pancreas, adrenal medulla and cortex and nasal mucosa. The antagonist of Y_1 (GW1229), PP acts as an agonist for receptor Y_4 but the antagonist is still under investigation. It inhibits exocrine secretions from the pancreas, relaxes gall bladder, stimulates LH secretion, regulates food intake and protects neurons. (Martin et al. 2015)

Receptor Y_5 situates in several limbic regions of brain like post-synaptic, hypothalamus, cortex, hippocampus, thalamus, amygdala, striatum and other organs like intestine, testis, ovary and prostate, pancreas, spleen, liver, skeletal muscle, kidney, heart and placenta. Y_1 agonists are $CPP_{1–7}$, $NPY_{19–23}$, [Ala^{31}, Aib^{32}] NPY and antagonist are acetylation of a-(3-pyridylmethyl)-h-aminotetralins, [D-Trp^{32}] NPY, GW438014A, L-152,804 and CGP71683A are responsible for appetite regulation, neuroprotection, etc. It regulates the food consumption and circadian rhythm, stimulates appetite, shows anticonvulsant, anxiolytic, neuroprotection, regulation and excites hippocampal activity. (Reichmann and Holzer 2016; Silva et al. 2002; Munglani et.al 1996; Martin et al. 2015)

Receptor Y_6 in post-synaptic areas mainly in pseudogene primates, small intestine and embryonic colon. An agonist or antagonist activities are not reported yet. (Pedrazzini et al. 2003)

2 Characterization of NPY

NPY is characterized by various parameters like *in-vitro* and *ex-vivo* drug release studies, mass spectroscopy, etc. A peptide, NPY, can be identified by MALDI-mass spectrometry which shows a peak at 4251.1 with C18 column retention time at 23.4 min. The detection of the therapeutic concentration of the drug for anticonvulsant effect was studied by using bursting model. In *in-vivo* experiments, rodents and human models are mostly recommended. Hippocampal slices are analyzed for seizure susceptibility and EEG records of the hippocampus are recorded of mice. *In vivo* target engagement with positron emissions tomography with radiotracers found NPY Y_1 PET tracer effective in rhesus monkey and humans. Blood-brain penetration was studied for neurochemical hormonal responses and stress models to show effect against peptide bonds and helical structure. Several studies revealed that striatum, hippocampus, amygdala and hypothalamus contain elevated levels of NPY and analysed by RIA for immunohistochemistry. (Ahrens et al. 2015; Liu et al. 2016; Reibel et al. 2001; El Bahh et al. 2005; Kautz et al. 2017; Sabban et al. 2016)

3 Applications of NPY

NPY functions in various conditions like anxiety, diabetes, cardiac contractility, hypertension, appetite, blood pressure, congestive heart failure, intestinal secretion, seizures, intestinal dysfunctions and neurodegenerative diseases. As NPY receptors bind to G- proteins, it inhibits cAMP synthesis and plays a major role in respiratory system, physiology and pathology of airway diseases, immune system, CNS, CVS, endocrine system, depression, vasoconstriction, etc. and performs various functions for regulating the food intake, energy storage, stress reduction, etc. (Groneberg et al. 2004; Loh et al. 2015)

3.1 CNS Diseases

(a) **Anxiety:** NPY regulates anxiety and emotional behaviour which mediates via receptor Y_1 and mainly amygdala by interacting between corticotrophin-releasing factor. Y_1 receptor antagonist or antisense like BIBP 3226 acts as an anxiogenic whereas Y_2 receptor stimulation and selective Y_1 receptor like [Leu^{31}, Pro^{34}] NPY agonist acts as an anxiolytic. Long-term NPY anxiolytic effect is inhibited by antagonist of calcineurin activity. A_2 adrenergic receptor antagonist like yohimbine, when administered intravenously, showed induction of anxiety by increasing plasma NPY as an anxiety regulator. On infusion of NPY in some brain regions like dorsal periaqueductal gray matter, lateral septum and locus coeruleus, it produces anxiolytic effect to facilitate cued and contextual fear extinction, within-session and also the retrieval of extinction. (Quirion et al. 2006; Wu et al. 2011; Sah and Geracioti 2013; Parker et al. 1998; Tasan et al. 2016)

(b) **Depression:** During depression, the NPY expression is reduced in hippocampal, cerebrospinal fluid and amygdalar but showed increase in the hypothalamus. NPY prevents amyloid-β (1–42)-induced depression-like behaviour or post-traumatic stress disorder (PTSD) releasing dopamine and glutamate acts as a neuromodulator on neurotransmitters.

(c) **Learning and memory:** For memory processing, several regions in the brain like amygdala and hippocampus are responsible to act as modulators for synaptic transmission, neuroplasticity and memory like explicit, implicit, sensory etc. NPY injection at caudal hippocampus or amygdala was found to induce amnesia while administrating into septum and rostral hippocampus was found to enhance memory retention. Depending on the type of memory, NPY exerts stimulatory or inhibitory effects like GABA, memory is enhanced by co-localising or enhancing the glutamate release in the hippocampus but glutamate release is inhibited by Y_2 receptor and prevents neurotransmission excitation, however, no activity is reported on presynaptic glutamate reduction and excitotoxic effects on injection to the thalamus, caudate/putamen, or cerebral cortex regions. (Gotzsche and Woldbye 2016)

(d) **Alcohol dependence**: NPY demonstrates an enhancement of extracellular dopamine and the brain activity regulated in extended amygdala and nucleus accumbens. (Robinson and Thiele 2017)

(e) **Stress:** Comorbid depression and PTSD are developed due to low levels of NPY but internal stress caused by visceral inflammation is affected by the cerebral NPY expression. Hence, in such cases administration of NPY externally is beneficial, especially administration into amygdala produces long-term stress resilience, protecting an individual from depression or PTSD.

(f) **Feeding behaviour:** NPY plays a role in food intake via various factors like signaling the CNS for a need of energy in hypothalamus by mediating appetite and shows orexigenic effect. Selective antagonist receptors of Y_1, Y_2 and Y_5 release stress-induced glucocorticoid and pituitary hormone secretion for inhibition of lipolysis and thermogenesis. Y_1 and Y_2 receptors play an important role in non-fasting and fasting conditions by acting on growth hormone. Suppression occurs by post-synaptic Y_1 receptor in fasting condition whereas, normal growth hormone output is maintained by the presynaptic Y_2 receptor under long-term *ad libitum*-fed conditions. (Shipp et al. 2016; Pandit et al. 2013; Huang et al. 2014)

(g) **Drug addiction:** The paraventricular direct injection of NPY helps in the withdrawal of alcohol. Drug abuse and eating disorder are caused by NPY dysfunction in the CNS, mostly nucleus accumbens and perifornical

hypothalamus. On experimentation, rats with non-consumption of alcohol showed lower NPY expression than rats with alcohol intake. Over expressive NPY mice showed less consumption of alcohol and more sedative effects whereas lack of NPY expression showed higher alcohol consumption and lower sedative effects.

(h) **Epilepsy:** NPY activates hippocampus which selectively inhibits excitatory synaptic transmission. Reduction in presynaptic terminals glutamate release is caused by activating presynaptic NPY receptors by supressing voltage-dependent Ca^{2+} currents influx and discharges epileptiform. Receptors Y_2 and Y_5 are known to regulate excitation responsible for mediating antiepileptic activity of NPY where BDNF-TrkB signaling factor inhibition and NPY system reinforcement occurs as a potential therapeutic strategy to prevent neuronal apoptosis. (Noe et al. 2008; Woldbye and Kokaia 2004; Iughetti et al. 2018; Marchese et al. 2018)

(i) **Alzheimer disorder:** It is caused by NPY depletion in CSF, cerebral cortex or hippocampus or amyloid-β peptide accumulation in neuritic plaques. [Leu^{31}, Pro^{34}]-NPY, a Y_1 receptor agonist is useful in the treatment of Alzheimer disease.

(j) **Parkinson disease:** Due to an increase in the level of NPY in striatum relates to dopaminergic loss on interneurons or decrease levels of NPY in CSF or cortical regions or NPY alteration in mRNA expression causes Parkinson's disease. NPY enhances tropic support of dopamine, modulates neurogenesis and stimulates autophagy. 6-OHDA induces toxicity and SH-SY5Y helps to prevent Parkinson by acting on dopaminergic neuroblastoma cells.

(k) **Huntington's disease:** An increased level of NPY in the striatum, frontal and temporal cortex, CSF, basal ganglia and subventricular zone causes Huntington's disease. It is treated by regulation of calcium homeostasis, increasing neurogenesis by acting on the subventricular zone and decreasing excitotoxicity.

(l) **Analgesic and hyperalgesic conditions**: Intrathecal administration of NPY causes analgesia to provide analgesic with a high sensory to motor therapeutic ratio and unaffected by opioid or *az* receptor antagonists or after thermal noxious stimuli. Y_1 receptor agonists act as a central analgesic and periaqueductal gray microinjection of [Leu^{31}, Pro^{34}]-NPY shows anti-fear effect. Hyperalgesia is a peripheral NPY dominant effect mediated by post-ganglionic sympathetic neurone. Y_1 and Y_5 receptor agonists modulate the nociception effects of NPY like [Leu^{31}, Pro^{34}] NPY but NPY_{13-36} whereas, Y_2 receptor agonists mediate an anti-nociceptive effect. (Vazquez-Leon et al. 2017).

3.2 Endocrine Disorders

(a) **Diabetes:** NPY regulates insulin secretion by disrupting hypothalamic signal pathway, moreover, reduction of NPY escalates insulin secretion. For the treatment of pancreatogenic diabetes, highly selective and long-acting Y_4 agonist was developed and useful in clinical applications. (Sun et al. 2017)

(b) **Obesity:** NPY acts as energy homeostasis and disrupts hypothalamic signal pathway plus hyperphagia associated with increased NPY signaling. NPY levels are elevated in the hypothalamus in fasted and obese rats and temporally correlated with intake of food. Inhibition of food intake and blood pressure, thermogenesis elevation, activation of lipolysis and reduction of activated gonadotrope axis affects metabolic syndrome by Y_1 antagonist. Y_1 and Y_5 receptors are down-regulators decreases exogenous NPY sensitivity in obese rats hence, a higher dose of NPY is required to induce food intake. NPY levels are affected by the composition of diet intake like before and after the obesity onset, high-fat diet intake lowers

hypothalamic NPY whereas high-fat high-sugar (choice) diet enhances hypothalamic NPY levels. A decrease in energy expenditure, body temperature and thermogenic capacity in adipose tissue are associated with the high level of NPY in the hypothalamus. (Van den heuvel et al. 2014; Zhang et al. 2011; Gumbs et al. 2016).

3.3 Cardiovascular Diseases

(a) **Cardiovascular effects:** NPY helps in neural regulation for functioning of cardiovascular conditions and glucose metabolism and found in high concentrations around the blood vessels in the nerve fibers. It acts as most potent vasoconstrictor peptides and affecting on blood pressure and cardiomyocytes, neurons and endocardium responsible for angiogenesis, vasoconstriction and cardiac remodelling. In vasoconstrictor peptides, NPY showed elevated blood pressure by Y_5 receptor on cardiomyocytes acts as a long and potent vasoconstrictor to control blood pressure by modulating sympathetic nervous activity. Y_1 receptor antagonist BRC-672 reduces the blood pressure in hypertensive rats whereas, selective Y_5 receptor agonist, [D-Trp4] NPY mimicks the NPY hypertrophic effects and treats cardiac abnormalities like hypertension, vascular endothelial cell proliferation, circadian rhythm and heart failure. NPY shows beneficial effects in arrhythmia, atherosclerosis and ischemia/infarction conditions by signal targeting calcium and potassium currents leads to cardiac excitability. In atherosclerosis, NPY levels are reduced due to disrupted hypothalamic signaling pathway as elevated NPY levels promote intake of food. The reduction in energy expenditure increases adiposity whereas NPY induces angiogenesis, proliferation and neural progenitor cells migrations. (Tan et al. 2018; Saraf et al. 2016).

3.4 Intestinal Diseases

(a) **Renal effects:** Co-localized NPY is found in the renal cortex, renal vasculature, corticomedullary interface and renal tubules. NPY is present in abundance in the human body possess some immunoreactivity but absent in the glomerulus. The direct tubular effects, mainly stimulation causes an excess of NPY and enhances natriuresis and diuresis followed by renal vasoconstriction. In hypersensitive people, the activation of the renin-angiotensin system and stimulation of the sympathetic nervous system develop high blood pressure.

(b) **Intestinal disorders:** Malabsorption disorders are treated by selective Y_2 receptors but not effective in chronic diarrhoea or intestinal bowel syndrome as NPY inhibits intestinal secretion but promotes absorption on luminal or intravenous administration. Thus, endogenous levels of NPY and PYY are considered as important stimulators to cure diarrhoea by facilitating Y receptor colonic absorption.

3.5 Respiratory Diseases

(a) **Respiratory disorders:** Local treatment of NPY shows vasoconstriction effects on mucosa by reducing mucus secretion and nasal obstruction induced by allergen on administration via intranasal route. A Y_2 receptor agonist called as TASP V influences the pre-treatment of intranasal or intrabronchial for functional response to histamine reacting on cross-section and nasal airway resistance area by reducing bronchoconstriction in chronic rhinosinusitis. In nasal mucosa, high concentrations of NPY is stored with noradrenaline in sympathetic nerve fibers. NPY Y_1 receptor mediates nitric oxide levels and reduction in blood flow in nasal mucosa of the human.

3.6 Others

(a) **Osteoblastic:** NPY expressions are found in bone-like the osteoblastic lineage in the suppression of bone formation by responding to mechanical stimuli, regulated by Y_1 receptor in progenitor commitment and bone formation. It also alters the secretion of beta cell mass and insulin modulates glucose tolerance in reducing obesity and type 2 diabetes. Hence, reduction in osteoblastic NPY production stimulates glucose tolerance, decreases adiposity and improves bone mass.

(b) **Retinal effects:** NPY acts as an accelerating factor in atherosclerosis by indirectly forming thrombus via changing vessel rheology or increase in NPY triggers high-stress levels and neointima formation and activating Y_1 and Y_5 receptor as it upregulates platelet and vascular NPY systems. It acts as a retinal neuroprotective by glutamate intravitreal injection or retinal explants and retinal synaptogenesis by modulating and developing of retinal circuit emerges as a new treatment for restenosis. NPY protects retinal cells against necrotic and apoptotic cell death due to reduction of calcium influx in retinal neurons. By activation of NPY Y_1, Y_2 and Y_5 receptors, the increase in progenitor cells proliferation and/or differentiation of neuronal retinal progenitor cells were also observed in the treatment of retinal degenerative diseases. (Li 2003)

(c) **Cancer:** NPY targets tumor cells by acting on endosomes releases cytolysin to break disulphide linkers in the cytoplasm which regulates tumor growth by induction or inhibition of apoptosis. A high density of human Y_1 receptor expressed in metastasis and breast cancer cells whereas selective Y_2 receptor analogs are effective on breast and pancreatic cancers by an increase of calcium concentration intracellularly and reduction of forskolin-induced cAMP accumulation. Expression of Y_2 and Y_5 receptors influences cancer progression to create hypoxia condition by modifying immune functions. CGP71683A, a Y_5 receptor antagonist induces apoptosis in a time-dependent manner. (Tilan and Kitlinska 2016)

(d) **Anti-aging**: NPY indicates positive effects of caloric restriction without affecting the lifespan. In hypothalamic neurons, autophagic flux is induced by NPY leading to damaged organelles and macromolecules accumulation and causing oxidant-antioxidant balance. It mediates nutrient-sensing pathways in hypothalamus possess a critical role in the development of whole-body aging. According to research, the overexpression of NPY transgenic rats lived longer whereas Y_2 receptor non-reactive mice showed a decline in cognitive function. Approximately 6 of 9 cellular hallmarks of aging is inhibited by NPY including intercellular communication alteration, proteostasis loss, exhaustion of stem cells, dysfunctioning mitochondria, nutrient sensing deregulation and cellular senescence.

(e) **Anti-inflammatory**: NPY shows anti-inflammatory action by reducing neuroinflammation. Overactivation of microglia affects CNS cells by pro-inflammatory factors due to continuous stimulus deregulates the immune response. Diverse effects of NPY are mediated by activation of different sub-types of Y receptor whereas pro- or anti-inflammatory actions are observed when the same receptor are expressed on different immune cell types, impacting the microbiota-gut-brain axis to exhibit pro-inflammatory activities in the intestine. In one experiment, NPY-HS on mouse splenocytes cell cytokine production showed a reduction in LPS-induced mouse splenocytes production of pro-inflammatory factors (TNF-α, IL-6, IFN-γ, and MCP-1) in a dose-dependent manner.

(f) **Stem cell (SC) therapy:** Several regulatory effects are exerted by NPY on stem cells due to its unique application of replacing damaged cells, modulating immunity and

promoting angiogenesis. NPY is useful in SC-based therapy by regulating effects like proliferation, cell survival and differentiation overcoming low transplanted SC integration rates. NPY acts by Y_1, Y_2 and Y_5 receptor in various body parts like it supports cell proliferation by Y_1 receptor acting as precursor cells in hippocamus, subventricular neural, olfactory neural, retinal neural, embryonic SC and adipose-derived SC. Y_2 receptors helps in differentiation and migration of cells by acting on subventricular neural, Retinal neural and mesenchymal SC whereas Y_5 helps in proliferation and self-renewal of cells via regulating retinal neural progenitor cells, mesenchymal SC and embryonic SC. On increasing NPY levels it regulates hematopoietic SC in bone marrow and helps to fight against nerve injury. NPY in SC-based therapy is found useful in treatment of various diseases like retinal degenerative diseases, myocardial infraction, osteoporosis, obesity and neurodegenerative diseases like Parkinson's, alzheimer's and multiple sclerosis. (Peng et al. 2017; Park et al. 2015; Geloso et al. 2015).

4 Toxicity

Gene of Y_2 receptors shows anti-anxiety effects and penetrates the brain whereas treating with NPY for cancer possess some unspecific toxicity. But selective antagonists of Y_2 receptor show non-significant cytotoxicity and penetrate BBB. The functions in retinal physiology are still unknown and NPY showed preventive action against increased cell death in retinal cell cultures. Y_1 receptors present in osteoblastic cells in murine studies are effective against osteogenesis and osteoclast genesis. High rates of comorbidity appear to be discrete neuropsychiatric and medical disorders results from stress exposure, such as PTSD and irritable bowel syndrome due to dysregulation of the NPY system which varies from an individual to genetic predisposition and environmental influences. Hence, the NPY system should be optimized before treating an individual. Some off-target effects occur while targeting the NPY system challenges to develop and recommend for the therapies. (Qiao et al. 2018; Rasmusson 2017).

5 Future Perspective

Various delivery systems can be formulated for the administration of NPY, its evaluation still lies as a major issue. NPY subtypes receptor clone generates a new pathway for various physiological, development and clinical usage. Yet, there are no reports on the clinical development or administration of NPY-based drugs for treatment and therapy of airways-based diseases. Further, various selective agonist, antagonist of NPY and its mechanism plus safety needs to be explored. The regulation of nervous system by NPY and its effect on different systems and organ have made significant progress though, in some areas like obesity management or metabolic disorders, its physiology and aetiology are unknown which requires a novel design and rational approach for ailment and prevention. Detailed investigation and study are required to unwind the details on functional protection, peptide interaction and influence, validation, side effects and involvement of subtypes receptors. It still remains a challenge to deal with NPY and its unknown role in the pathophysiology of several diseases and disorders like PTSD, bone disorders, etc. where performing trials like measuring NPY in body fluids like saliva, CSF, plasma may provide some significant insight. Thus, NPY can be recognised as a therapeutic target for management and therapy in several biological functions of the human body.

6 Conclusions

NPY can be cloned and developed into analogs to show pathophysiology for several conditions like anxiety, feeding disorder, cognition, seizures, depression, heart failure, depression, cancer, etc. Till date, four receptor subtypes of NPY are

cloned with their antagonists which can be used to treat various diseases like obesity, hypertension, epilepsy, etc. NPY emerges as a potential towards several fields still many applications are unknown. It arises as an attractive promising novel therapeutic target for the prevention and treatment of diseases like CVD, diabetes, PTSD, etc. Due to the homeostasis effects of the NPY receptor, it is useful as an anti-obesity agent, energy regulator and lipid metabolism. The kinetics of neuropeptide drugs with its ability to bypass the blood-brain barrier creates some novel therapeutic strategies. Combined therapies with NPY and its receptors open a new avenue for treating various diseases like diabetes, cancer, CNS disorders, etc.

Acknowledgements This study was supported by DST SERB (ECR/2017/001184). All authors declare that their funding source had no role in the writing of the report, and in the decision to submit the paper for publication.

References

Ahrens VM, Kostelnik KB, Rennert R et al (2015) A cleavable cytolysin-neuropeptide Y bioconjugate enables specific drug delivery and demonstrates intracellular mode of action. J Control Release 209:170–178

Balasubramaniam A (1997) Neuropeptide Y family of hormones: receptor subtypes and antagonists. Peptides 18(3):445–457

Balasubramaniam A (2002) Clinical potentials of neuropeptide Y family of hormones. Am J Surg 183:430–434

Baraban SC (2004) Neuropeptide Y and epilepsy: recent progress, prospect and controversies. Neuropeptides 38:261–265

Beck B (2006) Neuropeptide Y in normal eating and in genetic and dietary-induced obesity. Philos Trans R Soc Lond Ser B Biol Sci 361:1159–1185

Botelho M, Cavadas C (2015) Neuropeptide Y: an anti-aging player? Trends Neurosci 38(11):701–711

Colmers WF, El Bahh BE (2003) Neuropeptide Y and epilepsy. Epilepsy Curr 3(2):53–58

Cox HM (2007) Neuropeptide Y receptors; antisecretory control of intestinal epithelial function. Auton Neurosci 133(1):76–85

Decressac M, Barker RA (2012) Neuropeptide Y and its role in CNS disease and repair. Exp Neurol 238 (2):265–272

Duarte-Neves J, Almeida LP, Cavadas C (2016) Neuropeptide Y (NPY) as a therapeutic target for neurodegenerative diseases. Neurobiol Dis 95:210–224

Dumont Y, Martel JC, Fournier A, St-Pierre S, Quirion R (1992) Neuropeptide Y and neuropeptide Y receptor subtypes in brain and peripheral tissues. Prog Neurobiol 38(2):125–167

El Bahh BE, Balosso S, Hamilton T et al (2005) The anti-epileptic actions of neuropeptide Y in the hippocampus are mediated by Y2 and not Y5 receptors. Eur J Neurosci 22:1417–1430

Farzi A, Reichmann F, Holzar P (2015) The homeostatic role of neuropeptide Y in immune function and its impact on mood and behaviour. Acta Physiol (Oxford) 213(3):603–627

Geloso MC et al (2015) Cellular targets for neuropeptide Y-mediated control adult neurogenesis. Front Cell Neurosci 9:85

Gotzsche CR, Woldbye DP (2016) The role of NPY in learning and memory. Neuropeptides 55:79–89

Groneberg DA, Folkerts G, Peiser C (2004) Neuropeptide Y (NPY). Pulm Pharmacol Ther 17(4):173–180

Gumbs MC, van den Heuvel JK, la Fleur SE (2016) The effect of obesogenic diets on brain neuropeptide Y. Physiol Behav 162:161–173

Horsnell H, Baldock PA (2016) Osteoblastic actions of the neuropeptide Y system to regulate bone and energy homeostasis. Curr Osteoporos Rep 14(1):26–31

Huang L, Tan HY, Fogarty MJ et al (2014) Actions of NPY, and its Y1 and Y2 receptors on pulsatile growth hormone secretion during the fed and fasted state. J Neurosci 34(49):16309–16319

Iughetti L, Lucaccioni L, Fugetto F et al (2018) Brain-derived neurotrophic factor and epilepsy: a systematic review. Neuropeptides 72:23–29

Kautz M, Charney DS, Murrough JW (2017) Neuropeptide Y, resilience, and PTSD therapeutics. Neurosci Lett 649:164–169

Li L (2003) Neuropeptide Y- induced acceleration of postangioplasty occlusion of rat carotid artery. Arterioscler Thromb Vasc Biol 23(7):1204–1210

Liu W, Chen Y, Bai XW et al (2016) Identification and characterization of a novel neuropeptide (neuropeptide Y-HS) from leech salivary gland of *Haemadipsa sylvestris*. Chin J Nat Med 14(9):677–682

Loh K, Herzog H, Shi YC (2015) Regulation of energy homeostasis by the NPY system. Trends Endocrinol Metab 26(3):125–135

Malva JO, Xapelli S, Baptista S et al (2012) Multifaces of neuropeptide Y in the brain- neuroprotection, neurogenesis and neuroinflammation. Neuropeptides 46(6):299–308

Marchese E, Corvino V, Di Maria V et al (2018) The neuroprotective effects of 17-β-Estradiol Pretreatment in a model of neonatal hippocampal injury induced by trimethyltin. Front Cell Neurosci 12(385):1–18

Martin J, Elvas F, Brudzewsky D et al (2015) Activation of neuropeptide Y receptors modulates Retinal Ganglion

Cell physiology and exerts neuroprotective actions in vitro. ASN Neuro 7(4):1–21
Mittapalli GK, Roberts E (2014) Ligands of the neuropeotide Y Y2 receptor. Bioorg Med Chem Lett 24(2):430–441
Munglani R, Hudspith MJ, Hunt SP (1996) The therapeutic potential of neuropeptide Y. Analgesic, anxiolytic and antihypertensive. Drugs 52(3):371–389
Noe F, Pool AH, Nissinen J et al (2008) Neuropeptide Y gene therapy decreases chronic spontaneous seizures in a rat model of temporal lobe epilepsy. Brain 131 (Pt6):1506–1515
Pandit R, La Fleur SE, Adan RA (2013) The role of melanocortins and neuropeptide Y in food reward. Eur J Pharmacol 719(1–3):208–214
Park MH et al (2015) Neuropeptide Y regulates the hematopoietic stem cell microenvironment and prevents nerve injury in the bone marrow. EMBO J 34(12):1648–1660
Parker EM, Babij CK, Balasubramaniam A et al (1998) GR231118 (1229U91) and other analogues of the C-terminus of neuropeptide Y are potent neuropeptide Y Y_1 receptor antagonists and neuropeptide Y Y_4 receptor agonists. Eur J Pharmacol 349(1):97–105
Pedrazzini T, Pralong F, Grouzmann E (2003) Neuropeptide Y: the universal soldier. Cell Mol Life Sci 60 (2):350–377
Peng S, Zhou Y et al (2017) Effects of neuropeptide Y on stem cells and their potential applications in disease therapy. Stem Cells Int 2017:6823917
Qiao Y, Wang Y, Zhou Y et al (2018) The role of nervous system in adaptive response of bone to mechanical loading. J Cell Physiol 234(6):7771–7780
Quirion R, Dumont Y, Carvajal C (2006) Neuropeptide Y: role in emotion and alcohol dependence. CNS Neurol Disord Drug Targets 5(2):181–195
Rasmusson AN (2017) The gut peptide neuropeptide Y and post-traumatic stress disorder. Curr Opin Endocrinol Diabetes Obes 24(1):3–8
Reibel S, Nadi S, Benmaamar R et al (2001) Neuropeptide Y and epilepsy: varying effects according to seizure type and receptor activation. Peptides 22(3):529–539
Reichmann F, Holzer P (2016) Neuropeptide Y: A stressful review. Neuropeptides 55:99–109
Robinson SL, Thiele TE (2017) The role of neuropeptide Y (NPY) in alcohol and drug abuse disorders. Int Rev Neurobiol 136:177–197
Sabban EL, Alaluf LG, Serova LI (2016) Potential of neuropeptide Y for preventing or treating post-traumatic stress disorder. Neuropeptides 56:19–24
Sah R, Geracioti TD (2013) Neuropeptide Y and posttraumatic stress disorder. Mol Psychiatry 18(6):646–655
Santos-Carvalho A, Ambrosio AF, Cavadas C (2015) Neuropeptide Y system in the retina: from localization to function. Prog Retin Eye Res 47:19–37
Saraf R, Mahmood F, Amir R et al (2016) Neuropeptide Y is an angiogenic factor in cardiovascular regeneration. Eur J Pharmacol 776:64–70
Shipp SL, Cline MA, Gilbert ER (2016) Promotion of adipogenesis by neuropeptide Y during the later stages of chicken preadipocyte differentiation. Phys Rep 4 (21):1–12
Silva AP, Cavadas C, Grouzmann E (2002) Neuropeptide Y and its receptors as potential therapeutic drug targets. Clin Chim Acta 326:3–25
Sun WW, Zhu P, Shi YC et al (2017) Current views on neuropeptide Y and diabetes-related artherosclerosis. DiabVas Dis Res 14(4):277–284
Tan CMJ, Green P, Tapoulal N et al (2018) The role of neuropeptide Y in cardiovascular health and disease. Front Physiol 9(1281):1–13
Tasan RO, Verma D, Wood J et al (2016) The role of Neuropeptide Y in fear conditioning and extinction. Neuropeptides 55:111–126
Tilan J, Kitlinska J (2016) Neuropeptide Y (NPY) in tumor growth and progression: lessons learned from pediatric oncology. Neuropeptides 55:55–66
Van den Heuvel JK, Eggels L, van Rozen AJ et al (2014) Neuropeptide Y and leptin sensitivity is dependent on diet composition. J Neuroendocrinol 26(6):377–385
Vazquez-Leon P, Mendoza-Ruiz LG, Juan ER et al (2017) Analgesic and anxiolytic effects of [Leu^{31},Pro^{34}]-neuropeptide Y microinjected into the periaqueductal gray in rats. Neuropeptides 66:81–89
Woldbye DP, Kokaia M (2004) Neuropeptide Y and seizures: effects of exogenously applied ligands. Neuropeptides 38(4):253–360
Wu G, Feder A, Wegener G et al (2011) Central functions of neuropeptide Y in mood and anxiety disorders. Expert Opin Ther Targets 15(11):1317–1331
Zhang L, Bijker MS, Herzog H (2011) The neuropeptide Y system: pathophysiological and therapeutic implications in obesity and cancer. Pharmacol Ther 131(1):91–113

Adv Exp Med Biol - Cell Biology and Translational Medicine (2019) 7: 49–60
https://doi.org/10.1007/5584_2019_423
© Springer Nature Switzerland AG 2019
Published online: 3 August 2019

Rho Signaling-Directed YAP/TAZ Regulation Encourages 3D Spheroid Colony Formation and Boosts Plasticity of Parthenogenetic Stem Cells

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Abstract

Cell proliferation, apoptosis and differentiation are essential processes from the early phases of embryogenesis to adult tissue formation and maintenance. These mechanisms also play a key role in embryonic stem cells (ESCs) that are able to proliferate maintaining pluripotency and, at the same time, to give rise to all populations belonging to the three germ layers, in response to specific stimuli. ESCs are, therefore, considered a well-established in vitro model to study the complexity of these processes. In this perspective, we previously generated parthenogenetic embryonic stem cells (ParthESC), that showed many features and regulatory pathways common to bi-parental ESCs. However, we observed that mono-parental cells demonstrate a high ability to form outgrowths and generate 3D spheroid colonies, which are distinctive signs of high-plasticity. Furthermore, preliminary evidence obtained by WTA, revealed the presence of several differentially expressed genes belonging to the Rho and Hippo signaling pathways. In the present study, we compare bi-parental ESCs and ParthESC and analyze by Real-Time PCR the differentially expressed genes. We demonstrate up-regulation of the Rho signaling pathway and an increased expression of YAP and TAZ in ParthESC. We also show that YAP remains in a dephosphorylated form. This allows its nuclear translocation and its direct binding to TEADs and SMADs, that are up-regulated in ParthESC. Altogether, these complex regulatory interactions result in overexpression of pluripotency related genes, in a global DNA hypomethylation and a histone-dependent chromatin high permissive state that may account for

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ParthESC high potency, possibly related to their exclusive maternal origin.

Keywords

3D spheroid colonies · Hippo signaling pathway · Maternal · Parthenogenetic embryonic stem cells · Plasticity · Rho signaling pathway

Abbreviations

3D	three-dimensional
DNA	Deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
ESCs	Embryonic stem cells
ICMs	inner cell masses
IVF	in vitro fertilized
ParthESC	Parthenogenetic embryonic stem cells
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
WTA	Whole Transcriptome Analysis

1 Introduction

A coordinated balance and a tight control of cell growth, apoptosis and differentiation are essential for a normal and correct development, from the pre-implantation embryogenesis to the patterning of organs and tissue formation, as well as for the maintenance of homeostasis in adult body tissues. These mechanisms also play a key role in embryonic stem cells (ESCs), where, cell proliferation represents a fundamental process that guarantees long-term expansion in culture, maintains pluripotency and suppresses differentiation programs. At the same time, they control ESC potential to give rise to all populations belonging to the three germ layers, in response to specific differentiative stimuli. Because of this, ESCs are presently considered well-established in vitro model to study the complexity of these processes (Siggia and Warmflash 2018). In this perspective, we previously generated parthenogenetically activated embryos and cell lines and reported the existence of many features and regulatory pathways common between bi-parental ESCs and parthenogenetic embryonic stem cells (ParthESC), both in vitro and in vivo (Brevini et al. 2009, 2010, 2011, 2012; Pennarossa et al. 2015). On the other hand, we observed that parthenogenetic inner cell masses (ICMs) generated a significantly higher number of outgrowths than IVF ones (22.16% vs 4.82%) and formed stable three-dimensional (3D) spheroid colonies, which is a distinctive sign of high-plasticity.(Brevini et al. 2010). Furthermore, preliminary evidence obtained using the GeneChip™ Human Genome U133 Plus 2.0 Array, revealed the presence of several differentially expressed genes that may account for ParthESC distinct potency. Gene ontology and bioinformatic analysis highlighted changes in the intracellular signaling cluster, specifically involving the Rho and Hippo signaling pathways. These molecules play a key role in the preservation of self-renewal and the establishment and maintenance of pluripotency, strictly correlated to intrinsic and environmental cues, such as growth factors and cell-to-cell and cell-to-matrix interactions (Nichols and Smith 2009; Mullen 2014; Ohgushi et al. 2015). In particular, the two main effectors of the cascade, namely the Yes-associated protein (YAP) and the WW domain containing transcription regulator 1 (TAZ, also known as WWTR1), interact with specific transcription factors and elicit their functions, either maintaining pluripotency or driving cell differentiation (Fig. 1). Based on these observations, we reasoned that the differences in gene expression detected with the Whole Transcriptome Analysis (WTA) could account for the increased ability to form outgrowths/3D spheroid colonies of ParthESC and boost their plasticity.

In the present study, we compare bi-parental ESCs and ParthESC and analyze by Real-Time PCR the differentially expressed genes. We demonstrate up-regulation of the Rho signaling pathway and an increased expression of YAP and TAZ in ParthESC. Due to the increased Rho activity, we demonstrate that YAP remains in a dephosphorylated form, which is distinctive of their nuclear compartmentalization. This allows a direct interaction with the nuclear transcription

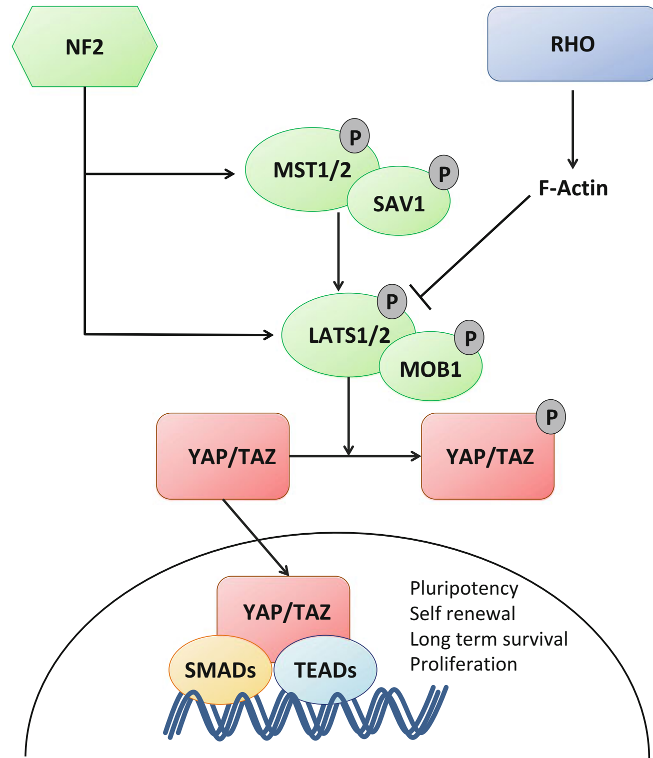

Fig. 1 Schematic representation of Rho and Hippo signaling pathways

factors TEADs and SMADs that regulate and maintain pluripotency, repress differentiation processes (Beyer et al. 2013), and that we show to be up-regulated in ParthESC. Altogether these complex regulatory interactions result in overexpression of pluripotency related genes, a global DNA hypomethylation and a histone-dependent chromatin high permissive state, boosting plasticity and increasing ability to form 3D spheroid colonies in mono-parental cell lines.

2 Methods

All chemicals were purchased from Life Technologies (Italy) unless otherwise indicated.

2.1 Ethics Statement

All the methods described in our study were approved by the Ethical Committee of the University of Milan and carried out in accordance with the approved guidelines. Oocytes were collected from adult patients (age range 32–39 years) after written informed consent, at the Infertility Unit of the Department of Obstetrics and Gynecology of the Ospedale Maggiore Policlinico Mangiagalli e Regina Elena (Milan, Italy).

2.2 Oocyte Activation, ParthESC Line Derivation and Culture

Human metaphase II oocytes were parthenogenetically activated and *in vitro* cultured up to the early blastocyst stage as previously described (Paffoni et al. 2007). ICMs were microsurgically removed from blastocysts and singly plated on freshly inactivated STO fibroblast feeder layers. ParthESC were passaged and culture as previously described (Brevini et al. 2009, 2010).

2.3 Gene Expression Analysis

RNA was extracted with the TaqManGene Expression Cells to Ct kit (Applied Biosystems), and DNase I was added in lysis solution at 1:100 concentration, as indicated by the manufacturer's instructions. Quantitative PCR was performed on a CFX96 Real-Time PCR detection system (Bio-Rad Laboratories) using predesigned gene-specific primers and probe sets from TaqManGene Expression Assays, listed in Table 1. GAPDH and ACTB were used as internal reference genes. CFX Manager software (Bio-Rad Laboratories) was used for target gene quantification. Human ESC lines, namely HUES13, HES 7, HES I-3 and HES I-6, were used as bi-parental counterpart. Gene expression levels are reported with the highest value set to 1 and the other relative to this.

2.4 ELISA Assays

Cell lysates obtained from bi-parental ESCs and ParthESC were analyzed using PathScan® Total YAP (Cell Signaling) and PathScan® Phospho-YAP (Ser127) Sandwich ELISA Kit (Cell Signaling) following the manufacturer's instructions. YAP levels are reported with the highest value set to 1 and the other relative to this.

2.5 Global DNA Methylation Analysis

PureLink® Genomic DNA Kits was used according to the manufacturer's instructions. Briefly, DNA was extracted and converted to single-stranded DNA by incubation at 95 °C for 5 min, followed by rapid chilling on ice. Samples were incubated with nuclease P1 for 2 h at 37 °C in 20 mM sodium acetate (pH 5.2) and, subsequently, with alkaline phosphatase for 1 h at 37 °C in 100 mM Tris (pH 7.5). After centrifugation, the supernatant was used for ELISA assay using Global DNA Methylation ELISA Kit (5′-methyl-2′-deoxycytidine Quantitation, CELL BIOLABS) according to the manufacturer's protocol. Global DNA methylation levels are reported with the highest value set to 1 and the other relative to this.

2.6 Statistical Analysis

All experiments were performed in triplicate. Data were presented as mean ± standard deviation (SD) of three independent experiments with three independent replicates. Statistical analysis was performed using Student t-test (SPSS 19.1; IBM). Differences of $p \leq 0.05$ were considered significant and were indicated with different superscripts.

3 Results

3.1 Up-Regulation of Rho Signaling Pathway in ParthESC

Expression analysis showed up-regulation of Rho GTPase family genes in ParthESC compared to bi-parental ESCs (Fig. 2a). In particular, RHOA, RHOB, and RHOC transcription levels were significantly higher in mono-parental cell lines, compared to their bi-parental counterpart. Consistent with these changes, the majority of Rho-GEF (guanine nucleotide-exchange factors) activators (12 out of 17) were significantly increased in ParthESC (Fig. 2b). MCF2 gene displayed no differences between mono- and bi-parental cell lines, while ARHGEF3, ARHGEF12, FARP1, and NET1 were down-regulated (Fig. 2b). This was also paralleled by a significant down-regulation of 8 out of 11 GAP (GTPase-activating protein) inhibitors (Fig. 2c), while ARHGAP18 and BRC did not show statistical differences between the two cell types and ARHGAP9 displayed higher expression levels in ParthESC compared to bi-parental ESCs (Fig. 2c).

Table 1 List of primers used for quantitative PCR analysis of human cells

Gene	Description	Catalog no.
ABR	Active BCR-related	Hs01077820_m1
ACTB	Actin,beta	Hs01060665_g1
AKAP13	A-kinase anchoring protein 13	Hs00180747_m1
ARAP1	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 1	Hs00362929_m1
ARAP3	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 3	Hs01078396_m1
ARHGAP9	Rho GTPase activating protein 9	Hs01037131_g1
ARHGAP10	Rho GTPase activating protein 10	Hs00226305_m1
ARHGAP11	Rho GTPase activating protein 11	Hs00207575_m1
ARHGAP18	Rho GTPase activating protein 18	Hs00364379_m1
ARHGAP22	Rho GTPase activating protein 22	Hs01098342_m1
ARHGAP28	Rho GTPase activating protein 28	Hs00229108_m1
ARHGAP29	Rho GTPase activating protein 29	Hs00191351_m1
ARHGEF1	Rho guanine nucleotide exchange factor 1	Hs00180327_m1
ARHGEF2	Rho/Rac guanine nucleotide exchange factor 2	Hs01064532_m1
ARHGEF3	Rho guanine nucleotide exchange factor 3	Hs00989814_m1
ARHGEF5	Rho guanine nucleotide exchange factor 5	Hs01026609_m1
ARHGEF11	Rho guanine nucleotide exchange factor 11	Hs01121959_m1
ARHGEF12	Rho guanine nucleotide exchange factor 12	Hs00209661_m1
BCR	BCR, RhoGEF and GTPase activating protein	Hs01036532_m1
DLC1	DLC1 rho GTPase activating protein	Hs00183436_m1
DNMT1	DNA (cytosine-5-)-methyltransferase 1	Hs00945875_m1
DNMT3	DNA (cytosine-5-)-methyltransferase 3	Hs00171876_m1
FARP1	FERM, ARH/RhoGEF and pleckstrin domain protein 1	Hs00195010_m1
FARP2	FERM, ARH/RhoGEF and pleckstrin domain protein 2	Hs00919572_m1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Hs02786624_g1
HAT1	Histone acetyltransferase 1	Hs00186320_m1
KAT7	Lysine acetyltransferase 7	Hs01561251_g1
HDAC1	Histone deacetylase 1	Hs02621185_s1
HDAC2	Histone deacetylase 2	Hs00231032_m1
HDAC3	Histone deacetylase 3	Hs00187320_m1
HDAC4	Histone deacetylase 4	Hs01041648_m1
HDAC5	Histone deacetylase 5	Hs00608351_m1
HDAC6	Histone deacetylase 6	Hs00997427_m1
HDAC7	Histone deacetylase 7	Hs01045864_m1
HDAC8	Histone deacetylase 8	Hs00954353_g1
HDAC9	Histone deacetylase 9	Hs01081558_m1
TERT	Telomerase reverse transcriptase	Hs00972650_m1
LATS1	Large tumor suppressor kinase 1	Hs01125524_m1
LATS2	Large tumor suppressor kinase 2	Hs01059009_m1
MCF2	MCF.2 cell line derived transforming sequence	Hs00930593_m1
MCF2L	MCF.2 cell line derived transforming sequence like	Hs00389845_m1
MOB1	MOB Kinase Activator 1	Hs00964416_m1
KAT6B	Lysine acetyltransferase 6B	Hs00202463_m1
MST1	Macrophage stimulating 1	Hs00360684_m1
STK3	Serine/threonine kinase 3	Hs01120604_m1
NANOG	Nanog homeobox	Hs02387400_g1
NET1	Neuroepithelial cell transforming 1	Hs01087884_m1
NF2	Neurofibromin 2	Hs00966302_m1
OCT4	POU Class 5 Homeobox 1	Hs04260367_gH

(continued)

Table 1 (continued)

Gene	Description	Catalog no.
REX1	ZFP42 zinc finger protein	Hs01938187_s1
RHOA	Ras homolog family member A	Hs00357608_m1
RHOB	Ras homolog family member B	Hs05051455_s1
RHOC	Ras homolog family member C	Hs00237129_m1
SAV1	Salvador family WW domain containing protein 1	Hs00560416_m1
SMAD2	SMAD family member 2	Hs00998187_m1
SMAD3	SMAD family member 3	Hs00969210_m1
SOX2	SRY-box 2	Hs01053049_s1
TAZ	WW domain containing transcription regulator 1	Hs00210007_m1
TEAD1	TEA domain transcription factor 1	Hs00173359_m1
TEAD3	TEA domain transcription factor 3	Hs00243231_m1
TEAD4	TEA domain transcription factor 4	Hs01125032_m1
TRIO	Trio rho guanine nucleotide exchange factor	Hs01125865_m1
UTF1	Undifferentiated embryonic cell transcription factor 1	Hs00864535_s1
VAV1	Vav guanine nucleotide exchange factor 1	Hs01041613_m1
VAV2	Vav guanine nucleotide exchange factor 2	Hs00610104_m1
VAV3	Vav guanine nucleotide exchange factor 3	Hs00916818_m1
YAP	Yes associated protein	Hs00902712_g1

3.2 Characterization of the Hippo Pathway in ParthESC

ParthESC displayed significantly higher expression levels of YAP and TAZ compared to bi-parental ESCs (Fig. 3a). In contrast, the analysis of the upstream genes involved in the Hippo signaling pathway revealed no differences between the two cell types. In particular, LATS 1/2, MOB1, MST1/2, NF2, and SAV1 expression levels were comparable in bi- and mono-parental lines (Fig. 3c). In agreement with these observations, a significantly higher total YAP protein content was detected in ParthESC, while the quantity of the phosphorylated form (Phospho-YAP-Ser127) is comparable in the two cell types (Fig. 3b).

Interestingly, the expression levels of the downstream nuclear co-activator genes, namely TEAD1/3/4 (Fig. 3d) and SMAD2/3 (Fig. 3e), were significantly higher in ParthESC, compared to their bi-parental counterparts.

3.3 YAP/TAZ-Boosted Plasticity in ParthESC

3.3.1 Up-regulation of pluripotency-related genes in ParthESC

Pluripotency gene expression analysis demonstrated the up-regulation of OCT4, NANOG, REX1, SOX2, UTF1, and TERT in ParthESC compared to bi-parental ESCs (Fig. 4a).

3.3.2 Global DNA Hypomethylation in ParthESC

Real-time PCR revealed a significant down-regulation of DNMT1 and DNMT3 (Fig. 4b) in ParthESC. This was accompanied by a significantly lower levels of global DNA methylation in parthenogenetic cell lines (Fig. 4c).

3.3.3 Histone-Dependent Chromatin High Permissive State in ParthESC

Mono-parental cells showed a significant decrease in transcription levels for 6 out of 9 HDAC genes (Fig. 4d). In contrast, HDAC7

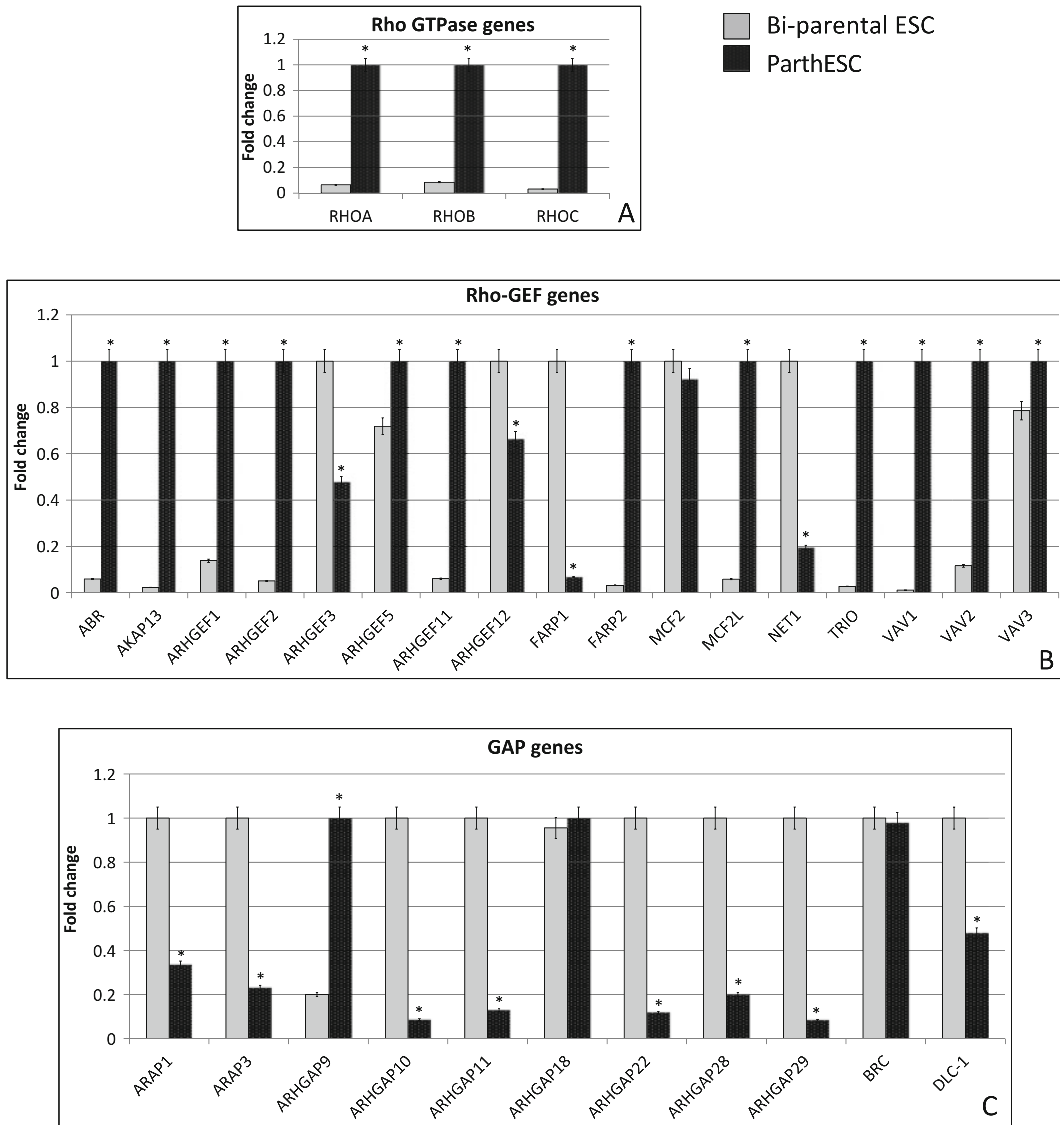

Fig. 2 Expression of the Rho signaling pathway-related molecules in ParthESC and bi-parental ESCs. (**a**) ParthESC display significantly higher expression levels of RHOA, RHOB, and RHOC compared to bi-parental counterpart. (**b**) Twelve out of 17 Rho-GEF activators are significantly up-regulated in ParthESC, while MCF2 gene displays no differences between the two cell types and 4 genes, namely ARHGEF3, ARHGEF12, FARP1, and NET1 are down-regulated. (**c**) Eight out of 11 GAP inhibitors are significantly down-regulated in ParthESC. ARHGAP18 and BRC do not show statistical differences and only ARHGAP9 is up-regulated. For each gene, the highest expression level is set to 1 and the other relative to this. Superscripts denote significant differences ($P < 0.05$)

was significantly higher, while HDAC3 and HDAC5 showed comparable levels in ParthESC and ESCs (Fig. 4d). Moreover, acetylating enzyme histone acetyltransferase 1 (HAT1) and the two lysine acetyltransferases KAT6B and KAT7 were up-regulated in ParthESC (Fig. 4e).

4 Discussion

In the present study we compare bi-parental and parthenogenetic ESCs, investigate the mechanisms allowing ParthESC increased ability to form outgrowths and generate 3D colonies,

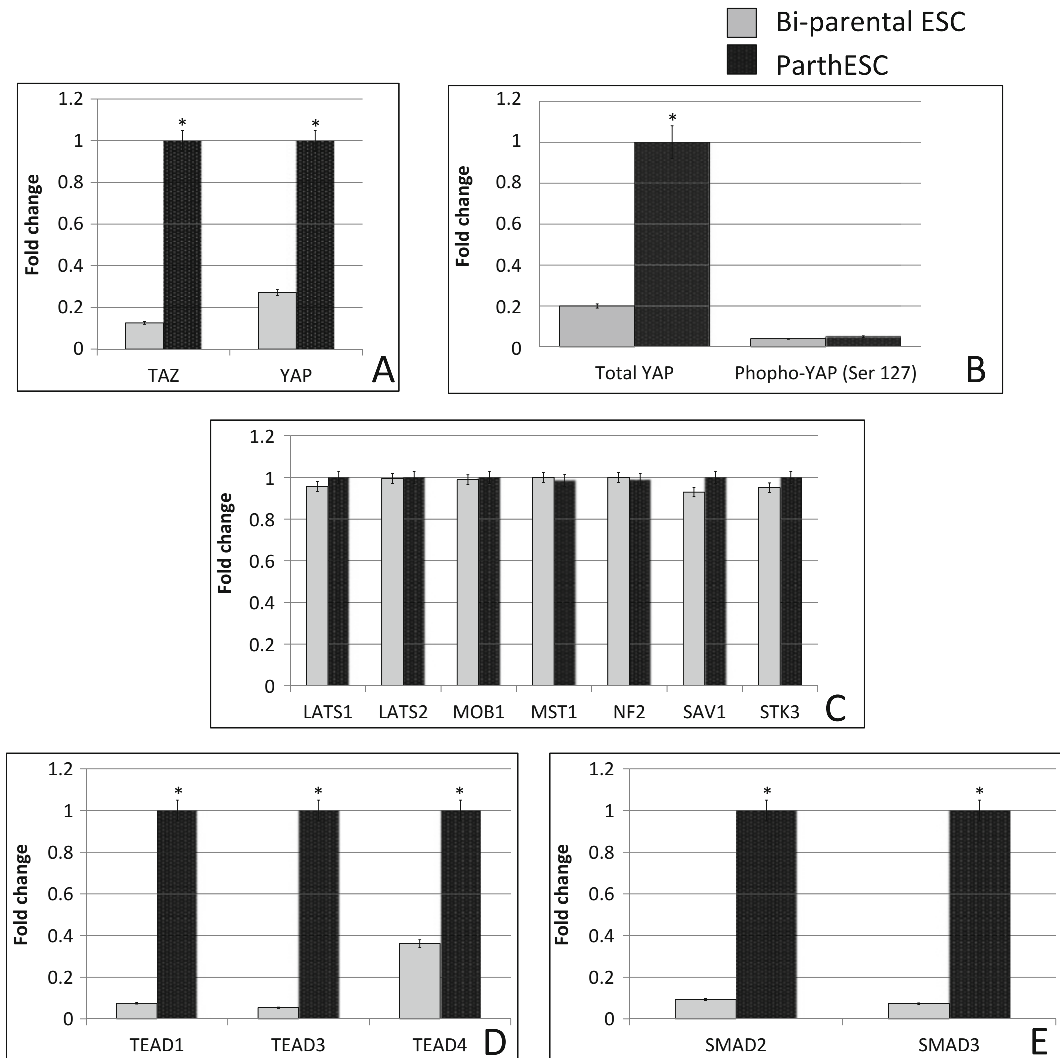

Fig. 3 Characterization of the Hippo pathway in ParthESC and bi-parental ESCs. (**a**) ParthESC display significantly higher expression levels of YAP and TAZ compared to bi-parental ESCs. (**b**) Similarly, total YAP protein content is significantly higher in ParthESC, while the quantity of the phosphorylated form (Phospho-YAP-Ser127) is comparable in the two cell types. YAP levels are reported with the highest value set to 1 and the other relative to this. Superscripts denote significant differences ($P < 0.05$). (**c**) The Hippo signaling pathway genes, namely LATS 1/2, MOB1, MST1/2, NF2, and SAV1 display comparable transcription levels in bi- and mono-parental cell lines. (**d**) TEAD1/3/4 expression levels are significantly higher in ParthESC. Also SMAD2/3 transcription is significantly up-regulated in ParthESC (**e**). For each gene, the highest expression level is set to 1 and the other relative to this. Superscripts denote significant differences ($P < 0.05$)

which directly correlates to a high plasticity state, and characterize the key signaling pathway controlling and boosting pluripotency in mono-parental cell lines. In particular, both WTA and gene expression studies demonstrated that the levels of the Rho GTPase family components, namely RHOA, RHOB, and RHOC, were significantly higher in ParthESC 3D spheroid colonies compared to bi-parental ESCs (Fig. 2a). These results are consistent with previous studies by Ohgushi et al. (Ohgushi et al. 2015) demonstrating that Rho is able to exert its activity

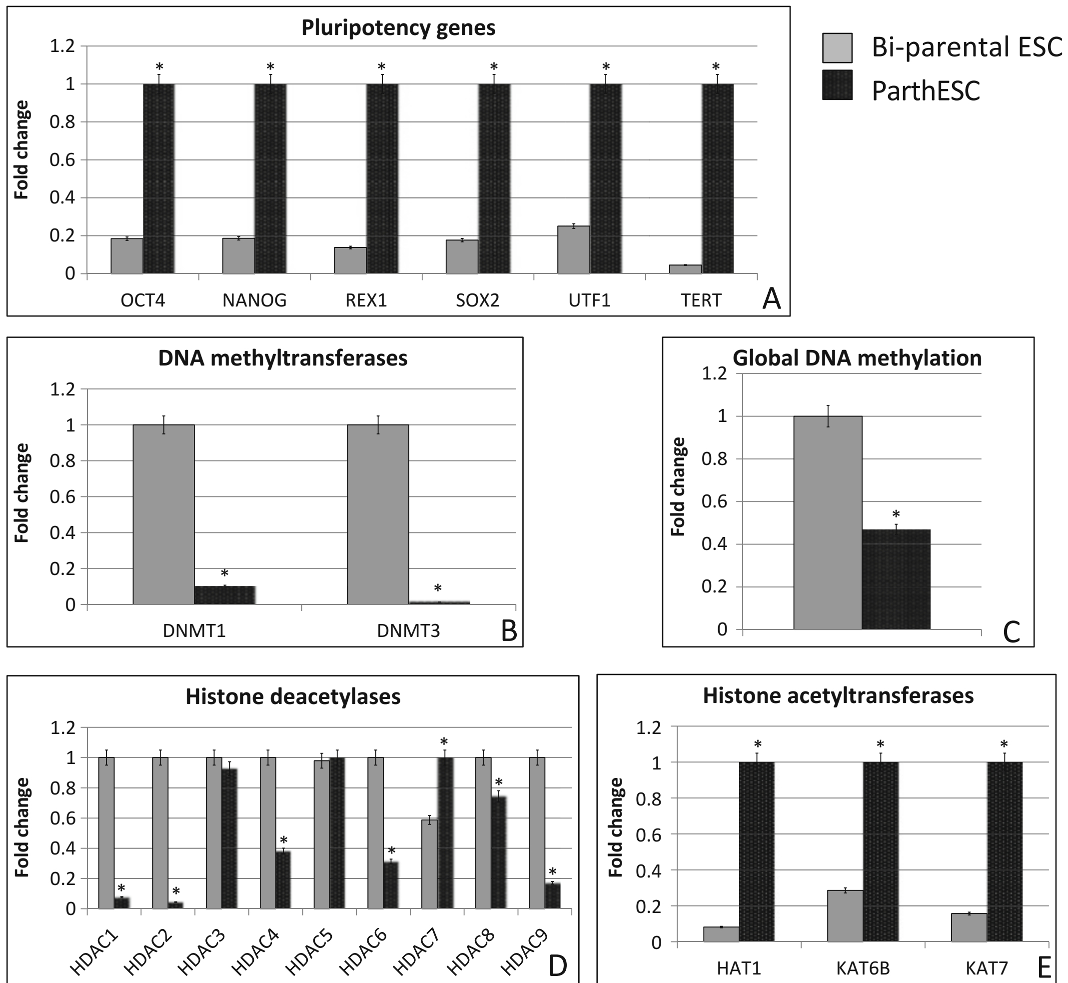

Fig. 4 Expression of pluripotency-related genes, global DNA methylation and transcription of histone acetylating/deacetylating molecules in ParthESC and bi-parental ESCs. (**a**) ParthESC display significantly higher expression levels of the pluripotency-related genes, OCT4, NANOG, REX1, SOX2, UTF1, and TERT, compared to ESCs. (**b**) DNMT1 and DNMT3 show significantly lower transcription in ParthESC. (**c**) Global DNA methylation levels are significantly lower in ParthESC compared to bi-parental ESCs. Highest expression set to 1 and the other relative to this. Superscripts denote significant differences ($P < 0.05$). (**d**) Mono-parental cells show a significantly lower transcription level for 6 out of 9 HDAC genes, while displaying a higher transcription for HDAC7. HDAC3 and HDAC5 show comparable levels in ParthESC and bi-parental ESCs. (**e**) HAT1 and the two lysine acetyltransferases KAT6B and KAT7 are up-regulated in ParthESC. For each gene, the highest expression level is set to 1 and the other relative to this. Superscripts denote significant differences ($P < 0.05$)

through two different independent mechanisms. Indeed, in 3D clumping cells or in the presence of connecting neighbor cells, Rho pathway is a pro-survival signal essential for ESC self-renewal. On the other hand, in dissociated single-cell, Rho plays a pro-apoptotic role. Therefore, the high expression levels of Rho genes detected in 3D spheroid colonies of ParthESC may represent one of the possible explanation for the elevated self-renewal ability displayed by mono-parental cell lines (Brevini et al. 2010).

The data obtained in our study by Real-time PCR also showed changes in the expression levels of the main Rho modulators, namely inhibitors and activators, suggesting a multi-step control acting on the Rho signaling cascade. In particular,

ParthESC displayed a down-regulation of 8 out of 11 GAP inhibitors (Fig. 2c) and a higher transcription level for the majority (12/17) of the GEF activators (Fig. 2b), when compared to their bi-parental counterpart. These complex interactions is coherent with the up-regulation of RHOA, RHOB, and RHOC we detected in mono-parental cell lines. It is also interesting to note that AKAP13 gene, which is one of the activator significantly up-regulated in ParthESC, has been previously described to be responsible for the survival-supporting activity played by Rho in ESCs *in vitro* (Ohgushi et al. 2015) and could have a similar role in mono-parental ones.

Comparison of ParthESC to bi-parental ESCs revealed a significantly higher expression level of YAP and TAZ in the former (Fig. 3a). In contrast, YAP/TAZ upstream genes (LATS 1/2, MOB1, MST1/2, NF2, and SAV1) showed no differences between the two cell types (Fig. 3c). This suggests that the Rho signaling pathway may regulate YAP/TAZ behavior, and, more in general, the Hippo pathway, via a LATS/MST/NF2-independent process in ParthESC. Similar mechanisms have been described in high plasticity cells (Sansores-Garcia et al. 2011; Zhao et al. 2012; Aragona et al. 2013; Johnson and Halder 2014) as well as in oocytes and embryos at early developmental stages (Cockburn et al. 2013; Posfai and Rossant 2016). The exact interactions are however still not known and further investigations are needed in order to clarify this aspect. In our study we detect an increase of the total YAP protein content but a low quantity of its phosphorylated form in mono-parental cell lines (Fig. 3b). This is strictly correlated with the up-regulation of Rho genes detected in ParthESC, since these molecules stimulate the assembly of contractile actin stress fibers that, in turn, inhibit LATS1/2 phosphorylation/activation, preventing YAP/TAZ subsequent phosphorylation (Yu et al. 2012; Miller et al. 2012; Yu and Guan 2013). Consistent with these results, several studies previously demonstrated an actin cytoskeleton-dependent Hippo-YAP regulatory mechanism tightly controlled by Rho (Sit and Manser 2011; Yu and Guan 2013; Low et al. 2014; Meng et al. 2016; Panciera et al. 2017; Seo and Kim 2018). These reports demonstrated Rho ability to strongly enhance YAP/TAZ activity in their dephosphorylated form and to induce their nuclear compartmentalization (Dupont et al. 2011; Zhao et al. 2012; Seo and Kim 2018). Once achieved a nuclear localization, the two factors are able to directly interact with SMAD2/3, forming a YAP/TAZ-SMAD2/3 complex. This newly formed complex binds to TEAD transcription factors as well as OCT4, induces pluripotency-related gene transcription, buffering pluripotency and repressing differentiation processes (Beyer et al. 2013; Mullen 2014) (Fig. 1). In line with this, it has been demonstrated that YAP/TAZ knockout results in loss of pluripotency and induction of differentiation (Varelas et al. 2008; Lian et al. 2010; Beyer et al. 2013; Varelas 2014; Hansen et al. 2015). Our data demonstrate significantly higher expression levels of TEAD1/3/4 (Fig. 3d) and SMAD2/3 (Fig. 3e) in ParthESC. This is accompanied by a significant upregulation of the pluripotency-related genes, OCT4, NANOG, REX1, SOX2, UTF1, and TERT (Fig. 4a) that boosts plasticity of mono-parental cells. These results are supported by a strong downregulation of DNMT1 and DNMT3 (Fig. 4b), that have been previously shown to modulate DNA methylation and chromatin modifications (Jin et al. 2012; Jeltsch et al. 2018), leading to a hypomethylated state in mono-parental cells compared to bi-parental ones (Fig. 4c). ParthESC increased high plasticity was also confirmed by up-regulation of the acetyltransferases, HAT1, KAT6B, and KAT7 (Fig. 4e) and the down-regulation of the majority (6/9) of the HDACs (Fig. 4d).

Altogether, our results demonstrate a significant up-regulation of Rho signaling pathway and YAP/TAZ activity in mono-parental cell lines. This may account for their higher ability to form outgrowths, generate 3D spheroid colonies and increase high plasticity, when compared to bi-parental counterpart. It is interesting to note that both Rho and Yap mRNAs are supplied, and expressed by the oocyte and maternally inherited (Clayton et al. 1999; Kawagishi et al. 2004; Zhang et al. 2014; Yu et al. 2016; Menchero et al. 2017). Although further clarifications are needed, we

hypothesize that their higher expression in ParthESC may be related to the strictly maternal origin of these cells.

Acknowledgments This work was funded by Carraresi Foundation. The Laboratory of Biomedical Embryology is member of the COST Action CA16119 In vitro 3-D total cell guidance and fitness (CellFit) and the COST Action CA17103 Delivery of Antisense RNA Therapeutics (DARTER).

Conflict of Interest The authors declare no conflict of interest in relation to this article.

References

Aragona M, Panciera T, Manfrin A et al (2013) A mechanical checkpoint controls multicellular growth through YAP/TAZ regulation by actin-processing factors. Cell 154:1047–1059. https://doi.org/10.1016/j.cell.2013.07.042

Beyer TA, Weiss A, Khomchuk Y et al (2013) Switch enhancers interpret TGF-beta and Hippo signaling to control cell fate in human embryonic stem cells. Cell Rep 5:1611–1624. https://doi.org/10.1016/j.celrep.2013.11.021

Brevini TAL, Pennarossa G, Antonini S et al (2009) Cell lines derived from human parthenogenetic embryos can display aberrant centriole distribution and altered expression levels of mitotic spindle check-point transcripts. Stem Cell Rev Rep 5. https://doi.org/10.1007/s12015-009-9086-9

Brevini TAL, Pennarossa G, Attanasio L et al (2010) Culture conditions and signalling networks promoting the establishment of cell lines from parthenogenetic and biparental pig embryos. Stem Cell Rev Rep 6. https://doi.org/10.1007/s12015-010-9153-2

Brevini TAL, Pennarossa G, de eguileor M et al (2011) Parthenogenetic cell lines: an unstable equilibrium between pluripotency and malignant transformation. Curr Pharm Biotechnol 12

Brevini TAL, Pennarossa G, Maffei S et al (2012) Centrosome amplification and chromosomal instability in human and animal parthenogenetic cell lines. Stem Cell Rev Rep 8. https://doi.org/10.1007/s12015-012-9379-2

Clayton L, Hall A, Johnson MH (1999) A role for rho-like GTPases in the polarisation of mouse eight-cell blastomeres. Dev Biol 205:322–331. https://doi.org/10.1006/dbio.1998.9117

Cockburn K, Biechele S, Garner J, Rossant J (2013) The hippo pathway member Nf2 is required for inner cell mass specification. Curr Biol 23:1195–1201

Dupont S, Morsut L, Aragona M et al (2011) Role of YAP/TAZ in mechanotransduction. Nature 474:179–183. https://doi.org/10.1038/nature10137

Hansen CG, Moroishi T, Guan K-L (2015) YAP and TAZ: a nexus for Hippo signaling and beyond. Trends Cell Biol 25:499–513. https://doi.org/10.1016/J.TCB.2015.05.002

Jeltsch A, Broche J, Bashtrykov P (2018) Molecular processes connecting DNA methylation patterns with DNA Methyltransferases and histone modifications in mammalian genomes. Genes (Basel) 9. https://doi.org/10.3390/genes9110566

Jin B, Ernst J, Tiedemann RL et al (2012) Cell reports resource linking DNA Methyltransferases to epigenetic marks and nucleosome structure genome-wide in human tumor cells. Cell Rep 2:1411–1424. https://doi.org/10.1016/j.celrep.2012.10.017

Johnson R, Halder G (2014) The two faces of hippo: targeting the hippo pathway for regenerative medicine and cancer treatment. Nat Rev Drug Discov 13:63. https://doi.org/10.1038/NRD4161

Kawagishi R, Tahara M, Sawada K et al (2004) Rho-kinase is involved in mouse blastocyst cavity formation. Biochem Biophys Res Commun 319:643–648. https://doi.org/10.1016/j.bbrc.2004.05.040

Lian I, Kim J, Okazawa H et al (2010) The role of YAP transcription coactivator in regulating stem cell self-renewal and differentiation. Genes Dev 24:1106–1118. https://doi.org/10.1101/gad.1903310

Low BC, Pan CQ, Shivashankar GV et al (2014) YAP/TAZ as mechanosensors and mechanotransducers in regulating organ size and tumor growth. FEBS Lett 588:2663–2670. https://doi.org/10.1016/j.febslet.2014.04.012

Menchero S, Rayon T, Andreu MJ, Manzanares M (2017) Signaling pathways in mammalian preimplantation development: linking cellular phenotypes to lineage decisions. Dev Dyn 246:245–261. https://doi.org/10.1002/dvdy.24471

Meng Z, Moroishi T, Guan K-L (2016) Mechanisms of Hippo pathway regulation. Genes Dev 30(1). https://doi.org/10.1101/GAD.274027.115

Miller E, Yang J, DeRan M et al (2012) Identification of serum-derived sphingosine-1-phosphate as a small molecule regulator of YAP. Chem Biol 19:955–962. https://doi.org/10.1016/j.chembiol.2012.07.005

Mullen AC (2014) Hippo tips the TGF-β scale in favor of pluripotency. Cell Stem Cell 14:6–8. https://doi.org/10.1016/j.stem.2013.12.009

Nichols J, Smith A (2009) Naive and primed pluripotent states. Cell Stem Cell 4:487–492. https://doi.org/10.1016/j.stem.2009.05.015

Ohgushi M, Minaguchi M, Sasai Y (2015) Rho-signaling-directed YAP/TAZ activity underlies the long-term survival and expansion of human embryonic stem cells. Cell Stem Cell 17:448–461. https://doi.org/10.1016/j.stem.2015.07.009

Paffoni A, Brevini TA, Somigliana E et al (2007) In vitro development of human oocytes after parthenogenetic activation or intracytoplasmic sperm injection. Fertil Steril 87:77–82

Panciera T, Azzolin L, Cordenonsi M, Piccolo S (2017) Mechanobiology of YAP and TAZ in physiology and disease. Nat Rev Mol Cell Biol 18:758–770. https://doi.org/10.1038/nrm.2017.87

Pennarossa G, Maffei S, Tettamanti G et al (2015) Intercellular bridges are essential for human parthenogenetic cell survival. Mech Dev 136. https://doi.org/10.1016/j.mod.2015.02.004

Posfai E, Rossant J (2016) Depending on maternal Yap. Cell Res 26:393–394. https://doi.org/10.1038/cr.2016.37

Sansores-Garcia L, Bossuyt W, Wada K et al (2011) Modulating F-actin organization induces organ growth by affecting the Hippo pathway. EMBO J 30:2325–2335. https://doi.org/10.1038/emboj.2011.157

Seo J, Kim J (2018) Regulation of hippo signaling by actin remodeling. BMB Rep 51:151–156. https://doi.org/10.5483/bmbrep.2018.51.3.012

Siggia ED, Warmflash A (2018) Modeling mammalian gastrulation with embryonic stem cells. Curr Top Dev Biol 129:1–23. https://doi.org/10.1016/bs.ctdb.2018.03.001

Sit S-T, Manser E (2011) Rho GTPases and their role in organizing the actin cytoskeleton. J Cell Sci 124:679–683. https://doi.org/10.1242/jcs.064964

Varelas X (2014) The Hippo pathway effectors TAZ and YAP in development, homeostasis and disease. Development 141:1614–1626. https://doi.org/10.1242/dev.102376

Varelas X, Sakuma R, Samavarchi-Tehrani P et al (2008) TAZ controls Smad nucleocytoplasmic shuttling and regulates human embryonic stem-cell self-renewal. Nat Cell Biol 10:837–848. https://doi.org/10.1038/ncb1748

Yu F-X, Guan K-L (2013) The hippo pathway: regulators and regulations. Genes Dev 27:355–371. https://doi.org/10.1101/gad.210773.112

Yu FX, Zhao B, Panupinthu N et al (2012) Regulation of the Hippo-YAP pathway by G-protein-coupled receptor signaling. Cell 150:780–791. https://doi.org/10.1016/j.cell.2012.06.037

Yu C, Ji S-Y, Dang Y-J et al (2016) Oocyte-expressed yes-associated protein is a key activator of the early zygotic genome in mouse. Cell Res 26:275–287. https://doi.org/10.1038/cr.2016.20

Zhang Y, Duan X, Cao R et al (2014) Small GTPase RhoA regulates cytoskeleton dynamics during porcine oocyte maturation and early embryo development. Cell Cycle. https://doi.org/10.4161/15384101.2014.952967

Zhao B, Li L, Wang L et al (2012) Cell detachment activates the Hippo pathway via cytoskeleton reorganization to induce anoikis. Genes Dev 26:54–68. https://doi.org/10.1101/gad.173435.111

Adv Exp Med Biol - Cell Biology and Translational Medicine (2019) 7: 61–74
https://doi.org/10.1007/5584_2019_416
© Springer Nature Switzerland AG 2019
Published online: 14 July 2019

Transamniotic Stem Cell Therapy

Stefanie P. Lazow and Dario O. Fauza

Abstract

Transamniotic stem cell therapy (TRASCET) is a novel prenatal therapeutic alternative for the treatment of congenital anomalies. It is based upon the principle of augmenting the pre-existing biological role of select populations of fetal stem cells for targeted therapeutic benefit. For example, amniotic fluid-derived mesenchymal stem cells (afMSCs) play an integral role in fetal tissue repair, validating the use of afMSCs in regenerative strategies. The simple intra-amniotic delivery of these cells in expanded numbers via TRASCET has been shown to promote the repair of and/or significantly ameliorate the effects associated with major congenital anomalies such as neural tube and abdominal wall defects. For example, TRASCET can induce partial or complete coverage of experimental spina bifida through the formation of a host-derived rudimentary neoskin, thus protecting the spinal cord from further damage secondary to amniotic fluid exposure. Furthermore, TRASCET can significantly reduce the bowel inflammation associated with gastroschisis, a common major abdominal wall defect. After intra-amniotic injection, donor stem cells home to the placenta and the fetal bone marrow in the spina bifida model, suggesting a role for hematogenous cell routing rather than direct defect seeding. Therefore, the expansion of TRASCET to congenital diseases without amniotic fluid exposure, such as congenital diaphragmatic hernia, as well as to maternal diseases, is currently under investigation in this emerging and evolving field of fetal stem cell therapy.

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Keywords

Transamniotic stem cell therapy · TRASCET · Amniotic mesenchymal stem cell · Amniotic stem cell · Amniotic neural stem cell · Fetal stem cell · Fetal cell therapy

Abbreviations

afMSC	amniotic fluid-derived mesenchymal stem cell
afNSC	amniotic fluid-derived neural stem cell
CD45	cluster of differentiation 45
CDH	congenital diaphragmatic hernia
CVS	chorionic villus sampling
Ece	endothelin converting enzyme
ECMO	extracorporeal membrane oxygenation
Egf	epidermal growth factor
Enos	endothelial nitric oxide synthase
Er-a	endothelin receptor-a
Er-b	endothelin receptor-b
FDA	Food and Drug Administration

Fgf-2	fibroblast growth factor-2
Fgf-10	fibroblast growth factor-10
H&E	hematoxin and eosin
HSC	hematopoietic stem cell
MOMS	management of myelomeningocele study
MRI	magnetic resonance imaging
MSC	mesenchymal stem cell
NSC	neural stem cell
NTD	Neural tube defect
PCR	polymerase chain reaction
pMSC	placental-derived mesenchymal stem cell
pPet-1	pre-Proendothelin-1
qRT-PCR	quantitative real time reverse transcription polymerase chain reaction
SPC	surfactant protein-C
Tgfb-1	transforming growth factor-b-1
TRASCET	transamniotic stem cell therapy
Vegf-a	vascular endothelial growth factor-a

1 Introduction

Transamniotic stem cell therapy (TRASCET) is a novel therapeutic paradigm for the treatment of a potentially wide range of congenital birth defects, first reported experimentally in 2014 (Fauza 2018; Dionigi et al. 2015a) It has not yet been translated to the bedside as of this writing, though the first clinical trial is already under review by the Food and Drug Administration (FDA). TRASCET's biological basis is centered on taking advantage of the natural role played by select populations of fetal stem cells, such as those occurring in the amniotic fluid or placenta in both normal and disease states. It involves simple intra-amniotic injections of large concentrations of stem cells to augment their normal biological activity for therapeutic gain, at minimal risk to the mother and fetus. The appeal and practicality of this strategy is self-evident. Despite the similar promise of a variety of new experimental cell-based therapies, most have yet to have widespread impact on patient care. The exceptions have been therapies that enhance the normal biological role of the donor cells, dispensing the need for any type of cell modification, such as blood transfusions and bone marrow transplants. This characteristic also applies to TRASCET, in that it is equally based on the normal biological activities of cells in their native environment.

The TRASCET approach also constitutes a minimally invasive intervention amenable to being performed as an outpatient procedure, which in turn could render it accessible to a sizeable proportion, if not the majority of pregnant women. It can also be performed starting at an early point in gestation, thus potentially maximizing impact. Ideally, TRASCET would be performed as an autologous therapy. However, allogeneic cell sourcing would also be conceivable and in fact already demonstrated in animal models, due to the immunotolerant fetal environment and unique immune reactivity of fetal cells. Amniotic cell banking could become a significant constituent of that perspective. While TRASCET's potential applications are broad, with several therapeutic targets currently under investigation, it is an exceedingly young development. There is experimental evidence currently available specifically in neural tube defects, abdominal wall defects, and congenital diaphragmatic hernia.

2 TRASCET's Biological Basis and Cell Sourcing

The mechanisms underlying the enhanced fetal wound healing process are not yet fully understood (Gurtner et al. 2008). Early studies focused on the role of local molecular pathways and gene expression patterns, leading to incomplete insights into the process (Tammi et al. 2005; David-Raoudi et al. 2008). In 2011, the role of the amniotic fluid-derived mesenchymal stem cell (afMSC) in fetal wound healing was first described, revealing a previously overlooked endogenous cellular component (Klein et al. 2011). Using an ovine model, that study demonstrated that afMSCs play an important, though not absolutely required, role by expediting fetal wound closure and enhancing the local extracellular matrix (Fig. 1) (Klein et al. 2011). This finding was consistent with

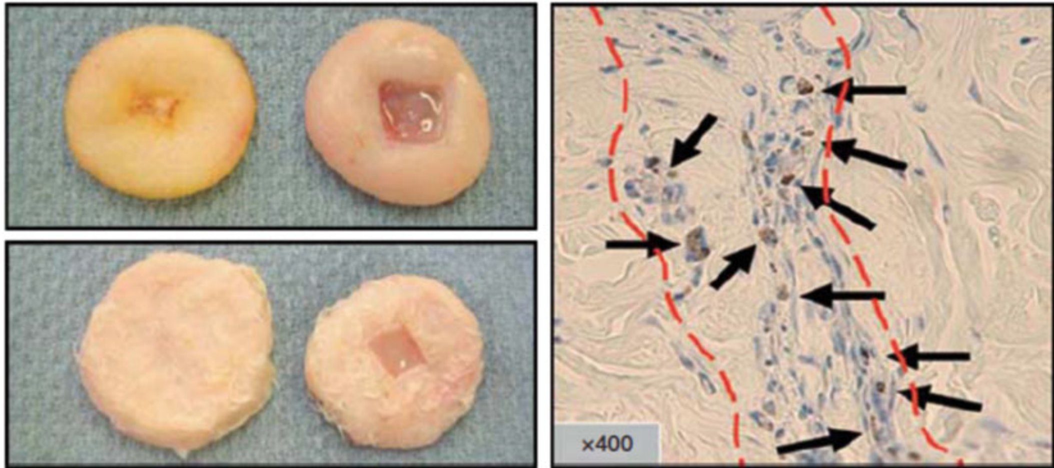


Fig. 1 Representative gross views of two sets of fetal wounds, each set from the same fetal lamb, 9 (top) and 20 (bottom) days after their creation, illustrating evident differences in healing rate. The excerpted wounds on the left were exposed to amniotic fluid cells, whereas those on the right were not. The micrograph shows a fetal wound (within the dotted lines) populated by labeled autologous amniotic mesenchymal stem cells identified by monoclonal anti-GFP immunohistochemistry (arrows; original magnification ×400). (Fauza 2018)

studies showing that mesenchymal stem cells (MSCs) from other sources, most notably bone marrow, can home to injured sites and promote postnatal wound repair (Wu et al. 2007; Kwong and Harris 2008; Fu and Li 2009). These results have provided biological support for the use of afMSCs in cell-based regenerative strategies, both perinatally and later in life.

The potential therapeutic applications of afMSCs have been explored in countless experiments since the early 2000s (Kaviani et al. 2001; Fuchs et al. 2004; Fauza and Bani 2016; Fukutake et al. 2019; Pratheesh et al. 2017). These cells secrete a variety of growth factors and pro-angiogenic factors thought to facilitate wound healing and promote immunomodulation (Shu et al. 2018; Yoon et al. 2010). The unique profile of afMSCs exosomes may also contribute to these effects (Tracy et al. 2019a). From a translational perspective, afMSCs may be the ideal cell type for use in TRASCET compared to other fetal stem cells in part because the amniotic cavity is their native environment. Additionally, afMSCs can be procured via a minimally invasive amniocentesis and have a robust proliferative capacity, expanding twice as quickly as other MSCs when grown under identical culture conditions *in vitro* (Kunisaki et al. 2007a). Therefore, only small volumes such as a 3–5 mL aliquot of amniotic fluid would suffice to generate hundreds of millions of cells within 3–4 weeks (Kunisaki et al. 2007b; Steigman et al. 2008).

Other fetal stem cells, such as placental-derived MSCs (pMSCs), can also be considered and in fact have already been used for TRASCET experimentally (Chalphin et al. 2019a; Feng et al. 2016a). Since chorionic villus sampling (CVS) is viable earlier in gestation than amniocentesis, pMSC-based TRASCET could supposedly be initiated earlier than afMSC-based TRASCET, should that be of benefit. Unique stem cells present in the amniotic fluid only in the presence of certain fetal diseases represent yet another potential sourcing option for TRASCET. For example, primitive neural stem cells (NSCs) can be isolated from the amniotic fluid in exposed neural tube defects (Aula et al. 1980; Chang et al. 2015; Gosden and Brock 1977; Greenebaum et al. 1997; Mendonca et al. 2005; Turner et al. 2013a). These amniotic fluid-derived NSCs (afNSCs) may represent not only a new cell source for TRASCET for spina bifida but have also been shown to have diagnostic value

(Pennington et al. 2013, 2015). While there is potential for many specific disease-associated amniotic stem cells to play a role in TRASCET in select clinical scenarios, there is a relative paucity of data on disease-specific amniotic fluid stem cells at present. Hence, this review will focus on MSC-based TRASCET.

3 Donor Mesenchymal Stem Cell Homing After TRASCET

The mechanisms underlying postnatal MSC trafficking in the setting of tissue repair and inflammation have yet to be fully understood (Hong et al. 2009). Similarly, MSC trafficking after TRASCET is an area of active research. Donor MSC engraftment patterns observed to date in TRASCET studies have shown that cells do not only act via direct seeding of the exposed defect, but they also home robustly to the placenta, fetal bone marrow, and even select sites of maternal injury, highlighting hematogenous routing as a central component of donor cell kinetics (Graham et al. 2017; Shieh et al. 2017). Donor cells have been found to home to the gestational membranes (both the chorion and amnion) *in vivo* as well as migrate through them *in vitro*, providing a possible route for transfer from the amniotic fluid to the placenta and from there to both the maternal and fetal circulations (Tracy et al. 2018). Cell routing through the gestational membranes and placenta has been found to follow a bimodal pattern in a rodent model, pointing to controlled cell trafficking rather than passive cell clearance (Tracy et al. 2018). Other studies have also shown similar evidence of active cell routing (Shieh et al. 2017; Derderian et al. 2016).

Prior work has shown that exogenous hematopoietic stem cells (HSCs) and MSCs engraft in the fetal bone marrow after either placental, fetal intravenous, or fetal intraperitoneal administration (Boelig and Flake 2016). To our knowledge, donor MSC engraftment in the host fetal bone marrow after simple intra-amniotic infection had not been described prior to the TRASCET studies mentioned. The donor afMSC ability to home to the fetal bone marrow rather than acting solely via direct defect seeding is of particular clinical relevance. From the bone marrow, donor MSCs could conceivably reach any other area of the body, much like postnatal bone marrow MSCs. This would significantly expand TRASCET's therapeutic potential well beyond only defects exposed to the amniotic cavity.

The fetal bone marrow is a complex, shifting microenvironment with close interaction between two distinct multipotent cell populations: HSCs and MSCs. There are several variables that could affect donor cell trafficking, including the location of the exogenous administration. Additionally, the homing of donor HSCs and MSCs in the fetal environment is likely influenced by the predictable spatiotemporal and quantitative development of prenatal hematopoiesis, with temporal overlaps at different anatomical sites (Mendes et al. 2005). These sites include the yolk sac, aorta-gonad mesonephros region, placenta, fetal liver, fetal spleen, and ultimately the fetal bone marrow in murine models (Christensen et al. 2004; Medvinsky and Dzierzak 1996; Mikkola et al. 2005). Donor HSCs have been shown to join host HSCs in this migratory pattern in the fetus (Vrecenak and Flake 2013). If donor HSCs and MSCs were to show similar engraftment patterns after intra-amniotic injection, TRASCET could theoretically also become a route of administration for donor HSCs with the goal of achieving donor HSC fetal bone marrow engraftment for therapeutic purposes. This interesting perspective, however, remains purely speculative at this time.

There is also a possibility of fetal microchimerism within the maternal circulation after TRASCET, given evidence of the consistent cell homing to the placenta and then to select sites of maternal injury (Graham et al. 2017; Shieh et al. 2017). While a recent study of long-term cell homing at 16 days after TRASCET in normal rodents showed that donor cells were no longer present in any of the screened fetal tissues, including the fetal bone marrow, donor cells were still weakly present in the maternal skin incision. Longer-term engraftment studies in the setting of different disease models will be required to better address TRASCET's safety profile in both mother and fetus.

4 TRASCET Applications

Of approximately four million live births per year in the United States, congenital anomalies are reported in 3–4% (Egbe et al. 2015; Mohamed and Aly 2012; Parker et al. 2010). They contribute to 20% of neonatal deaths and cause significant morbidity (Parker et al. 2010; Cragan and Gilboa 2009). TRASCET represents a biologically plausible, practical, minimally invasive, and ethically unobjectionable option to improve perinatal management of these disorders. It was initially introduced experimentally for the treatment of neural tube defects and gastroschisis, which are both defects directly exposed to the amniotic fluid. After donor afMSC homing to the placenta and fetal bone marrow was discovered, expanding TRASCET's applicability to non-exposed defects has been pursued in experiments involving congenital diaphragmatic hernia.

4.1 Neural Tube Defects

Neural tube defects (NTDs) are a common congenital disease occurring secondary to failure of normal neural tube closure by the fourth week of embryonic development. These defects can involve any portion of the brain and/or spinal cord and can be either open or closed, depending upon the presence or absence of exposed neural tissue. While the etiology of NTDs is multifactorial, folic acid deficiency is a well-established risk factor (Main and Mennuti 1986; Botto et al. 1999). Folic acid supplementation during the first trimester is reported to reduce the risk of NTDs by 50–70% (MRC Vitamin Study Research Group 1991; Czeizel and Dudas 1992). However, the incidence of neural tube defects in the United States has remained stable at 3–4 per 10,000 live births despite mandatory folate supplementation in all cereal grain products (Mohamed and Aly 2012; Boulet et al. 2008).

Spina bifida, the most common survivable NTD, is a caudal defect with exposure of spinal cord tissue. The most severe form of spina bifida is myelomeningocele, in which both the meningeal sac and the spinal cord protrude through the defect into the amniotic cavity. This leads to significant spinal cord damage by a two-hit pathophysiology, resulting in life-long neurological morbidity such as paraplegia, bowel and bladder incontinence, sexual dysfunction, and secondary musculoskeletal deformities (Danzer and Johnson 2014). The first hit results from abnormal spinal cord development associated with the incomplete closure of the neural tube. The second hit results from neural tissue exposure to the surrounding amniotic fluid, inducing both chemical and mechanical insults. Several studies report that the second hit contributes to the majority of neurological morbidity (Patten 1953; Osaka et al. 1978; Hutchins et al. 1996; Meuli et al. 1997; Korenromp et al. 1986; Sival et al. 1997; Luthy et al. 1991; Shurtleff et al. 1994; Meuli et al. 1995a, b, 1996).

Overall, the benefits of either pre- or postnatal therapy remain modest, and the majority of current treatments are only supportive. Given the significant neurological morbidity of this disease, the first prospective randomized control trial of prenatal surgical repair of a non-life threatening birth defect was performed for spina bifida. It was a National Institutes of Health-funded study known as the Management of Myelomeningocele Study, or MOMS trial (Adzick et al. 2011). The goal of that study was to prospectively compare outcomes between prenatal and postnatal initial spina bifida surgical coverage. The trial showed that prenatal repair does offer some benefit, however not without many limitations. Firstly, it can only be safely performed starting in the second half of the second trimester of gestation. Given that NTDs form by the fourth week of gestation, this allows for a long period in the interim during which the spinal cord experiences the damaging effects of amniotic fluid exposure. Further, it entails significant maternal and fetal risks, most notably preterm labor and prematurity. A more effective strategy for prenatal defect coverage should increase accessibility, allow repair earlier in gestation, and minimize the risk of maternal and fetal complications via a more minimally invasive approach. TRASCET is compatible with these requirements.

4.2 TRASCET for Neural Tube Defects

In several different studies, TRASCET has demonstrated therapeutic benefit in rodent and leporine models of spina bifida (Dionigi et al. 2015a, b; Feng et al. 2016a; Shieh et al. 2019). In the rodent studies, time-dated pregnant Sprague Dawley dams were exposed to oral retinoic acid at a specific time of gestation to induce fetal neural tube defects based upon a widely used model (Danzer et al. 2005). Fetuses then underwent either no further treatment or intra-amniotic injections of volume-matched saline or a concentrated suspension of afMSCs (Fig. 2). Among fetuses with isolated spina bifida, there were no significant differences in the size of the defect across all groups. However, TRASCET was associated with a statistically significant increase in the presence of coverage, either partial (most commonly) or complete, of the spina bifida by a thin, rudimentary neoskin, confirmed histologically (Fig. 3). This neoskin defect coverage after TRASCET was also noted in the surgically created spina bifida model in rabbits, with cells injected at the time of operation (Shieh et al. 2019). In the rodent model, TRASCET also lessened the Chiari-II malformation that is almost universally associated with spina bifida, as shown by high resolution MRI measurements of brainstem displacement (Dionigi et al. 2015b). It can be theorized that the TRASCET-induced neoskin coverage may have reduced leakage of cerebrospinal fluid through the defect and therefore mitigated the associated brainstem herniation.

Interestingly, after injecting GFP-labeled afMSCs in the rodent model, donor cells were identified on immunohistochemistry within bone near the defect, but not within the neoskin itself (Dionigi et al. 2015a). Such an engraftment pattern was subsequently supported by the results from comprehensive cell homing analyses in which donor MSCs were found to traffic hematogenously to the fetal bone marrow (Shieh et al. 2018). An initial molecular mechanistic screening was performed in the rodent model using quantitative reverse transcription PCR (qRT-PCR) to further explore local and distant processes contributing to the coverage. Paracrine factor expression was measured locally at the defect and distantly at the bone marrow in covered versus uncovered defects in all treatment groups. Covered defects exhibited downregulation of Epidermal growth factor (*Egf*) and Fibroblast growth factor-2 (*Fgf-2)* expressions at the defect level as well as decreased bone marrow expression of Transforming growth factor-b-1 (*Tgfb-1*); this suggested a possible role for donor cell-related paracrine activity with negative feedback after coverage had been achieved. In addition, covered defects exhibited decreased *CD45* expression in the bone marrow, suggesting increased relative

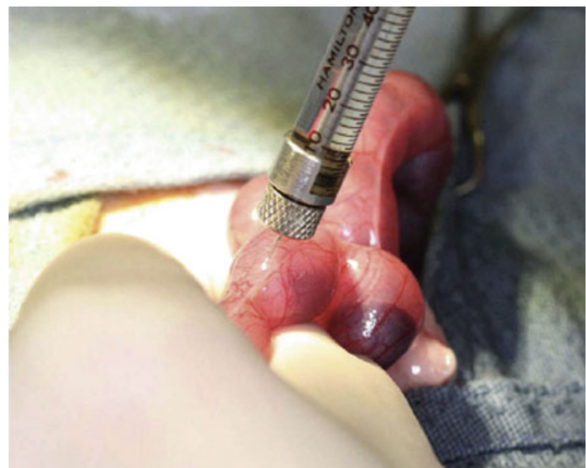


Fig. 2 Gross view of the intra-amniotic injection by the ventral aspect of the fetus in the rodent model, carefully avoiding it and the umbilical cord, using a 33G non-coring needle on a 100 μL syringe. (Dionigi et al. 2015a)

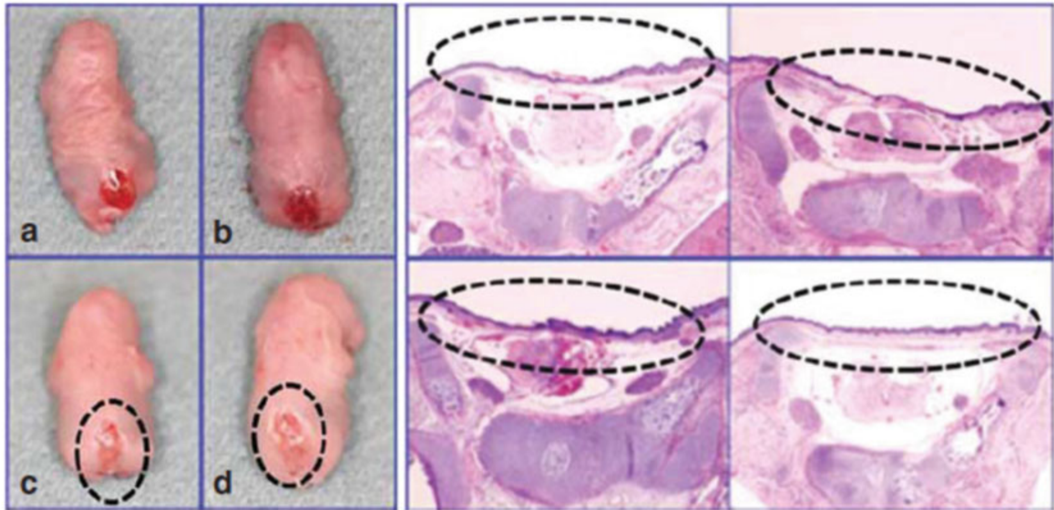


Fig. 3 Gross views of spina bifida defects in term fetal rats at the time of killing. (**a**, **b**) Typical appearance from untreated and sham animals; (**c**, **d**) appearance found in some animals that received TRASCET, in which the defect appeared covered (dotted perimeters). The micrographs are from some of the TRASCET animals, showing the typical widely open vertebral arches, the spinal cord variably deformed, and coverage of the defect by a rudimentary neoskin with a paucity of adnexa (dotted perimeters; H&E, original magnification ×200). (Fauza 2018)

mesenchymal to hematopoietic activity in the setting of coverage. Of note, in several of these rodent studies, a small percentage of partial defect coverage (none complete) was found in control animals not undergoing TRASCET (Feng et al. 2016a; Tracy et al. 2019b). This suggests that TRASCET essentially amplifies an already existing host response.

Another recent study, also in the rodent model, explored whether TRASCET's effects are dose-dependent, comparing one versus two intra-amniotic injections at different time points (Tracy et al. 2019b). It found higher rates of coverage in the one injection versus double injection group, with significantly higher mortality in the latter. These results suggest that TRASCET leads to a lasting host response, without improvement after a second injection, at least in that model. However, time is an inherent limiting factor of that retinoic acid model since the rat gestation is only 22 days long, so that injections could only be performed late in gestation and in close proximity to each other. One cannot yet rule out the possibility that multiple injections could enhance the results in a larger animal model or in humans. Further work is required.

In another study, TRASCET with afMSCs versus pMSCs was compared to further optimize cell sourcing. The afMSC and pMSC-injected groups had similar rates of either partial or complete coverage of the spina bifida defect, suggesting that either cell population could be options for TRASCET for that anomaly (Feng et al. 2016a). This finding adds to the notion that afMSCs and pMSCs may actually represent the same cell pool, simply trafficking between the amniotic and placental compartments (Kunisaki et al. 2007a; Tracy et al. 2018; Fauza 2004; Klein and Fauza 2011).

Additional studies have also explored afNSCs present in the amniotic fluid in the setting of neural tube defects as additional donor cells applicable to TRASCET. In the rodent model, intra-amniotic injection of expanded afNSCs resulted in cell homing to both the superficial and deeper portions of the exposed spinal cord tissue (Turner et al. 2013b). However, neurological outcomes could not be compared in that initial work. Technically, autologous afNSC and afMSC (or pMSC) injections could eventually be combined, though this has yet to be attempted.

The aforementioned rodent and leporine studies represented some of the first *in vivo* reports of intra-amniotic injection of fetal cells in mammals. Prior to them, intra-amniotic injection of other cells to promote experimental spina bifida coverage had only been described once, in an *ex vivo* avian model (i.e. in eggs) using either embryonic stem cells or bone marrow stem cells (Lee et al. 2004, 2006, 2010). From a clinical perspective, fetal MSCs represent a more appealing cell population due to their ease of procurement with minimal, if any, ethical restrictions. While further mechanistic and large animal investigations are still in progress, these data support TRASCET's potential as a minimally invasive alternative for the prenatal management of spina bifida.

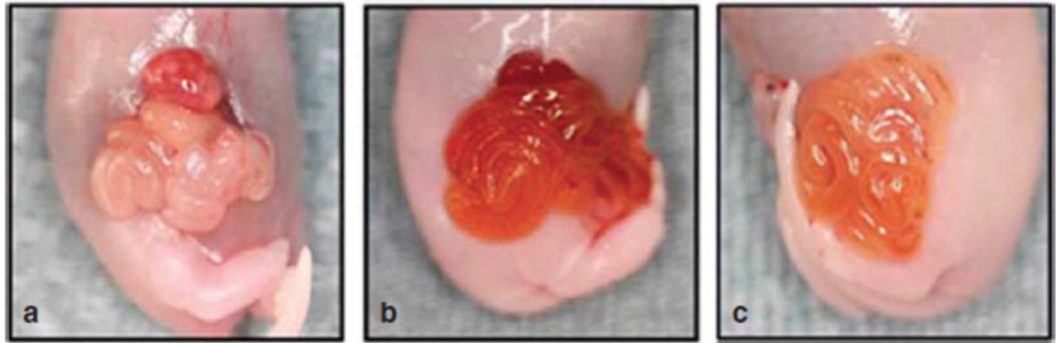
4.3 Abdominal Wall Defects

Abdominal wall defects, including omphalocele and gastroschisis, are very common major congenital anomalies, with a combined incidence of approximately 1 in 2,100 live births (Egbe et al. 2015; Mohamed and Aly 2012). While the prevalence of omphalocele is higher, gastroschisis rates have been increasing in recent years. The etiology of both conditions remains unknown. Omphalocele is a central anterior abdominal wall defect located at the site of the umbilicus. A sac composed of peritoneum, Wharton's jelly, and amnion covers the herniated contents, providing protection from the amniotic fluid. In contrast, gastroschisis is as an abdominal wall defect to the right of the umbilicus that is typically small (under 4 cm in diameter). The eviscerated contents (typically large and small bowel) have no covering and thus are directly exposed to the amniotic fluid, leading to significant chemical and mechanical insults. These insults are compounded by a time-dependent constriction of the abdominal wall defect diameter, leading to vascular congestion and/or ischemia, bowel strangulation, and possible bowel atresia (Langer et al. 1989; Logghe et al. 2005). Therefore, gastroschisis is associated with significantly higher intestinal morbidity than omphalocele, secondary to amniotic fluid peritonitis and severe bowel inflammation, with the eviscerated bowel noted to be both grossly and histologically abnormal. From a functional perspective, patients with gastroschisis suffer from severe gastrointestinal dysmotility and a delayed return to normal gastrointestinal function that correlates with the intensity of bowel damage (Luton et al. 1999).

Congenital abdominal wall defects can be prenatally diagnosed by ultrasound as early as 10 weeks gestation. Despite advances in prenatal imaging, however, there have not been corresponding improvements in the prenatal management of these anomalies. Instead, family counseling on the optimal timing and type of delivery is the focus of prenatal care. In addition to early delivery, several prenatal therapies have been proposed, most only experimentally, with the goal of minimizing amniotic fluid peritonitis and subsequent intestinal damage in gastroschisis. These therapies have included amnio-exchange, amniotic fluid dilution, prenatal steroid administration, induced fetal diuresis, nitric oxide donors, and even intra-uterine repair of the defect, all with minimal success and substantial risks (Bittencourt et al. 2006; Goncalves et al. 2015; Hakguder et al. 2002; Till et al. 2003; Yu et al. 2003). TRASCET represents a less risky, minimally invasive alternative for the treatment of gastroschisis.

4.4 TRASCET for Gastroschisis

Recent studies on TRASCET in the setting of experimental gastroschisis in rodent and leporine models represented the first investigations into prenatal cell-based therapy for abdominal wall defects (Feng et al. 2016b, 2017). They involved the surgical creation of a gastroschisis in the fetuses, with animals receiving either afMSC injection, volume-matched saline injection, or no further treatment. In both models, TRASCET was associated with significantly reduced total bowel wall, serosal, muscular, and mucosal thicknesses, which are known surrogates for bowel inflammation and damage (Fig. 4). In a subsequent study focusing on comprehensive donor cell homing analysis, donor cells were



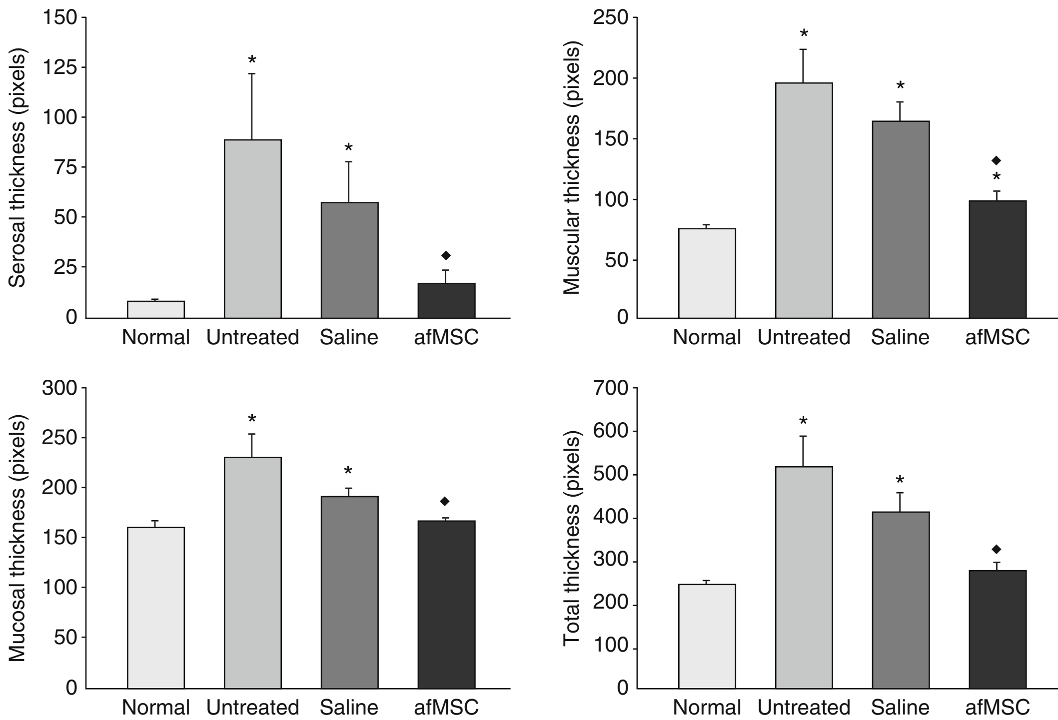


Fig. 4 Representative gross views of term rat fetuses with gastroschisis. The exposed intestine in the (**a**) untreated and (**b**) saline groups appeared heterogeneous, edematous, and thickened. The intestine in the (**c**) TRASCET group seemed less affected. This was confirmed by multiple histological measurements of bowel wall thickness (graph), showing the mean total and segmental thicknesses of the intestinal wall compared across the groups. There were significant decreases in serosal, muscular, and mucosal layer thicknesses and in total bowel wall thickness in the TRASCET group vs. the untreated and saline groups. There were no such differences between the untreated and saline groups. There were no differences between the TRASCET group and normal controls, except for a significantly thicker muscular layer in the former. (Fauza 2018)

found to independently home to exposed bowel and to the placenta (Chalphin et al. 2018). This finding suggests that both direct seeding and hematogenous routing of donor cells take place.

More recently, the optimal cell source for TRASCET for gastroschisis was examined, comparing the effects of afMSCs and pMSCs (Chalphin et al. 2019a). It was shown that either

cell type promoted significantly reduced bowel wall thickness indexes, as listed above, compared to sham and untreated control animals. However, the pMSC-injected group showed significantly more variation in the outcome, while the afMSC-injected group was both more consistent and somewhat more effective. Unlike in the spina bifida model where afMSCs and pMSCs had equivalent outcomes, this finding suggests that afMSCs may be the optimal cell source for TRASCET in gastroschisis. Initial mechanistic screening has also been performed using qRT-PCR to scrutinize the expression of paracrine and immunomodulatory factors after afMSC-based TRASCET for that disease, but has yet to uncover any conclusive insights. Further work, including in the ovine model, is ongoing.

4.5 Congenital Diaphragmatic Hernia

Congenital diaphragmatic hernia (CDH) is a prenatally diagnosable congenital anomaly occurring in between 1 in 2000 to 1 in 5000 live births (Chiu 2014; Langham Jr. et al. 1996; Malowitz et al. 2015; McGivern et al. 2015; Doyle and Lally 2004). Contemporary mortality rates range from 20% to 30%, reduced from around 50% in prior decades (Downard et al. 2003). While markedly improved, mortality and related cardiopulmonary morbidity still remain substantial (Hollinger et al. 2017). CDH involves a defect in the diaphragm, most commonly located in the left posterolateral region, allowing for the herniation of intra-abdominal contents into the chest cavity, which worsens an underlying primary pulmonary hypoplasia. While more pronounced in the lung ipsilateral to the hernia, the disease process affects both lungs. Right cardiac failure secondary to pulmonary hypertension is a major component of the pathophysiology (Gien and Kinsella 2016). Besides surgical repair, current postnatal therapies involve variable degrees of cardiorespiratory support, including extracorporeal membrane oxygenation (ECMO) in the most severe cases. The role for videofetoscopic tracheal occlusion to promote lung growth, long performed clinically, remains controversial, with patient selection for this invasive procedure still unclear (Grivell et al. 2015; Seravalli et al. 2017).

In light of the hematogeous routing of donor cells after TRASCET, the invasiveness and controversy of current prenatal treatment options, and the continuing high postnatal morbidity and mortality of CDH, this disease was the first anomaly not exposed to the amniotic cavity selected for investigation as a potential target for TRASCET.

4.6 TRASCET for Congenital Diaphragmatic Hernia

Studies exploring a potential role for TRASCET in the prenatal management of CDH are underway, with the first two not yet published as of this writing (Chalphin et al. 2019b). Using the nitrofen rat model, TRASCET with afMSCs was compared with untreated and sham treated controls, as well as healthy fetuses. First, gene expression of surrogate markers for select constituent processes of pulmonary development, including: alveolar growth and development (Fibroblast growth factor-10 - *Fgf-10*), lung vascularization (Vascular endothelial growth factor-a - *Vegf-a*), and alveolar maturation/surfactant production (surfactant protein-C - *SPC*) were quantified by qRT-PCR in lungs both ipsi- and contra-lateral to the CDH. The TRASCET group showed significant downregulation of *Fgf-10* and *Vegf-a* gene expressions compared to the untreated and sham groups. *SPC* expression was narrowly significantly higher among TRASCET fetuses compared to untreated animals, but not compared to the saline group. Subsequently, the effects of TRASCET on mediators of pulmonary vascular tone were examined. TRASCET led to significant downregulation of Endothelial nitric oxide synthase (*Enos)* and Endothelin receptor-a (*Er-a)* expressions compared to the untreated and sham groups. The TRASCET group showed downregulation of Endothelin receptor-b (*Er-b*) and pre-Proendothelin-1 (*pPet-1*) compared to the sham group, but not the untreated group. Endothelin converting enzyme (*Ece*) expression was unchanged. Lung laterality

had minimal impact on any of these comparisons. These initial findings suggest that TRASCET affects select fundamental processes of lung development and fetal pulmonary vascular tone homeostasis in experimental CDH, warranting further scrutiny into this novel therapy as a potential component of the prenatal management of this disease.

5 Safety, Regulatory Considerations, and Future Perspectives

From a safety perspective, there have been no reports of tumorigenesis or other harmful effects in any animal models in which native afMSCs have been employed for different purposes, for almost two decades now. These cells are also genetically and phenotypically stable during extensive cell processing under FDA guidelines for clinical use (Kunisaki et al. 2007b; Steigman et al. 2008). Such a robust and predictable behavior during standard *in vitro* expansion, combined with the fact that TRASCET is based on the use of afMSCs in their native, undifferentiated state and reintroduced via simple injection into their own environment, further underlines the safety, practicality and potential reach of the TRASCET approach. At the same time, the hematogenous routing of donor cells both opens new avenues of exploration for clinical applicability, as well as raises questions about safety, which demand addressing.

Certainly, fetal MSCs are less primitive than embryonic stem cells. As they apply to TRASCET, in an as of yet unpublished study, afMSCs could no longer be detected at 16 days of postnatal life in healthy rodents submitted to intra-amniotic injections, further supporting their safety profile. However, donor cells remained weakly present in maternal skin incisions at that time, pointing to a need for longer-term studies of fetal-maternal microchimerism.

Further work on TRASCET in larger animals is needed, if not required, and indeed is currently underway. Larger animals have longer gestation, allowing for potentially repeated cell injections at higher volumes and/or concentrations. These models also often constitute prerequisites for the FDA to approve clinical trials.

In summary, as expected for a new development still in its infancy, there are multiple facets of TRASCET requiring further investigation. Still, the results reported so far, along with the biological basis for TRASCET, justify the expectation that it may become a practical and accessible constituent of novel strategies for the prenatal management of various birth defects.

References

Adzick NS, Thom EA, Spong CY, Brock JW 3rd, Burrows PK, Johnson MP et al (2011) A randomized trial of prenatal versus postnatal repair of myelomeningocele. N Engl J Med 364(11):993–1004

Aula P, von Koskull H, Teramo K, Karjalainen O, Virtanen I, Lehto VP et al (1980) Glial origin of rapidly adhering amniotic fluid cells. Br Med J 281 (6253):1456–1457

Bittencourt DG, Barreto MW, Franca WM, Goncalves A, Pereira LA, Sbragia L (2006) Impact of corticosteroid on intestinal injury in a gastroschisis rat model: morphometric analysis. J Pediatr Surg 41(3):547–553

Boelig MM, Flake AW (2016) In utero stem cell transplantation. In: Fauza DO, Bani M (eds) Fetal stem cells in regenerative medicine: principles and translational strategies. Springer/Humana Press, New York, pp 317–337

Botto LD, Moore CA, Khoury MJ, Erickson JD (1999) Neural-tube defects. N Engl J Med 341(20):1509–1519

Boulet SL, Yang Q, Mai C, Kirby RS, Collins JS, Robbins JM et al (2008) Trends in the postfortification prevalence of spina bifida and anencephaly in the United States. Birth Defects Res A Clin Mol Teratol 82 (7):527–532

Chalphin AV, Tracy SA, Kycia I, Chan C, Finkelstein A, Zurakowski D et al (eds) (2018) Donor mesenchymal stem cell kinetics in transamniotic stem cell therapy for experimental gastroschisis American Academy of Pediatrics annual meeting, Orlando, FL

Chalphin AV, Tracy SA, Lazow SP, Kycia I, Zurakowski D, Fauza DO (eds) (2019a) Comparison between placental and amniotic mesenchymal stem cells in transamniotic stem cell therapy for experimental gastroschisis. American Pediatric Surgical Assocation annual meeting, Boston, MA

Chalphin AV, Tracy SA, Lazow SP, Kycia I, 789 Zurakowski D, Fauza DO (eds) (2019b) Congenital diaphragmatic hernia as a potential target for transamniotic stem cell therapy. British Association of Pediatric Surgeons annual meeting, Nottingham, UK

Chang YJ, Su HL, Hsu LF, Huang PJ, Wang TH, Cheng FC et al (2015) Isolation of human neural stem cells from the amniotic fluid with diagnosed neural tube defects. Stem Cells Dev 24(15):1740–1750

Chiu PP (2014) New insights into congenital diaphragmatic hernia – a surgeon's introduction to CDH animal models. Front Pediatr 2:36

Christensen JL, Wright DE, Wagers AJ, Weissman IL (2004) Circulation and chemotaxis of fetal hematopoietic stem cells. PLoS Biol 2(3):E75

Cragan JD, Gilboa SM (2009) Including prenatal diagnoses in birth defects monitoring: experience of the metropolitan Atlanta congenital defects program. Birth Defects Res A Clin Mol Teratol 85(1):20–29

Czeizel AE, Dudas I (1992) Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. N Engl J Med 327 (26):1832–1835

Danzer E, Johnson MP (2014) Fetal surgery for neural tube defects. Semin Fetal Neonatal Med 19(1):2–8

Danzer E, Schwarz U, Wehrli S, Radu A, Adzick NS, Flake AW (2005) Retinoic acid induced myelomeningocele in fetal rats: characterization by histopathological analysis and magnetic resonance imaging. Exp Neurol 194(2):467–475

David-Raoudi M, Tranchepain F, Deschrevel B, Vincent JC, Bogdanowicz P, Boumediene K et al (2008) Differential effects of hyaluronan and its fragments on fibroblasts: relation to wound healing. Wound Repair Regen 16(2):274–287

Derderian SC, Jeanty C, MacKenzie TC (2016) Fet-maternal cell trafficking and labor. In: Fauza DO, Bani M (eds) Fetal stem cells in regenerative medicine: principles and translational strategies. Springer/ Humana Press, New York, pp 33–45

Dionigi B, Ahmed A, Brazzo J 3rd, Connors JP, Zurakowski D, Fauza DO (2015a) Partial or complete coverage of experimental spina bifida by simple intra-amniotic injection of concentrated amniotic mesenchymal stem cells. J Pediatr Surg 50(1):69–73

Dionigi B, Brazzo JA 3rd, Ahmed A, Feng C, Wu Y, Zurakowski D et al (2015b) Trans-amniotic stem cell therapy (TRASCET) minimizes Chiari-II malformation in experimental spina bifida. J Pediatr Surg 50 (6):1037–1041

Downard CD, Jaksic T, Garza JJ, Dzakovic A, Nemes L, Jennings RW et al (2003) Analysis of an improved survival rate for congenital diaphragmatic hernia. J Pediatr Surg 38(5):729–732

Doyle NM, Lally KP (2004) The CDH Study Group and advances in the clinical care of the patient with congenital diaphragmatic hernia. Semin Perinatol 28 (3):174–184

Egbe A, Lee S, Ho D, Uppu S, Srivastava S (2015) Racial/ ethnic differences in the birth prevalence of congenital anomalies in the United States. J Perinat Med 43 (1):111–117

Fauza DO (2004) Amniotic fluid and placental stem cells. Best Pract Res Clin Obstet Gynaecol 18(6):877–891

Fauza DO (2018) Transamniotic stem cell therapy: a novel strategy for the prenatal management of congenital anomalies. Pediatr Res 83(1–2):241–248

Fauza DO, Bani M (2016) Fetal stem cells in regenerative medicine: principles and translational strategies. Springer Science, New York, 453 p

Feng C, Graham CD, Connors JP, Brazzo J 3rd, Zurakowski D, Fauza DO (2016a) A comparison between placental and amniotic mesenchymal stem cells for transamniotic stem cell therapy (TRASCET) in experimental spina bifida. J Pediatr Surg 51 (6):1010–1013

Feng C, Graham CD, Connors JP, Brazzo J 3rd, Pan AH, Hamilton JR et al (2016b) Transamniotic stem cell therapy (TRASCET) mitigates bowel damage in a model of gastroschisis. J Pediatr Surg 51(1):56–61

Feng C, Graham CD, Shieh HF, Brazzo JA 3rd, Connors JP, Rohrer L et al (2017) Transamniotic stem cell therapy (TRASCET) in a leporine model of gastroschisis. J Pediatr Surg 52(1):30–34

Fu X, Li H (2009) Mesenchymal stem cells and skin wound repair and regeneration: possibilities and questions. Cell Tissue Res 335(2):317–321

Fuchs JR, Kaviani A, Oh JT, LaVan D, Udagawa T, Jennings RW et al (2004) Diaphragmatic reconstruction with autologous tendon engineered from mesenchymal amniocytes. J Pediatr Surg 39(6):834–838. discussion -8

Fukutake M, Ochiai D, Masuda H, Abe Y, Sato Y, Otani T et al (2019) Human amniotic fluid stem cells have a unique potential to accelerate cutaneous wound healing with reduced fibrotic scarring like a fetus. Hum Cell 32 (1):51–63

Gien J, Kinsella JP (2016) Management of pulmonary hypertension in infants with congenital diaphragmatic hernia. J Perinatol 36(Suppl 2):S28–S31

Goncalves FL, Bueno MP, Schmidt AF, Figueira RL, Sbragia L (2015) Treatment of bowel in experimental gastroschisis with a nitric oxide donor. Am J Obstet Gynecol 212(3):383 e1-7

Gosden CM, Brock DJ (1977) Morphology of rapidly adhering amniotic-fluid cells as an aid to the diagnosis of neural-tube defects. Lancet (London, England) 1 (8018):919–922

Graham CD, Shieh HF, Brazzo JA 3rd, Zurakowski D, Fauza DO (2017) Donor mesenchymal stem cells home to maternal wounds after transamniotic stem cell therapy (TRASCET) in a rodent model. J Pediatr Surg 52(6):1006–1009

Greenebaum E, Mansukhani MM, Heller DS, Timor-Tristsch I (1997) Open neural tube defects: immunocytochemical demonstration of neuroepithelial cells in amniotic fluid. Diagn Cytopathol 16(2):143–144

Grivell RM, Andersen C, Dodd JM (2015) Prenatal interventions for congenital diaphragmatic hernia for improving outcomes. Cochrane Database Syst Rev 11: Cd008925

Gurtner GC, Werner S, Barrandon Y, Longaker MT (2008) Wound repair and regeneration. Nature 453 (7193):314–321

Hakguder G, Ates O, Olguner M, Api A, Ozdogan O, Degirmenci B et al (2002) Induction of fetal diuresis with intraamniotic furosemide increases the clearance of intraamniotic substances: an alternative therapy aimed at reducing intraamniotic meconium concentration. J Pediatr Surg 37(9):1337–1342

Hollinger LE, Harting MT, Lally KP (2017) Long-term follow-up of congenital diaphragmatic hernia. Semin Pediatr Surg 26(3):178–184

Hong HS, Lee J, Lee E, Kwon YS, Ahn W, Jiang MH et al (2009) A new role of substance P as an injury-inducible messenger for mobilization of CD29(+) stromal-like cells. Nat Med 15(4):425–435

Hutchins GM, Meuli M, Meuli-Simmen C, Jordan MA, Heffez DS, Blakemore KJ (1996) Acquired spinal cord injury in human fetuses with myelomeningocele. Pediatr Pathol Lab Med 16(5):701–712

Kaviani A, Perry TE, Dzakovic A, Jennings RW, Ziegler MM, Fauza DO (2001) The amniotic fluid as a source of cells for fetal tissue engineering. J Pediatr Surg 36 (11):1662–1665

Klein JD, Fauza DO (2011) Amniotic and placental mesenchymal stem cell isolation and culture. Methods Mol Biol 698:75–88

Klein JD, Turner CG, Steigman SA, Ahmed A, Zurakowski D, Eriksson E et al (2011) Amniotic mesenchymal stem cells enhance normal fetal wound healing. Stem Cells Dev 20(6):969–976

Korenromp MJ, van Gool JD, Bruinese HW, Kriek R (1986) Early fetal leg movements in myelomeningocele. Lancet (London, England) 1 (8486):917–918

Kunisaki SM, Fuchs JR, Steigman SA, Fauza DO (2007a) A comparative analysis of cartilage engineered from different perinatal mesenchymal progenitor cells. Tissue Eng 13(11):2633–2644

Kunisaki SM, Armant M, Kao GS, Stevenson K, Kim H, Fauza DO (2007b) Tissue engineering from human mesenchymal amniocytes: a prelude to clinical trials. J Pediatr Surg 42(6):974–979. discussion 9-80

Kwong FN, Harris MB (2008) Recent developments in the biology of fracture repair. J Am Acad Orthop Surg 16 (11):619–625

Langer JC, Longaker MT, Crombleholme TM, Bond SJ, Finkbeiner WE, Rudolph CA et al (1989) Etiology of intestinal damage in gastroschisis. I: effects of amniotic fluid exposure and bowel constriction in a fetal lamb model. J Pediatr Surg 24(10):992–997

Langham MR Jr, Kays DW, Ledbetter DJ, Frentzen B, Sanford LL, Richards DS (1996) Congenital diaphragmatic hernia. Epidemiol Outcome Clin Perinatol 23 (4):671–688

Lee DH, Park S, Kim EY, Kim SK, Chung YN, Cho BK et al (2004) Enhancement of re-closure capacity by the intra-amniotic injection of human embryonic stem cells in surgically induced spinal open neural tube defects in chick embryos. Neurosci Lett 364(2):98–100

Lee DH, Kim EY, Park S, Phi JH, Kim SK, Cho BK et al (2006) Reclosure of surgically induced spinal open neural tube defects by the intraamniotic injection of human embryonic stem cells in chick embryos 24 hours after lesion induction. J Neurosurg 105 (2 Suppl):127–133

Lee DH, Phi JH, Kim SK, Cho BK, Kim SU, Wang KC (2010) Enhanced reclosure of surgically induced spinal open neural tube defects in chick embryos by injecting human bone marrow stem cells into the amniotic cavity. Neurosurgery 67(1):129–135. discussion 35

Logghe HL, Mason GC, Thornton JG, Stringer MD (2005) A randomized controlled trial of elective preterm delivery of fetuses with gastroschisis. J Pediatr Surg 40 (11):1726–1731

Luthy DA, Wardinsky T, Shurtleff DB, Hollenbach KA, Hickok DE, Nyberg DA et al (1991) Cesarean section before the onset of labor and subsequent motor function in infants with meningomyelocele diagnosed antenatally. N Engl J Med 324(10):662–666

Luton D, de Lagausie P, Guibourdenche J, Oury J, Sibony O, Vuillard E et al (1999) Effect of amnioinfusion on the outcome of prenatally diagnosed gastroschisis. Fetal Diagn Ther 14(3):152–155

Main DM, Mennuti MT (1986) Neural tube defects: issues in prenatal diagnosis and counselling. Obstet Gynecol 67(1):1–16

Malowitz JR, Hornik CP, Laughon MM, Testoni D, Cotten CM, Clark RH et al (2015) Management practice and mortality for infants with congenital diaphragmatic hernia. Am J Perinatol 32(9):887–894

McGivern MR, Best KE, Rankin J, Wellesley D, Greenlees R, Addor MC et al (2015) Epidemiology of congenital diaphragmatic hernia in Europe: a register-based study. Arch Dis Child Fetal Neonatal Ed 100(2):F137–F144

Medvinsky A, Dzierzak E (1996) Definitive hematopoiesis is autonomously initiated by the AGM region. Cell 86 (6):897–906

Mendes SC, Robin C, Dzierzak E (2005) Mesenchymal progenitor cells localize within hematopoietic sites throughout ontogeny. Development 132 (5):1127–1136

Mendonca ED, Gutierrez CM, Peres LC (2005) Brain tissue fragments in the amniotic fluid of rats with neural tube defect. Pathology 37(2):152–156

Meuli M, Meuli-Simmen C, Yingling CD, Hutchins GM, Hoffman KM, Harrison MR et al (1995a) Creation of myelomeningocele in utero: a model of functional damage from spinal cord exposure in fetal sheep. J Pediatr Surg 30(7):1028–1032. discussion 32-3

Meuli M, Meuli-Simmen C, Hutchins GM, Yingling CD, Hoffman KM, Harrison MR et al (1995b) In utero surgery rescues neurological function at birth in sheep with spina bifida. Nat Med 1(4):342–347

Meuli M, Meuli-Simmen C, Yingling CD, Hutchins GM, Timmel GB, Harrison MR et al (1996) In utero repair of experimental myelomeningocele saves neurological function at birth. J Pediatr Surg 31(3):397–402

Meuli M, Meuli-Simmen C, Hutchins GM, Seller MJ, Harrison MR, Adzick NS (1997) The spinal cord lesion

in human fetuses with myelomeningocele: implications for fetal surgery. J Pediatr Surg 32(3):448–452

Mikkola HK, Gekas C, Orkin SH, Dieterlen-Lievre F (2005) Placenta as a site for hematopoietic stem cell development. Exp Hematol 33(9):1048–1054

Mohamed MA, Aly H (2012) Birth region, race and sex may affect the prevalence of congenital diaphragmatic hernia, abdominal wall and neural tube defects among US newborns. J Perinatol 32(11):861–868

MRC Vitamin Study Research Group (1991) Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. Lancet 338(8760):131–137

Osaka K, Tanimura T, Hirayama A, Matsumoto S (1978) Myelomeningocele before birth. J Neurosurg 49 (5):711–724

Parker SE, Mai CT, Canfield MA, Rickard R, Wang Y, Meyer RE et al (2010) Updated National Birth Prevalence estimates for selected birth defects in the United States, 2004–2006. Birth Defects Res A Clin Mol Teratol 88(12):1008–1016

Patten BM (1953) Embryological stages in the establishing of myeloschisis with spina bifida. Am J Anat 93 (3):365–395

Pennington EC, Gray FL, Ahmed A, Zurakowski D, Fauza DO (2013) Targeted quantitative amniotic cell profiling: a potential diagnostic tool in the prenatal management of neural tube defects. J Pediatr Surg 48 (6):1205–1210

Pennington EC, Rialon KL, Dionigi B, Ahmed A, Zurakowski D, Fauza DO (2015) The impact of gestational age on targeted amniotic cell profiling in experimental neural tube defects. Fetal Diagn Ther 37 (1):65–69

Pratheesh MD, Dubey PK, Gade NE, Nath A, Sivanarayanan TB, Madhu DN et al (2017) Comparative study on characterization and wound healing potential of goat (Capra hircus) mesenchymal stem cells derived from fetal origin amniotic fluid and adult bone marrow. Res Vet Sci 112:81–88

Seravalli V, Jelin EB, Miller JL, Tekes A, Vricella L, Baschat AA (2017) Fetoscopic tracheal occlusion for treatment of non-isolated congenital diaphragmatic hernia. Prenat Diagn 37(10):1046–1049

Shieh HF, Ahmed A, Tracy SA, Zurakowski D, Fauza DO (2017) Fetal bone marrow homing of donor mesenchymal stem cells after Transamniotic stem cell therapy (TRASCET). J Pediatr Surg 53(1):174–177

Shieh HF, Ahmed A, Rohrer L, Zurakowski D, Fauza DO (2018) Donor mesenchymal stem cell linetics after transamniotic stem cell therapy (TRASCET) for experimental spina bifida. J Pediatr Surg 53(6):1134–1136

Shieh HF, Tracy SA, Hong CR, Chalphin AV, Ahmed A, Rohrer L et al (2019) Transamniotic stem cell therapy (TRASCET) in a rabbit model of spina bifida. J Pediatr Surg 54(2):293–296

Shu J, He X, Li H, Liu X, Qiu X, Zhou T et al (2018) The beneficial effect of human amnion mesenchymal cells in inhibition of inflammation and induction of neuronal repair in EAE mice. J Immunol Res 2018:5083797

Shurtleff DB, Luthy DA, Nyberg DA, Benedetti TJ, Mack LA (1994) Meningomyelocele: management in utero and post natum. CIBA Found Symp 181:270–280. discussion 80-6

Sival DA, Begeer JH, Staal-Schreinemachers AL, Vos-Niel JM, Beekhuis JR, Prechtl HF (1997) Perinatal motor behaviour and neurological outcome in spina bifida aperta. Early Hum Dev 50(1):27–37

Steigman SA, Armant M, Bayer-Zwirello L, Kao GS, Silberstein L, Ritz J et al (2008) Preclinical regulatory validation of a 3-stage amniotic mesenchymal stem cell manufacturing protocol. J Pediatr Surg 43 (6):1164–1169

Tammi R, Pasonen-Seppanen S, Kolehmainen E, Tammi M (2005) Hyaluronan synthase induction and hyaluronan accumulation in mouse epidermis following skin injury. J Invest Dermatol 124(5):898–905

Till H, Muensterer O, Mueller M, Klis V, Klotz S, Metzger R et al (2003) Intrauterine repair of gastroschisis in fetal rabbits. Fetal Diagn Ther 18(5):297–300

Tracy SA, Chalphin AV, Kycia I, Chan C, Finkelstein A, Zurakowski D et al (eds) (2018) Insights into hematogenous donor cell routing after transamniotic stem cell therapy American Academy of Pediatrics annual meeting, Orlando, FL

Tracy SA, Ahmed A, Tigges JC, Ericsson M, Pal AK, Zurakowski D et al (2019a) A comparison of clinically relevant sources of mesenchymal stem cell-derived exosomes: bone marrow and amniotic fluid. J Pediatr Surg 54(1):86–90

Tracy SA, Chalphin AV, Lazow SP, Kycia I, Chan C, Finkelstein A et al (eds) (2019b) Repeat dosing in the retinoic acid model of transamniotic stem cell therapy for spina bifida. American Pediatric Surgical Assocation Annual Meeting, Boston, MA

Turner CG, Klein JD, Wang J, Thakor D, Benedict D, Ahmed A et al (2013a) The amniotic fluid as a source of neural stem cells in the setting of experimental neural tube defects. Stem Cells Dev 22(4):548–553

Turner CG, Pennington EC, Gray FL, Ahmed A, Teng YD, Fauza DO (2013b) Intra-amniotic delivery of amniotic-derived neural stem cells in a syngeneic model of spina bifida. Fetal Diagn Ther 34(1):38–43

Vrecenak JD, Flake AW (2013) In utero hematopoietic cell transplantation--recent progress and the potential for clinical application. Cytotherapy 15(5):525–535

Wu Y, Wang J, Scott PG, Tredget EE (2007) Bone marrow-derived stem cells in wound healing: a review. Wound Repair Regen 15(Suppl 1):S18–S26

Yoon BS, Moon JH, Jun EK, Kim J, Maeng I, Kim JS et al (2010) Secretory profiles and wound healing effects of human amniotic fluid-derived mesenchymal stem cells. Stem Cells Dev 19(6):887–902

Yu J, Gonzalez-Reyes S, Diez-Pardo JA, Tovar JA (2003) Effects of prenatal dexamethasone on the intestine of rats with gastroschisis. J Pediatr Surg 38(7):1032–1035

Adv Exp Med Biol - Cell Biology and Translational Medicine (2019) 7: 75–95
https://doi.org/10.1007/5584_2019_412
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Published online: 14 July 2019

Regenerative Medicine: Injectable Cell-Based Therapeutics and Approved Products

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Abstract

Cellular-based therapies have drawn a great deal of attention thanks to their regenerative medicine approaches to treat incurable diseases and specific injuries. In this regard, injectable cell delivery systems could actualize the therapeutically beneficial outcomes of cell-based therapeutic products. These systems have found considerable clinical uses. Hence, the recent studies have focused on developing injectable bio-constructs to protect transplanted cells during delivery and stimulating endogenous regeneration through interactions of these cells and host tissue. This paper introduces a framework, as a general concept, to improve cell delivery systems for cell-based therapeutic products. Studies on stable injectable carriers can enhance cell homing, proliferation, viability, dressing of irregular shape of target sites, and subsequently support transplanted cell functionality. However, more studies should be conducted on new technologies for the injectable cell-based product for cell delivery and the clinical applications.

Keywords

Cellular therapy · Injectable product · Regenerative medicine

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Abbreviations

CAR-T cell	Chimeric antigen receptor T cells
CBER	Center for Biologics Evaluation and Research
CT	Cell-based therapy
DMEM	Dulbecco's Modified Eagle ́s Medium
FDA	U.S. food and drug administration
GvHD	Graft versus host disease
MSCs	Mesenchymal Stem Cells
OTAT	Tissues and Advanced Therapies
RG	Regenerative medicine

1 Introduction

New medical sciences have drawn a great deal of attention among medical science researchers and scientific societies. The hope for finding treatments for incurable and/or un-definitive cure diseases and further improvement of human's health is growing thanks to new medical technologies. Among the modern medical fields that have been extensively covered by medical and academic societies are cell and tissue-based therapy that are categorized under Regenerative medicine (RG) field (Golchin and Farahany 2019).

Regenerative medicine is one of the branches of medicine that develops methods to regrow, repair, restore or replace damaged or miss-functional cells, tissues, or organs. Therefore, it includes the generation and use of biological products such as therapeutic stem cells, cell and/or tissue engineering, and the artificial organs productions. Regenerative medicine that are categorized in the biological product section of U.S. food and drug administration (FDA) products include stem cell therapies, therapeutic tissue engineering products, human cell and tissue products, and combinations of products using any such therapies, such as genetic engineering cells that lead to a stable modification of cells or tissues. The development and approval process of these products are regulated by the FDA's Office of Tissues and Advanced Therapies (OTAT) in the Center for Biologics Evaluation and Research (CBER) (Golchin and Farahany 2019; Lapteva et al. 2018). According to the majority of researchers and physicians, regenerative therapies will permeate clinical outlook, in particular for diseases that have been proven incurable based on the current management strategies (Wyles et al. 2019).

Cell sources of cell-based therapies (CT) (also called cellular therapy, cell therapy or cytotherapy) include stem cell products that may be derived from different sources, somatic functionally differentiated cells, various types of cell lineages, and genetically engineered cells (Golchin and Farahany 2019; Lapteva et al. 2018). Hematopoietic stem cell (also called bone marrow transplant) and mesenchymal stem cell transplantation are the most frequently used CT that are applied to treat a variety of hematologic diseases. Generally, cell-based therapy refers to medicinal products containing cells as the main functional compartment that are typically injected into patients. Cell-based therapy has potential applications in a variety of common, incurable and rare diseases including cancers, autoimmune disease, urinary and reproductive system problems, infectious disease, regenerating damaged cartilage in joints, modulating the immune system, helping patients with neurological disorders, skin diseases, and aesthetic therapy. Given that the costs of cell-based therapy are considerably high compared with traditional and usual therapies, cost drivers need to be identified and understood to lead improvement efforts to achieve viable costs (Bandeiras et al. 2018; Lopes et al. 2018). Recently, several research projects with cell-based therapy products have received grants via FDA's Orphan Products Clinical Trials Grants Program (Press Announcements 2019).

As mentioned earlier, CT includes therapeutic cell-based products. Therefore, using these cells for therapeutic purposes needs specific tools (Fig. 1). One of the main items is the presentation of a suitable delivery system of cells to target sites. Using scaffold as a cell delivery system has been always an option in tissue engineering field. However, direct and indirect injection are the most commonly used methods of the cell delivery system in cell-based therapy approaches (Golchin et al. 2017). Generally, approximately 1–20% of transplanted cells survive, which significantly limits the therapeutic potentials (Golchin et al. 2017, 2018a). Therefore, more studies on new technology development are needed for the injectable cell-based products for cell delivery systems.

This study is designed to amass and present of applied information about injectable cell-based therapeutics for RG field especially FDA approved cellular therapy products. Therefore, in the first section of this review, the cell tracking and cell homing that are key points in the design of injectable cell products are described. Then,

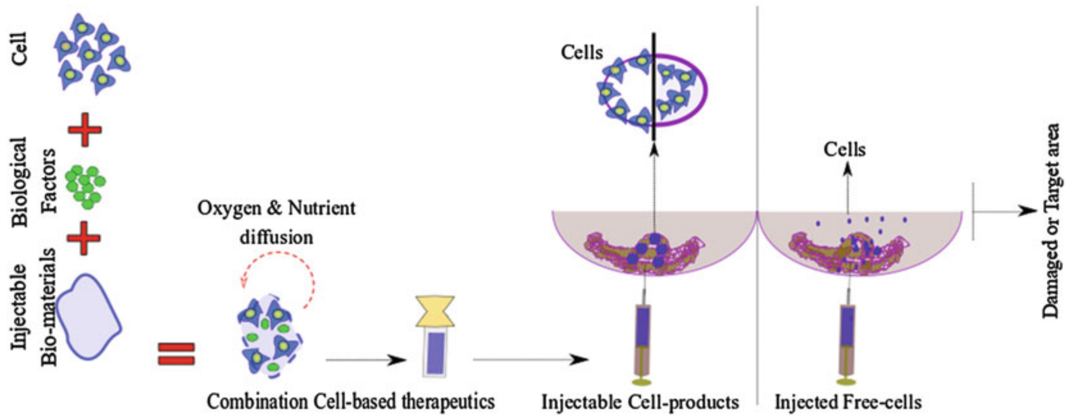

Fig. 1 Summary of a schematic design of an injectable cell-based product and its advantage as a cell delivery system

different types of injection methods and the current applications in this field are reviewed. In the section two, injectable products and materials for developing injectable cell-based products are discussed. The main aim is to introduce injectable cell-based therapeutics products.

2 Cell Tracking and Cell Homing

Among various injectable cell-based products, reported in studies, several stem cell-based products, two CAR-T cell products, and several other cellular therapy products can be approved by the FDA for clinical applications. However, the number of cellular products have been progressively approved and withdrawn in some other cases. In this section, stem cell homing and immune CAR-T cells reaction as two main features of cell and gene products field are reviewed and described. This information can help us in engineering more effective cell injectable products.

2.1 Stem Cell Homing for Performing Regenerative and Therapeutic Functions

Stem cells have a considerable potential to differentiate into various cell types in the body during their primary life and growth (Golchin and Farahany 2019). Four types of stem cells exist on the basis of transdifferentiation, including (Golchin and Farahany 2019) unipotent, (Lapteva et al. 2018) multipotent, (Wyles et al. 2019) pluripotent, and (Bandeiras et al. 2018) totipotent. Since stem cells have the ability to create different tissues in the human body, they have a substantial potential for repair and therapeutic uses in cellular based therapy. On the basis of regenerative applications, stem cells can be classified as embryonic stem cells (ESCs), tissue specific progenitor stem cells (TSPSCs), mesenchymal stem cells (MSCs), umbilical cord stem cells (UCSCs), bone marrow stem cells (BMSCs), iPSCs (Mahla 2016), and some other sources.

Stem cell homing is one of the most important factors in the process of using stem cells for therapeutic purposes. Homing is an essential and rapid process in clinical stem cell transplantation in which circulating cells actively cross the blood endothelium barrier and domicile at the target site by activation of adhesion interactions before their proliferation. Migration and homing entail cells attachment and migration from endothelial cells (ECs) to enter the target tissue. For example, it is well established that leukocytes attach to ECs, roll over the ECs, and then transmigrate between ECs (Sohni and Verfaillie 2013). Components of stem cell homing that have been characterized include cell adhesion molecules, their ligands, extracellular matrix components, and chemokines. Although homing of stem cells induces a cascade of

happenings, "rolling" is the main initial step. Rolling is a necessity for cell homing because it selectively decelerates branches of circulating cells and allows their following endothelial adhesion and transmigration under situations of physiological shear pressure (Chute 2006). There are different factors effective in each group of stem cells; however, the majority of studies have focused on the mechanisms underlying migration and homing that assessed leukocyte migration into inflamed tissues, hematopoietic stem cell (HSCs), mesenchymal stem cell (MSCs) and metastatic cancer cells.

A significant part of the studies is related to the mechanism of MSCs migration towards the target tissue and the role of cell surface receptors and molecules in aiding homing and migration to tissue of interest (Golchin et al. 2018b, 2019). Moreover, other studies have demonstrated the control of hematopoietic stem cell homing to the bone marrow and regulation of hematopoietic stem cell migrating to the metastasis of cancer stem cells and extra-medullary tissues. As MSCs are extremely used in cell-based therapies, the enduring problem in this field is the delivery of the cells to the site of injury (Golchin et al. 2017), a process that is termed "homing." The MSC homing is explained as detention of MSCs within the vasculature of a tissue pursued by transmigration through the endothelium (Karp and Leng Teo 2009). Moreover, the therapeutic effect of MSCs is considerably depended on their ability to produce paracrine or juxtacrine factors that enhance regeneration from endogenous (stem) cells (Golchin et al. 2018b).

Migration and homing to the tissue of injury is affected by many factors containing passage number of the cells, the delivery method, and culture conditions. However, MSCs express many receptors and cell adhesion molecules that help in migration and homing to target tissues. The exact mechanisms that MSCs use are not clear completely. Homing receptors, such as CXCR4, are chemokine receptors for stromal derived factor-1(SDF-1) that is upregulated in ischemic tissues and the bone marrow. In this regard, Wynn et al. showed that CXCR4 is present in a subpopulation of MSCs, which assists in CXCL12-dependent homing and migration. Expression of CXCR4 enhances homing ability and this receptor is not present on the surface of culture-expanded MSCs (Ruster et al. 2006). Besides CXCR4, cultured MSCs and isolated BM-MSCs also express CCR1, CCR4, CCR7, CCR10, CCR9, CXCR5, and CXCR6 that are also associated with MSC migration (Sohni and Verfaillie 2013). In this process, MSCs express molecules as adhesion molecules like integrins and selectins. The rolling and binding of MSCs are mediated by the P-selectin adhesion molecule. Rüster et al. displayed that MSCs like HSCs bind to endothelial cells (ECs) derived from human umbilical cord vein (HUVECs). Moreover, ECs were activated by TNF-α and the binding was tightened (Ruster et al. 2006).

To improve homing of MSCs, the corresponding receptors on MSCs and the right combination of signaling molecules from the injured tissue are required. The expression of chemokine receptors on MSCs is controlled by many factors. For instance, pretreatment of cultured MSCs with cytokines such as IL-6, HGF and others can increase the expression of chemokine receptors (CXCR4) and improve their homing and migration both in vivo and in vitro (Ponte et al. 2007). In addition, Hung et al. demonstrated an increased expression of chemokine receptors (CX3CR1 and CXCR4) by exposing MSCs to hypoxia in a short term (Hung et al. 2007). Another agent uses engineered MSCs that overexpress chemokine receptors like integrin-alpha4 and CXCR4 to control their homing ability. Recently, retrovirus vectors that encode homing receptors (CXCR4) have been used to enhance homing and engraftment of MSCs and HSCs via increasing cell invasion in response to SDF-1. Kumar et al. showed a transduced MSCs with an adenovirus encoding integrin-alpha4, which enhanced their ability for homing to bone (Kumar and Ponnazhagan 2007). Although these genetically modified MSCs can be used for pre-treatment method, they are not yet accessible for therapeutic use in human (Sohni and Verfaillie 2013). While homing of MSC to specific sites is mediated by adhesive interactions and chemokines, possible biomaterials liberated from endothelial cells or tissue increase activation of trans-endothelial migration, adhesion ligands,

subsequent retention in surrounding tissue, and chemotaxis.

Moreover, endothelial cells and transmigration via the endothelium are required for homing of transplanted HSCs to the bone marrow and the interaction of transplanted stem cells with bone marrow. Cell adhesion molecules on the surface of HSCs bind to different kinds of ligands on endothelial cells (ECs) and allows rolling and firm adhesion. Hematopoietic stem cells represent several adhesion molecules, containing integrins (consisting α and β subunits), CD_{44} isoforms, and sialomucins (Chute 2006). Endothelial cells within the bone marrow express P and E selectins, which are membrane bound to C-type lectins that are in turn bound to cell surface glycosylated ligands expressed on HSCs (Xia et al. 2004).

Recent investigations have shown that α4β7 integrin and the α6 integrin participated to homing of transplanted bone marrow cells to the recipient marrow independently. Moreover, expression of CD_{44} on hematopoietic progenitor cells plays an important role in HSC homing to the bone marrow. The role of selectins, integrins, and CD_{44} in the primary adhesion and tethering of stem cells to bone marrow endothelial cells is controlled by the chemokine and SDF-1. The SDF-1 is a component of the chemokine family and it is expressed by both endothelial cells and bone marrow stromal cells. A receptor of SDF-1 is the CXCR4 and the interactions between them are known to be the main pivot controlling stem cell homing in the bone marrow following transplantation (Lapidot et al. 2005). Moreover, several studies have demonstrated a notable relationship between CXCR4 expression in stem cells and successful engraftment and homing in vivo. In addition, SDF-1 can induce CD_{44} binding to hyaluronic acid, which is represented on the surface of bone marrow endothelial cells. Many studies have displayed a modulatory and collaboration role for SDF-1 in increasing the ability of adhesion of HSCs to the bone marrow endothelium through its factors in CD_{44} and integrin activity (Chute 2006). Therefore, HSCs transmigrate the endothelial cells, following a stromal derived factor (SDF)-1 gradient into the bone marrow wherein they take up domicile within the endosteal niche, next to osteoblast cells (OCs) (Lapidot et al. 2005). Accessory cells like CD_{34}^{-} and CD_{34}^{+} CD_{38}^{+} progenitor cells have an important role in the efficiency of stem cells homing to the bone marrow. These cells have been found along with cytokine administration for simplifying human HSC engraftment in NOD/SCID marrow (Chute 2006).

Since quantification of homing in a target tissue is important, there are two techniques for appraising them: (Golchin and Farahany 2019) quantification of the comparative level of radioactivity in segregated organs and tissues or (Lapteva et al. 2018) counting the number of fluorescently labeled cells in microscopic fields per tissue sample (Barbash et al. 2003). Moreover, using retroviral vectors to express fluorescent proteins is a classical method to label cells, which helps to understand MSC homing and engraftment (Belema-Bedada et al. 2008). There are more progressive techniques in order to detect the injected cells in vivo, like single-photon emission CT (SPECT), bioluminescence imaging (BLI), multiple photon microscopy, magnetic resonance imaging (MRI), and positron emission tomography (PET) (Sohni and Verfaillie 2013).

2.2 Interaction of (CAR) T Cells with Cancer Cells

Chimeric antigen receptor (CAR) T cells that are created by genetic engineering can improve some of hematological cancers and solid tumors. CARs consist of three constituents: (i) a genetically engineered receptor for identifying a tumor associated antigen (TAA) in an HLA-independent system, (ii) an activation domain acquired from the $CD_3ζ$ chain of the T cell receptor complex, and (iii) co-stimulator domain(s) to increase initial activation by CAR-$CD_3ζ$ signaling. For desirable effector function, CAR T cells need three signals: (i) activating signal induced by CAR identification of tumor antigen and the next signal transduction via $CD_3ζ$, (ii) co-stimulation enabled via one or more domains engineered into the CAR pattern, and (iii) stimulatory cytokines for effector

function and continued growth (DeRenzo and Gottschalk 2019). Until now, CAR T cells have been successful in treating acute lymphoblastic leukemia and large B-cell lymphoma. For treating chronic lymphoblastic leukemia (CLL), a CAR targeted to the B cell antigen CD_{19} was used successfully for the first time. The FDA approved the use of CART19 (Kymriah) to treat pediatric recurred or resistant acute lymphoblastic leukemia (ALL) in 2017 (Golchin and Farahany 2019). In the same year, another CD_{19}-targeting CAR (Yescarta) was approved by the FDA for adult recurred or refractory large B cell lymphoma (Golchin and Farahany 2019). Despite the broad studies, CAR T cell therapy has not been successful for solid tumors. The major difference between hematological tumors and solid tumors is the difficulty of detecting perfect target antigen in solid tumors. In cancers like ALL or CLL, the tumor cells generally express the B-cell marker CD_{19}. While solid tumors seldom express tumor specific antigen. In most solid tumors, a tumor associated antigen (TAA), it is easier to find and enrich tumors while they are expressed on normal tissues at low levels (Knochelmann et al. 2018). Hence, the important step in a successful adoptive T cell therapy for solid tumor is to select an optimal TAA for CAR T cell targeting. The perfect target should have two properties.

First, the TAA must be expressed selectively on tumor cells at high levels and not be expressed on other normal tissues surfaces (if expressed, it should be at a very low level). Second, since the CAR can only attack cells that have the targeted antigen, the perfect TAA should be expressed on 100% of the tumor cells surface. It is necessary to mention that the tendency of scFv to TAA plays an important role and immune-editing and subsequent elimination of the most immunogenic epitopes can cause tumor escape (Knochelmann et al. 2018). As a target antigen for B cell malignancies, CD_{19} demonstrates the both properties. All acute lymphoblastic leukemia cells with intravenous immunoglobulin support express CD_{19} at high levels and the other targets of CD_{19} like normal B cells are relatively dispensable. These features are the main reasons for the enormous success of CD_{19} CARs in leukemia.

So far, about 30 solid tumor antigens have been appraised for CAR T cell therapy such as neoantigens (e.g., mutated sequences), or tumor selective antigens (i.e., those with enriched expression on neoplastic cells but low expression on normal cells), and oncofetal or developmental antigens. The tumor-selective antigen is an attractive antigen in this list as it includes targets that are overexpressed on transformed cells, while expressed at low levels on normal tissues. Unfortunately, most of the antigens are intracellular and not available for CARs (Gill et al. 2016). Antigen heterogeneity is a major limitation to all of TAAs in solid tumors; it means the expression of the antigen on the cells within a certain tumor is variable. For instance, mesothelin is expressed on >90% of epithelial malignant mesothelioma tumor cells, however this antigen is expressed on lower percentages of tumor cells in breast, ovarian, and lung cancer tumors. A critical agent with strong relationship for solving the heterogeneity problem is the extent to which CAR-cell therapy can trigger "antigen spreading" and/or can induce indirect tumor killing. Epitope or antigen spreading is an action in which CAR T cells induce the generation of other endogenous antitumor CD_8 T cells.

Indirect killing is done by activation of macrophages, tumoricidal neutrophils, or natural killer (NK) cells via cytokines released after CAR engagement. Another problem with solid tumors is successful trafficking. Despite hematologic malignancies, CAR T cells should pass from the blood into solid tumor sites successfully. Therefore, and despite antigen heterogeneity or loss, CAR T cells must infiltrate the stromal components of solid tumors in order to evoke TAA-specific cytotoxicity. Successful trafficking depends on coupling of adhesion receptors on T cells and tumor endothelium, a suitable expression, and a match between the chemokines secreted by the tumors and the chemokine receptors on the CAR (primarily CCR5 and CXCR3). Unfortunately, tumors produce very small amounts of CCR5 and CXCR3 ligands and there is often an incompatibility between chemokine and receptor that results in inefficient targeting of the CXCR3 high CD_8 CAR T cells to

tumor sites. Hence, an approach to overcome this obstacle is to design CAR T cells that coexpress better-matched chemokine receptors. In order to solve this problem, several research teams have demonstrated the use of oncolytic viruses armed with chemotactic chemokines to attract CAR T cells to tumor sites Fortunately, oncolytic viruses (OVs) are effective agents for the treatment of solid tumors. Herpes virus expressing GM-CSF is approved by the FDA for advanced melanoma oncolytic (Andtbacka et al. 2015). Moreover, two research teams have demonstrated that introducing CCR2b into CARs and injection of these CARs into tumors that produce large amounts of CCL2 causes an increased intratumoral migration of CAR T cells and better tumor destruction (Knochelmann et al. 2018). Although, early trials of CAR T cells for solid tumors have not indicated the same success as seen in the leukemia trials, a better intuition of the various obstacles seen in solid tumors can lead to advances in engineering or designing effective CAR in clinical trial.

One procedure is to design CAR-T cells to work only in the tumor site. Han et al. developed the "masked CAR" system, which includes a masking peptide that blocks a protease-sensitive linker and the antigen-binding site. Researches have demonstrated that proteases that are generally active in the tumor microenvironment (TME) (probably inactive in normal tissue) can split the linker and release the masking peptide. This permits CAR T cells to identify target antigens only at the tumor site (Han et al. 2017). Another strategy that may be effective to improve CAR-T cell anti-tumor activity is performance of switch receptors that alter pro-tumor into anti-tumor signals. For instance, transfer of a PD1-CD_{28} receptor such as a shorten extracellular domain of PD1 and cytoplasmic signaling domains and the transmembrane of CD_{28} into CAR-T cells can increase CAR-T cell anti-tumor activity, which leads to a promising result for future clinical research (Morgan and Schambach 2018). Hence, CAR-T cells can be the "next generation" of cancer hallmarks by increasing CAR-T cells anti-tumor activity via engineering methods such as T cell persistence, T cell infiltration into solid tumors, and recruitment/activation of additional anti-tumor immune cells that can handle the mechanisms of tumors for creating an immunosuppressive niche. Researches on the future of the CAR T cell including the design and construct of general CAR T cells using novel gene-editing techniques like CRISPR/Cas9 are ongoing. These cells represent strategies to ameliorate optimize T cell signaling, antigen-binding, and decrease immunogenicity. With the optimization of gene editing techniques, CRISPR/Cas9 edited CARs are leading into clinical trials: a PD-1 knockout CD_{19} CAR has been studied in phase I clinical trial (Martinez and Moon 2019).

3 Injection Route for Cell Delivery System

Generally, there are three routes for cell transplantation including injection, spray, and using bio-scaffold. Injection is a technique for delivering medicines and it is categorized into subcutaneous (SC), intramuscular (IM), intravenous (IV), intraperitoneal (IP), intraarticular (IA), intracavernous (IC), intracardiac and other candidate injection routes. The injection route is selected based on the type of disease and therapeutic targets. For instance, subcutaneous injections are used for several reasons, including immunizations and target site (Cooper et al. 2000). An injection system needs a suitable injection device and the injectable medical fluid products. Injection methods through each route have advantages and disadvantages that are beyond the scope of this paper. However, and generally, it has been accepted that injections are among the most common medical procedures as the delivery system. The injection devices can be classified based on their function, site of delivery, and mechanism of action for cell delivery. Injection can be applied for free cell suspension and/or encapsulated cells in combinational cell-based products. However, many of the approved cellular products for clinical applications do not have a homing improvement injection.

Injection can be administered for two distinct purposes including the systematical and local

delivery aims (Golchin and Farahany 2019). Systematical injection is administered for several FDA approved cellular products and it can be the best and ultimate cell delivery system for cell-based therapeutic approaches if its shortcomings are solved (Golchin and Farahany 2019). Transplanted cells in systemic injection, that is done via IV injection, is not free of drawbacks such as exposure of cells to the inflammatory microenvironment, immune destruction, dispersion through the impaired local vascular system, trap in other organs, loss of performance, apoptosis, and less homing in the target site. For instance, MSCs can be delivered systemically; however, the majority of cells will be trapped by the lungs after infusion (Peltzer et al. 2018). Therefore, most of the injected MSCs are eliminated, and that was not detectable 1–4 weeks after transplantation (Peltzer et al. 2018; Kurtz 2008). Therefore, the lung, lymph nodes, kidney, spleen, liver, heart are the common organs for trapping injected cells, especially stem cells, in systemically injection (Golchin et al. 2017; Shin et al. 2016; Farrell et al. 2017). In this route, cell dosage and site of injection are the important options for therapeutic efficacy enhancement. For instance, hMSC injection in border zone (BZ) of ischemic limb in mice showed a significant enhancement of cell engraftment and paracrine factor secretion compared with injection in ischemic region (IR) (Shin et al. 2016). Azficel-T as the first cellular therapy autologous FDA approved product that is used to improve the appearance of wrinkles that appear from the sides of the nose to the corners of the mouth (smile lines) is injected ID through a specific therapeutic protocol (Golchin and Farahany 2019; Schmidt 2011). The site of injection effectively stimulated vessel generation, enhanced blood perfusion in the IR, and enabled the cell dosage reduction as well. However, local injection has limited usage for some special diseases and again and immunological responses make it responsible for some of shortcoming for injected cell efficacy.

Specifically, the design of a suitable injectable cell-based product needs to takes into account options, tools, and materials (Amer et al. 2017). The important options for the design of an acceptable injectable cell delivery system include cell density, injection volume, cell suspension vehicle, engineering of injector device, and optimization of its features (See Fig. 2). Sterility, stability, the viscosity of the product, safety, and effective dose of cells are the main components which should be taken into account.

Generally, there is no agreement regarding the optimal cell number to be transplanted, because that depends on cell type, disease, and properties of product. For instance, azficel-T that contains 1.8×10^7 autologous fibroblasts in 1.2 mL of its suspension, has been administered for injection of

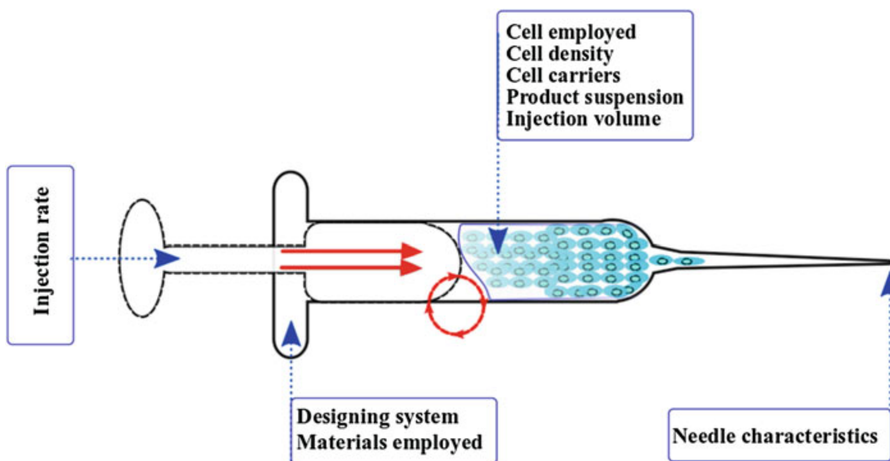

Fig. 2 Schematic design of injectable cell-based product and its important items

0.1 mL ID per linear centimeter into the nasolabial fold wrinkles, whereas Allocord minimum dose contains 2.5×10^7 nucleated cells/kg at cryopreservation in which multiple units may be required in order to achieve the appropriate therapeutic dose (Research C for BE and. Cellular and Gene Therapy Products 2018).

4 Injectable Based Cell Delivery Systems and Cell-Based Therapy Products

As mentioned above, among various types of cell carriers for therapeutic application, injectable carriers are highly preferred in cell and tissue-based therapy scenarios. In addition to the cell homing facilitation, injectable carriers can enhance cell proliferation, viability, functionality, and dressing of irregular shape of target sites. An injectable cell carrier allows replacing invasive surgical procedures with a simple injection with minimized surgical intervention, discomfort, risk of infection and cost of treatment (Zhang 2017). Although, injectable biomaterials offer many advantages for cell product administration, there are challenges in biomaterials science.

Microcarriers and hydrogels form two main groups of cell carriers that are extensively explored as cell carriers in RG. These constructs can encapsulate cells and regulate cells function and their fate. Perhaps the most important step to facilitate cell delivery through injection is biomaterial selection. There are various biomaterials with different properties that can be used depending on objective (Liow et al. 2016; Devasani et al. 2016; Dimatteo et al. 2018; Kim and Cho 2018). There are reports of clinical use of hydrogels including hyaluronic acid, fibrin, collagen, gelatin, alginate, poly (2-hydroxypropyl methacrylate), poly (acrylic acid), poly (ethylene glycol), hydroxyethyl cellulose, carboxymethylcellulose, and poly (2-hydroxyethyl methacrylate) in the recent regenerative medicine studies (Li and Mooney 2016). Some of the common biomaterials used in the design of cell injectable products are listed below (Marquardt and Heilshorn 2016a).

- Cardiovascular: Alginate, Chitosan/β-glycerophosphate (β-GP) gel, poly Nisopropylacrylamide (PNIPAM)/acrylic acid/2-hydroxylethyl methacrylate-poly(ε--caprolactone), Collagen.
- Cartilage: Methacrylate-chitosan-based material (MeCG), Methacrylate-gelatin hydrogel, poly (ethylene oxide) diacrylate, chitosan/β-GP/hydroxyethyl cellulose hydrogel, chitosan-poly (vinyl alcohol) copolymer hydrogel.
- Nervous system: fibrin hydrogels, Matrigel, hyaluronic acid-methylcellulose (HAMC) hydrogel, HA hydrogel, HA-PLL gel, self-assembling peptide K2(QL)6 K2 (QL6).
- Bone: PNIPAM, Gelatin, Alginate hydrogel, Pluronic F127, chitosan/collagen/β-GP, calcium phosphate cement.
- Retinal: hyaluronan-methyl cellulose (HAMC) hydrogel.
- Muscle: collagen/chitosan/βGP, composite synthetic polymers PNIPAM/acrylic acid/2-hydroxyethyl methacrylate-oligomers, small intestinal submucosa, and fibrin.
- Cutaneous wound: gelatin microspheres, poly (ether urethane) hydrogel, Carboxymethyl-hexanoyl chitosan hydrogel, chitosan chloride hydrogel.

As mentioned, there are several approved injectable cell-based therapeutics that recently are used in clinical treatment. These injectable cell-based therapeutics are listed in Table 1 and a summary of their important specification was given. Several products are currently available in different countries but have the same marketing authorization holder, including in YESCARTA, KYMRIAH, IMLYGIC, RMS Ossron/OSSGROW and Chodron/CARTIGROW.

5 Conclusion and Outlook

Ideal cell delivery strategies for injectable cell-based products are required to boost the efficacy

Table 1 List of injectable Cell/Tissue/Gene based products with marketing authorization (MA)

Name (MA holder)	Product description and indication(s)	Dosage and administration	Date of MA	Additional information
Chondrocytes -T – Ortho-ACI (Orth cell Pty Ltd)	Autologous cultured chondrocytes for use in treatment of cartilage lesions associated with the knee, patella and ankle. Each single-use vial contains approximately 2–5 $\times$ 10^6 cells aseptically processed and suspended in 1.0 ml of assembly medium, which consists of sterile, buffered, phenol red-free Dulbecco's modified eagle medium nutrient mixture F12 (DMEM F12) supplemented with 0.02% gentamicin, 0.3% ascorbic acid and 10% autologous serum. The chondrocyte cell suspension is applied to a porcine-derived type I/III collagen scaffold for implantation into the cartilage defect(s)	The cell dose is dependent on the extent of cartilage lesion (s), with patients generally receiving between 4 and 10 $\times$ 10^6 cells /implantation procedure	26-Mar-2017 Australia	Click Here
Prochymal (mesoblast international sarl)	The world's first stem cell drug Allogeneic ex-vivo cultured adult human MSC s for the management of acute graft versus host disease (GvHD) in pediatric patients The important nonmedicinal ingredients are dimethyl sulfoxide (DMSO), human serum albumin (HSA), and Plasmalyte A	Liquid containing 100 $\times$ 10^6hMSCs /15 mL The diluted product contains 2.5 $\times$ 10^6hMSCs Route of administration is intravenous infusion The dose of cells that is delivered to a patient is based on the patient's weight. The frozen cells are thawed, diluted, and transferred to an infusion bag for intravenous administration. The recommended dose of PROCHYMAL is 2 $\times$ 10^6hMSC/kg (actual body weight) administered intravenously at a controlled rate of 4–6 mL/min by infusion pump for patients weighing 35 kg and over.	02-May-2015 Canada	Click Here
CARTIGROW™ (Chondron ACI) (RMS Regrow)	Autologous cultured cartilage cells for treatment of articular cartilage defects	Autologous chondrocytes are removed from the patient's (knee, ankle or shoulder), and cultured to proliferate and multiply in the in vitro condition. After 3–5 weeks to obtain sufficient number of cells (usually between 12–48 $\times$ 10^6), then re-implanted in the area of	Apr-2017 India	Click Here

(continued)

Table 1 (continued)

Name (MA holder)	Product description and indication(s)	Dosage and administration	Date of MA	Additional information
		damaged cartilage in a minimally invasive surgical procedure called autologous chondrocyte implantation (ACI)		
ALOFISEL (Takeda pharma A/S)	Expanded allogeneic adipose stem cells as a suspension for injection for the treatment of complex perianal fistulas in patients with Crohn's disease **List of excipients:** Dulbecco's modified Eagle´s medium (DMEM) (containing amino acids, vitamins, salts and carbohydrates), human albumin **Incompatibilities:** In the absence of compatibility studies, this medicinal product must not be mixed with other medicinal products	For intralesional use in a surgical environment under anesthesia (general or regional). A single dose of Alofisel consists of 120×10^6 cells distributed in 4 vials. Each vial contains 30×10^6 cells in 6 mL of suspension. The full content of the 4 vials must be administered for the treatment of up to two internal openings and up to three external openings. This means that with a dose of 120×10^6 cells it is possible to treat up to three fistula tracts that open to the perianal area	27-Mar-2018 European Union	Click Here
TEMCELL HS (JCR Pharmaceuticals Co. Ltd.)	Allogeneic mesenchymal stromal cells for treatment of acute graft versus host disease (aGvHD)	For all patients, eight doses of 2×10^6 cells/kg, delivered as an intravenous infusion. For patients with persistent symptoms beyond 4 weeks, a further weekly dose of 2×10^6 cells/kg may be given for four additional weeks. Subcutaneously administered	Sept- 2015 Japan	Click here Click here
CARTISTEM	Allogeneic umbilical cord blood-derived MSC (hUCB-MSC) **Indication:** Treatment of knee cartilage defects caused by degenerative osteoarthritis or repeated trauma	**Dosage form**: a suspension of hUCB-MSC in hyaluronic acid (gel matrix) **DOSAGE:** 2.5×10^6 cells/ 500 μl/cm^2 **Active Ingredient:** hUCB-MSC ls, 7.5×10^6 cells/vial **Rout of administration**: Local injection in area of the knee cartilage defect	18-Jan-2012 South Korea	Click Here Click Here
Cellgram	Autologous bone marrow-derived MSC s for acute myocardial infarction patients (improvement of LVEF (left ventricular ejection fraction) **Active ingredient:** Autologous bone marrow-derived MSC	**Injection route:** Intramuscular **Dosage:** 50×10^6cells/10 ml, 2-time injection**.** Under 60 kg = 10 mL/5×10^7 cells 61 ~ 80 kg = 14 mL/7×10^7 cells (over 81 kg = 18 mL/ 9×10^7 cells)	July 2011 South Korea	Click Here Click Here
CardioRel	This cellular product is an autologous product designed for early or planned intervention in patients of	10^6 cells injection to target cite	2010 India	Click Here Click Here

(continued)

Table 1 (continued)

Name (MA holder)	Product description and indication(s)	Dosage and administration	Date of MA	Additional information
	post-acute myocardial infarction (MI) providing mono-nuclear and BM-MSCs for cardiac regeneration			
CureSkin Injection (S.Biomedics Co., Ltd.)	Autologous dermal fibroblasts Improvement of depressed scars associated with acne healing **Ingredients:** Self-derived dermal fibroblast **Package Unit:** 1 ml vial	**Injection route**: Intra-dermal injection 3 times injection biweekly (recommend) **Dose:** Dose: 2×10^7cells/ml	11-May-2010 South Korea	Click Here Click Here Click here
Cupistem (Anterogen)	Autologous adipose tissue-derived MSC for Crohn's fistula	**Composition:** 3×10^7cell/mL. Orphan drug	18-Jan-2012 South Korea	Click Here
NEURONATA-R® (Corestem, Inc.)	Autologous BM-MSC therapy for Amyotrophic Lateral Sclerosis	Approximately 8×10^7 for an adult patient	30-Jul-2014	Click Here
	Acts a neuroprotective effect and relieves progression of a disease through prevention of motor nerve cell, survival extension of motor neurons, releasing nerves inflammatory and immune regulation function	Orphan product	South Korea	
Immuncell-LC (GC Cell Corp.)	Autologous activated T-cell for liver cancer (hepatocellular carcinoma)	**Usage/dosage:** Gently shake the bag 3–4 times so that the cells become completely suspended in the agent	6-Aug-2007 South Korea	Click Here Click Here
	Ingredients: In 200 mL Activated T-lymphocyte Human plasma albumin	Use a needle smaller than 22G for IV injection. Make sure dose is fully administered to the patient in 1 h		
	Physiological saline	A 200 mL dose contains 1×10^9–2×10^{10} cells Administer 1 dose every week for the first 4 weeks, 1 dose every 2 weeks for the next 8 weeks. 1 dose every 4 weeks for the next 16 weeks, and 1 dose every 8 weeks for the next 32 weeks, for a total of 16 doses		
OSSGROW™ (Ossron ABI) (RMS Regrow)	Autologous cultured osteoblasts for avascular necrosis of hip	–	Apr-2017 India	(Cuende et al. 2018)
APCEDEN (APAC Biotech)	Autologous monocyte-derived dendritic cell for treatment of prostate, ovarian, colorectal and non-small cell lung carcinoma	The complete treatment regimen consists of six doses administered over a period of 14 weeks. The cell suspension will be injected with 100 ml of	Mar-2017 India	Click here (Kumar et al. 2017)

(continued)

Table 1 (continued)

Name (MA holder)	Product description and indication(s)	Dosage and administration	Date of MA	Additional information
		normal saline and patient will be infused intravenously **Infusion procedure for apceden** Normal saline (100 ml), IV set, Emeset injection, disposable gloves, disposable syringes, sprit swab 4 packets		
Stempeucel® (Stempeutics Research)	Ex-vivo cultured adult allogeneic MSCs for treatment of critical limb ischemia due to Thromboangiitis Obliterans (Buerger's disease). Fifth "off-the-shelf" stem cell product which received approval by any regulatory body in the world. The first fully approved "off-the-shelf" allogeneic cell therapy was a MSC product. A Phase III read product.	**Dose-finding phase II study:** 2×10^6 cells/kg **Administration rout:** Intramuscular administration of allogeneic human bone marrow-derived MSC population (Stempeucel) in critical limb ischemia due to Buerger's disease	2017, In market, limited release, India	Click here Click here
CreaVax-RCC® (JW CreaGene Corporation)	Autologous dendritic cells for metastatic renal cell carcinoma	5 × 107 cells (1 vial of CreaVax-RCC Inj.) are to be administered 4 times at the interval of once every 2 weeks (The duration of administration may be adjusted in accordance with the decision of the physician	15-May-2007 South Korea	Click here
Chondron™ (Sewon Cellontech Co., Ltd.)	Cultured autologous chondrocytes for focal cartilage defect of knee, can be used with or without fibrin glue	One to six vials, which contains more than 12 × 106 chondrocytes /vial, are provided for implantation	30-Jan-2001 South Korea	Click Here
1.HPC, Cord Blood (MD Anderson Cord Blood Bank) 2. Ducord (HPC, Cord Blood) (Duke University School of Medicine)	For use in unrelated donor hematopoietic progenitor cell transplantation procedures in conjunction with an appropriate preparative regimen for hematopoietic and immunologic reconstitution in patients with disorders affecting the hematopoietic system that are inherited, acquired, or result from myeloablative treatment	**Dosage and administration:** Each unit contains a minimum of 9×10^8 total nucleated cells with at least 1.25×10^6 viable $CD34^+$ cells at the time of cryopreser vation. The exact pre-cryopreser vation nucleated cell content of each unit is provided on the container label and accompanying records. For intravenous use only. Do not irradiate. **HPC, Cord Blood has the following inactive ingredients**: dimethyl sulfoxide (DMSO), citratephosphatedextrose (CPD), hydroxyethyl starch, and Dextran 40.	06-Jun-2018 4-Oct-2012 USA	Click Here Click Here

(continued)

Table 1 (continued)

Name (MA holder)	Product description and indication(s)	Dosage and administration	Date of MA	Additional information
1. ALLOCORD (SSM cardinal Glennon Children's Medical Center)	For use in unrelated donor hematopoietic progenitor cell transplantation procedures in conjunction	**Dosage and administration:** Each unit contains a minimum of 5×10^8 total nucleated cells with at least 1.25×10^6 viable $CD34^+$ cells at the time of cryopreservation. The exact pre-cryopreservation nucleated cell content of each unit is provided on the accompanying records	30-May-2013	Click here
2. Clevecord (HPC, Cord Blood) (Cleveland Cord	**The active ingredient** is hematopoietic progenitor cells which express the cell surface marker CD_{34}. The potency of cord blood is determined by measuring the numbers of total nucleated cells (TNC) and CD34+ cells, and cell viability.		1-Sep-2016	Click here
3. HPC, Cord Blood (Bloodworks)			28-Jan-2016	Click Here
4. HPC, Cord Blood (LifeSouth Community Blood Centers, Inc.)	**Inactive ingredients**: PrepaCyte-CB separation solution, citrate-phosphate-dextrose, dimethyl sulfoxide (DMSO) and Dextran 40. When prepared for infusion according to instructions, the product contains the following inactive ingredients: PrepaCyte-CB separation solution, citrate-phosphate-dextrose, Dextran 40, human serum albumin, and residual DMSO	For intravenous use only. Do not irradiate	13-Jun-2013	Click Here
5. Hemacord (HPC, Cord Blood) (New York Blood Center, Inc.)		**The active ingredient** is hematopoietic progenitor cells which express the cell surface marker CD_{34}. The potency of cord blood is determined by measuring the numbers of total nucleated 325 cells (TNC) and CD34+ cells, and cell viability	1-Nov-2011	Click Here
		Inactive ingredients: PrepaCyte-CB separation solution, citrate- phosphate-dextrose, dimethyl sulfoxide (DMSO) and Dextran 40. When prepared for infusion 334 according to instructions, the product contains the following inactive ingredients: PrepaCyte-CB separation solution, citrate-phosphate-dextrose, Dextran 40, human serum albumin, and residual 336 DMSO	USA	
Laviv® (Azficel-T) (Fibrocell Technologies, Inc.)	Autologous fibroblasts for improvement of the appearance of moderate to severe nasolabial fold wrinkles in adults	**Dosage and administration**	21-Jun-2011	Click here
	The ingredients in LAVIV:	A single vial of LAVIV contains approximately 18×10^6 autologous fibroblasts in a 1.2 ml suspension, sufficient to administer 1 ml of product	USA	
	LAVIV is made from your own skin cells placed in a mixture of water and salts. Antibiotics (amphotericin and gentamicin), bovine serum (from cattle) and dimethyl sulfoxide (DMSO) are used during processing. Trace amounts of these ingredients may be present in LAVIV	For autologous intradermal injection only		
		Only healthcare providers who have completed a Fibrocell-approved training program should administer LAVIV		
		Inject LAVIV at 0.1 ml / linear centimeter into the nasolabial fold wrinkles. The recommended treatment regimen is three treatment		

(continued)

Table 1 (continued)

Name (MA holder)	Product description and indication(s)	Dosage and administration	Date of MA	Additional information
		sessions at 3–6 week intervals.		
PROVENGE (sipuleucel-T) (Dendreon Corporation)	Autologous cellular immunotherapy indicated for the treatment of asymptomatic or minimally symptomatic metastatic castrate resistant (hormone refractory) prostate cancer	**Dosage and administration:** Each dose of PROVENGE contains a minimum of 50×10^6 autologous $CD54^+$ cells activated with PAPGM-CSF, suspended in 250 mL of Lactated Ringer's Injection, USP in a sealed, patient-specific infusion bag. For Autologous Use Only.	29-Apr-2010	Click here
		Administer 3 doses at approximately 2 week intervals	USA	
		Premedicate patients with oral acetaminophen and an antihistamine such as diphenhydramine		
		Before infusion, confirm that the patient's identity matches the patient identifiers on the infusion bag		
		Do not initiate infusion of expired PROVENGE		
		Infuse PROVENGE intravenously over a period of approximately 60 min. Do Not Use a Cell Filter		
		Interrupt or slow infusion for acute infusion reactions, depending on the severity of the reaction		
1.Kymriah (Novartis Pharmaceuticals Australia Pty Ltd (	CD19-directed genetically modified autologous T-cell immunotherapy indicated for the treatment of pediatric and young adult patients up to 25 years with B-cell precursor acute lymphoblastic leukemia (ALL) that is refractory, in relapse post-transplant or in second or later relapse and for the treatment of adult patients with relapsed or refractory diffuse large B-cell lymphoma (DLBCL) after two or more lines of systemic therapy	**Dosage and administration**	19-Dec-2018	Click here Click here
2. KYMRIAH (NOVARTIS PHARMACEUTICAL S CANADA INC)		For autologous use only. For intravenous use only		
		Administer a lymphodepleting regimen if needed before infusion of KYMRIAH. Do NOT use a leukodepleting filter	USA	
		Verify the patient's identity prior to infusion		
		Premedicate with acetaminophen and an H1-antihistamine. Confirm availability of tocilizumab prior to infusion		
		Dosing of KYMRIAH is based on the number of chimeric antigen receptor (CAR)-positive viable T cells		

(continued)

Table 1 (continued)

Name (MA holder)	Product description and indication(s)	Dosage and administration	Date of MA	Additional information
		Pediatric and young adult B-cell ALL (up to 25 years of age)		
		For patients 50 kg or less, administer 0.2–5.0 × 10^6CAR-positive viable T cells /kg body weight intravenously		
		For patients above 50 kg, administer 0.1–2.5 × 10^8 Total CAR-positive viable T cells (non-weight based) intravenously		
		Adult relapsed or refractory diffuse large B-cell lymphoma		
		Administer 0.6–6.0 × 10^8 CAR-positive viable T cells intravenously		
Gendicine (Shenzhen SiBiono GeneTech Co. Ltd.)	Recombinant adenovirus expressing p53 for treatment of head and neck squamous cell carcinoma	169,571 vials (1.0 × 10^{12} vector particles /vial) have been used to treat patients	Oct-2003 China	Click here
LUXTURNA (Spark Therapeutics Ireland Ltd.)	Adeno-associated virus vector-based gene therapy indicated for the treatment of patients with confirmed biallelic RPE65 mutation-associated retinal dystrophy	**Dosage and administration:** The recommended dose of LUXTURNA for each eye is 1.5 × 10^{11}vector genomes (vg), administered by sub retinal injection in a total volume of 0.3 mL	23-Nov-2018 USA	Click here
		Perform sub retinal administration of LUXTURNA to each eye on separate days within a close interval, but no fewer than 6 days apart.		
		Dosage forms and strengths		
		LUXTURNA is a suspension for sub retinal injection, supplied in a 0.5 mL extractable volume in a single-dose 2 mL vial for a single administration in one eye. The supplied concentration (5 × 10^{12}vg/mL) requires a 1:10 dilution prior to administration. The diluent is supplied in two single-use 2-mL vials		
		Component of vial: Each single-dose vial of LUXTURNA contains 5 × 10^{12} vector genomes (vg) /mL, and the excipients		

(continued)

Table 1 (continued)

Name (MA holder)	Product description and indication(s)	Dosage and administration	Date of MA	Additional information
		180 mM sodium chloride, 10 mM sodium phosphate, and 0.001% Poloxamer 188 (pH 7.3), in a 0.5-mL extractable volume. LUXTURNA requires a 1:10 dilution prior to administration. After dilution, each dose of LUXTURNA consists of 1.5×10^{11} vg in a deliverable volume of 0.3 mL. The diluent, supplied in 1.7 mL extractable volume /vial in two 2-mL vials, is composed of sterile water containing 180 mM sodium chloride, 10 mM sodium phosphate, and 0.001% Poloxamer 188 (pH 7.3). LUXTURNA may also contain residual components of HEK293 cells including DNA and protein and trace quantities of fetal bovine serum		
YESCARTA (axicabtagene ciloleucel) (Kite Pharma, Incorporated)	A CD19-directed genetically modified autologous T cell immunotherapy indicated for the treatment of adult patients with relapsed or refractory large B-cell lymphoma after two or more lines of systemic therapy, including diffuse large B-cell lymphoma (DLBCL) not otherwise specified, primary mediastinal large B-cell lymphoma, high grade B-cell lymphoma, and DLBCL arising from follicular lymphoma	**Dosage And Administration:** For autologous use. For IV use only	18-Oct-2017	Click here
		Do NOT use a leukodepleting filter	USA	
		Administer a lymphodepleting regimen of cyclophosphamide and fludarabine before infusion of YESCARTA		
		Verify the patient's identity prior to infusion		
		Premedicate with acetaminophen and an H1-antihistamine		
		Confirm availability of tocilizumab prior to infusion.		
		Dosing of YESCARTA is based on the number of chimeric antigen receptor (CAR)-positive viable T cells		
		The target YESCARTA dose is 2×10^{6} CAR-positive viable T cells /kg body weight, with a maximum of 2×10^{8} CAR-positive viable T cells		
		Administer YESCARTA in a certified healthcare facility		

(continued)

Table 1 (continued)

Name (MA holder)	Product description and indication(s)	Dosage and administration	Date of MA	Additional information
		Dosage forms and strengths		
		YESCARTA is available as a cell suspension for infusion		
		YESCARTA comprises a suspension of 2×10^6 CAR-positive viable T cells / kg of body weight, with a maximum of 2×10^8 CAR-positive viable T cells in approximately 68 ml		
IMLYGIC (Talimogene laherparepvec) (Amgen Inc.)	Genetically modified oncolytic viral therapy indicated for the local treatment of un-resect able cutaneous, subcutaneous, and nodal lesions in patients with melanoma recurrent after initial surgery. **Component of vial:** Each vial contains 1 mL deliverable volume of IMLYGIC at either 1×106 (1×10^6) PFU/mL or1×108 (100×10^6) PFU /mL concentrations and the following excipients: d i-sodium hydrogen phosphate dehydrate (15.4 mg), sodium dihydrogen phosphate dehydrate (2.44 mg), sodium chloride (8.5 mg), myo-inositol (40 mg), sorbitol (20 mg), and water for injection. The 10^6 (1×10^6) PFU /mL vial of IMLYGIC contains a clear to semi-translucent liquid following thaw from its frozen state. The 10^8 (100×10^6) PFU /mL vial of IMLYGIC contains a semi-translucent to opaque liquid following thaw from its frozen state. The liquid in each vial may contain white, visible, variously shaped, virus-containing particles. Each vial of IMLYGIC may also contain residual components of VERO cells including DNA and protein and trace quantities of fetal bovine serum	**Dosage and administration**	16-Dec-2015	Click here
		Administer IMLYGIC by injection into cutaneous, subcutaneous, and/or nodal lesions. Recommended starting dose is up to a maximum of 4 mL of IMLYGIC at a concentration of 10^6 (1×10^6) plaque-forming units (PFU) /mL. Subsequent doses should be administered up to 4 mL of IMLYGIC at a concentration of 108 (100×10^6) PFU /mL	USA	
		Dosage forms and strengths: Injection: 10^6 (1×10^6) PFU/mL, 10^8 (100×10^6) PFU/mL in single-use vials		

and reproducibility of the products. Studies have shown that several characters of injection system including injection rate, needle size, and cell carrier have a significant impact on the percentage of cell dose delivered as viable cells, cellular health, and functionality in post-ejection (Amer 2017). As mentioned, unpleasant conditions in injury or disease, and the delivery process itself too, can significantly hinder the viability and functional impact of transplanted cells (Marquardt and Heilshorn 2016b). Recently, stem cell therapy has opened a new viewpoint in RG. Hence, there are more studies on different phases of preclinical and clinical stages focused on introducing cell-based products to market. The use of injectable stem cell carriers can improve stem cell viability and function during the transplantation and treatment.

Many of the currently approved cell-based products are free of biomaterials or especial adjuvants for enhancing cell homing and functionality. However, it is predicted that injectable materials can improve the rate of clinical translation for the CT by increasing grafted cell survival and functionality. Therefore, it is clear that an ideal cell-based therapy is comprised of using a suitable cell source, biomaterials (as adjuvant or native niche stimulator) and biological factors (Golchin and Farahany 2019). The combination of the effective factors, among others, may also influence the fate of injected cells, and subsequently the success of cell-based therapies.

Acknowledgement This study is related to the project NO. 1397/69252 From Student Research Committee, Shahid Beheshti University of Medical Sciences, Tehran, Iran. We also appreciate the "Student Research Committee" and "Research & Technology Chancellor" in Shahid Beheshti University of Medical Sciences for their financial support of this study.

Author Contributions A.G. conceptualized the outline and contents of the article. All authors contributed to writing and editing the manuscript. S.H. had an advisory role.

Conflict of Interest Authors don't have conflict of interest.

References

Amer MH (2017) Development of injectable cell delivery systems for high accuracy cell therapy applications. [cited 2019 May 5]. Available from: http://eprints.nottingham.ac.uk/39839/

Amer MH, Rose FRAJ, Shakesheff KM, Modo M, White LJ (2017) Translational considerations in injectable cell-based therapeutics for neurological applications: concepts, progress and challenges. npj Regen Med 2 (1):23. [Internet] [cited 2019 Feb 27]. Available from: http://www.nature.com/articles/s41536-017-0028-x

Andtbacka RHI, Kaufman HL, Collichio F, Amatruda T, Senzer N, Chesney J et al (2015) Talimogene Laherparepvec improves durable response rate in patients with advanced melanoma. J Clin Oncol 33 (25):2780–2788. [Internet] [cited 2019 May 2]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26014293

Bandeiras C, Cabral JM, Finkelstein SN, Ferreira FC (2018) Modeling biological and economic uncertainty on cell therapy manufacturing: the choice of culture media supplementation. Regen Med 13(8):917–933. [Internet] [cited 2019 Feb 8]. Available from: https://www.futuremedicine.com/doi/10.2217/rme-2018-0034

Barbash IM, Chouraqui P, Baron J, Feinberg MS, Etzion S, Tessone A et al (2003) Systemic delivery of bone marrow–derived mesenchymal stem cells to the infarcted myocardium. Circulation 108(7):863–868. [Internet] [cited 2019 May 2]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12900340

Belema-Bedada F, Uchida S, Martire A, Kostin S, Braun T (2008) Efficient homing of multipotent adult mesenchymal stem cells depends on FROUNT-mediated clustering of CCR2. Cell Stem Cell 2(6):566–575. [Internet] [cited 2019 May 2]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18522849

Chute JP (2006) Stem cell homing. Curr Opin Hematol 13 (6):399–406. [Internet] [cited 2019 May 2]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17053451

Cooper JA, Bromley LM, Baranowski AP, Barker SGE (2000) Evaluation of a needle-free injection system for local anaesthesia prior to venous cannulation. Anaesthesia 55(3):247–250. [Internet] [cited 2019 Feb 26]. Available from: http://doi.wiley.com/10.1046/j.1365-2044.2000.01210.x

Cuende N, Rasko JEJ, Koh MBC, Dominici M, Ikonomou L (2018) Cell, tissue and gene products with marketing authorization in 2018 worldwide. Cytotherapy 20 (11):1401–1413. [Internet] [cited 2019 Jun 3]. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1465324918306194

DeRenzo C, Gottschalk S (2019) Genetic modification strategies to enhance CAR T cell persistence for patients with solid tumors. Front Immunol 10:218. [Internet] [cited 2019 May 2]. Available from: https://www.frontiersin.org/article/10.3389/fimmu.2019.00218/full

Devasani S, Dev A, Rathod S, Deshmukh GR (2016) An overview of in situ gelling systems. [Internet] [cited 2019 May 4]. Available from: https://www.semanticscholar.org/paper/An-overview-of-in-situ-gelling-systems-Devasani-Dev/6c239f86991156ad22cdc4af8dcdbbd231902ab5

Dimatteo R, Darling NJ, Segura T (2018) In situ forming injectable hydrogels for drug delivery and wound repair. Adv Drug Deliv Rev 127:167–184. [Internet] [cited 2019 May 4]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29567395

Farrell K, Joshi J, Kothapalli CR (2017) Injectable uncrosslinked biomimetic hydrogels as candidate scaffolds for neural stem cell delivery. J Biomed Mater Res Part A 105(3):790–805. [Internet] [cited 2017 Oct 25]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27798959

Gill S, Maus MV, Porter DL (2016) Chimeric antigen receptor T cell therapy: 25 years in the making. Blood Rev 30(3):157–167. [Internet] [cited 2019 May 2]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26574053

Golchin A, Farahany TZ (2019) Biological products: cellular therapy and FDA approved products. Stem Cell Rev Rep 15:1–10. [Internet]. [cited 2019 Jan 10]. Available from: http://link.springer.com/10.1007/s12015-018-9866-1

Golchin A, Hosseinzadeh S, Roshangar L (2017) The role of nanomaterials in cell delivery systems. Med Mol Morphol 51(1):1–12. [Internet] [cited 2018 Jan 17]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29170827

Golchin A, Hosseinzadeh S, Ardeshirylajimi A (2018a) The exosomes released from different cell types and their effects in wound healing. J Cell Biochem 119(7):5043–5052. [Internet] [cited 2018 Mar 28]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29377240

Golchin A, Rekabgardan M, Taheri RA, Nourani MR (2018b) Promotion of cell-based therapy: special focus on the cooperation of mesenchymal stem cell therapy and gene therapy for clinical trial studies. In: Turksen K (ed) Cell Biology and Translational Medicine, Volume 4 Advances in Experimental Medicine and Biology, vol 1119. Springer, Cham, pp 103–118. [Internet] [cited 2019 Jun 4]. Available from: http://link.springer.com/10.1007/5584_2018_256

Golchin A, Hosseinzadeh S, Staji M, Soleimani M, Ardeshirylajimi A, Khojasteh A (2019) Biological behavior of the curcumin incorporated chitosan/poly(vinyl alcohol) nanofibers for biomedical applications. J Cell Biochem. [Internet] [cited 2019 Jun 4]. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1002/jcb.28808

Han X, Bryson PD, Zhao Y, Cinay GE, Li S, Guo Y et al (2017) Masked chimeric antigen receptor for tumor-specific activation. Mol Ther 25(1):274–284. [Internet] [cited 2019 May 2]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28129121

Hung S-C, Pochampally RR, Hsu S-C, Sanchez C, Chen S-C, Spees J et al (2007) Short-term exposure of multipotent stromal cells to low oxygen increases their expression of CX3CR1 and CXCR4 and their engraftment in vivo. PLoS One 2(5):e416. [Internet] [cited 2019 May 2]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17476338

Karp JM, Leng Teo GS (2009) Mesenchymal stem cell homing: the devil is in the details. Cell Stem Cell 4(3):206–216. [Internet] [cited 2019 May 2]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19265660

Kim B-S, Cho C-S (2018) Injectable hydrogels for regenerative medicine, Tissue Eng Regen Med. 15(5):511–512. [Internet] [cited 2019 May 4]. Available from: http://link.springer.com/10.1007/s13770-018-0161-7

Knochelmann HM, Smith AS, Dwyer CJ, Wyatt MM, Mehrotra S, Paulos CM (2018) CAR T Cells in solid tumors: blueprints for building effective therapies. Front Immunol 9:1740. [Internet] [cited 2019 May 2]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/30140266

Kumar S, Ponnazhagan S (2007) Bone homing of mesenchymal stem cells by ectopic α4 integrin expression. FASEB J 21(14):3917–3927. [Internet] [cited 2019 May 2]. Available from: http://www.fasebj.org/doi/10.1096/fj.07-8275com

Kumar C, Kohli S, Chiliveru S, Bapsy PP, Jain M, Suresh Attili VS et al (2017) A retrospective analysis comparing APCEDEN® dendritic cell immunotherapy with best supportive care in refractory cancer. Immunotherapy 9(11):889–897. [Internet] [cited 2019 Jun 3]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28838282

Kurtz A (2008) Mesenchymal stem cell delivery routes and fate. Int J Stem Cells 1(1):1–7. [Internet] [cited 2017 Apr 23]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24855503

Lapidot T, Dar A, Kollet O (2005) How do stem cells find their way home? Blood 106(6):1901–1910. [Internet] [cited 2019 May 2]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15890683

Lapteva L, Vatsan R, Purohit-Sheth T (2018) Regenerative medicine therapies for rare diseases. Transl Sci Rare Dis 3(3–4):121–132. [Internet] [cited 2019 Feb 8]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/30613470

Li J, Mooney DJ (2016) Designing hydrogels for controlled drug delivery. Nat Rev Mater 1(12):16071. [Internet] [cited 2019 May 4]. Available from: http://www.nature.com/articles/natrevmats201671

Liow SS, Dou Q, Kai D, Karim AA, Zhang K, Xu F et al (2016) Thermogels: in situ gelling biomaterial. ACS Biomater Sci Eng 2(3):295–316. [Internet] [cited 2019 May 4]. Available from: http://pubs.acs.org/doi/10.1021/acsbiomaterials.5b00515

Lopes AG, Sinclair A, Frohlich B (2018) Cost analysis of cell therapy manufacture: autologous cell therapies, part 2. BioProcess Int. [Internet] [cited 2019 Feb 8].

Available from: https://bioprocessintl.com/manufacturing/cell-therapies/cost-analysis-of-cell-therapy-manufacturing-autologous-cell-therapies-part-2/

Mahla RS (2016) Stem cells applications in regenerative medicine and disease therapeutics. Int J Cell Biol 2016 (1):–24. [Internet] [cited 2019 May 2]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27516776

Marquardt LM, Heilshorn SC (2016a) Design of injectable materials to improve stem cell transplantation. Curr Stem Cell Rep 2(3):207–220. [Internet] [cited 2019 May 4]. Available from: http://link.springer.com/10.1007/s40778-016-0058-0

Marquardt LM, Heilshorn SC (2016b) Design of injectable materials to improve stem cell transplantation. Curr Stem Cell Rep 2(3):207–220. [Internet] [cited 2019 May 5]. Available from: http://link.springer.com/10.1007/s40778-016-0058-0

Martinez M, Moon EK (2019) CAR T cells for solid tumors: new strategies for finding, infiltrating, and surviving in the tumor microenvironment. Front Immunol 10:128. [Internet] [cited 2019 May 2]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/30804938

Morgan MA, Schambach A (2018) Engineering CAR-T cells for improved function against solid tumors. Front Immunol 9. [Internet] [cited 2019 May 2]. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6217729/

Peltzer J, Aletti M, Frescaline N, Busson E, Lataillade J-J, Martinaud C (2018) Mesenchymal stromal cells based therapy in systemic sclerosis: rational and challenges. Front Immunol 9:2013. [Internet] [cited 2019 Feb 25]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/30271402

Ponte AL, Marais E, Gallay N, Langonné A, Delorme B, Hérault O et al (2007) The in vitro migration capacity of human bone marrow mesenchymal stem cells: comparison of chemokine and growth factor chemotactic activities. Stem Cells 25(7):1737–1745. [Internet] [cited 2019 May 2] Available from: http://www.ncbi.nlm.nih.gov/pubmed/17395768

Press Announcements – FDA awards 12 grants to fund new clinical trials to advance the development of medical products for the treatment of rare diseases. [cited 2019 Feb 8]; Available from: https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm621490.htm

Research C for BE and. Cellular and Gene Therapy Products. 2018 [cited 2018 May 6]. Available from: https://www.fda.gov/BiologicsBloodVaccines/CellularGeneTherapyProducts/default.htm

Ruster B, Gottig S, Ludwig RJ, Bistrian R, Muller S, Seifried E et al (2006) Mesenchymal stem cells display coordinated rolling and adhesion behavior on endothelial cells. Blood 108(12):3938–3944. [Internet] [cited 2019 May 2]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16896152

Schmidt C (2011) FDA approves first cell therapy for wrinkle-free visage. Nat Biotechnol 29(8):674–675. [Internet] [cited 2018 May 6]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21822225

Shin J-Y, Yoon J-K, Noh MK, Bhang SH, Kim B-S (2016) Enhancing therapeutic efficacy and reducing cell dosage in stem cell transplantation therapy for ischemic limb diseases by modifying the cell injection site. Tissue Eng Part A 22(3–4):349–362. [Internet] [cited 2019 Feb 25]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26824782

Sohni A, Verfaillie CM (2013) Mesenchymal stem cells migration homing and tracking. Stem Cells Int 2013:130763. [Internet] [cited 2019 May 2]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24194766

Wyles SP, Hayden RE, Meyer FB, Terzic A (2019) Regenerative medicine curriculum for next-generation physicians. npj Regen Med 4(1):3. [Internet] [cited 2019 Feb 7]. Available from: http://www.nature.com/articles/s41536-019-0065-8

Xia L, McDaniel JM, Yago T, Doeden A, McEver RP (2004) Surface fucosylation of human cord blood cells augments binding to P-selectin and E-selectin and enhances engraftment in bone marrow. Blood 104 (10):3091–3096. [Internet] [cited 2019 May 2]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15280192

Zhang Z (2017) Injectable biomaterials for stem cell delivery and tissue regeneration. Expert Opin Biol Ther 17 (1):49–62. [Internet] [cited 2019 May 4]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27805430

Adv Exp Med Biol - Cell Biology and Translational Medicine (2019) 7: 97–119
https://doi.org/10.1007/5584_2019_441
© Springer Nature Switzerland AG 2019
Published online: 15 November 2019

Stem Cell Therapy for Hepatocellular Carcinoma: Future Perspectives

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common types of cancer and results in a high mortality rate worldwide. Unfortunately, most cases of HCC are diagnosed in an advanced stage, resulting in a poor prognosis and ineffective treatment. HCC is often resistant to both radiotherapy and chemotherapy, resulting in a high recurrence rate. Although the use of stem cells is evolving into a potentially effective approach for the treatment of cancer, few studies on stem cell therapy in HCC have been published. The administration of stem cells from bone marrow, adipose tissue, the amnion, and the umbilical cord to experimental animal models of HCC has not yielded consistent responses. However, it is possible to induce the apoptosis of cancer cells, repress angiogenesis, and cause tumor regression by administration of genetically modified stem cells. New alternative approaches to cancer therapy, such as the use of stem cell derivatives, exosomes or stem cell extracts, have been proposed. In this review, we highlight these experimental approaches for the use of stem cells as a vehicle for local drug delivery.

Keywords

Conditioned media · Engineered MSCs · Exosomes · HCC · Stem cells

Authors Hoda Elkhenany and Ahmed Shekshek have equally contributed to this chapter.

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Abbreviations

ABCG2	ATP-binding cassette sub-family G member 2
ADC	Apparent diffusion coefficient
ADSCs	Adipose-derived stem cells
AFP	Alpha-fetoprotein
AM	Amniotic membrane
BM-MSCs	Bone marrow-derived mesenchymal stem cells
CAF	Cancer-associated fibroblasts

CA-MSCs	Cancer-associated MSCs
CDKN2B	Cyclin-dependent kinase inhibitor 2B transcript
CDX2	Homeobox transcription factor 2
CM	Conditioned medium
CSCs	Cancer stem cells
DIRAS3	GTP-binding RAS-like 3
EMT	Epithelial-mesenchymal transition
GATA6	GATA-binding protein 6
hAECs	Amniotic membrane-derived epithelial stem cells
hAMPE	hAECs protein extract
hAMSCs	Human amniotic membrane-derived mesenchymal stem cells
HCC	Hepatocellular carcinoma
HPCs	Hepatic progenitor cells
HSP	Heat shock protein
MDR	Multidrug resistance
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
MSCs	Mesenchymal stem cells
NLK	Nemo-like kinase
PACDC	Pancreatic adenocarcinoma-derived cell lines
PCNA	Proliferating cell nuclear antigen
PEDF	Pigment epithelium-derived factor
PI3K	Phosphatide inositol 3 kinase
Rbl-1	Retinoblastoma-like 1
SPIO	Superparamagnetic iron oxide
TAK1	TGF β-activated kinase-1
TAMs	Tumor-associated macrophages
TERT	Telomerase reverse transcriptase
TGF-β	Transforming growth factor Beta
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
UC-MSCs	Umbilical cord-derived mesenchymal stem cells

1 Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer and is associated with a high mortality rate worldwide (Ferlay et al. 2010). HCC represents approximately 90% of all primary hepatic cancers (El–Serag and Rudolph 2007). Annually, half a million patients develop HCC, primarily in Africa and Asia (Llovet et al. 2003). Certain lesions, including chronic viral infection, alcoholism and bilharziasis, can contribute to the development of HCC. In addition, at least 80% of HCC cases are associated with viral infection by hepatitis B and C (Perz et al. 2006).

The therapeutic options for HCC depend primarily on the disease stage at the time of diagnosis. Unfortunately, most cases are diagnosed during advanced stages, leading to poor prognoses and ineffective treatment. Surgical interference by local resection, radiofrequency ablation, and hepatic transplantation remain the standard treatment options for HCC, primarily targeting the small, localized tumors that are identified in the early disease stages of patients with good liver function (Blum 2005). The surgical option is less effective during advanced stages of HCC (Tsilimigras et al. 2019). Furthermore, a shortage in the number of donors for liver transplantation is considered a major challenge. Other treatment options, such as systemic chemotherapy, immunotherapy and interferon treatment, have not resulted in significant cure rates (Llovet et al. 2000; Su and Ioannou 2018; Jindal et al. 2019). Sorafenib was the first chemotherapeutic agent approved for HCC cases. However, its clinical application for advanced cases showed drug resistance and sever adverse side effects (Zhu et al. 2017). Recently, other chemotherapeutics such as lenvatinib and regorafenib have shown enhanced overall survival and reduced adverse effects, but they are still under investigation (Tovoli et al. 2018). Although some herbals and dietary remedies were proposed to be effective complimentary therapies for HCC (Lv et al. 2019; Zang et al. 2019), there are doubts about their efficacy when used alone (Abdel-Hamid et al. 2018; Abdelmoneem et al. 2019).

Similar to many other cancers, HCC may acquire resistance to both radiotherapy and chemotherapy, even after surgical resection. The five-year recurrence rate of HCC has been

reported to be as high as 70% (Imamura et al. 2003), primarily due to metastatic tumors and more pronounced hepatitis (Imamura et al. 2003). Furthermore, cancer cells have been found to express multidrug resistance (MDR) genes (Nooter and Herweijer 1991), exhibit high levels of glutathione (known for its capacity to conjugate with drugs) (Balendiran et al. 2004) and harbour mutations in the p53 gene, which is responsible for regulating the cell cycle and apoptosis (Morgan and Kastan 1997).

Cancer stem cells (CSCs) are a small population of cells that have been proposed to be responsible for the heterogeneity observed in HCC growth, resistance to therapy, invasion, metastasis and an increased recurrence rate (Ji and Wang 2012; Llovet and Bruix 2008). As there is no approved second-line treatment for HCC (Ji and Wang 2012), targeting CSCs has become the goal of many researchers. In some cases, CSCs have been targeted without surgical interference; however, in most studies, CSC targeting has been performed in a complementary fashion to surgical excision, to remove any residual tumors and to support liver regeneration (Furst et al. 2007; Esch et al. 2005; Yuan et al. 2016).

Stem cells have been proposed to compensate for degenerated tissues by means of their robust differentiation capacities as well as their ability to secrete cytokines that promote cell proliferation and vasculogenesis (Song et al. 2016; Dominici et al. 2006). Despite a paucity of evidence of the role of stem cell therapy in many cancers, preclinical studies using bone marrow, adipose tissue, and amnion mesenchymal stem cells for HCC treatment have been promising (Li et al. 2010; Zhao et al. 2012; Mamede et al. 2012). However, important questions remain unanswered, including the identification of the best source for stem cells, the most effective doses, the best route of injection, and their mechanisms of action.

In this review, we evaluate the current status of stem cell therapy for HCC treatment and the outcomes of experimental and pre-clinical studies that have been conducted in the field over the past few years. We will specifically focus on the role of CSCs as a promising new treatment target.

2 Pathogenesis of Hepatocellular Carcinoma

Carcinogenesis is the process of transforming normal cells into pre-neoplastic lesions that subsequently develop into malignant tumors (Severi et al. 2010). Carcinogenesis is thought to be initiated and maintained by the following factors: first, the tumor microenvironment, which produces growth factors, cytokines, chemokine, free radicals, and other tumorigenic substrates that contribute to tumor initiation and progression (Wu et al. 2012); second, cancer-associated fibroblasts (CAFs) (Pietras and Ostman 2010); third, tumor-associated macrophages (TAMs) (Leonardi et al. 2012); fourth, hypoxia, which enhances proliferation, angiogenesis, metastasis, chemo-resistance, and radio-resistance in cancer cells (Wu et al. 2012); fifth, epithelial-mesenchymal transition (EMT) (Chen et al. 2010); sixth, oxidative stress (Chuma et al. 2008); seventh, inflammation (Elsharkawy and Mann 2007); and eighth, cancer stem cells. Moreover, liver cirrhosis, resulting from chronic liver diseases such as viral hepatitis, represents a major risk factor for the development of HCC (Fattovich et al. 2002; Fattovich et al. 2004).

3 Molecular Pathogenesis of Hepatocellular Carcinoma

At the molecular level, disruption of several pathways is involved in the pathogenesis of HCC. These pathways include the Wnt–βcatenin, transforming growth factorβ, JAK-STAT and AKT signalling pathways (Tornesello et al. 2016) (Fig. 1).

3.1 Wnt-β-Catenin

The Wnt-β-catenin pathway plays a vital role in many cellular regulatory processes, such as cell proliferation, survival and self-renewal (Anastas and Moon 2013). The activation of this pathway

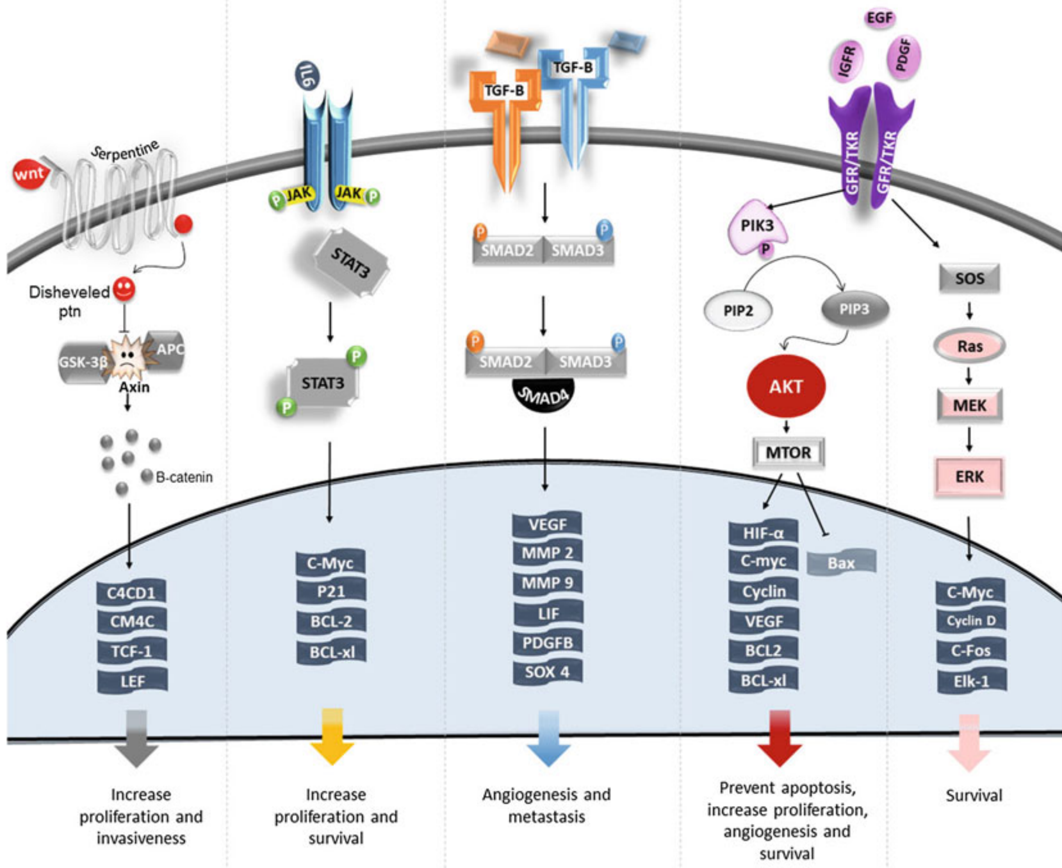

Fig. 1 Key signalling pathways that regulate the function of hepatic CSCs
Wnt β-Catenin, IL6-JAK-STAT, TGF-β, AKT and Ras/Raf/MEK/ERK signaling pathway

is initiated by the binding of the Wnt ligand to its Frizzled-family serpentine transmembrane receptor, which leads to the activation of disheveled. Consequently, β-catenin accumulates in the cytosol, as its degradation is prohibited by the β-catenin destruction complex (APC, axin, and GSK3β). The accumulated β-catenin is then translocated to the nucleus, where it activates the transcription of cyclin D, c-Myc, TCF and LEF, which are responsible for the regulation of many cellular processes (Fig. 1) (Gordon and Nusse 2006; Wang et al. 2006). In nearly 95% of HCC cell lines, abnormally high expression levels of Wnt, its receptor and another component of this pathway has been detected (Bengochea et al. 2008; Protiva et al. 2015; Yuzugullu et al. 2009).

It is worth mentioning that the activation of the Wnt-β-catenin pathway is correlated with the invasiveness and progression of HCC through several mechanisms. These mechanisms include regulation of the CTNNB1 gene, which promotes HCC proliferation and invasion (Lai et al. 2011; Rebouissou et al. 2016), the recruitment of EpCAM^{+} hepatic CSCs (Yamashita et al. 2007; Yamashita et al. 2009) and the expression of the microRNA 181 family in EPCAM^{+} AFP^{+} liver CSCs. miR181 is critical for the maintenance of stemness in EPCAM^{+} AFP^{+} liver CSCs and acts by targeting the Wnt-β-Catenin inhibitors nemo-like kinase (NLK), homeobox transcription factor 2 (CDX2) and GATA-binding protein 6 (GATA6). In addition, inhibition of miR181

results in a reduction of the number and tumorigenicity of EPCAM+ AFP+ liver CSCs (Ji et al. 2011).

3.2 Transforming Growth Factor Beta (TGF-β)

TGF-β plays an important role in the regulation of HCC. When the TGF-β ligand binds to its cell membrane receptor, TGF-β II/III, the intracellular kinase domain of TGF-R1 is activated. This kinase, in turn, activates Smad2 and Smad3, resulting in the formation of a complex with Smad4, which then translocates to the nucleus to regulate gene transcription (Heldin and Moustakas 2012). This pathway was found to orchestrate HCC progression through either the intrinsic activity of growth factors or micro-environmental changes, such as alteration of inflammatory mediators and the production of cancer-associated fibroblasts (CAF) (Giannelli et al. 2014).

Interestingly, downregulation of TGF-β signalling results in spontaneous HCC development via upregulation of IL6-STAT3 signalling (Tang et al. 2008). However, excessive and persistently high levels of TGF-β promote malignancy and cause the number of HCC metastases to multiply, as the expression levels of TGF-β in patients with aggressive metastatic HCC are higher than those in non-metastatic HCC patients (Shirai et al. 1994). Tumor formation is further facilitated by TGF-β-mediated epithelial to mesenchymal transition (EMT) through the upregulation of SNAI1 and downregulation of E-cadherin (Giannelli et al. 2005). Moreover, TGF-β has been shown to increase the expression of anti-apoptotic genes in primary cultures of human foetal hepatocytes and HCC cell lines by inhibiting the protein kinase CKII (Caja et al. 2011). TGF-β can therefore mediate opposing effects in HCC, as it has the ability to induce apoptosis and inhibit cellular growth, while also being able to enhance EMT, which induces cell invasion and metastasis, leading to tumor progression.

3.3 IL6-JAK-STAT Signalling Pathway

The binding of IL-6 to its specific cell membrane receptor induces receptor dimerization, which activates JAK phosphorylation. Free STAT in the cytoplasm becomes dimerized when it binds to phosphorylated JAK. This dimerized STAT is then translocated to the nucleus to regulate the transcription of many genes responsible for cell growth, proliferation and survival (Bournazou and Bromberg 2013). The overactivation of this pathway plays an important role in the transformation of hepatic CSCs in HCC, along with disrupted TGF-β signalling (Tang et al. 2008).

Patients with HCC have been found to exhibit higher levels of IL-6 than cirrhotic patients (Malaguarnera et al. 1996). Furthermore, hepatic progenitor cells, which initiate tumorigenesis, have been shown to acquire autocrine IL-6 signalling, which stimulates tumor growth and progression in different HCC models, including MUP-uPA transgenic mice, $Il6^{\Delta hep}$ mice, DEN-injected BL/6 mice and retrorsine-CCL4-treated BL/6 mice (He et al. 2013). Targeting the IL6-JAK-STAT signalling pathway may therefore prove to be significant for the inhibition of HCC progression. This hypothesis is supported by the remarkable *in vivo* regression of tumor size observed in an HCC-induced mouse model after injection with a STAT3 inhibitor, which targets the IL6-STAT3 signalling pathway (Lin et al. 2009a).

3.4 AKT Signalling Pathway

Phosphatide inositol 3 kinase (PI3K) is an important enzyme that regulates cell growth, survival, angiogenesis, chemoresistance and differentiation. Abnormal activation of this signalling pathway occurs in approximately 45% of HCC (Sahin et al. 2004). The stimulation of receptors such as G-protein-coupled receptors and tyrosine kinase receptors activates PI3K. PI3K in turn generates phosphatidylinositol (3,4,5)-trisphosphate (PIP3) from PIP2 in the lipid membrane, which acts as a

second messenger. AKT, which is responsible for the crosstalk between kinases, binds to PIP3 and promotes its phosphorylation by specific kinases. Downstream signalling is activated following AKT phosphorylation to regulate cell cycle progression, survival and growth (Yap et al. 2008). Furthermore, AKT activates mTOR, which further activates downstream effectors that regulate the transcription of many angiogenic and proliferative factors, such as HIF1α, VEGF, c-MYC and Cyclin D1 (Dunlop and Tee 2009). Normally this pathway is inhibited by PTEN (a tumor suppressor gene), which targets the lipid products of PI3K (Roberts and Gores 2005).

Several studies have shown that upregulation or downregulation of this pathway results in mutations (Bénistant et al. 2000; Brugge et al. 2007). The persistent activation of the PI3K/AKT/mTOR pathway is a major determinant of malignant cell growth and survival (Chen et al. 2005). Substantial evidence has shown that AKT signalling plays a role in the development of HCC (Sahin et al. 2004; Villanueva et al. 2008). AKT signalling can be activated by several factors, including the mutation or downregulation of PTEN (Hu et al. 2003) and hepatitis viral infection. Hepatitis viral infection acts through the activation of N-RAS (Mannová and Beretta 2005) and PIK3CA mutations, as it has been shown to increase PIP3 levels and induce cellular transformations (Bader et al. 2006; Bader et al. 2005). Interestingly, a correlation between the activation of this pathway and high-grade HCC with a poor prognosis, leading to drug resistance, has been reported (Zhou et al. 2010; Alexia et al. 2004).

3.5 Ras/Raf/MEK/ERK Pathway

The Ras/Raf/MEK/ERK pathway plays an important role in the regulation of cell survival and proliferation, along with the PI3K/AKT pathways (Huynh et al. 2010; Yu et al. 2015). However, the underlying mechanism through which the activation of this pathway occurs is not yet understood. Overactivity of the PI3K/AKT pathway has been reported in HCC (Hoffmann et al. 2011; Ito et al. 1998; Liu et al. 2006; Zuo et al. 2012), and overexpression of Raf-1 has been reported in almost all HCC patients (Gollob et al. 2006). HCC genomic sequencing studies have identified somatic missense mutations in many associated genes, such as B-Raf, N-Ras, B-Ras and H-Ras (Colombino et al. 2012; Kalinina et al. 2013).

Hepatitis viral infection (HBV, HCV), which is considered to be one of the most common causes of HCC, has been associated with activation of the PI3K/AKT pathway. For instance, HBV protein X has been reported to further activate the Ras/Raf/MEK/ERK pathway and inhibit the P53 (tumor suppressor) gene (Giambartolomei et al. 2001; Wang 2001), while the HCV core protein has been found to activate Raf-1 (Sahin et al. 2004; Nakamura et al. 2011).

4 Cancer Stem Cells

The recent discovery of CSCs has contributed to a better understanding of the pathophysiology of HCC and its histological characteristics and functional heterogeneity (Nowell 1976). CSCs are a small population of cells that have been proposed to be responsible for tumor growth and progression. Many laboratories are working on the isolation and proper identification of CSCs in HCC (Yamashita et al. 2009; Suetsugu et al. 2006; Yang et al. 2008; Zhu et al. 2010; Haraguchi et al. 2010; Sugihara and Saya 2013). These cells appear to share common surface markers such as CD133, CD90, CD44 CD13 and EpCAM (Table 1).

CSCs show heterogeneity in HCC based on their cell surface markers, tumorigenic potential and invasiveness. The $CD133^+$ HCC cell line HuH7 exhibits higher proliferative and neoplastic capacities than $CD133^-$ cells (Suetsugu et al. 2006), while $CD90^+$ cells display a higher tumorigenic and metastatic potential than $CD90^-$ cells (Yang et al. 2008). The $CD44^+CD133^+$ HCC cell lines HuH7, SMMC-7721, MHCC-LM3 and MHCC-97 L show increased resistance to chemotherapy and increased expression levels of stem cell genes compared to $CD44^-CD133^+$ cell lines (Zhu et al. 2010). $CD13^+$ CSCs in HCC are a

Table 1 Surface markers expressed by CSCs and their functions

	Cell marker	HCC cell line	Role in HCC	Ref.
1	EpCAM+	Huh7, HepG2	Proliferation and tumorigenicity	[49]
2	CD133+	Huh7, SMMC7721	Tumorigenicity and metastases	[88]
3	CD90+	HepG2, Hep3B2	Metastasis and invasion	[89]
4	CD44+	Huh7	Invasion	[90]
5	CD24+	HepG2	Chemo resistance, self-renewal, metastasis	[218]
6	CD13+	Huh7	Recurrence, dormancy, chemo resistance	[219]
7	Hoechst 33342	Huh7	Stemeness genes expression, tumorigenicity	[220]
8	ALDH+	PLc8024	Chemoresistanc	[221]

dormant population of cells at the G0 stage of the cell cycle (Haraguchi et al. 2010). Comparison of the $EpCAM^+$ and $EpCAM^-$ subpopulations of CSCs revealed that this marker plays roles in invasiveness and tumorigenicity (Yamashita et al. 2009). In the laboratory, HCC CSCs can be sorted based on their ability to efflux the DNA-binding dye Hoechst 33342 (Sugihara and Saya 2013). These criteria are useful for the identification of CSCS in HCC as well as therapeutic targeting approaches (He et al. 2015).

5 Origin of CSCs in HCC

The origin of CSCs in HCC is proposed to follow a stochastic or hierarchical clonal model. The stochastic theory dictates that any cell in a neoplastic clone presents an equal chance of tumorigenic initiation and progression, while the hierarchical theory states that only certain cells (CSCs) have this ability (Reya et al. 2001; Soltysova et al. 2005). Proper identification of the origin of CSCs requires a full understanding of embryogenesis and the normal cell hierarchy during liver development. This hierarchy is believed to start from hepatic stem cells, which give rise to committed progenitors that further develop into mature hepatocytes and cholangiocytes (Fig. 2).

Some researchers have proposed that hepatic progenitor cells (HPCs) play a role and may be a source of CSCs in HCC (Chiba et al. 2007; Kitade et al. 2013; Tschaharganeh et al. 2014). Supporting this hypothesis, chronic liver diseases have been demonstrated to be associated with the activation of HPCs located in the canal of Hering near the distal biliary tract (Alison et al. 2009; Russo and Parola 2011). When these HPCs are activated, they give rise to small dysplastic oval cells that contribute to liver cancer (de Lima et al. 2008; Grozdanov et al. 2006). In contrast, others have shown that mature hepatocytes undergo neoplastic transformation and dedifferentiate to produce HCC (Font-Burgada et al. 2015; He et al. 2013; Jors et al. 2015; Marquardt 2016). Disruption of p53 along with a mutation in the Wnt-β-catenin pathway has been suggested to result in the dedifferentiation of mature hepatocytes into progenitor-like cells, driving the generation of HCC (Tschaharganeh et al. 2014). These data were supported by *in vivo* studies in which fluorescently labelled progenitor cells and hepatocytes were tracked in $MDR2^{-/-}$ mice. The results showed that HCC did not originate from HPCs but rather from adult hepatocytes (Mu et al. 2015). These data were later confirmed by Shin et al. using a similar tracking protocol in another mouse model ($Rosa^{YFP}$ mice) (Shin et al. 2016).

Most previous studies have provided evidence to support the premise of a single-lineage origin of CSCs. Thorgerisson and colleagues, however, have proposed that multiple hepatic cell lineages have the ability to be transformed into CSCs. These authors transfected murine HPCs, hepatoblasts and adult hepatocytes with H-Ras and the Simion virus 40 large-T antigen. Surprisingly, all of the transduced cells expressed CSC markers, showed the capacity for self-renewal and were able to develop into cancer (Holczbauer et al. 2013). HCC could

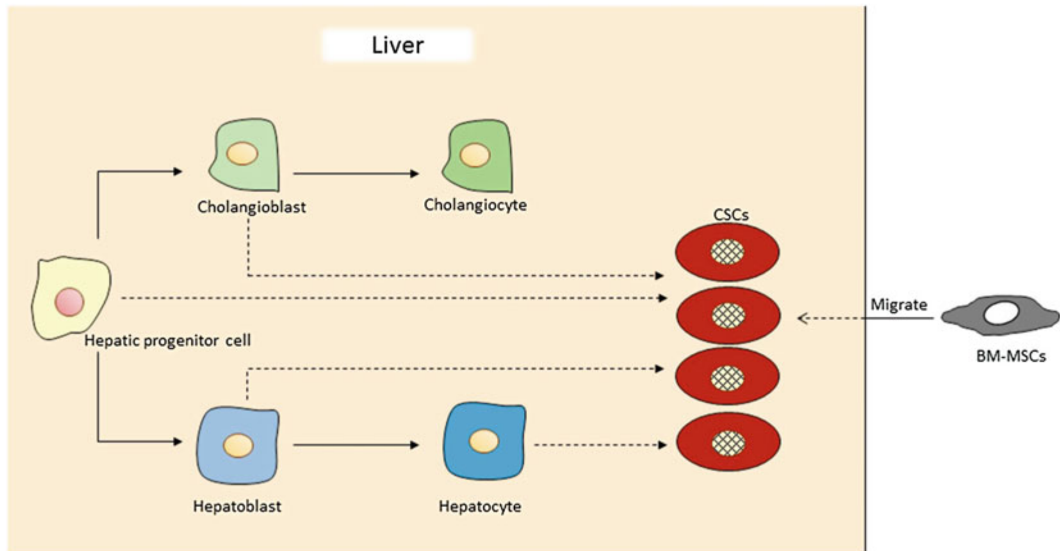

Fig. 2 Origin of CSCs in HCC
CSCs may originate from hepatic progenitor cells, cholangioblasts, hepatoblasts, hebatocytes, or BM-MSCs migrating to the tumor microenvironment. Abbreviations: *CSCs* Cancer stem cells, *BM-MSCs* Bone marrow mesenchymal stem cells

therefore originate from multiple cell types, such as HPCs, hepatoblasts or mature hepatocytes. These findings explain the heterogeneity and different phenotypes of HCC.

6 Mesenchymal Stem Cells and HCC

Mesenchymal stem cells (MSCs) have been administered for the treatment of many intractable diseases, in both experimental settings and clinical applications, because of their relative safety, abundance, ease of access, and lack of ethical concerns. These cells have also been applied as a potentially promising cell therapy approach for cancer because of their immunosuppressive functions and high tumor tropism. However, controversial data on the effects of MSC therapy on tumor progression have been reported. While studies aiming to use MSCs as a safe approach for treating cancer have shown some promise (Galderisi et al. 2010), other studies have reported the deleterious effect of enhanced tumor growth (Hogan et al. 2012; Ridge et al. 2017). Most of these studies will be discussed below and are summarized in Table 2.

6.1 Bone Marrow-Derived MSCs

Bone marrow-derived mesenchymal stem cells (BM-MSCs) are a rich source of well-characterized MSCs that have been used for cancer therapy in experimental settings. The administration of BM-MSCs to a non-Hodgkin's lymphoma mouse model resulted in slow tumor development, and histopathological sections exhibited extensive intratumor necrosis (Secchiero et al. 2010). The same antitumorigenic effect was reported in a Kaposi's sarcoma mouse model resulting from the inhibition of AKT protein kinase (Khakoo et al. 2006). The anticancer effects of BMSCs were further studied in the murine hepatoma H22 cell line *in vitro* and *in vivo* (Lu et al. 2008). *In vitro,* MSCs displayed an inhibitory effect on cancer cells via the upregulation of P21 (a negative regulator of the cell cycle) and caspase 3 (an apoptosis-associated protease). Moreover, the administration of MSCs *in vivo* in a mouse model of hepatoma associated with

Table 2 Published studies on HCC treatment using stem cells (naïve and engineered), or stem cell extracts and products

Stem cells used			In vitro (Cell line)	In vivo model	Dose	No. of inj.	F/M	Site	Results	Mechanism	Ref
BMMSCs	Cells		-	Nude mice	$5*10^5$	3	-	IV	Inhibit invasion &metastasis Increase tumor growth	Down regulation of TGFB1 & MMP-2	[27]
BMMSCs	Cells		H_{22}	inbred BALB/c mice	$1\text{-}2*10^5$	1	M	IP	Inhibitory effect on tumor cell growth *in vitro* and *in vivo*	Upregulation of P21 and caspase 3 in tumor cells	[222]
BMMSCs	Cells		Bel7404/LM3 cells	Mice	$5*10^5$	1	-	SC/ liver	Enhance cell proliferation and metastasis *in vitro* and *in vivo*	Activating of MAPK signaling pathway, upregulation of TNF-α, IL-6 and suppression of NK cell.	[117]
BMMSCs	Conditioned medium		HepG2, Bel 7402, Bel 7404	-					Promote invasion and metastasis of HCC cell lines	Activation of IL6/STAT3 signaling pathway	[223]
BMMSCs	Conditioned medium		MHCC97-H	-					Enhance cell proliferation and suppress the invasive ability of HCC cells	Down-regulation of TGFB1 & MMP-2	[27]
BMMSCs	Engineered MSCs	TRAIL	N1-S1, HepG2, MHCC97-H, and L02	Nude Mice	200K	1	F	SC	It resulted in inhibition of tumor growth and could be useful in prevention of the recurrence	Trails activated caspase 3 which resulted in apoptosis. Also, it resulted in down-regulation of DcR1 and DcR2 in cancer cells	[173]
BMMSCs	Engineered MSCs	TRAIL	MHCC97-H	Balb/c nude mice	$1*10^6$	1	-	IV	TRAIL-MSCs in combination with cisplatin exhibit a potent antitumor effect	Cisplatin + TRAIL-MSCs resulted in up-regulation of DR5	[175]
BMMSCs	Engineered MSCs	IL12	-	BALB/c mice	$2*10^6$ - $1*10^6$	5	-	IV	Tumor regression and prevent its metastasis.	Inhibit Akt phosphorylation and block VEGF-D	[178]
BMMSCs	Engineered MSCs	PEDF	MHCC-97H HepG2	BALB/c-nu/nu mice	$10*10^6$	1	M	IV	Suppression of the tumor and its metastasis to lung	Inhibit angiogenesis and migration	[179]
BMMSCs	Engineered MSCs	Apoptin	HepG2	BALB/c-nu mice	$20*10^6$	1	F	TP	Inhibition of the growth of tumor at *in vitro* and *in vivo* level	Pro-apoptosis effect and blocking Wnt pathway	[181]
BMMSCs	Engineered MSCs	s Flt- 1	SMMC- 7721	BALB/C nude mice	600K	3	M	IV	Inhibition of tube formation *in vitro* and angiogenesis *in vivo*.	Inactivation of VEGFR-2	[180]
BMMSCs	Engineered MSCs	IFN-γ & IL-10	HepG2	Nude/SCID mice	-	1	M	IV	Resulted in cell cycle arrest and inhibition of cell proliferation	Modulation of MAPK pathway with the upregulation of p38 and JNK, and suppression of ERK	[187]
BMMSCs	BMSCs pulsed with TEX		H_{22}	-					Enhanced migratory capacity and exhibited more effective antitumor activities	Cell cycle arrest	[210]

Stem cells used	In vitro (Cell line)	In vivo model	Dose	No. of inj.	F/M	Site	Results	Mechanism	Ref
Exosomes	HepG2	SCID mice	100 – 50 μg	2	-	IT	Arrest cell cycle, decrease viability and promote apoptosis Tumor regression	Up-regulation of DIRAS3, Rbl-1 and CDKN2B	[212]
Cells	-	-					-	-	-
Conditioned medium	Huh7, HepG2, Bel 7404, SMMC7721	-					Reduce proliferation and induce apoptosis of HCC	Inhibition of PI3K-AKT Signaling Pathway	[28]
Engineered MSCs SPIO@AuNP	Hepatoblastoma, HepG2	Nude Mice	-	1	-	Spleen	ADSC can act as a good carrier for the theranostic agent delivery to HCC.	Thermal ablation, in which it leads temperature increase for up to 55C during laser treatment	[191]
Exosomes	-	HCC rat model	2000 μg	1	M	Penile vein	Suppression of HCC and decrease the tumor volume	Activation of NKT cells	[211]
Exosomes from engineered MSCs	miR 122 HepG2	Balb/c nude mice	50 μg	1	M	IT	Induction of apoptosis and cell cycle arrest Increase chemosensitivity Decrease dose of chemotherapy	Down-regulation of the following genes CCNG1, ADAM10 and IGF1R in HCC cells which are responsible for tumor initiation, progression and drug resistance	[217]
Cells	-	-					-	-	-
Conditioned medium	-	-					-	-	-
Cell extract	HepG2, Hep3B2.1-7	-					Reduction in metabolic activity	-	[150]
Cell extract	HuH7, Hep3B2.1-7 and HepG2	BALB/c nu/nu mice	60 mg/kg	6	M	IP	It could induce apoptosis and had a selective cytotoxicity	Activation of caspase 3 and 8 leads to apoptosis. Increase in BAX/BCL2 ratio leads to mitochondrial destabilization.	[151]
Cell extract	HuH7, Hep3B2.1-7 and HepG2	-					Regression of HCC	Interfere with oxidative stress, cell cycle, its associated proteins, multidrug resistant proteins, DNA, P21&53 and B-Catenin	[152]
Engineered MSCs	-	-					-	-	-
Exosomes	-	-					-	-	-

(continued)

Table 2 (continued)

Umbilical cord MSCs	Cells		HepG-2	-					Inhibited proliferation and promoted apoptosis of HepG2 cells in a time dose-dependent manner	Down-regulation mRNA and protein expression of alpha-fetoprotein (AFP), Bcl2 and Survivin	[224]
			HepG-2	-					1- Condition medium of HepG-2 cells induces the trans-differentiation of hUC-MSCs into CA-MSCs. 2- supernatant of induced CA-MSCs significantly promote proliferation and migration of HepG-2	Differentiation of hUCMSCs into CAFs was through TGF-β/Smad signaling pathway.	[163]
			HCCLM3	-					Metastatic enhancement of 3D cultured HCC	Activation of TGF-B Leading to EMT	[225]
	UC CD34+ human hematopoietic stem cell		-	Chemically induced HCC rat model					Progression of HCC	Upregulation of wnt4, p53 and increased the cancer progenitor cells	[161]
	Conditioned medium		BEL7402	-					Suppression of HCC cell proliferation and migration	Downregulation of BCL-2, pro-caspase-7 β-catenin and c-Myc. Increase of cancer cell dormancy biomarker EphA5.	[164]
	Engineered MSCs	IL-24	HepG2	BALB/c nude mice	$1*10^6$	1	F	IV	E1A-UC-MSCs have significant anti-tumor activity in vivo and in vitro	Increase in the expression of Bax, PARP, caspase-3 and 9. Decrease the expression of Bcl-2. Activation of the p38MAPK pathway.	[186]
		miR122	HepG2 and Huh7	Balb/c athymic nude mice	$1*10^6$	1	F	IV	MiR122 engineered MSCs showed anti-tumor effect on the orthotropic and subcutaneous xenograft tumor models.	-	[24]
		TRAIL	HepG2	Balb/c athymic nude mice	500K	1	M	IV	Significant antitumor activity specially when combined with 5-FU.	Regulate the expression of Bcl-2 family members and activation of caspase signal pathway.	[174]
	Exosomes		-	-					-	-	-

Sources of stem cells include bone marrow (BM), adipose (AD), amnion and umbilical cord mesenchymal stem cells (MSCs) as a therapy for HCC. (-) No data available. *IV* Intravenous, *IT* intra-tumor, *TP* tumor pleura, *SC* subcutaneous, *IP* intra-peritoneal

ascites inhibited the growth of the hepatoma and complicating ascites, and no immunosuppressive effect was observed. Li et al. have shown that BM-MSCs significantly inhibit the invasiveness of HCC and its metastasis to the lungs in an *in vivo* mouse model, but these cells did not decrease tumor size (Li et al. 2010). Further investigation of the underlying mechanism through the culture of the MHCC47-H cell line using conditioned medium from BM-MSCs revealed downregulation of TGFβ1 (Fig. 3). Recently, BM-MSCs was found to promote the proliferation and metastasis of human hepatoma cell line Bel7407 both in vitro and in vivo through upregulation of MAPK signaling pathway (Chen et al. 2019).

Other studies, however, have shown that BM-MSCs enhance the proliferation, metastasis and angiogenesis of colon cancer (Zhu et al. 2006; Shinagawa et al. 2010) and breast cancer (Karnoub et al. 2007). Interestingly, BM-MSCs were shown to migrate and differentiate into CAFs in the tumor stroma, which has also been observed in *in vitro* studies (Karnoub et al. 2007; El-Badawy et al. 2017a). Moreover, BM-MSC-conditioned medium has been demonstrated to promote the invasion of HCC (Bel7404 cell line) through the activation of IL6-STAT3 signalling (Mi and Gong 2017) (Fig. 3).

6.2 Adipose-Derived MSCs

Adipose tissue represents a significant source of MSCs (Fraser et al. 2008; El-Badawy et al. 2016). Recently, experimental studies in which adipose-derived stem cells (ADSCs) have been administered to treat diseases such as diabetes, myocardial infarction and neurodegenerative diseases have shown promising results (Lin et al. 2009b; Marei et al. 2017; Meliga et al. 2007; El-Badawy et al. 2017b; Gabr et al. 2017; El-Badri 2016; El-Badawy and El-Badri 2016). However, the roles of ADSCs in cancer regression or progression are still under investigation.

Similar to marrow MSCs, the few studies on the role of adipose MSCs in tumor progression have been controversial. While some studies have shown that human ADSCs increase the size of lung gliomas (Yu et al. 2008), prostate carcinomas (Lin et al. 2010), melanomas and glioblastomas (Kucerova et al. 2010), the role of ADSCs in promoting the supportive tumor

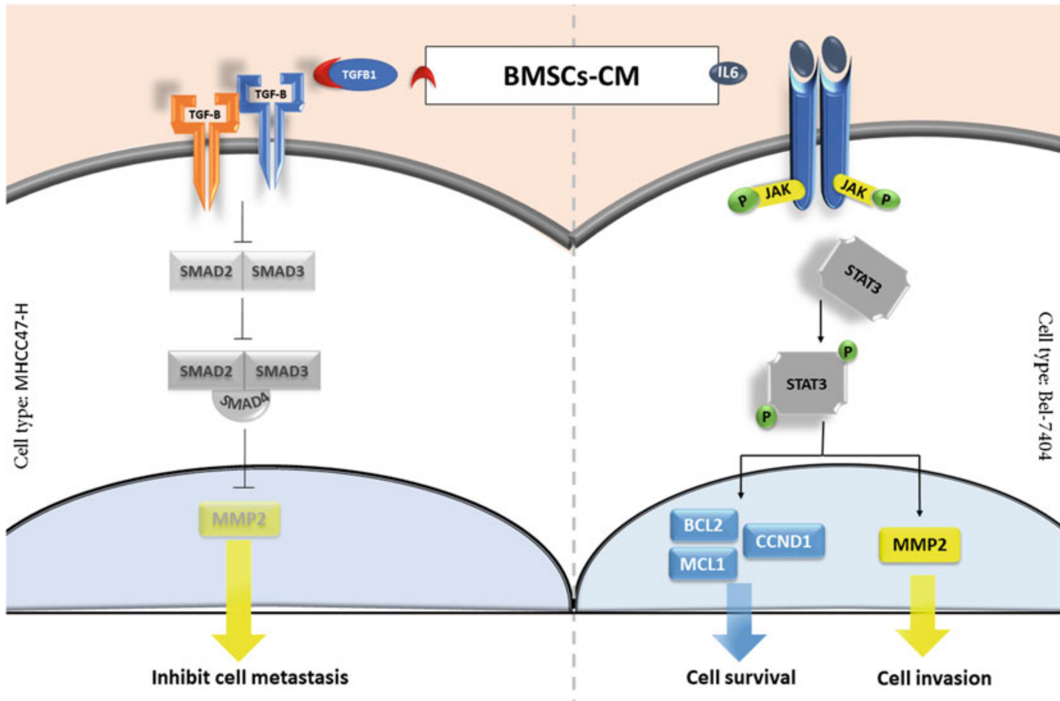

Fig. 3 Schematic representation of the mechanism of action of BMSCs-conditioned medium
(→) denotes activation, (⊢) denotes suppression. Faint colors denote diminishing or disappearance of effector. The left side of the diagram represents the mechanism of BMSCs-CM to inhibit the metastasis of MHCC47-H cells through inhibition of TGF-β pathway (Li et al. 2010). However, according to Mi and Gong (2017), the right side of the diagrams shows that culturing of Bel7404 cells with the conditioned medium of BMSCs promote cell invasion and survival through activation of through activation of IL6-STAT3 signalling pathway

microenvironment has been documented in other studies. The administration of ADSCs along with lung cancer, glioblastoma, prostate tumor and melanoma cell lines has been shown to increase tumor cell viability, reduce cell apoptosis, and promote vasculogenesis and migration, in addition to other immunomodulatory effects caused by proinflammatory cytokines (Yu et al. 2008; Lin et al. 2010; Kucerova et al. 2010).

On the other hand, a study by Zhu et al. showed that ADSCs suppressed the growth of leukaemia cell lines (K562 and HL60) and a breast adenocarcinoma cell line (MCF7) (Zhu et al. 2009). These researchers implanted Cellmax artificial capillary devices filled with K562 cells, with or without ADSCs, intraperitoneally in mice. In this model, which was designed to avoid potential disturbance by the immune system, mADSCs inhibited K562 cell proliferation by inducing cell cycle arrest. This inhibitory effect of ADSCs on tumor growth and progress was attributed to direct cell-cell contact or to the secretion of tumor-inhibiting factors. ADSCs were found to secret DKK-1 molecules, which are known to attenuate cellular proliferation via their negative effect on the Wnt signalling pathway. In another disease model, the co-culture of ADSCs with several pancreatic adenocarcinoma-derived cell lines (PACDC) (Capan-1, Capan-2, BxPC3, and Miapaca-2) resulted in apoptosis and cell cycle arrest (Cousin et al. 2009). Interestingly, a single-dose injection of ADSCs in a PDAC mouse model exerted a transient anti-proliferative effect on PDAC, leading to the supposition that the injection of multiple doses of ADSCs could be more beneficial than a single dose. ADSC-conditioned medium had a similar anti-proliferative effect and resulted in pancreatic adenocarcinoma cell death by inducing cell cycle arrest (Cousin et al. 2009).

Although no reported studies have used ADSCs as a therapeutic approach for HCC, the administration of ADMSC-conditioned medium

was shown to result in inhibition of HCC proliferation and to induce tumor cell death and apoptosis. Downregulation of the AKT signalling pathway results in downregulation of both angiogenesis and cell survival genes. The study showed that certain factors in the ADSC-conditioned medium were able to block the ATP-binding cassette sub-family G member 2 (ABCG2), which is known to play a role in multidrug resistance in cancer chemotherapy. The sensitivity of cancer stem cells to anticancer drugs should be increased by targeting this pathway (Zhao et al. 2012). The mechanism through which the conditioned medium (CM) influences the HCC cancer cell line is outlined in Fig. 4.

6.3 Amnion-Derived MSCs

The placenta is a vital embryological tissue consisting of the outer chorion, which comes in contact with the maternal decidua, and the inner amnion, which is an avascular thin membrane that completely surrounds the amniotic fluid and the foetus (Carlson 2004). Histologically, the amnion consists of an inner epithelial layer and an outer mesenchymal layer (Marongiu et al. 2010). The availability and unique characteristics of the amniotic membrane (AM), and the lack of ethical concerns regarding its use (as it is regularly discarded) have made it a suitable therapeutic alternative to other types of stem cells. The regenerative capacities of the AM are mediated through its stem cell content and immunomodulatory functions, in addition to its anti-scarring, anti-neoplastic and anti-inflammatory properties (Mamede et al. 2012). The anti-neoplastic properties of the AM have been proposed to be due to its capacity to produce pro-apoptotic agents (Seo et al. 2008) and the secretion of anti-angiogenic cytokines (Shao et al. 2004), leading to diminished tumor vascularization. AM-derived cells exhibit anti-angiogenic (Niknejad et al. 2013; Yazdanpanah et al. 2015), pro-apoptotic (Jiao et al. 2012) and immunomodulatory properties (Kubo et al. 2001). Stem cells

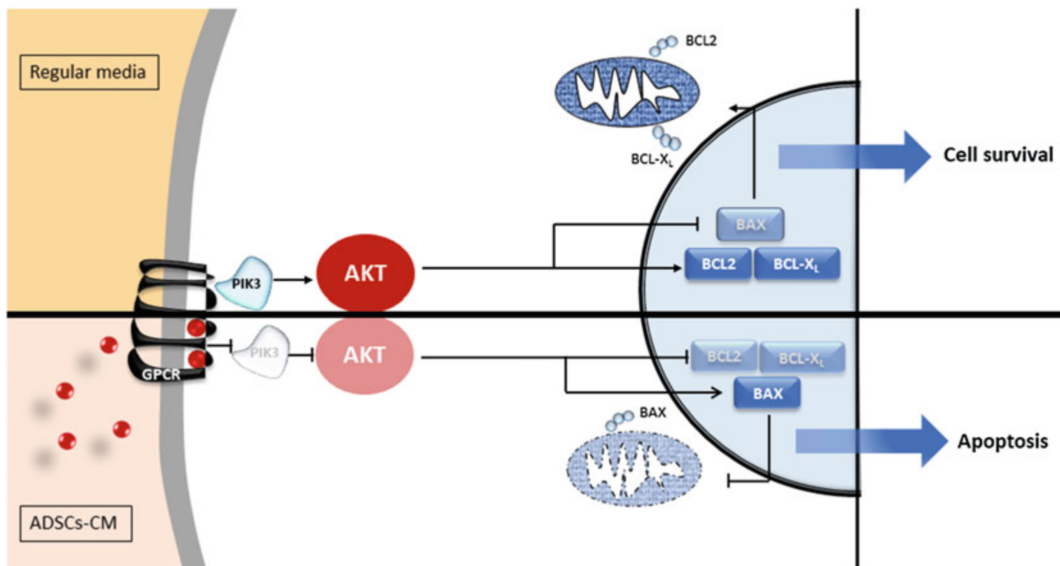

Fig. 4 Schematic representation of the mechanism of action of ADSC-conditioned medium
(→) denotes activation, (⊢) denotes suppression. Faint colors denote diminishing or disappearance of effector. The upper part of the diagram represents the increased survival rate of HCC cells in regular media. However, according to Zhao et al (2012), the lower part of the diagrams shows that culturing of HCC cells with the conditioned medium of ADSCs revealed in inhibition of this pathway and hence results in apoptosis

derived from the amniotic membrane express the pluripotency markers Oct3/4, Sox2, Klf4, Nanog, TRA1–60, TA1–81 and SSEA-4 and have been used experimentally in regenerative medicine (Barboni et al. 2014).

6.3.1 Human Amniotic Membrane-Derived Mesenchymal Stem Cells (hAMSCs)

Amniotic-derived mesenchymal stem cells (AMSCs) have been proposed to exert an anti-tumor effect. For example, the co-culture of hAMSCs with cancer cell lines of either haematopoietic or non-haematopoietic origins significantly reduces tumor proliferation and induces cell cycle arrest (Magatti et al. 2012). The same anti-tumor effect was achieved using hAMSC-conditioned medium, which was shown to inhibit prostate cancer cell proliferation and induce cell cycle arrest (Rolfo et al. 2014). Similarly, Jiao et al. reported that intra-tumor injection of hAMSCs inhibited glioma growth and induced apoptosis via activation of the proapoptotic genes Bax and caspases 3 and 8 and suppression of the Bcl2 survival gene (Jiao et al. 2012).

6.3.2 Amniotic Membrane-Derived Epithelial Stem Cells (hAECs)

Kang et al. were the first to demonstrate the ability of naïve, non-engineered hAECs to reduce the viability of breast cancer cells, both *in vivo* and *in vitro* (Kang et al. 2012). Furthermore, the hAEC culture supernatant was demonstrated to exert an apoptotic effect on both HeLa and breast cancer cell lines via the upregulation of caspases 3 and 8 (Niknejad et al. 2014). Moreover, an intensive antiangiogenic effect was observed when epithelial cells were cultured with AM using the aortic ring assay. However, there have been no published studies reporting the effects of hAECs on HCC.

Mamede et al. (2014) evaluated the proliferation of cancer cells after being cultured with a protein extract from hAECs (hAMPE). The metabolic activity was reduced by 64% and 83% in HepG2 and Hep3B2.1–7 cells, respectively. The anti-tumor effect of hAMPE is achieved via the induction of apoptosis through the activation of caspases 3 and 8. Mitochondrial destabilization is also observed, as measured by an increasing BAX/BCL2 ratio and interference in oxidative stress and the cell cycle. The levels of multidrug-resistant proteins are also reduced, leading to increased sensitivity to chemotherapy and attenuated expression of P21, P53 and β-catenin (Mamede et al. 2015, 2016). Furthermore, hAMPE does not show a toxic effect on normal, non-tumorigenic, control cells, indicating selective cytotoxicity for cancer cells. These therapeutic approaches are not equally effective in all tested HCC cell lines, as HuH7 cells are resistant to hAMPE treatment. In a mouse model, BALB/c nu/nu mice were injected with either HuH7 or HepG2 cells to initiate a tumor model, followed by the intraperitoneal injection of 6 doses of hAMPE over 12 days. HepG2-induced tumors responded favourably to hAMPE therapy, while the HuH7-induced tumors did not show significant differences from the control group.

6.4 Umbilical Cord-Derived MSCs

In 1991, McElreavey et al. isolated fibroblast-like cells from human umbilical cord Wharton's jelly; these cells are referred to as umbilical cord-derived mesenchymal stem cells (UC-MSCs) (McElreavey et al. 1991). These cells share common characteristics with other MSCs, such as multi-lineage differentiation (into adipocytes, osteocytes and chondrocytes), immunosuppression and the expression of specific mesenchymal surface markers (CD90, CD105, and CD73) (Dominici et al. 2006). UC-MSCs also show *in vivo* migration towards the site of inflammation and tumor tropism (Kim et al. 2013a). Studies by Ma et al. have demonstrated that hUC-MSCs significantly inhibit the growth of breast CSCs through the induction of apoptosis, cell cycle arrest and inhibition of the PI3K-AKT signalling pathway (Ma et al. 2012a). Both hUC-MSCs and their extracts (conditioned media and cell lysates) show inhibitory effects on the growth of breast adenocarcinomas, ovarian carcinomas and osteosarcomas. The mechanism of this effect was proposed to occur through upregulation of

the pro-apoptotic BAX gene and downregulation of the anti-apoptotic BCL2 and SURVIVIN genes, in addition to the upregulation of autophagy genes (ATG5, ATG7, and BECLIN1) (Gauthaman et al. 2012).

Consistently, naïve rat UC-MSCs markedly attenuate the growth of both lung and pancreatic carcinoma cells, both *in vitro* and in mouse models, without evidence of differentiation or adverse effects (Maurya et al. 2010; Doi et al. 2010). A comparative analysis of the gene expression profiles of the two types of UC-MSCs suggested that rat UC-MSC-dependent growth regulation is significantly stronger than that of human UC-MSCs when co-cultured with species-matched breast carcinoma cells. This process appears to be mediated by enhancing tumor suppressor gene expression (Ohta et al. 2015). Moreover, UC-MSCs inhibit the proliferation of HepG2 cells and promote their apoptosis in a time- and dose-dependent manner through downregulation of the mRNA and protein expression levels of alpha-fetoprotein (AFP), Bcl2 and survivin (Tang et al. 2016).

Conversely, administration of UC-derived hematopoietic stem cells, and not MSCs, to HCC induced rat model resulted in upregulation of Wnt4, p53 and increased the cancer progenitor cells percentage (Sherif et al. 2018). Liu et al. have shown that hUC-MSCs significantly enhanced the metastasis of 3D-cultured HCC cells through upregulation of matrix metalloproteinase (MMP) and EMT-related genes and TGF-β-induced migration (Liu et al. 2016). Further support for the adverse effects of UC-MSCs on cancer progression was provided by Yang et al., who demonstrated that hUC-MSCs transdifferentiate into cancer-associated MSCs (CA-MSCs) after 2 days of incubation with HepG-2 conditioned medium. Moreover, the co-culture of HepG-2 cells with the supernatant from induced CA-MSCs significantly promotes the proliferation and migration of HepG-2 cells (Yang et al. 2016).

Culture of UC-MSCs-CM with BEL7402 hepatoma cell line showed significant decrease in cell proliferation and migration through downregulation of BCL-2, pro-caspase-7 β-catenin and c-Myc (Yuan et al. 2018).

7 Engineered MSCS

Engineered MSCs are produced through the introduction of certain genes to construct MSCs with specialized functions. BM-MSCs, adipose-derived MSCs (AD-MSCs), and gingival-derived MSCs (G-MSCs) that are engineered to produce TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) have been shown to target cancer cells and to induce necrosis and apoptosis (Grisendi et al. 2010; Tang et al. 2014; Menon et al. 2009). TRAIL-producing stem cells have been shown to target cell lines derived from lung cancers (Grisendi et al. 2010; Loebinger et al. 2010; Loebinger et al. 2009), glioblastomas (Tang et al. 2014; Sasportas et al. 2009), squamous cancers (Loebinger et al. 2009, 2010), breast cancer (Loebinger et al. 2009), cervical cancer, (Loebinger et al. 2009) malignant mesotheliomas, (Lathrop et al. 2015) and renal carcinomas (Kim et al. 2013b). TRAIL-engineered stem cells derived from bone marrow, the umbilical cord, and the pancreas exert especially potent anticancer effects on liver malignancies (Deng et al. 2014; Yan et al. 2014; Zhang et al. 2012a; Sun et al. 2011; Yang et al. 2014). BM-MSCs, engineered to overexpress certain cytokines, such as IL-12, pigment epithelium-derived factor (PEDF), soluble FLT-1 and apoptin, have been shown to induce cancer apoptosis and anti-angiogenesis and to inhibit the formation of metastasis, leading to the proposed application of these factors for the targeted therapy of HCC (Chen et al. 2008; Gao et al. 2010; Li et al. 2017; Zhang et al. 2016).

IL-12-engineered MSCs were tested in a heterotopic HCC mouse model. The administration of 5 intravenous injections at 5-day intervals led to tumor damage and inhibition of lymph node metastasis. This process was achieved via the inhibition of Akt phosphorylation and blockage of VEGF-D ligands (Chen et al. 2008). Similarly, the application of PEDF to engineered MSCs resulted in the suppression of tumors and lung

metastasis after only one IV injection in an HCC mouse model (Gao et al. 2010). The potent antiangiogenic property of PEDF was shown to contribute to this anticancer effect (Dawson et al. 1999). Similar results were reported from another study, in which murine BM-MSCs loaded with soluble Flt-1, an antiangiogenic factor, were administered to treat an HCC mouse model. Engineered MSCs were administered through IV injection of 3 doses at one-week intervals (Li et al. 2017).

Apoptin has previously been reported to have a selective apoptotic effect on tumor cells (Taebunpakul et al. 2012; Danen-Van Oorschot et al. 2003; Maddika et al. 2005). The injection of a single dose of Apoptin-loaded BM-MSCs in HepG2-induced tumors resulted in tumor regression, apoptosis and the inhibition of angiogenesis (Zhang et al. 2016). Similar to Apoptin, IL-24 engineered MSCs show an apoptotic effect on HCCs, both *in vitro* and *in vivo*. Combined therapy with engineered MSCs and chemotherapy showed synergistic augmentation of apoptosis and enhanced chemosensitivity (Li et al. 2016).

Furthermore, combining more than one cytokine can work synergistically to guaranty effective outcome. This strategy has been proposed by Wang, Wang (Wang et al. 2017) who genitically modified BM-MSCs to coexpress IFN-γ and IL-10 cytokines. Promising results showed a potentiating anticancer effect of IFN-γ and IL-10 genetically modified BM-MSCs as it resulted in cell cycle arrest and inhibition of cell proliferation. Similar therapeutic aapproaches maybe proposed targetting genes such as Telomerase reverse transcriptase (TERT), which has shown high efficacy in inducing apoptosis (Xu et al. 2015; Kim et al. 2016), inhibiting cell migration and EMT transition (El-Badawy et al. 2018) in HCC cell lines. So, we suggest testing the efficacy of the stem cells to deliver anti-TERT to the site of the HCC rather than using adenovirus strategy.

Adipose-derived stem cells have also been used as a vehicle for gold nanoparticles. Using ADSCs for the targeted delivery of nanoparticles to the tumor site enhances safety and efficacy, leading to complete thermal ablation of the tumor. In this study, a combination of 2 theranostic nanoparticles, superparamagnetic iron oxide (SPIO) and gold nanoparticles, was loaded into ADSCs. *In vivo*, cells loaded with nanoparticles could be detected at the tumor site using magnetic resonance imaging (MRI). Interestingly, thermal ablation of HCC cells was histologically detected when the cells were subjected to laser irradiation (Zhao et al. 2014). Using stem cells as a vehicle for therapy could therefore overcome the side effects of instability and dispersion of nanoparticles to the spleen, liver, lungs and blood (De Jong et al. 2008).

8 Exosomes

Exosomes are cellular particles (proteins, lipids, mRNAs or microRNAs), characterized by a small size that ranges from 30 to 100 nm. These particles are secreted into the extracellular space and then carried by the blood to distant cells to help in cellular communication (Zhang et al. 2012b; Clayton et al. 2001). Exosomes secreted from tumor cells were found to provide a microenvironment that is usually favorable for tumor progression and aggressiveness, through several mechanisms, and dependinging on the tissue of origin. For example, exosomes secreted from glioma tumor cells were shown to suppress T-cell immune responses (Domenis et al. 2017). Preliminary studies showed that BM-MSCs exosomes stimulated invasiveness of colorectal cancer cells, promoted their survival and enhanced tumorigenesis. Exosomes of ADSCs, on the other hand, were found to have an antitumor effect on metastatic prostate cancer (De Boeck et al. 2013; Zhou et al. 2016; Takahara et al. 2016; Zhu et al. 2012). The role of exosomes in aggrevating tumor metastasis was reported in melanoma of the lymph node and lung, in pancreatic tumors (Weidle et al. 2017) and in HCC cell lines. The effect of HCC-derived exosomes was mediated by targeting HCC inhibitor, TGF β-activated kinase-1 (TAK1) leading to tumor progression and expansion (Kogure et al. 2011). Exosomes derived from tumor cells also exhibited an immunosuppressive effect, promoted apoptosis and inhibited lymphocyte and

dendritic cell functions (Yu et al. 2007). Exosomes may also promote tumor angiogenesis, by increasing the blood supply of a tumor (Lang et al. 2017a, b; Hsu et al. 2017). And finally, HCC cell-derived exosomes induced chemoresistance to sorafenib (Kinase inhibitor drug) and protected tumor cells from apoptosis through activation of Akt pathway. The efficacy of the HCC-derived exosomes was correlated to the invasiveness of the tumor cells (Qu et al. 2016).

Due to these properties, exosomes are proposed as a cell-free approach to the treatment of cancerous tumors. Therapeutic approaches depend on interference with the production of exosomes by cancer cells at the stage of their formation, their release or even later at the stage of uptake. Other approaches include producing exosomes that are known to enhance the immunity and stimulate the antitumor effect of dendritic cells and cytotoxic T lymphocytes (Yao et al. 2013). Furthermore, the administration of certain anticancer drugs could result in the production of cancer exosomes with specific immune boosting effect. Using this approach, HepG2 cell lines were treated with MS-275 or anticancer drugs. The cells produced exosomes that expressed high levels of heat shock protein (HSP70) and major histocompatibility complex (MHC) class I polypeptide-related sequence A (MICB). Those tumor-specific exosomes increased the cytotoxicity of natural killer cells (Xiao et al. 2013; Lv et al. 2012), and were the basis for the development of vaccine for HCC immunotherapy (Lv et al. 2012). Similarly, exosomes derived from tumors have been shown to enhance the immune effect of MSCs against HCC. In this study, exosomes derived from tumors were shown to increase the efficacy of theanti-tumorogenic effect of BM-MSCs through inhibition of proliferating cell nuclear antigen (PCNA) protein and cancer cell cycle arrest (Ma et al. 2012b).

In animal experimentation, exosomes derived from ADSCs suppressed HCC and increased the apparent diffusion coefficient (ADC) via the promotion of NKT-cell antitumor responses (Ko et al. 2015). ADC is a cellular biomarker to assess HCC by quantification of changes in water diffusion of tissues using MRI. Similarly, exosomes derived from BM-MSCs showed antitumor effect mediated by inhibition of angiogenesis (Bruno et al. 2013). In a mouse model of induced HCC tumor, BM-MSC-derived exosomes arrested the cell cycle, decreased cell viability and promoted tumor apoptosis. These effects were achieved by upregulation of GTP-binding RAS-like 3 (DIRAS3), retinoblastoma-like 1 (Rbl-1) and cyclin-dependent kinase inhibitor 2B transcript (CDKN2B). In HCC mouse model treated with an intra-tumor injection of 2 doses of exosomes within one-week intervals, tumor regression and growth inhibition were observed.

MicroRNAs (miRNAs, short non-coding RNAs) are considered to be important diagnostic and prognostic biomarkers of cancer (Schwarzenbach et al. 2014; Iorio and Croce 2012). Recently, microRNA-122 (miR-122) was found to be downregulated in HCC patients (Wu et al. 2015). It was therefore suggested that over-expression of miR-122 overexpression can suppress tumorigenic properties in HCC cell lines (Pineau et al. 2010). Engineered UC-MSCs containing miR-122 promoters have been found to show promising inhibitory effect on HCC cells, but not breast cancer cells (Yuan et al. 2016). Lou et al. followed the same strategy using ADSCs transfected with microRNA-122 (Lou et al. 2015). In this study, while miR-122 exosome therapy itself was not effective, combining exosomes with sorafenib led to a reduction in tumor size, suggesting a role in promoting the chemosensitivity of HCC to chemotherapy.

9 Conclusion & Future Perspectives

Conflicting data support the role of MSCs in both cancer progression and therapy. There is growing evidence that the administration of BM-MSCs directly to cancer subjects could have deleterious effects on the disease, although similarly conclusive evidence has not been provided for adipose stem cells. Direct cell contact between cancer cells and stem cells from BM-MSCs and

UC-MSCs results in aggravated tumor invasion and metastasis. Soluble factors produced by cancer cells may also contribute to the generation of cancer stem cells, leading to increased tumor progression, invasiveness, and metastasis (El-Badawy et al. 2017a).

Alternatively, stem cell derivatives may prove to be beneficial for cancer patients. The co-administration of MSCs along with MSC-conditioned medium and the administration of MSC exosomes have shown the greatest promise. Immune therapy using exosomes derived from MSCs or using MSCs as a vehicle for gene therapy are other promising approaches, especially when co-administered with chemotherapy.

In conclusion, the available data on stem cell therapy in HCC are controversial. The low immunogenicity of MSCs, their ability to home to a tumor site, engraftment as well as their stability, ability to escape immune attack and relatively long half-life all suggest that MSCs are an attractive choice for drug delivery in HCC treatment. The flexible approach of using MSCs as a "relatively safe" vehicle for gene therapy, cytokine production, nanoparticles or drug delivery further supports the expansion of preclinical experimentation in cancer therapy. However, data providing evidence of accelerated tumor growth, spreading and/or invasiveness following the administration of MSCs call for extreme caution and careful consideration of the role of stem cells in cancer development and therapy.

Acknowledgement This work is supported by grant #5300, funded by the Science and Technology Development Fund (STDF) and the Sawiris Foundation for Social Development.

Conflict of Interest The authors declare that they have no competing interests.

References

Abdel-Hamid NM et al (2018) Herbal management of hepatocellular carcinoma through cutting the pathways of the common risk factors. Biomed Pharmacother 107:1246–1258

Abdelmoneem M et al (2019) Dual-targeted Lactoferrin Shell-Oily Core Nanocapsules for synergistic targeted/herbal therapy of hepatocellular carcinoma. ACS Appl Mater Interfaces 11(30):26731–26744

Alexia C et al (2004) An evaluation of the role of insulin-like growth factors (IGF) and of type-I IGF receptor signalling in hepatocarcinogenesis and in the resistance of hepatocarcinoma cells against drug-induced apoptosis. Biochem Pharmacol 68(6):1003–1015

Alison M, Islam S, Lim S (2009) Stem cells in liver regeneration, fibrosis and cancer: the good, the bad and the ugly. J Pathol 217(2):282–298

Anastas JN, Moon RT (2013) WNT signalling pathways as therapeutic targets in cancer. Nat Rev Cancer 13 (1):11

Bader AG et al (2005) Oncogenic PI3K deregulates transcription and translation. Nat Rev Cancer 5(12):921

Bader AG, Kang S, Vogt PK (2006) Cancer-specific mutations in PIK3CA are oncogenic in vivo. Proc Natl Acad Sci 103(5):1475–1479

Balendiran GK, Dabur R, Fraser D (2004) The role of glutathione in cancer. Cell Biochem Funct 22 (6):343–352

Barboni B et al (2014) Gestational stage affects amniotic epithelial cells phenotype, methylation status, immunomodulatory and stemness properties. Stem Cell Rev Rep 10(5):725–741

Bengochea A et al (2008) Common dysregulation of Wnt/Frizzled receptor elements in human hepatocellular carcinoma. Br J Cancer 99(1):143

Bénistant C, Chapuis H, Roche S (2000) A specific function for phosphatidylinositol 3-kinase [alpha](p85 [alpha]-p110 [alpha]) in cell survival and for phosphatidylinositol 3-kinase [beta](p85 [alpha]-p110 [beta]) in de novo DNA synthesis of human colon carcinoma cells. Oncogene 19(44):5083

Blum HE (2005) Hepatocellular carcinoma: therapy and prevention. World J Gastroenterol 11(47):7391–7400

Bournazou E, Bromberg J (2013) Targeting the tumor microenvironment: JAK-STAT3 signaling. JAKSTAT 2(2):e23828

Brugge J, Hung M-C, Mills GB (2007) A new mutational AKTivation in the PI3K pathway. Cancer Cell 12 (2):104–107

Bruno S et al (2013) Microvesicles derived from human bone marrow mesenchymal stem cells inhibit tumor growth. Stem Cells Dev 22(5):758–771

Caja L et al (2011) The transforming growth factor-beta (TGF-β) mediates acquisition of a mesenchymal stem cell-like phenotype in human liver cells. J Cell Physiol 226(5):1214–1223

Carlson B (2004) Placenta and extraembryonic membranes. In: Human embryology and developmental biology, 3rd edn. Mosby, Philadelphia, pp 106–127

Chen YL, Law P-Y, Loh HH (2005) Inhibition of PI3K/Akt signaling: an emerging paradigm for targeted cancer therapy. Curr Med Chem Anticancer Agents 5 (6):575–589

Chen X et al (2008) A tumor-selective biotherapy with prolonged impact on established metastases based on cytokine gene-engineered MSCs. Mol Ther 16 (4):749–756

Chen L et al (2010) CHD1L promotes hepatocellular carcinoma progression and metastasis in mice and is associated with these processes in human patients. J Clin Invest 120(4):1178–1191

Chen J et al (2019) Human mesenchymal stem cells promote tumor growth via MAPK pathway and metastasis by epithelial mesenchymal transition and integrin alpha5 in hepatocellular carcinoma. Cell Death Dis 10(6):425

Chiba T et al (2007) Enhanced self-renewal capability in hepatic stem/progenitor cells drives cancer initiation. Gastroenterology 133(3):937–950

Chuma M et al (2008) 8-Hydroxy-2′-deoxy-guanosine is a risk factor for development of hepatocellular carcinoma in patients with chronic hepatitis C virus infection. J Gastroenterol Hepatol 23(9):1431–1436

Clayton A et al (2001) Analysis of antigen presenting cell derived exosomes, based on immuno-magnetic isolation and flow cytometry. J Immunol Methods 247 (1–2):163–174

Colombino M et al (2012) BRAF and PIK3CA genes are somatically mutated in hepatocellular carcinoma among patients from South Italy. Cell Death Dis 3(1): e259

Cousin B et al (2009) Adult stromal cells derived from human adipose tissue provoke pancreatic cancer cell death both in vitro and in vivo. PLoS One 4(7):e6278

Danen-Van Oorschot AA et al (2003) Importance of nuclear localization of apoptin for tumor-specific induction of apoptosis. J Biol Chem 278 (30):27729–27736

Dawson D et al (1999) Pigment epithelium-derived factor: a potent inhibitor of angiogenesis. Science 285 (5425):245–248

De Boeck A et al (2013) Bone marrow-derived mesenchymal stem cells promote colorectal cancer progression through paracrine neuregulin 1/HER3 signalling. Gut 62(4):550–560

De Jong WH et al (2008) Particle size-dependent organ distribution of gold nanoparticles after intravenous administration. Biomaterials 29(12):1912–1919

de Lima VM et al (2008) A rodent model of NASH with cirrhosis, oval cell proliferation and hepatocellular carcinoma. J Hepatol 49(6):1055–1061

Deng Q et al (2014) TRAIL-secreting mesenchymal stem cells promote apoptosis in heat-shock-treated liver cancer cells and inhibit tumor growth in nude mice. Gene Ther 21(3):317–327

Doi C et al (2010) Cytotherapy with naive rat umbilical cord matrix stem cells significantly attenuates growth of murine pancreatic cancer cells and increases survival in syngeneic mice. Cytotherapy 12(3):408–417

Domenis R et al (2017) Systemic T cells immunosuppression of Glioma stem cell-derived Exosomes is mediated by Monocytic myeloid-derived suppressor cells. PLoS One 12(1):e0169932

Dominici M et al (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8(4):315–317

Dunlop E, Tee A (2009) Mammalian target of rapamycin complex 1: signalling inputs, substrates and feedback mechanisms. Cell Signal 21(6):827–835

El-Badawy A, El-Badri N (2016) Clinical efficacy of stem cell therapy for diabetes mellitus: a meta-analysis. PLoS One 11(4):e0151938

El-Badawy A et al (2016) Adipose stem cells display higher regenerative capacities and more adaptable electro-kinetic properties compared to bone marrow-derived mesenchymal stromal cells. Sci Rep 6:37801

El-Badawy A et al (2017a) Cancer cell-soluble factors reprogram mesenchymal stromal cells to slow cycling, chemoresistant cells with a more stem-like state. Stem Cell Res Ther 8(1):254

El-Badawy A, Ahmed SM, El-Badri N (2017b) Adipose-derived stem cell-based therapies in regenerative medicine. In: El-Badri N (ed) Advances in stem cell therapy: bench to bedside. Springer International Publishing, Cham, pp 117–138

El-Badawy A et al (2018) Telomerase reverse transcriptase coordinates with the epithelial-to-mesenchymal transition through a feedback loop to define properties of breast cancer stem cells. Biol Open 7(7):bio034181

El-Badri N (2016) Advances in stem cell therapy: bench to bedside. Springer, Humana Press

El–Serag HB, Rudolph KL (2007) Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology 132(7):2557–2576

Elsharkawy AM, Mann DA (2007) Nuclear factor-kappaB and the hepatic inflammation-fibrosis-cancer axis. Hepatology 46(2):590–597

Esch JSA 2nd et al (2005) Portal application of autologous CD133+ bone marrow cells to the liver: a novel concept to support hepatic regeneration. Stem Cells 23 (4):463–470

Fattovich G et al (2002) Effect of hepatitis B and C virus infections on the natural history of compensated cirrhosis: a cohort study of 297 patients. Am J Gastroenterol 97(11):2886–2895

Fattovich G et al (2004) Hepatocellular carcinoma in cirrhosis: incidence and risk factors. Gastroenterology 127(5 Suppl 1):S35–S50

Ferlay J et al (2010) GLOBOCAN 2008, cancer incidence and mortality worldwide: IARC CancerBase No. 10 [Internet]. International Agency for Research on Cancer, Lyon, p 2

Font-Burgada J et al (2015) Hybrid periportal hepatocytes regenerate the injured liver without giving rise to cancer. Cell 162(4):766–779

Fraser JK et al (2008) Adipose-derived stem cells. Methods Mol Biol 449:59–67

Furst G et al (2007) Portal vein embolization and autologous CD133+ bone marrow stem cells for liver regeneration: initial experience. Radiology 243(1):171–179

Gabr MM et al (2017) From human Mesenchymal stem cells to insulin-producing cells: comparison between bone marrow- and adipose tissue-derived cells. Biomed Res Int 2017:3854232

Galderisi U, Giordano A, Paggi MG (2010) The bad and the good of mesenchymal stem cells in cancer: boosters

of tumor growth and vehicles for targeted delivery of anticancer agents. World J Stem Cells 2(1):5

Gao Y et al (2010) Human mesenchymal stem cells overexpressing pigment epithelium-derived factor inhibit hepatocellular carcinoma in nude mice. Oncogene 29(19):2784–2794

Gauthaman K et al (2012) Human umbilical cord Wharton's jelly stem cell (hWJSC) extracts inhibit cancer cell growth in vitro. J Cell Biochem 113 (6):2027–2039

Giambartolomei S et al (2001) Sustained activation of the Raf/MEK/Erk pathway in response to EGF in stable cell lines expressing the Hepatitis C Virus (HCV) core protein. Oncogene 20(20):2606

Giannelli G et al (2005) Laminin-5 with transforming growth factor-β1 induces epithelial to mesenchymal transition in hepatocellular carcinoma. Gastroenterology 129(5):1375–1383

Giannelli G, Villa E, Lahn M (2014) Transforming growth factor-β as a therapeutic target in hepatocellular carcinoma. Cancer Res 74(7):1890–1894

Gollob JA et al (2006) Role of Raf kinase in cancer: therapeutic potential of targeting the Raf/MEK/ERK signal transduction pathway. In: Seminars in oncology. Elsevier, WB Saunders

Gordon MD, Nusse R (2006) Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors. J Biol Chem 281(32):22429–22433

Grisendi G et al (2010) Adipose-derived mesenchymal stem cells as stable source of tumor necrosis factor-related apoptosis-inducing ligand delivery for cancer therapy. Cancer Res 70(9):3718–3729

Grozdanov PN, Yovchev MI, Dabeva MD (2006) The oncofetal protein glypican-3 is a novel marker of hepatic progenitor/oval cells. Lab Investig 86 (12):1272–1284

Haraguchi N et al (2010) CD13 is a therapeutic target in human liver cancer stem cells. J Clin Invest 120 (9):3326–3339

He G et al (2013) Identification of liver cancer progenitors whose malignant progression depends on autocrine IL-6 signaling. Cell 155(2):384–396

He AR, Smith D, Mishra L (2015) Targeting cancer stem cells in hepatocellular carcinoma. Gastrointest Cancer Targets Ther 5:1–10

Heldin C-H, Moustakas A (2012) Role of Smads in TGFβ signaling. Cell Tissue Res 347(1):21–36

Hoffmann K et al (2011) Correlation of gene expression of ATP-binding cassette protein and tyrosine kinase signaling pathway in patients with hepatocellular carcinoma. Anticancer Res 31(11):3883–3890

Hogan NM et al (2012) Mesenchymal stem cells in the colorectal tumor microenvironment: recent progress and implications. Int J Cancer 131(1):1–7

Holczbauer A et al (2013) Modeling pathogenesis of primary liver cancer in lineage-specific mouse cell types. Gastroenterology 145(1):221–231

Hsu YL et al (2017) Hypoxic lung cancer-secreted exosomal miR-23a increased angiogenesis and vascular permeability by targeting prolyl hydroxylase and tight junction protein ZO-1. Oncogene 36 (34):4929–4942

Hu TH et al (2003) Expression and prognostic role of tumor suppressor gene PTEN/MMAC1/TEP1 in hepatocellular carcinoma. Cancer 97(8):1929–1940

Huynh H et al (2010) AZD6244 enhances the anti-tumor activity of sorafenib in ectopic and orthotopic models of human hepatocellular carcinoma (HCC). J Hepatol 52(1):79–87

Imamura H et al (2003) Risk factors contributing to early and late phase intrahepatic recurrence of hepatocellular carcinoma after hepatectomy. J Hepatol 38(2):200–207

Iorio MV, Croce CM (2012) MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. EMBO Mol Med 4 (3):143–159

Ito Y et al (1998) Activation of mitogen-activated protein kinases/extracellular signal-regulated kinases in human hepatocellular carcinoma. Hepatology 27(4):951–958

Ji J, Wang XW (2012) Clinical implications of cancer stem cell biology in hepatocellular carcinoma. Semin Oncol 39(4):461–472

Ji J, Yamashita T, Wang XW (2011) Wnt/beta-catenin signaling activates microRNA-181 expression in hepatocellular carcinoma. Cell Biosci 1(1):4

Jiao H et al (2012) Human amniotic membrane derived-mesenchymal stem cells induce C6 glioma apoptosis in vivo through the Bcl-2/caspase pathways. Mol Biol Rep 39(1):467–473

Jindal A, Thadi A, Shailubhai K (2019) Hepatocellular carcinoma: etiology and current and future drugs. J Clin Exp Hepatol 9(2):221–232

Jors S et al (2015) Lineage fate of ductular reactions in liver injury and carcinogenesis. J Clin Invest 125 (6):2445–2457

Kalinina O et al (2013) Somatic changes in primary liver cancer in Russia: a pilot study. Mutat Res Genet Toxicol Environ Mutagen 755(2):90–99

Kang N-H et al (2012) Human amniotic membrane-derived epithelial stem cells display anticancer activity in BALB/c female nude mice bearing disseminated breast cancer xenografts. Int J Oncol 40(6):2022–2028

Karnoub AE et al (2007) Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. Nature 449(7162):557–563

Khakoo AY et al (2006) Human mesenchymal stem cells exert potent antitumorigenic effects in a model of Kaposi's sarcoma. J Exp Med 203(5):1235–1247

Kim DW et al (2013a) Wharton's jelly-derived Mesenchymal stem cells: phenotypic characterization and optimizing their therapeutic potential for clinical applications. Int J Mol Sci 14(6):11692–11712

Kim SW et al (2013b) Complete regression of metastatic renal cell carcinoma by multiple injections of engineered mesenchymal stem cells expressing dodecameric TRAIL and HSV-TK. Clin Cancer Res 19(2):415–427

Kim YH et al (2016) Image-aided suicide gene therapy utilizing multifunctional hTERT-targeting adenovirus for clinical translation in hepatocellular carcinoma. Theranostics 6(3):357–368

Kitade M et al (2013) Specific fate decisions in adult hepatic progenitor cells driven by MET and EGFR signaling. Genes Dev 27(15):1706–1717

Ko SF et al (2015) Adipose-derived Mesenchymal stem cell exosomes suppress hepatocellular carcinoma growth in a rat model: apparent diffusion coefficient, natural killer T-cell responses, and histopathological features. Stem Cells Int 2015:853506

Kogure T et al (2011) Intercellular nanovesicle-mediated microRNA transfer: a mechanism of environmental modulation of hepatocellular cancer cell growth. Hepatology 54(4):1237–1248

Kubo M et al (2001) Immunogenicity of human amniotic membrane in experimental xenotransplantation. Invest Ophthalmol Vis Sci 42(7):1539–1546

Kucerova L et al (2010) Tumor cell behaviour modulation by mesenchymal stromal cells. Mol Cancer 9:129

Lai T-Y et al (2011) β-catenin plays a key role in metastasis of human hepatocellular carcinoma. Oncol Rep 26 (2):415–422

Lang HL et al (2017a) Glioma cells enhance angiogenesis and inhibit endothelial cell apoptosis through the release of exosomes that contain long non-coding RNA CCAT2. Oncol Rep 38(2):785–798

Lang HL et al (2017b) Glioma cells promote angiogenesis through the release of exosomes containing long non-coding RNA POU3F3. Eur Rev Med Pharmacol Sci 21(5):959–972

Lathrop MJ et al (2015) Antitumor effects of TRAIL-expressing mesenchymal stromal cells in a mouse xenograft model of human mesothelioma. Cancer Gene Ther 22(1):44–54

Leonardi GC et al (2012) The tumor microenvironment in hepatocellular carcinoma (review). Int J Oncol 40 (6):1733–1747

Li GC et al (2010) Human mesenchymal stem cells inhibit metastasis of a hepatocellular carcinoma model using the MHCC97-H cell line. Cancer Sci 101 (12):2546–2553

Li Z et al (2016) E1A-engineered human umbilical cord mesenchymal stem cells as carriers and amplifiers for adenovirus suppress hepatocarcinoma in mice. Oncotarget 7(32):51815–51828

Li G et al (2017) Anti-angiogenesis gene therapy for hepatocellular carcinoma via systemic injection of mesenchymal stem cells engineered to secrete soluble Flt-1. Mol Med Rep 16(5):5799–5806

Lin L et al (2009a) The STAT3 inhibitor NSC 74859 is effective in hepatocellular cancers with disrupted TGF-β signaling. Oncogene 28(7):961

Lin G et al (2009b) Treatment of type 1 diabetes with adipose tissue-derived stem cells expressing pancreatic duodenal homeobox 1. Stem Cells Dev 18 (10):1399–1406

Lin G et al (2010) Effects of transplantation of adipose tissue-derived stem cells on prostate tumor. Prostate 70 (10):1066–1073

Liu L et al (2006) Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. Cancer Res 66(24):11851–11858

Liu C et al (2016) Mesenchymal stem cells enhance the metastasis of 3D-cultured hepatocellular carcinoma cells. BMC Cancer 16:566

Llovet JM, Bruix J (2008) Molecular targeted therapies in hepatocellular carcinoma. Hepatology 48 (4):1312–1327

Llovet JM et al (2000) Randomized controlled trial of interferon treatment for advanced hepatocellular carcinoma. Hepatology 31(1):54–58

Llovet JM, Burroughs A, Bruix J (2003) Hepatocellular carcinoma. Lancet 362(9399):1907–1917

Loebinger MR et al (2009) Mesenchymal stem cell delivery of TRAIL can eliminate metastatic cancer. Cancer Res 69(10):4134–4142

Loebinger MR et al (2010) TRAIL-expressing mesenchymal stem cells kill the putative cancer stem cell population. Br J Cancer 103(11):1692–1697

Lou G et al (2015) Exosomes derived from miR-122-modified adipose tissue-derived MSCs increase chemosensitivity of hepatocellular carcinoma. J Hematol Oncol 8(1):122

Lu YR et al (2008) The growth inhibitory effect of mesenchymal stem cells on tumor cells in vitro and in vivo. Cancer Biol Ther 7(2):245–251

Lv LH et al (2012) Anticancer drugs cause release of exosomes with heat shock proteins from human hepatocellular carcinoma cells that elicit effective natural killer cell antitumor responses in vitro. J Biol Chem 287(19):15874–15885

Lv MY et al (2019) Urolithin B suppresses tumor growth in hepatocellular carcinoma through inducing the inactivation of Wnt/beta-catenin signaling. J Cell Biochem 120(10):17273–17282

Ma Y et al (2012a) The in vitro and in vivo effects of human umbilical cord mesenchymal stem cells on the growth of breast cancer cells. Breast Cancer Res Treat 133(2):473–485

Ma B et al (2012b) Murine bone marrow stromal cells pulsed with homologous tumor-derived exosomes inhibit proliferation of liver cancer cells. Clin Transl Oncol 14(10):764–773

Maddika S et al (2005) Cancer-specific toxicity of apoptin is independent of death receptors but involves the loss of mitochondrial membrane potential and the release of mitochondrial cell-death mediators by a Nur77-dependent pathway. J Cell Sci 118(Pt 19):4485–4493

Magatti M et al (2012) Amniotic membrane-derived cells inhibit proliferation of cancer cell lines by inducing cell cycle arrest. J Cell Mol Med 16(9):2208–2218

Malaguarnera M et al (1996) Role of interleukin 6 in hepatocellular carcinoma. Bull Cancer 83(5):379–384

Mamede A et al (2012) Amniotic membrane: from structure and functions to clinical applications. Cell Tissue Res 349(2):447–458

Mamede AC et al (2014) Effect of amniotic membrane proteins in human cancer cell lines: an exploratory study. J Membr Biol 247(4):357–360

Mamede A et al (2015) Selective cytotoxicity and cell death induced by human amniotic membrane in hepatocellular carcinoma. Med Oncol 32(12):257

Mamede A et al (2016) Oxidative stress, DNA, cell cycle/cell cycle associated proteins and multidrug resistance proteins: targets of human amniotic membrane in hepatocellular carcinoma. Pathol Oncol Res 22(4):689–697

Mannová P, Beretta L (2005) Activation of the N-Ras–PI3K–Akt-mTOR pathway by hepatitis C virus: control of cell survival and viral replication. J Virol 79 (14):8742–8749

Marei HES et al (2017) Cholinergic and dopaminergic neuronal differentiation of human adipose tissue derived mesenchymal stem cells. J Cell Physiol 233 (2):936–945

Marongiu F et al (2010) Isolation of amniotic mesenchymal stem cells. Curr Protoc Stem Cell Biol 12:1E. 5.1–1E. 5.11

Marquardt JU (2016) Deconvolution of the cellular origin in hepatocellular carcinoma: hepatocytes take the center stage. Hepatology 64(4):1020–1023

Maurya DK et al (2010) Therapy with un-engineered naive rat umbilical cord matrix stem cells markedly inhibits growth of murine lung adenocarcinoma. BMC Cancer 10:590

McElreavey KD et al (1991) Isolation, culture and characterisation of fibroblast-like cells derived from the Wharton's jelly portion of human umbilical cord. Biochem Soc Trans 19(1):29s

Meliga E et al (2007) Adipose-derived cells. Cell Transplant 16(9):963–970

Menon LG et al (2009) Human bone marrow-derived mesenchymal stromal cells expressing S-TRAIL as a cellular delivery vehicle for human glioma therapy. Stem Cells 27(9):2320–2330

Mi F, Gong L (2017) Secretion of interleukin-6 by bone marrow mesenchymal stem cells promotes metastasis in hepatocellular carcinoma. Biosci Rep 37(4): BSR20170181

Morgan SE, Kastan MB (1997) p53 and ATM: cell cycle, cell death, and cancer. Adv Cancer Res 71:1–25

Mu X et al (2015) Hepatocellular carcinoma originates from hepatocytes and not from the progenitor/biliary compartment. J Clin Invest 125(10):3891–3903

Nakamura H et al (2011) HCV core protein promotes heparin binding EGF-like growth factor expression and activates Akt. Hepatol Res 41(5):455–462

Niknejad H et al (2013) Side dependent effects of the human amnion on angiogenesis. Placenta 34 (4):340–345

Niknejad H et al (2014) Human amniotic epithelial cells induce apoptosis of cancer cells: a new anti-tumor therapeutic strategy. Cytotherapy 16(1):33–40

Nooter K, Herweijer H (1991) Multidrug resistance (mdr) genes in human cancer. Br J Cancer 63(5):663–669

Nowell PC (1976) The clonal evolution of tumor cell populations. Science 194(4260):23–28

Ohta N et al (2015) Human umbilical cord matrix mesenchymal stem cells suppress the growth of breast cancer by expression of tumor suppressor genes. PLoS One 10 (5):e0123756

Perz JF et al (2006) The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. J Hepatol 45(4):529–538

Pietras K, Ostman A (2010) Hallmarks of cancer: interactions with the tumor stroma. Exp Cell Res 316 (8):1324–1331

Pineau P et al (2010) miR-221 overexpression contributes to liver tumorigenesis. Proc Natl Acad Sci U S A 107 (1):264–269

Protiva P, Gong J, Sreekumar B, Torres R, Zhang X, Belinsky GS, Cornwell M, Crawford SE, Iwakiri Y, Chung C (2015) Pigment epithelium-derived factor (PEDF) inhibits Wnt/beta-catenin signaling in the liver. Cell Mol Gastroenterol Hepatol 1:535–549.e514

Qu Z et al (2016) Exosomes derived from HCC cells induce sorafenib resistance in hepatocellular carcinoma both in vivo and in vitro. J Exp Clin Cancer Res 35:159

Rebouissou S et al (2016) Genotype-phenotype correlation of CTNNB1 mutations reveals different ß-catenin activity associated with liver tumor progression. Hepatology 64(6):2047–2061

Reya T et al (2001) Stem cells, cancer, and cancer stem cells. Nature 414(6859):105

Ridge SM, Sullivan FJ, Glynn SA (2017) Mesenchymal stem cells: key players in cancer progression. Mol Cancer 16(1):31

Roberts LR, Gores GJ (2005) Hepatocellular carcinoma: molecular pathways and new therapeutic targets. In: Seminars in liver disease. Copyright© 2005 by Thieme Medical Publishers, Inc., 333 Seventh Avenue, New York, NY 10001, USA

Rolfo A et al (2014) New perspectives for prostate cancer treatment: in vitro inhibition of LNCaP and PC3 cell proliferation by amnion-derived mesenchymal stromal cells conditioned media. Aging Male 17(2):94–101

Russo FP, Parola M (2011) Stem and progenitor cells in liver regeneration and repair. Cytotherapy 13 (2):135–144

Sahin F et al (2004) mTOR and P70 S6 kinase expression in primary liver neoplasms. Clin Cancer Res 10 (24):8421–8425

Sasportas LS et al (2009) Assessment of therapeutic efficacy and fate of engineered human mesenchymal stem cells for cancer therapy. Proc Natl Acad Sci U S A 106 (12):4822–4827

Schwarzenbach H et al (2014) Clinical relevance of circulating cell-free microRNAs in cancer. Nat Rev Clin Oncol 11:145

Secchiero P et al (2010) Human bone marrow mesenchymal stem cells display anti-cancer activity in SCID mice bearing disseminated non-Hodgkin's lymphoma xenografts. PLoS One 5(6):e11140

Seo JH, Kim YH, Kim JS (2008) Properties of the amniotic membrane may be applicable in cancer therapy. Med Hypotheses 70(4):812–814

Severi T et al (2010) Tumor initiation and progression in hepatocellular carcinoma: risk factors, classification, and therapeutic targets. Acta Pharmacol Sin 31 (11):1409–1420

Shao C et al (2004) Suppression of corneal neovascularization by PEDF release from human amniotic membranes. Invest Ophthalmol Vis Sci 45 (6):1758–1762
Sherif RN et al (2018) Effect of human umbilical cord blood derived CD34(+) hematopoietic stem cell on the expression of Wnt4 and P53 genes in a rat model of hepatocellular carcinoma. Tissue Cell 50:125–132
Shin S et al (2016) Genetic lineage tracing analysis of the cell of origin of Hepatotoxin-induced liver tumors in mice. Hepatology (Baltimore, Md) 64(4):1163–1177
Shinagawa K et al (2010) Mesenchymal stem cells enhance growth and metastasis of colon cancer. Int J Cancer 127(10):2323–2333
Shirai Y et al (1994) Plasma transforming growth factor-β 1 in patients with hepatocellular carcinoma. Comparison with chronic liver diseases. Cancer 73 (9):2275–2279
Soltysova A, Altanerova V, Altaner C (2005) Cancer stem cells. Neoplasma 52(6):435
Song YH et al (2016) Adipose-derived stem cells increase angiogenesis through matrix metalloproteinase-dependent collagen Remodeling. Integr Biol Quant Biosci Nano Macro 8(2):205–215
Su F, Ioannou GN (2018) The impact of direct-acting antiviral therapy for hepatitis C on hepatocellular carcinoma risk. Curr Hepatol Rep 17(4):377–384
Suetsugu A et al (2006) Characterization of CD133+ hepatocellular carcinoma cells as cancer stem/progenitor cells. Biochem Biophys Res Commun 351 (4):820–824
Sugihara E, Saya H (2013) Complexity of cancer stem cells. Int J Cancer 132(6):1249–1259
Sun XY et al (2011) MSC(TRAIL)-mediated HepG2 cell death in direct and indirect co-cultures. Anticancer Res 31(11):3705–3712
Taebunpakul P et al (2012) Apoptin induces apoptosis by changing the equilibrium between the stability of TAp73 and DeltaNp73 isoforms through ubiquitin ligase PIR2. Apoptosis 17(8):762–776
Takahara K et al (2016) microRNA-145 mediates the inhibitory effect of adipose tissue-derived stromal cells on prostate Cancer. Stem Cells Dev 25 (17):1290–1298
Tang Y et al (2008) Progenitor/stem cells give rise to liver cancer due to aberrant TGF-β and IL-6 signaling. Proc Natl Acad Sci 105(7):2445–2450
Tang XJ et al (2014) TRAIL-engineered bone marrow-derived mesenchymal stem cells: TRAIL expression and cytotoxic effects on C6 glioma cells. Anticancer Res 34(2):729–734
Tang YM et al (2016) Umbilical cord-derived mesenchymal stem cells inhibit growth and promote apoptosis of HepG2 cells. Mol Med Rep 14(3):2717–2724
Tornesello ML et al (2016) Molecular alterations in hepatocellular carcinoma associated with hepatitis B and hepatitis C infections. Oncotarget 7(18):25087–25102
Tovoli F et al (2018) Systemic treatments for hepatocellular carcinoma: challenges and future perspectives. Hepat Oncol 5(1):HEP01–HEP01
Tschaharganeh DF et al (2014) p53-dependent Nestin regulation links tumor suppression to cellular plasticity in liver cancer. Cell 158(3):579–592
Tsilimigras DI et al (2019) Prognosis after resection of Barcelona Clinic Liver Cancer (BCLC) stage 0, A, and B hepatocellular carcinoma: a comprehensive assessment of the current BCLC classification. Ann Surg Oncol 1–8
Villanueva A et al (2008) Pivotal role of mTOR signaling in hepatocellular carcinoma. Gastroenterology 135 (6):1972–1983. e11
Wang XW (2001) Microinjection technique used to study functional interaction between p53 and hepatitis B virus X gene in apoptosis. Mol Biotechnol 18 (2):169–177
Wang H-Y, Liu T, Malbon CC (2006) Structure-function analysis of Frizzleds. Cell Signal 18(7):934–941
Wang H et al (2017) Genetically engineered bone marrow-derived mesenchymal stem cells co-expressing IFN-gamma and IL-10 inhibit hepatocellular carcinoma by modulating MAPK pathway. J BUON 22 (6):1517–1524
Weidle HU et al (2017) The multiple roles of Exosomes in metastasis. Cancer Genomics Proteomics 14(1):1–16
Wu SD et al (2012) Role of the microenvironment in hepatocellular carcinoma development and progression. Cancer Treat Rev 38(3):218–225
Wu Q et al (2015) Decreased expression of hepatocyte nuclear factor 4alpha (Hnf4alpha)/microRNA-122 (miR-122) axis in hepatitis B virus-associated hepatocellular carcinoma enhances potential oncogenic GALNT10 protein activity. J Biol Chem 290 (2):1170–1185
Xiao W et al (2013) Effects of the epigenetic drug MS-275 on the release and function of exosome-related immune molecules in hepatocellular carcinoma cells. Eur J Med Res 18:61
Xu H et al (2015) Targeting human telomerase reverse transcriptase by a simple siRNA expression cassette in HepG2 cells. Hepat Mon 15(3):e24343
Yamashita T et al (2007) Activation of hepatic stem cell marker EpCAM by Wnt–β-catenin signaling in hepatocellular carcinoma. Cancer Res 67(22):10831–10839
Yamashita T et al (2009) EpCAM-positive hepatocellular carcinoma cells are tumor-initiating cells with stem/progenitor cell features. Gastroenterology 136 (3):1012–1024. e4
Yan C et al (2014) Suppression of orthotopically implanted hepatocarcinoma in mice by umbilical cord-derived mesenchymal stem cells with sTRAIL gene expression driven by AFP promoter. Biomaterials 35(9):3035–3043
Yang ZF et al (2008) Identification of local and circulating cancer stem cells in human liver cancer. Hepatology 47 (3):919–928

Yang X et al (2014) IFN-γ-secreting-Mesenchymal stem cells exert an antitumor effect in vivo via the TRAIL pathway. J Immunol Res 2014:9

Yang J et al (2016) Condition medium of HepG-2 cells induces the transdifferentiation of human umbilical cord mesenchymal stem cells into cancerous mesenchymal stem cells. Am J Transl Res 8(8):3429

Yao Y et al (2013) Tumor cell-derived exosome-targeted dendritic cells stimulate stronger CD8+ CTL responses and antitumor immunities. Biochem Biophys Res Commun 436(1):60–65

Yap TA et al (2008) Targeting the PI3K–AKT–mTOR pathway: progress, pitfalls, and promises. Curr Opin Pharmacol 8(4):393–412

Yazdanpanah G et al (2015) The effects of cryopreservation on angiogenesis modulation activity of human amniotic membrane. Cryobiology 71(3):413–418

Yu S et al (2007) Tumor exosomes inhibit differentiation of bone marrow dendritic cells. J Immunol 178 (11):6867–6875

Yu JM et al (2008) Mesenchymal stem cells derived from human adipose tissues favor tumor cell growth in vivo. Stem Cells Dev 17(3):463–473

Yu P et al (2015) NSK-01105 inhibits proliferation and induces apoptosis of prostate cancer cells by blocking the Raf/MEK/ERK and PI3K/Akt/mTOR signal pathways. Tumor Biol 36(3):2143–2153

Yuan X et al (2016) Mesenchymal stem cells deliver and release conditionally replicative adenovirus depending on hepatic differentiation to eliminate hepatocellular carcinoma cells specifically. Cancer Lett 381(1):85–95

Yuan Y et al (2018) Suppression of tumor cell proliferation and migration by human umbilical cord mesenchymal stem cells: a possible role for apoptosis and Wnt signaling. Oncol Lett 15(6):8536–8544

Yuzugullu H et al (2009) Canonical Wnt signaling is antagonized by noncanonical Wnt5a in hepatocellular carcinoma cells. Mol Cancer 8(1):90

Zang W et al (2019) Traditional Chinese Medicine (TCM) Astragalus Membranaceus and Curcuma Wenyujin promote vascular normalization in tumor-derived endothelial cells of human hepatocellular carcinoma. Anticancer Res 39(6):2739–2747

Zhang B et al (2012a) The inhibitory effect of MSCs expressing TRAIL as a cellular delivery vehicle in combination with cisplatin on hepatocellular carcinoma. Cancer Biol Ther 13(12):1175–1184

Zhang HG et al (2012b) Exosomes and immune surveillance of neoplastic lesions: a review. Biotech Histochem 87(3):161–168

Zhang J et al (2016) Inhibitory effect of genetically engineered mesenchymal stem cells with Apoptin on hepatoma cells in vitro and in vivo. Mol Cell Biochem 416(1–2):193–203

Zhao W et al (2012) Efficacy of mesenchymal stem cells derived from human adipose tissue in inhibition of hepatocellular carcinoma cells in vitro. Cancer Biother Radiopharm 27(9):606–613

Zhao J et al (2014) Stem cell-mediated delivery of SPIO-loaded gold nanoparticles for the theranosis of liver injury and hepatocellular carcinoma. Nanotechnology 25(40):405101

Zhou L et al (2010) The mTOR pathway is associated with the poor prognosis of human hepatocellular carcinoma. Med Oncol 27(2):255–261

Zhou HS et al (2016) Mesenchymal stem cells promote pancreatic adenocarcinoma cells invasion by transforming growth factor-beta1 induced epithelial-mesenchymal transition. Oncotarget 7 (27):41294–41305

Zhu W et al (2006) Mesenchymal stem cells derived from bone marrow favor tumor cell growth in vivo. Exp Mol Pathol 80(3):267–274

Zhu Y et al (2009) Human mesenchymal stem cells inhibit cancer cell proliferation by secreting DKK-1. Leukemia 23(5):925–933

Zhu Z et al (2010) Cancer stem/progenitor cells are highly enriched in CD133+CD44+ population in hepatocellular carcinoma. Int J Cancer 126(9):2067–2078

Zhu W et al (2012) Exosomes derived from human bone marrow mesenchymal stem cells promote tumor growth in vivo. Cancer Lett 315:28–37

Zhu YJ et al (2017) New knowledge of the mechanisms of sorafenib resistance in liver cancer. Acta Pharmacol Sin 38(5):614–622

Zuo Q et al (2012) Multivariate analysis of several molecular markers and clinicopathological features in postoperative prognosis of hepatocellular carcinoma. Anat Rec 295(3):423–431

Adv Exp Med Biol - Cell Biology and Translational Medicine (2019) 7: 121–134
https://doi.org/10.1007/5584_2019_440
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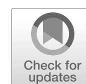
Published online: 5 December 2019



Potential of Tribological Properties of Metal Nanomaterials in Biomedical Applications

Pravin Shende and Drashti Patel

Abstract

Metallic nanomaterials show tremendous applications in biomedical devices due to compatible integration into the most of the biological systems as they are nanostructured. Metallic nanomaterials are capable of mimicking all the three major antioxidant enzymes such as catalase (CAT), peroxidase and oxidase, to control the level of reactive oxygen species (ROS) inside the cell as an alternative strategy over conventional one which has biological toxicity and have several adverse effects, if accumulation takes places during the treatment. This anti-oxidant property of metallic nanomaterials demonstrates as a promising candidate for its biomedical application in disease conditions where the excessive level of ROS causes damage to DNA, lipids and protein in several conditions such as diabetes, cancer and neurodegenerative diseases. Tribology is the study of interacting surfaces in motion and the measurement of properties such as friction, wear-tear and abrasion. While designing nano-scale biomedical devices, the consideration of tribology is particularly important because the high surface area ratio enhances problems with friction and wear-tear which can further affects its function as well as longevity.

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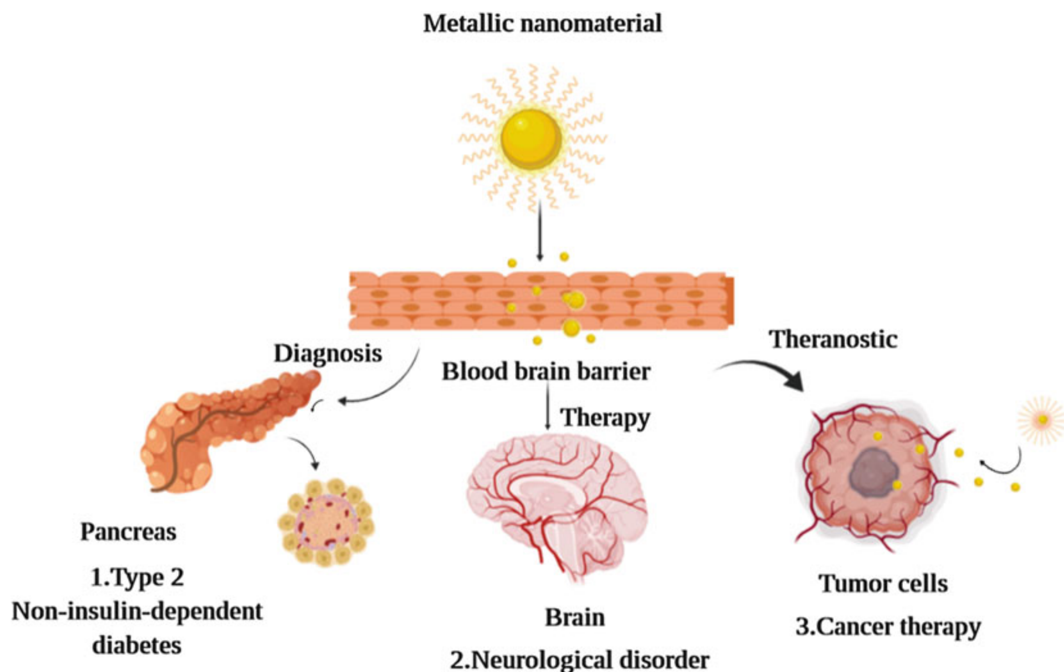
Graphical Abstract



Keywords
Biomedical application · Metallic nanomaterial · Tribological testing · Tribology

Abbreviations

AD	Alzheimer's disease
Aβ	Amyloid beta
CAT	Catalase
CLP	Regulation concerning **Classification, Labeling and Packaging** of Substances and Mixtures
EGF	Epidermal growth factor
FGH	fibroblast growth factor
LSPR	Large Surface Area Plasmon resonance
Mn_3O_4	Manganese oxide
PBCA-	Polybutylcyanoacrylate polymer-
Ti/Au	Titanium/Aluminum
PDGF	Platelet-derived growth factor
REACH	Registration, Evaluation, Authorization and Restrictions of Chemicals
ROS	Reactive oxygen species
SERs	Surface-enhanced Raman Scattering
SNC	Substantial Nigra pars Compacta
SNURs	Significant New Use Rules
TSCA	Toxic Substance Control Act
VEGF	Vascular endothelial growth factor

1 Introduction

Nanotechnology is considered as the engineering of matter on an atomic or molecular scale for the development of composite, structure and grafted material in nano dimensions (1–100 nm). This modifies the properties of the materials such as tiny size with large surface area to exhibit novel

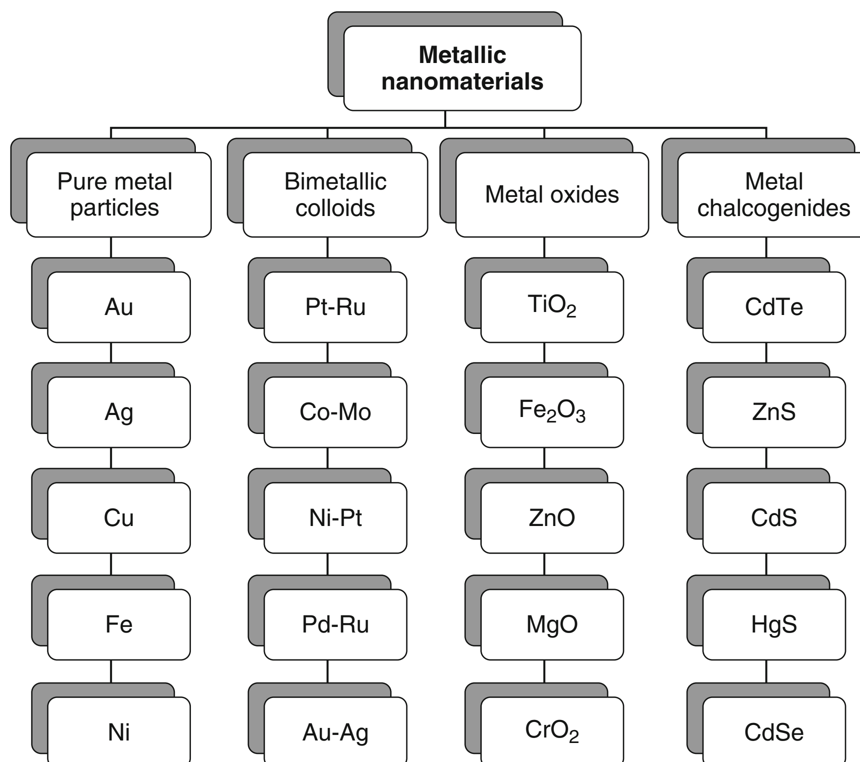


Fig. 1 Examples of metallic nanomaterials

optical, magnetic, mechanical and electric properties (Valavanidis and Vlachogianni 2016). Nanomaterials like gold and silver exhibit distinct properties due to the high surface area to volume ratio and new quantum mechanical effects (i.e. Plasmon resonance). Metallic nanomaterials are metals with a size range of 1–100 nm (Venkatesh et al. 2018). For example, copper at macro-size is considered as hard metal, opaque, transparent but exhibits malleability and ductility at the nano-scale. Similarly, the changes in the melting point of gold at the nano-scale from 200 °C to 1,060 °C and optical property induce color changes from yellow to violet at nano-size apart from catalytic property. Noble metals like gold and silver are widely used in for various applications as a catalyst, anti-microbial agents and anti-cancer agents (Alistair et al. 2010; Atom et al. 1999) as shown in Fig. 1. Tribology is the science and engineering of interacting surfaces in relative motion. It includes the study and application of the principles of friction and wear-tear (Friedrich 2018). Friction between metallic nanomaterials on the cellular surface shows essential influence on the biological function. It also helps to predict the effect of metallic nanomaterials with interacting cellular surfaces and the modifications which are responsible for tuning their response when in contact with biological environment (Yang et al. 2015).

1.1 Advantages and Disadvantages of Metallic Nanomaterials

Metallic nanomaterials alter their properties due to their inert nature, high surface to volume ratio for reaction kinetics, faster diffusion and higher feasibility at lower temperatures. The bio-adhesion and bio-conjugation facilitate easy interaction with other particles and cellular surface (Harish et al. 2018). The disadvantages of

metallic nanomaterials include instability of metallic particles as they situated in high energy minima region and are thermodynamically unstable if accumulated and aggregated which lead to toxicity to the body as well to the environment, expensive technology and scaling-up (Montaño and Ranville 2014). However, the alternatives are present to synthesize metallic nanomaterials via biosynthetic pathways utilizing organisms like fungi, bacteria as well as by using plant extract. Silver is the most widely synthesized metallic nanomaterial using alternatives pathways of *Escherichia coli, Bacillus licheniformis, Bacillus strain CS 11; fungi like Fusarium oxysporum, Verticillium sp, S. pombe* and algae like *S. longifolium, P. capillacae, Microcoleus sp*, etc. (Shende and Basarkar 2019)

1.2 Classification of Metallic Nanomaterials

Metallic nanomaterials are further classified based on their types such as;

(a) **Pure metal particles**: Nanomaterials comprising of submicron entities of pure metals like gold, silver, copper, platinum, etc.
(b) **Bimetallic colloids:** Colloids consist of metallic core is encapsulated by a protective shell and show steric hindrance or electrostatic stabilization function.
(c) **Metal oxides**: Metal oxides are chemical compounds; typically contain an anion of oxygen in the oxidation state. Titanium oxide, nickel oxide, copper oxide and manganese dioxide are versatile materials mostly preferred for catalytic properties (Jacques 2017).
(d) **Metal chalcogenides**: In the periodic table, group 16 elements are referred as chalcogenides, mostly reserved for tellurides, selenium and sulfides rather than oxides.

2 Tribology of Metallic Nanomaterial

Tribology is the science and engineering of chemically interactive surfaces in relative motion and related subjects and the measurements of properties such as abrasion, wear-tear and friction (Jinga et al. 2016). In the year 1966, Josh first reported the word tribology from Greek word "trios" meaning rubbing which is equivalent to friction and wear-tear used alternatively and "bology" means between surfaces/bodies. The consideration of tribological attributes of metallic nanomaterials plays an important role in designing and manufacturing biomedical devices. Since metallic nanomaterials exhibit a larger surface to volume ratio they enhance problems of wear-tear as well as friction. Commonly used metals are aluminum, copper and its oxides. In order to reduce the friction coefficient, understanding nanotribological attributes surface changes of tribological properties will also allow for either increase or decrease friction at a greater scale helps to predict the effects of nanomaterial interrelationship with dynamic surfaces. The emergent characteristics of metallic nanomaterials of tribology-based properties useful in quantitative detection for the formation of new analytical tools such as biosensors. Metallic nanomaterials when fabricated into DNA biosensor by implementing properties such as friction, wear-tear and cohesive forces can use be control biosensors and improve the biochemical reactions necessary for biosensing. It will also improve the sensitivity of biomolecule by enhancing signal amplification due to the electric property and strengthening the ability to control these properties by increasing the probability of device within the body (Credits and Artem 2017).

2.1 Evaluation of Metallic Nanomaterials and Its Tribological Properties

The synthesis and utilization of metallic nanomaterial are increased recently in the

different fields of nanotechnology like biomedical, textile and pharmaceutical engineering. With an increasing market share of nano-products, it is important to assess its potential for contamination, toxic exposure to human as well for environmental hazards. In order to determine the safety of nanomaterials, they are intended for use or possess possible health or/and environmental effects (transport, fate, interaction with living organism), a number of physical and chemical parameters should be evaluated. An exhaustive characterization of metallic nanomaterial is often time-consuming, expensive and complex.

I. **Size and shape:** UV-Visible spectroscopy is a powerful tool for identification, analysis, and characterization of metallic nanomaterials in particular size and shape. The measurement of metallic nanomaterial is because of localized surface plasmon resonance property which arises due to scattering and absorption of photons (Bakalova et al. 2014). Metallic nanomaterial is prepared using reduction method in an aqueous solution or suspensions; this solution is used in UV-Visible spectrophotometer to measure the wavelength maxima of metallic nanomaterials. The red shift in the λ_{max} of copper, gold, and silver show prominent absorption peaks (My et al. 2011). It also enables to measure the amount of metal ion precursor used during the formation of a metallic nanomaterial as well binding of biomolecule to the surface of metallic nanomaterial.

II. **Chemical property:** The chemical bonds of metallic nanomaterial depend on the chemical composition of the metal. Energy dispersive X-ray spectroscopy measures purity and chemical composition. The chemical composition (elemental and chemical state) of metallic nanomaterials surfaces are evaluated with SEM which examines the consistency of nanomaterial surface equipped with energy dispersive X-ray spectroscopy. FTIR spectroscopy evaluates the chemical bonds and functional atoms on the metallic surface (Rasmussen et al. 2018).

III. **Surface chemistry and charge:** Surface charge influences the physical state of metallic nanomaterial. The dynamic light scattering is used for the measurement of surface charge, indirect measurement to measure the net charge on metallic nanomaterial surface.

IV. **Tribological property:** It directly influences the surface chemistry environment and body during relative motion (Bakalova et al. 2014). The evaluation of tribology attributes for metallic nanomaterials includes the determination of friction, wear-tear, interaction with conducting surfaces in relative motion (Thirumalaikumaran 2017). Table 1 shows different tribological testing method, mechanism and the determination of characteristics properties.

3 Biomedical Applications-Based on Tribological Properties of Metallic Nanomaterial

Metals, the essential cell components, present in enzymes, catalyst domain, cellular activities and also involve in multiple biological processes. For example, zinc acts as a vital as a co-factor in the various metabolic process of proteins, lipids and carbohydrates while copper is an essential constituent of several enzymes such as catalase, superoxide dismutase and monoamine oxidase, etc. (Al-fartusie and Mohssan 2017) Metals when fabricated into nano scale, it tunes well into biomedical devices since most biological systems are also nano-sized (Mody et al. 2010; Ndagi et al. 2017). Metals are capable to mimic all three major antioxidant enzymes to control ROS levels inside the cell. In addition the enhanced surface area and increase reactivity on

Table 1 Tribological testing method

Test	Mechanism	Characteristics determine
Ball on disc	Measurement involves the injection of fixed attachment of a test (ball) of the selected material with a defined force to drive (test sample)	Determines wear-tear, extreme pressure properties and friction behaviour of lubricants (Jinga et al. 2016; Bakalova et al. 2014; Thirumalaikumaran 2017)
Light optical microscope	The structures of the test sample are analyzed in reflected light depending on the contrast of images and obtained based on the individual structural reflectivity due to surface topography	Determines the coefficient of friction (Jinga et al. 2016)
Disc- on disc tribotester	One rotating plate is connected with a motor and another plate is fixed with a load cell	Determines the coefficient of friction (Jinga et al. 2016)
Ball-on ring wear tester	A ball-on-ring test determines the resistance of materials on metal-to-metal point contact similar to the block-on-ring wear tester	Evaluates the lubrication characteristics of lubricating oil and nanoparticle mixed lubricating oil (Thirumalaikumaran 2017)
Block on ring tester	The block-on-ring testing machine is utilized to rank pair of materials according to their sliding-wear compatibility characteristics which replicate metal-to-metal wear	Determines the resistance of materials on metal-metal point contact (Jinga et al. 2016)
Dektak™ xt mechanical profilometer	The test sample is placed on the substrate and direct contact of the stationary tip and scans the surface	Determines the resistance of material on metal to metal sliding wear (Jinga et al. 2016)
Pin-on-disk tribometer	A stationary pin is under an applied load which contact with a rotating disc	Determines the mechanical profile of a material (Jinga et al. 2016)

the cellular surfaces of metallic nanomaterials based on its tribological properties. In diseases conditions like diabetes, cancer, obesity and neurodegenerative disorders, generation of an excess level of ROS causes damage to DNA. Lipids and proteins can be detected and treated by using metallic nanomaterials like copper, zinc, iron, manganese, etc. (Chen et al. 2008; Osredkar and Sustar 2011)

3.1 Type 2 Non-insulin-Dependent Diabetes

Diabetes is characterized by impaired insulin secretion or a lack of response of insulin by β-cells of pancreas (Lushchak et al. 2018). The conventional therapy includes oral or injectable function by improving the insulin production in the body and/or suppressing hepatic gluconeogenesis. However, over a period of time, conventional therapies are ineffective due to the resistance caused by traditional medications, inability to maintain blood-glucose levels in the body as well several side effects caused by various available marketed drugs, for example, metformin widely used drug causes nausea, diarrhoea, and weight loss, as well as vitamin B12 deficiency as few side effects, encountered during drug regimen. The other adverse effects mainly in diabetes type 2 are the occurrence of non-kenotic hyperglycaemia. To overcome such problems, an alternative is the utilization of metallic nanomaterials due to their unique physiochemical altered tribological properties to modulate the levels of oxidative stress in diabetes management therapy (Disanto et al. 2018).

Metallic nanomaterials act as scavenging agents by mimicking the peroxidise, catalase (CAT) and oxidase activities which enable excess free radical in the body, reduction in ROS levels and prevents further inflammation. These metallic nanomaterials modified with diverse functionalities such as antibodies, potent drugs, ligands, alloys, peptides, proteins, DNA/RNA specific target cells to conjugate with cellular surfaces. Tribology of metallic nanomaterial aids in the interaction of metal-protein-cell surfaces which provides a medium to influence the enzyme response to a disease state. Metallic

nanomaterials increase the bioavailability, specificity and minimal side effects by reducing dose frequency. The novel metals like gold, silver and traces of metals like selenium, chromium, molybdenum, vanadium, etc. One of the methods of diabetes management where insulin encapsulation in a zinc-silica matrix for drug protection and controlled released. Silica in the formulation prevents the denaturation and aggregation whereas the zinc oxide improves the stability of the matrix system. Moreover, the formation of insulin hexamers in the presence of zinc ions improves stability of insulin during preparation and storage (Woldu and Lenjisa 2014).

In type 2 diabetes, trace metal such as chromium aids in poorly controlled diabetes. Since the tribology of chromium helps the metal to overcome multiple barriers and site-specificity which directly influences the interaction of the metal with the β-cell surface due to its large surface area ratio. Chromium acts as a dietary supplement and improves pharmacological response on β-cell surface (Lushchak et al. 2018). In 2007, Balk used chromium as a supplement on glucose metabolism (Rafique et al. 2010). β-Cells of pancreas within the nanopore (20 nm in diameter) device and implanted in the recipient's body (Chen et al. 2008). Elements like vanadium and zinc are administered in the form of inorganic salts to control glucose level in the plasma. Vanadium-ligand organic complex is shown to improve solubility, lipophilicity and reduce toxicity. In 1999, Badmaey developed a number of vanadium complexes possess insulin-mimetic properties which involve activation of insulin-signalling pathway. Mehdi explained the vanadium complex to enhance tyrosine phosphorylation of insulin receptor substrate (Rafique et al. 2010).

3.2 Cancer Therapy

Metallic nanomaterials are used over conventional methods due to effectiveness and specificity to differentiate between normal and mutated cancerous cells (Baptista 2012). The use of engineered metallic nanomaterials is a less invasive alternative, increase life expectancy and quality of life by early diagnosis when interact with biomolecule both at the surface and within the cells; yielding better signals and target specificity (Baptista 2012). Metallic nanomaterials are easily fabricated in nanoshells, nanorods and quantum dots which are tuned to desirable wavelength according to shape, size and chemical composition to enable bio-imaging, contrast agents, biosensors and biomarkers. Nanoshells, nanoclusters, nanocages, nanorods, etc. are used for *in-vivo* imaging which can absorb and scatter in IR region and its surface interaction is enhanced by tribological properties of metal (Baptista 2012). The properties of the metal complex like its structure, charge variation, variable coordination modes reactivity, bonding metal-ligand interactions, redox activity, partially filled d-shell are considered as a potential candidate in cancer therapy (Press 2017). An experiment conducted at Rice University by Naomi Halas and Peter Nordlander developed gold-silica nanosphere by using amine-terminated spheres. The amine-terminated spheres were then treated with a gold suspension (1–2 nm). They found that irreversible photo-thermal ablation of localized tumor tissue successfully in presence of gold-silica nanospheres (Mody et al. 2010). Gold (I) and gold (II) NHC complexes induced apoptosis of cancerous cells as a anti-cancer agent (Press 2017). Zhang developed a fluorescent metal composed of silica spheres with encapsulated Ru $(bpy)_3^{2+}$ in the silver shell, RU(II) complex mimic iron and also interact with plasimidic DNA (Ndagi et al. 2017), which enhances emission intensity up to six-fold and photostability by two-fold, as well as achieve longer emission signals that overcome cellular autofluorescence interference (Siddiqi et al. 2018). Surface-enhanced Raman Scattering (SERs) is used to obtain information related to cellular surfaces (Yeung 2010).

Doxorubicin encapsulated with gold nanomaterial in the core showed large surface area plasmon resonance (LSPR) in the near infrared region 600–1100 nm was suggested for

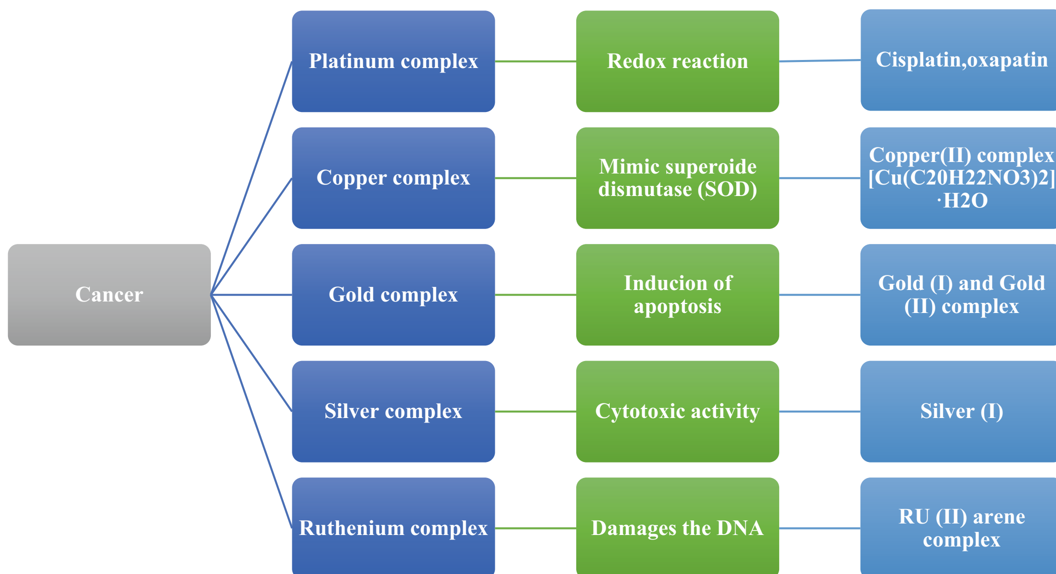


Fig. 2 Metal complex used in cancer treatment

imaging and therapy of neoplasm. In clinical trials, it was employed in murine rat models as contrast agent as well as drug carrier *in-vitro* (MCF-7). Jin prepared gold nanomaterial containing graphene oxide and polyallylamine hydrochloride (Au-PLA) via layer-by-layer technique (Cassano and Voliani 2018). Hembury et al. produced hollow mesoporous silica spheres with a shell thickness of 25 nm composed of gold nanoparticle (AuNP<8 nm) and fluorescent gold quantum dots (AuQD <2 nm). The gold-silica nanomaterial complex was biocompatible and enhanced capsulation efficiency. Gold QD which emit lights in NIR region and paramagnetic resulting in promising agents for multimodal imaging, MRI and NIR fluroscence (Cassano and Voliani 2018).

Metallic nanomaterials act also as anti-angiogenesis, angiogenesis is the formation of new blood vessels (capillaries) which provide oxygen and nutrients to a cancerous cell. It occurs during the development of most metastases cancer in order to grow and spread. It is regulated by chemical signals, imbalance in these signals leads to angiogenesis in cancer. The growth factors like platelet-derived growth factor (PDGF), fibroblast growth factor (FGH), epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) are imperative for angiogenesis. Metallic nanoparticles include quantum dots, gold, silver, iron oxide as well nanocomposite like viral capsids ferritin are utilized for targeting growth factors and inhibiting by acting as anti-angiogenesis agent (Chowdhury et al. 2017). Figure 2 shows the metal complex used in cancer treatment.

3.3 Neurological Disorder

Engineered metallic nanomaterials are recently used to treat neurological conditions like Parkinsonism and Alzheimer's diseases. Nanomaterials like metal and their oxides are used because they show the ability to penetrate multiple barriers, bio-conjugation, and bio-adhesion to the cellular surface based on the tribological property. For example, targeted drug rivastigmine of nanoparticles can effectively cross BBB with minimal side effects (Leszek et al. 2017). The

treatment and management of neurological disease conditions are limited because of BBB restrict for crossing the poorly soluble drug to reach the targeted site (Siddiqi et al. 2018). In order to overcome such problem, metallic nanomaterials are fabricated as an alternative therapy for diagnosis and management by acting as a nanomedicine drug delivery system. As metallic nanomaterials are smaller in size, engulfed by cells and stored in peripheral tissues for a longer period as sustained release formulations. The increase bioavailability *in-vivo* since metallic nanomaterials can interact due to its tribological property with physiological systems at the supramolecular level. This occurs with reduced adverse effects as nanomaterials do not interact with the body's defense mechanism. Various nanomaterials like nanoshell, nanorods, nanocage, and nanospheres can be conjugated with polymers like hydraulic acid (HA), PEG, silica, chitosan, gelatin, polyethylene glycol, cyclodextrin as well as with metals like gold, silver, titanium, iron and copper in the treatment and management of neurological disorders as theranostic agents (Siddiqi et al. 2018).

Marty quantified the band gap between EC of rat BBB generated by the ultrasound using superparamagnetic iron oxide nanoparticles of 65 nm and gadolinium-based MRI contrast agents. It was demonstrated that gold nanoparticles (50 nm) when encapsulated into PEG thiolate enhances the potential of the drug delivery system in rat model. The uses of transition metals like lithium are used in the treatment of neurological disorders. The mechanism based on inhibiting the scavenging pathway for capturing the inositol in the resynthesis of polyphoshnositides in the brain. Lithium in combination with valproate delays disease onset, prolongs survival in an amyotrophic lateral sclerosis mouse model and reduces neurological deficits (Rafique et al. 2010).

Alzheimer's Disease

Alzheimer's disease (AD), a cognitive dysfunction, is characterized by irreversible neurological conditions. The most commonly described pathophysiology of AD is the amyloid cascade hypothesis which implies that Aβ aggregation is the major cause of neurodegeneration in disorder. Recently multiple metallic nanomaterials are used in the diagnosis of Alzheimer's disease which is encapsulated using functionalized hydrophilic polymers, proteins and peptides. Some approaches intended to reduce Amyloid beta (Aβ) clusters using a metallic nanomaterial which has improved affinity toward the amyloid-β surface. This can be achieved by tribological property of a metal which increases the interaction of metallic nanomaterial conjugated with proteins with the amyloid-β surface. The potential application of metallic nanomaterial is detection primarily by novel properties at nanoscale such as optical, electrical and magnetic. DNA-Au conjugate detects Aβ protein biomarker at low concentration (10–18 per molar litre) technique called bio-barcode magnetic iron oxide and gold attached to DNA strands. It also prevents the aggregation by dissolving Aβ clusters by localized thermal effect (Ahmad et al. 2018). Metallic nanomaterials such as polysorbate 80, hydrophilic polymer PEG (lactic acid) to stabilize the nano-metallic core which can be used as a fluorescent probe which is highly selective as well sensitive for the early detection of amyloid- β cells in Alzheimer's disease (Siddiqi et al. 2018). Bolisetty synthesized metals such as palladium, gold, and silver, a food protein derived amyloid fibril (β-lactoglobulin) that effectively internalization into dendritic cells (Karthivashan et al. 2018). One approach to control progressive of AD for the usage of metal chelation since brains contain a certain amount of trace metal ions like iron (Fe^{+2}), zinc (Zn^{+2}) and copper (Cu^{+2}), trace metal ion also participates in various physiological activities as trace essential nutrients. The compart-

mentalization as well as maintaining homeostasis balance of trace metal ions is essential. Any disturbance in homeostasis of trace ions leads to produce excess ROS which results in affecting various proteins and damages lipid membrane. Amyloid beta (Aβ) proteins possess few selective binding trace ions for Zn^{+2} and Cu^{+2} therefore, considered as metalloprotein. When the trace ions of Cu^{+2} and Zn^{+2} are released in synaptic terminal of brain cortical neuron might induce Aβ aggregation by interacting with Aβ histidine. This aggregation can be reversed by metal chelation, it is reported that zinc competes with copper for Aβ binding and inhibits copper-mediated Aβ redox chemistry prior to fibrillization and hence reduction in Alzheimer disease progression (Osredkar and Sustar 2011; Ahmad et al. 2018).

Parkinson Disease

Parkinsonism is the most common and progressive neuro-degenerative disease with high prevalence in the elderly population, which is characterized by extensive and progressive loss of dopaminergic neurons in substantial nigra pars compacta (SNC) (Kaushik et al. 2018). The conventional therapy includes drugs that accelerates dopamine level or stimulate central dopamine receptors. However, drugs are limited to dopaminergic targeting, with several side effects which include discomfort, anxiety, hallucination and arrhythmia. Recently, there are no effective biomarkers which can detect Parkinsonism at an early stage. Therefore, metallic nanomaterials are engineered in diagnosis which is conjugated with peptides. Titanium dioxide and gold conjugated with polybutylcyanoacrylate polymer (PBCA-Ti/Au) are used as chemical biosensor which helps in neuron α-synuclein clearance (Siddiqi et al. 2018).

The team of researchers at Indian Institute of science in Bengaluru, 2017 has fabricated a manganese oxide (Mn_3O_4) nanomaterial that is capable to mimic all three major antioxidants such as catalase, superoxide dismutase and glutathione peroxidase enzymes. Mn_3O_4 was able to control the levels of excess ROS; they scavenge ROS and optimize the level without affecting normal physiology of the cell. Zinc (II) complex is involved in neuronal signalling pathways. Aras reported, zinc (II) can be responsible for the initiation of a neuroprotective pathway. Zinc oxide wire of 100 nm diameter selectively detect levels of dopamine and uric acid in the serum of Parkinsonism patient with enhancing the sensitivity of electrochemical biosensor which makes potential biomarker in Parkinson diagnosis (Adhikary et al. 2015). Cerium dioxide at 20 nm is used for diagnosis in Parkinsonism (Kaushik et al. 2018). In experiment conducted on Drosophila melanogaster suggested that metals such as iron, manganese and copper in combination are capable to destroy dopaminergic neurons in the brain (Osredkar and Sustar 2011).

3.4 Miscellaneous

Nanosized silica can be used as filler in a range of cosmetic products and in dental fillings (Noraihan et al. 2011). In cosmetic preparations, nanomaterials impart them new characteristics (Chaudhri et al. 2015) such as excellent dispersibility, smooth texture and transparency to the skin. For example, titanium dioxide and zinc oxide are incorporated in sunscreens and sunscreen lotions for UV absorption (Katz 2007).

In order to overcome drug resistance problem, silver is used in nanosize as an alternative therapy. Since the properties of the metallic nanomaterials like bio-adhesion and bio-conjugation which interfere in bacterial physiology and inhibiting further bacterial growth. Silver nanoparticles are used for their antimicrobials property against gram-positive strains, at the minimum concentration (10 nm) complex with PEG or chitosan. Silver ions bind to DNA and inhibit bacterial enzymes which damage bacterial cell wall and cytoplasmic membrane as well promotes the wound healing process (Nikalje 2015). Iron is the co-factor of many enzymes and play a vital role in cell physiology

process to increase in the amount of iron is harmful to bacteria and hence preventing further infection due to bacterial cell lysis. Zinc oxide bimetallic composite is prepared using polyurethane biocompatible polymer (Zn:PU) demonstrates significant antimicrobial activity against *E.coli (*Vimbela et al. 2017*)*. Copper oxide (CuO) is used against gram positive and gram negative strains of bacteria can be incorporated into medical device (Santos et al. 2013). Metallic nanomaterials are widely used as diagnostic tools; cellular functions can be measured as metals possess unique properties like redox activity, valency and radiochemical properties for example, Gold nanorods used for photoacoustic molecular imaging. De la Presa synthesized gold nanoparticle inside ethosomes which can be used as a diagnostic tool (Rafique et al. 2010).

Nanotoxicity of metallic nanomaterials is dependent on their size, shape, chemical composition, surface chemistry and route of administration (Raj et al. 2012). Two types of nanotoxicities of metallic nanomaterials include biological and environmental toxicity. The interaction of nanomaterials with biological systems is termed as biological nanotoxicity. The relationship between physical and chemical properties (size, shape, composition, and aggregation) of nanomaterials with the induction of toxic biological response. Nanotoxicity is commonly precipitated in the respiratory system and gastrointestinal tract. Environmental toxicity is defined as the presence of nanomaterial in the environment which further leads to accumulation of nanomaterials on soil or water bodies which is further indirectly harmful to humans (Buzea et al. 2017).

4 Nanotoxicity

Metallic nanomaterials based on their tribological property directly interact with biomolecules at the cellular surface and within the cells which contribute to the specificity, but also causes potential nanotoxicity of nanomaterials (Contado 2015). The tendency of nanomaterials to accumulate *in-vivo* and *in-vitro* which can lead to nanotoxicity due to the high degree of bio-accumulation in tissues like lymph nodes, lungs, kidney, spleen, bone marrow etc. (Radomska et al. 2016) The smaller size of engineered metallic materials helps to avoid renal clearance since kidney filters particles <10 nm while nanomaterials range from 1–100 nm remains in the systemic circulation, penetrate into tissues via capillaries and triggers an immune reaction. Silver nanoshells provide camouflage to the cellular barrier and its accumulation leads to undesired effects due to their size similarity (Chen et al. 2008). Metallic nanomaterials induce DNA damage and oxidative damage.

5 Regulatory Aspects

Currently, an increase in the need to understand engineered metallic nanomaterials at the cellular and systemic levels not only to optimize the therapeutic applications but also to minimize their potential side effects. Efforts are in process to develop different methods for safety evaluation during the development of nanomaterials. Some lipophilic and polymeric nanomaterials are engineered to biodegrade *in-vivo*, but other metals and metal oxides are sparingly soluble which may accumulate *in-vivo* and leads to cellular toxicity by the generation of reactive species, cytotoxicity, and genotoxicity. The urgent need to standardize and validate the analytical methods to determine the physiochemical properties important to describe nanomaterials biological interactions *in-vivo.* An emerging need to create reference materials and developing a reference protocol mainly because of the increasing complexity of nanomaterial (Contado 2015). The potential health risk following exposure to a substance also depends on the disease conditions like Parkinsonism and

Table 2 Regulatory body in different countries

Country	Regulatory body
USA	Toxic Substance Control Act (TSCA);
	SNURs (Significant New Use Rules)
Europe	Registration, Evaluation, Authorisation and Restrictions of Chemicals (REACH);
	Regulation concerning Classification, Labeling and Packaging of Substances and Mixtures (CLP)
Korea	Toxic Chemical Control Act and the Chemical Registration and Assessment Act
China	Regulation on the Safety Management of Hazardous Chemicals

Alzheimer disease magnitude and duration of the exposure, the persistence of the material in the body, the inherent toxicity of the material and the susceptibility or health status of the person. The results of existing studies in animals or humans on exposure and response to ultrafine or other respirable particles provides a base for preliminary estimates of the possible adverse health effects from exposures to similar materials on the nanoscale. It is important to recognize and understand the influence of metallic nanomaterials, including size and surface area. Existing toxicity information about a nanomaterial provides a baseline for anticipating the possible adverse health effects that occur from exposure to that same nanomaterial on the nano-scale. The collection of more data with regards to the health risks associated with exposure to engineered nanomaterial is required. The existing legislation (Park 2012) for the regulation of nanomaterial is shown in the Table 2.

6 Conclusions

Owing to the ever-increasing need of nanotechnology in the field of the health care industry, it is very critical to understand the behaviour of materials at the nano-size. Metallic nanomaterials are studied exhaustively considering its inertness, high surface to volume ratio, bio-adhesion and bio-conjugation. It is strictly important to study the tribological properties of nanomaterials which will help the pharmaceutical scientists to explore them better. As metals are inherent components of biological processes in the human body, metallic nanomaterials tune well with the biomedical devices. The role of metallic nanomaterials based on its tribological properties helps to predict the effect of nanomaterials with interacting surfaces in type 2 diabetes, cancer and neurological diseases and used as analytical tools in biomedical applications (Yapar and Inal 2012). As far as the regulatory aspects of metallic nanomaterials are concerned, stringent guidelines are needed for the maximum utilization of tribological properties of nanomaterials as the future pillars of the healthcare industry.

References

Adhikary RR, Sandbhor P, Banerjee R (2015) Nanotechnology platforms in Parkinson's disease. ADMET DMPK 3:155–181

Ahmad J, Akhter S, Khan MA (2018) Nanotechnology based Theranostic approaches in Alzheimer's disease management: current status and future perspective. Curr Alzheimer Res 14:1164–1181

Alistair C, Morris ER, Horcajada P, Ferey G, Gref R, Couvreur P, Serre C (2010) BioMOFs: metal–organic frameworks for biological and medical applications. Angew Chem 49:6260–6266

Al-fartusie FS, Mohssan SN (2017) Essential trace elements and their vital roles in human body. Indian J Adv Chem Sci:127–136. https://doi.org/10.22607/IJACS.2017.503003

Atom C, Metal N, Corma A, Kumar D (1999) Learn more about metal catalyst adsorption and its applications in industry and environmental protection mesoporous molecular sieves 1998

Bakalova T, Voleský L, Škodová P, Louda (2014) Effect of natural fillers in the form of nanoparticles on the tribological properties of process fluids. Nano 5–10

Baptista P (2012) Noble metal nanoparticles applications in cancer. J Drug Deliv 2012:1–13

Buzea C, Pacheco II, Robbie K (2017) Nanomaterials and nanoparticles: sources and toxicity. Biointerphases 2:1–57. https://doi.org/10.1116/1.2815690

Cassano D, Voliani V (2018) Ultrasmall-in-nano approach: enabling the translation of metal nanomaterials to clinics. Bioconjug Chem 29 (1):4–16. https://doi.org/10.1021/acs.bioconjchem.7b00664

Chaudhri N, Soni GC, Prajapati SK (2015) Nanotechnology: an advance tool for nano-cosmetics preparation. Int J Pharma Res Rev 4:28–40

Chen PC, Mwakwari SC, Oyelere AK (2008) Gold nanoparticles: from nanomedicine to nanosensing. Nanotechnol Sci Appl 1:45–66

Chowdhury A, Kunjiappan S, Panneerselvam T, Somasundaram B, Bhattacharjee C (2017) Nanotechnology and nanocarrier-based approaches on treatment of degenerative diseases. Int Nano Lett 7:91–122

Contado C (2015) Nanomaterials in consumer products: a challenging analytical problem. Front Chem 3:1–20

Credits I, Artem E (2017) Tribology for nanotechnology applications nanotechnology applications of tribology for biosensors and drug delivery nanotechnology applications of tribology for data storage. Azo Nano, AZoNetwork UK Ltd, UK, pp 1–3

Disanto RM, Subramanian V, Gu Z (2018) Recent advances in nanotechnology for diabetes treatment. *Wiley* Interdiscip Rev Nanomed Nanobiotechnol 7:1–24

Friedrich K (2018) Advanced industrial and engineering polymer research polymer composites for tribological applications. Adv Ind Eng Polymer Res 1:3–39

Harish Kumar K, Venkatesh N, Bhowmik H, Kuila A (2018) Metallic nanoparticle: a review. Biomed J Sci Tech Res 4(2):3765–3775. https://doi.org/10.26717/BJSTR.2018.04.001011

Harish KK, Nagasamy V, Himangshu B, Anuttam K (2018) Metallic nanoparticle: a review. Biomed J Sci Tech Res 4:1–11

Jacques CV (2017) Heterogeneous catalysis on metal oxides. Catalysts 7:1–25. https://doi.org/10.3390/catal7110341

Jinga V et al (2016) The influence of nanoadditives on the tribological properties of process fluids. J Phys Conf Ser:1–11. https://doi.org/10.1088/1742-6596/709/1/012001

Karthivashan G, Ganesan P, Park S, Choi D (2018) Therapeutic strategies and nano-drug delivery applications in management of ageing Alzheimer's disease. Drug Deliv 0:307–320

Katz LM (2007) Nanotechnology and applications in cosmetics: general overview. ACS Symp Ser:193–200. https://doi.org/10.1021/bk-2007-0961.ch011

Kaushik AC, Bharadwaj S, Kumar S, Wei D (2018) Nanoparticle mediated inhibition of Parkinson's disease using computational biology approach. Sci Rep:1–8. https://doi.org/10.1038/s41598-018-27580-1

Leszek J, Ashraf G, Tse WH, Zhang J, Kazimierz G (2017) Nanotechnology for Alzheimer disease. Curr Alzheimer Res 14(11):1182–1118. https://doi.org/10.2174/1567205014666170203l

Lushchak O, Zayachkivska A, Vaiserman A (2018) Metal846 lic nanoantioxidants as potential therapeutics for type 847 2 diabetes: a hypothetical background and translational AU6 848 perspectives. Oxidative Med Cell Longev 1–9

Mody VV, Siwale R, Singh A, Mody HR (2010) Introduction to metallic nanoparticles. J Pharm Bioallied Sci 2:282–289

Montaño MD, Ranville J (2014) Detection and characterization of engineered nanomaterials in the environment: current state-of-the-art and future directions. U.S. Environmental Protection Agency, Washington, DC

My T, Dang D, Tuyet T, Le T, Fribourg-blanc E (2011) Synthesis and optical properties of copper nanoparticles prepared by a chemical reduction process. Adv Nat Sci Nanosci Nanotechnol:1–7. https://doi.org/10.1088/2043-6262/2/1/015009

Ndagi U, Mhlongo N, Soliman ME (2017) Metal complexes in cancer therapy – an update from drug design perspective. Drug Des Devel Ther 11:599–616

Nikalje AP (2015) Nanotechnology and its applications in medicine. Med Chem 5(2):81–89. https://doi.org/10.4172/2161-0444.1000247

Noraihan T, Tuan A, Mohamad D, Ismail AR (2011) Synthesis of nanosilica fillers for experimental dental nanocomposites and their characterisations. J Phys Sci 22:93–105

Osredkar J, Sustar N (2011) Copper and zinc, biological role and significance of copper/zinc imbalance. J Clin Toxicol:1–18. https://doi.org/10.4172/2161-0495.S3-001

Park Y (2012) Nano-regulation in China, Japan, Korea, USA and EU. http://www.egg2012.De/tl_files/pdf/paper/EGG2012

Press D (2017) Metal complexes in cancer therapy – an update from drug design perspective. Drug Des Devel Ther 11:599–616

Radomska A, Leszczyszyn J, Radomski M (2016) The nanopharmacology and nanotoxicology of nanomaterials: new opportunities and challenges. Adv Clin Exp Med 25:151–162. https://doi.org/10.17219/acem/60879

Rafique S, Idrees M, Nasim A, Akbar H, Athar A (2010) Transition metal complexes as potential therapeutic agents. Biotechnol Mol Biol Rev 5:38–45

Raj S, Sumod U, Jose S, Sabitha M (2012) Nanotechnology in cosmetics: opportunities and challenges. J Pharm Bioallied Sci 4:186–193

Rasmussen K et al (2018) Physico-chemical properties of manufactured nanomaterials – characterisation and relevant methods. An outlook based on the OECD testing Programme. Regul Toxicol Pharmacol 92:8–28

Santos CL, Albuquerque AJR, Sampaio FC, Keyson D (2013) Nanomaterials with antimicrobial properties: applications in health sciences. In: Méndez-Vilas A (ed) Microbial pathogens and strategies for combating them: science, technology and education, 1st edn. Formatex Research Center, Badajoz, pp 143–154

Shende P, Basarkar V (2019) Recent trends and advances in microbe-based drug delivery systems. DARU J Pharm Sci. https://doi.org/10.1007/s40199-019-00291-2

Siddiqi KS, Husen A, Sohrab SS, Yassin MO (2018) Recent status of nanomaterial fabrication and their potential applications in neurological disease management. Nanoscale Res Lett 13(15–18)

Thirumalaikumaran A (2017) The tribological behaviour of nanoparticles mixed lubricating oil – review. Int Res J Eng Technol 4:3220–3228

Valavanidis A, Vlachogianni T (2016) Engineered nanomaterials for pharmaceutical and biomedical products new trends, benefits and opportunities. Pharm Bioprocess 4(1):13–24

Vimbela GV, Ngo SM, Fraze C, Yang L, Stout DA (2017) Antibacterial properties and toxicity from metallic nanomaterials. Int J Nanomedicine 12:3941–3965

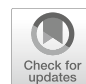
Woldu MA, Lenjisa JL (2014) Nanoparticles and the new era in diabetes management. Int J Basic Clin Pharmacol 3:277–284

Yang L, Liu H, Lin Y (2015) Biomaterial nanotopography-mediated cell responses: experiment and modeling. Int J Smart Nano Mater 5:227–256

Yapar EA, Inal O (2012) Nanomaterials and cosmetics. J Pharm Istanbul Univ 42(1):43–70

Yeung KWK (2010) Nanotechnology in biomedical applications: a review. Int J Nano Biomater 3 (2):119–132. https://doi.org/10.1504/IJNBM.2010.037801

Adv Exp Med Biol - Cell Biology and Translational Medicine (2019) 7: 135–149
https://doi.org/10.1007/5584_2019_420
© Springer Nature Switzerland AG 2019
Published online: 3 August 2019



The Impact of the Low Frequency of the Electromagnetic Field on Human

Kawthar A. Diab

Abstract

Recently, there has been attention and controversial debate topic about the effect of low-frequency electromagnetic fields (EMFs) on human beings. The catalyst for public awareness initiated from the first epidemiological study in 1979 that reported an association between residential EMFs exposure and the incidence of childhood leukemia. For over 40 years, many epidemiological and laboratory investigations were conducted to identify the possible biological effects of low-frequency EMF. Several studies conducted at frequencies 50/60 Hz, which related to generating of electricity from electrical appliances. Experimental studies on low-frequency EMF have provided conflicting data under specific *"in vivo" and "in vitro"* environments. Some original papers have reported the damaging effect on DNA molecule in EMF-exposed cells. Other studies have suggested no such damage in EMF-exposed cells. Also, the conclusions from other studies were inconclusive. These conflicting findings may attribute to the differences in the apparatus used to generate electromagnetic fields, experimental design, exposure time, genetic endpoints, and biological materials such as cell lines and animal species, strain, and age. As DNA damage is frequently a prerequisite for cancer disease, this review provided an experimental body of evidence on the effect of EMF on genetic material.

Keywords

Carcinogenicity · Epidemiological studies · Genotoxicity · Low-frequency electromagnetic field · Mammalian cells

Abbreviations

2dG	2-Deoxyguanosine
8-OHdG	8-Hydroxy-2′-deoxyguanosine
ALL	Acute lymphoblastic leukemia
BNU	n-butylnitrosourea
BP	Benzo(a)pyrene
CAs	Chromosomal aberrations
CHO	Chinese hamster ovary
CREST	Antikinetochore antibody staining
DMBA	7, 12-dimethylbenz[a]anthracene
EMFs	Electromagnetic fields
ENU	N-ethyl-N nitrosourea
G	Gauss
Gd	Gadolinium
GMF	Gradient magnetic field
HF-EMFs	Higher-frequency-electromagnetic fields
HLECs	Human lens epithelial cells
HMSC	Human mesenchymal stromal cells
Hz	Hertz
LM-EFs	Low- to mid-frequency EMFs

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MN	Micronuclei
M054	Human brain glioma
MRI	Magnetic resonance imaging
SCE	Sister chromatid exchange
SI units	International System of Units
T	Tesla

1 Introduction

Recently, much attention has increased in electromagnetic fields (EMFs) due to concerns about the possible adverse effects of low-frequency EMF on humans and animals (WHO 2007). All living organisms surrounded by the Earth's magnetic field and electromagnetic pollution that resulted from man-made EMF sources such as electrical wiring, appliances, and power lines. The possible damaging effect of EMF depends on the density of the field, the wavelength or frequency, and the exposure period (Phillips et al. 2009). Low-frequency EMFs emit non-ionizing radiations that produce long wavelengths and small frequencies (Furse et al. 2009). Most experimental studies performed at a frequency range between 50 and 60 hertz to generate electricity from electrical appliances at homes (WHO 2007). Fifty Hertz matched to a wavelength of 3500 km, which is near to the Earth's radius (Furse et al. 2009; WHO 2007).

Mutation alteration of the genome is considered as the main key in the cancerous process. The chromatin integrity under low-frequency EMF exposure conditions has been assessed in different model systems with inconsistent outcomes (Vijayalaxmi and Prihoda 2009). These contradictory data may be due to the differences in the animal model, type of cell line, experimental design, biomarker assays and equipment used for generation EMF (Jin et al. 2014). Furthermore, many epidemiological studies pointed out the presence link between low-frequency EMF exposure and increased incidence of cancer in children and adults (Marcilio et al. 2011; Sermage-Faure et al. 2013). Other studies reported no such associations (Koeman et al. 2014; Sorahan 2012). The present review presented the following points: (1) The basic background to EMF; (2) The potential effects of EMF on the human health; (3) The published literature and future research.

2 Basic Background of EMF

2.1 Definition of EMF

Both electric and magnetic fields are invisible regions of energy that are formed by electricity, which is the movement of electrons through the electrical wiring. The electric field strength is measured by the voltage that is the force used to push the electrons through the electrical wires, similar to pushing water through a pipe. While the voltage increases, the electric field strength increases. The magnetic field is generated during the flow of electric current in wires or electrical devices and increases in strength as the current increases. The SI units of electrical potential differences and electric current are measured in volts per meter (V/m) and amperes (A), respectively. The units of magnetic intensity (flux density) are measured in either Tesla (T) or Gauss (G). The strength of a magnetic field decreases rapidly with increasing distance from its source (Furse et al. 2009).

The EMF, invisible energy, is generated from the charged particles and is indefinitely expanded throughout the space. Electromagnetic waves are waves carrying an electric field, a magnetic field, and quanta energy. These waves can travel at the

Units of magnetic intensity
Tesla (T) = 1000 mT (milli tesla) = 10^6μT (micro tesla) = 10^9 nT (nano tesla)
Gauss (G) = 1,000 mG (milli gauss) = 10^6μG (micro gauss) = 10^9nG (nano guass)
Tesla (T) = 10,000 G = 1000 mT
Gauss (G) = 10^{-4} T = 100 μT

speed of the light in space and can travel at a slower speed through a medium. These waves have a snake-like figure that makes them as transverse waves. The highest peak of a wave is known as a crest, while the lowest peak of a wave is known as a trough. Electromagnetic waves are measured by their height (amplitude) or by their wavelength, which is the distance between the crest of one wave to the crest of the next wave. One complete wave, from trough to trough, or from crest to crest is called a cycle. The number of complete cycles that occur per second is called the wave's frequency. The hertz (Hz) is the standard of wave's frequency (Furse et al. 2009).

2.2 Types of Electromagnetic Field (EMFs)

Basically, EMFs can be classified into two main types: Higher-frequency EMFs and low to mid-frequency EMFs (Table 1, Fig. 1). The electromagnetic waves possess two major effects on the human body namely, thermal effect (heat-dependent damage) and non-thermal effect (chemical) effect (WHO 2007).

2.2.1 Higher-Frequency EMFs (HF-EMFs)

HF-EMFs, which include gamma rays, X-rays, and higher ultraviolet, are in the ionizing radiation part of the electromagnetic spectrum and cause DNA damage directly (Furse et al. 2009). The lower ultraviolet part, invisible light and infrared are considered high frequency and are in the non-ionizing radiation part of the electromagnetic spectrum.

2.2.2 Low- To Mid-Frequency EMFs (LM-EMFs)

LM-EMFs include static fields (electric or magnetic fields that do not vary with time), magnetic fields from power lines and electrical equipment, visible light, infrared radiation, microwaves, and radio waves. These LM-EMFs are in the non-ionizing radiation part of the electromagnetic

Table 1 Types of EMFs and their frequencies

Types of EMFs	Wavelength	Frequency	Designation
I-Higher frequency EMFs			
1-High frequency	100–10 m	3–30 MHz	Radio waves
2-Very high frequency	10–1 m	30–300 MHz	Infrared ray
3-Ultra high frequency	1 m–10 cm	300 MHz –3 GHz	Visible light
4-Super high frequency	10–1 cm	3–30 GHz	Ultraviolet ray
5-Extremely high frequency	1 cm–1 mm	30–300 GHz	X-ray
6-Tremendously high frequency	1 mm–0.1 mm	300 GHz–3THz	Gamma ray
II-Low to mid frequency EMFs			
1-Extremely low frequency	10^5–10^4 km	3–30 Hz	Lightning and natural disturbances in the geomagnetic field
2-Super low frequency	10^4–10^3 km	30–300 Hz	Power cables and electronic instruments
3-Ultra low frequency	10^3–100 km	300–3,000 Hz	Military communication through the ground
4-Very low frequency	100–10 km	3–30 kHz	Radio navigation service; secure military with submarines; computer monitors and TV sets
5-Radiofrequency	10–1 km	30–300 kHz	Radar signals
6-Medium frequency	1 km–100 m	300 kHz–3 MHz	Radio broadcasting; navigation radio beacons; maritime ship-to-shore communication

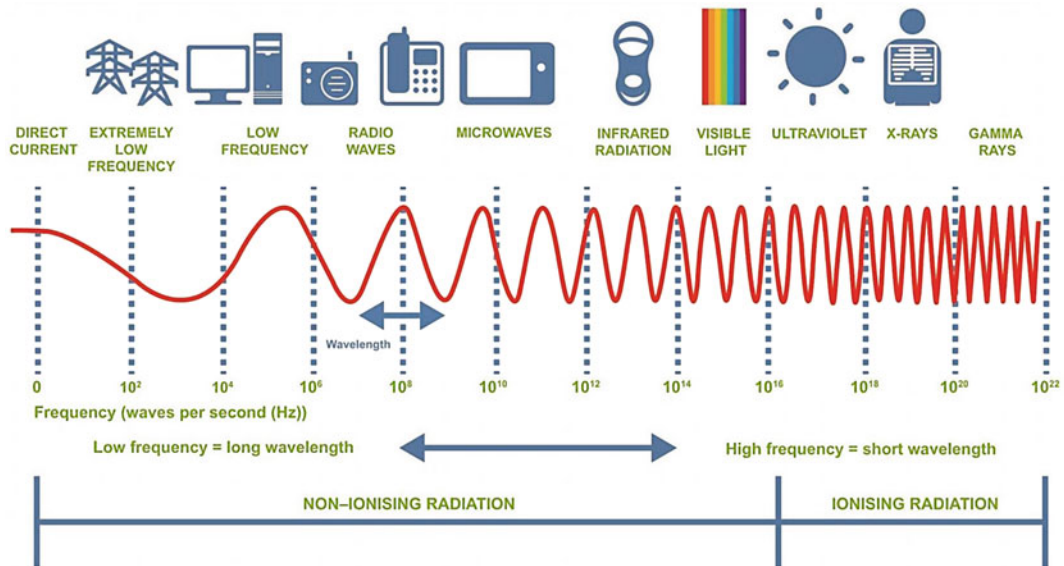


Fig. 1 Electromagnetic spectrum

spectrum and are not known to damage DNA or cells directly (WHO 2007). LM-EMFs include different frequencies of EMFs that ranged from extremely low-frequency EMFs and radiofrequency EMFs (Table 1). Radiofrequency EMFs have frequencies from 30 kHz to 300 KHz which corresponds to the frequency of electrical singles used to produce and detect radio waves.

2.3 Sources of Non-ionizing EMFs

2.3.1 Natural Source of EMFs

Before the invention of electricity, human beings were exposed only to the magnetic field of the earth. The electric field is produced by charges in the clouds or by the static electricity of two items abrasion together, or the unexpected electric and magnetic fields caused by lighting. Geomagnetic field or earth's magnetic field originates in earth's core, a region of iron alloys extending to about 3400 km (the earth's radius is 6370 km). This region consists of a solid inner core and liquid outer core (Livermore et al. 2013). The magnetic field of the earth generates from the motion of liquid iron alloy in the outer core. This motion is driven by heat flow from the solid inner core, which its thermal conductivity is about 6000 K (5730 °C; 10,340 °F), to the core-mantle boundary, which is about 3800 K (3530 °C; 6380 °F). The geomagnetic field is organized by the rotation of the earth around the sun and the presence of the solid inner core (Finlay et al. 2010).

Earth's magnetic field that extends from the Earth's interior out into space meets the solar wind, a stream of energetic particle emanating from the sun. Its magnitude at the earth's surface ranges from 25 to 65 μT (0.25–0.65 G) (Finlay et al. 2010). Geomagnetic field deflects the solar wind, whose charged particles would otherwise strip away the ozone layer that protects the living organism from harmful ultraviolet radiation (Randall et al. 2005). The earth's magnetic field causes a compass needle to orient in a North-South direction and is used by birds and fish for navigation (Ng 2003). The electric field is caused by charges in the clouds or by the static electricity of two items abrasion together, or the unexpected electric and magnetic fields caused by lighting (Ng 2003).

2.3.2 Human-Made Source of Non-ionizing EMFs

After invention of electricity, humans have been increasingly surrounded by man-made EMFs which included extremely low-frequency and radiofrequency categories of non-ionizing part

of the electromagnetic spectrum. These EMFs can come from a number of sources (Ng 2003).

Low-frequency EMFs: The most common sources of ELF-EMFs are included power lines, electrical wiring in buildings, electricity emerged from power socket, and electrical equipments such as shavers, hair dryers, computer monitor, and electric blankets (WHO 2007).

Radiofrequency EMF: The most common sources of radiofrequency EMF are microwave ovens, cell phones, tablets, and portable wireless devices (IARC 2013). Other sources for radiofrequency EMF are magnetic resonance imaging (MRI), radio and television waves, radar, satellite stations, cordless telephones, wireless telecommunication devices televisions and computer monitors, wireless local area networks (Wi-Fi), antenna towers (radio and television broadcasting), mobile phone networks and smart meters such as digital electric and gas meters (IARC 2013).

3 Effect of Low-Frequency EMFs on Health

A static magnetic field is created during the direct flow of electric current while a time-varying gradient magnetic field (GMF) is created by alternating current supply. Household electronic devices produce a 4 μT EMF which extend from 0.01 to 1 μT inside and outside of house respectively. The strength of low frequency-EMF depends on the electrical current and distance from the conductor. Therefore, low frequency-EMFs are the highest near the power cable and decrease rapidly by distance. Without doubt, our bodies are exposed daily to a huge amount of EMFs in all over the place (outdoors, indoors, and workplaces). EMFs are considered too-weak to influence on human biological systems in the short-term, but in the long-term they have accumulative effects which could lead to different damages in the human genome, causing dangerous diseases such as cancers.

3.1 Cancer Epidemiological Researches

Most studies focused on the effects of low-frequency EMFs on human health, mostly focused on cancer (Hug et al. 2010; Pedersen et al. 2014). Since, Wertheimer and Leeper (1979) demonstrated the presence of a relationship between the population who lived near power-lines and risk of childhood leukemia. At the time, several epidemiological studies have been reported an association between residential or occupational exposure to low-frequency EMF and potentially human health. The probable association between exposure to low-frequency EMF and human cancer risk has extensively studied in the past decades. Leukemia, breast, and brain cancers have received more attention than other types of cancers (Calvente et al. 2010; Kaszuba-Zwoinska et al. 2015).

Brain Cancer

Brain cancer has become a topic of interest after Lin et al. (1985) reported a possible relationship between workers in electrical factories and increased brain cancer risk. According to information available from IARC (2002) and WHO (2007), the effect of low-frequency EMF on the incidence of cancer was inadequate. Some studies reported a positive correlation between occupational exposure to low-frequency EMF and brain cancer. For example, a small increase of 10–20% in the incidence of brain cancer was recorded among broad workers of electrical occupations (Ahlbom et al. 2001). Furthermore, Kheifets et al. (2008) observed occupation low-frequency EMF induced a small significant increase of 10% in the brain tumor (gliomas). On the other hand, other studies supported no correlation between occupational exposure low-frequency EMF and central nervous tumors such as brain cancer, glioma, and meningioma (Carlberg et al. 2018; Koeman et al. 2014; Marcilio et al. 2011).

Hemo-Lymphoproliferative Malignancies

Leukemia cancer is characterized by the abnormal proliferation of lymphocytes. Human beings who have dysfunction or deregulation of lymphocytes are susceptible to grow a blood or bone marrow cancer (Calvente et al. 2010). Leukemia has gained great attention since childhood acute lymphoblastic leukemia has been found to be consistently associated with low-frequency EMF exposure (Schuz 2011). In England and Wales, during the period 1962–1995, Draper et al. (2005) studied the relationship between childhood leukemia risk and distance of birth from the high-voltage power lines. The authors found that leukemia was increased within 600 m of the powerlines compared to children residing away from 600 m.

In large population-based-control study, children whose fathers were occupationally exposed to low-frequency EMF (50/60 Hz) either preconceptionally or during pregnancy did induce an increase in leukemia or non-Hodgkin's lymphoma. Regarding maternal exposure, the number of causes was so small to conclude firm findings (Hug et al. 2010). A study for the period 2002–2007 in France recorded elevated childhood leukemia within 50 m, confined to the higher-voltage power lines and to younger children but not extending outside 50 m (Sermage-Faure et al. 2013). It is noteworthy, the positive correlation between EMF and childhood leukemia might be due to selection bias and exposure misclassification. A study in Denmark found no overall pattern of increased risk childhood leukemia living 200–599 m of overhead powerline (132–400 kV) (Pedersen et al. 2014).

These findings in children have raised question about the existence of a similar relationship for adult leukemia. For example, Kheifets et al. (2006) found positive association between occupational exposure to low-frequency EMF and adult leukemia particularly chronic lymphocytic leukemia and acute myeloid leukemia for the people living around power lines. Furthermore, Marcilio et al. (2011) pointed out the presence of positive correlation between adult leukemia and exposure to low-frequency EMF. Negative correlation between adult leukemia and EMF was recorded in several reports (Koeman et al. 2014; Willett et al. 2003). In United Kingdom, a study found that no increased rate of leukemia among electricity and transmission workers. However, it was observed and increased trend for workers (Sorahan 2012).

Breast Cancer

Interest in breast cancer based on a hypothesized inhibition of nighttime melatonin level due to nighttime low-frequency EMF exposure, which in turn might increase breast cancer occurrence (Ahlbom et al. 2001). It well known that low-frequency EMF at night disrupts normal sleep (Juutilainen and Kumlin 2006). Melatonin is a hormone secreted by the pineal gland in response to darkness. It is act as a powerful, endogenously antioxidant which responsible for scavenger of free radical species (Juutilainen and Kumlin 2006). Low-frequency EMF decreased the melatonin level during sleep leading to oxidative damage through disturbance between the pro-oxidants and antioxidants (Irmak et al. 2002)

The possible association between exposure to low-frequency of EMFs (50–60 Hz) and breast cancer risk has generated significant controversy. Several studies have reported an increase breast cancer risk in women and men working in electrical occupational that involve presumed high level of EMFs (Feychting and Forssen 2006; McElroy et al. 2007; Zhu et al. 2016). Other studies did not support the hypothesis of an association between occupational exposures to EMFs in the electric utility industry and the risk for breast cancer (Johansen et al. 2007; Koeman et al. 2014).

Interpretation of Contradictory Epidemiological Outcomes

Epidemiological studies have not able to prove a clear relationship between cancer risk and the effect of low-frequency EMF. Suppose a hypothetical study exhibited an association between an increased occurrence of cancer and occupational exposure to EMF to workers in electronic

factories. Presence of a significant positive correlation between the occurrence of cancer and the exposure to EMF does not necessarily mean that EMF is the chief cause of cancer. As the factories workers were not only exposed to EMF, but also they were exposed to other factors such as chemical solvents, smoking, and alcohol. All these factors have affected together on the incidence of cancer. Therefore, the positive association may result in statistical effects or may be due to some problem in the study design (WHO 2007).

Discover the causes of the disease require that the researchers take into account many factors such as clear dose-response relationship, a credible biological justification, evidence provided by experimental animal studies, and consistency between results. These factors have been not present in the epidemiological studies of the effect of low- frequency EMF and cancer risk. Accordingly, scientists have hesitated to conclude that low-frequency EMF has induced the occurrence of cancer (WHO 2007).

3.2 Animal Carcinogenicity Studies

Cancer epidemiological studies are contradictory; thus, it difficult to conclude the effect of EMF on the occurrence of cancer. Therefore, the scientists turn toward laboratory animal to determine whether EMF can initiate, promote or co-promote cancer in experimental animals.

There is no evidence that EMF cause tumors with the possible exception of lymphomas arising after chronic exposure to very strong EMF (60 Hz, 25 mT) exposing CFW mice for EMF at high-strength fields (60 kHz, 25 mT) for prolonged period induced the development of malignant lymphoma (Fam and Mikhail 1996). Overall, no persuasive findings of animal carcinogenesis have been supported the hypothesis that exposure to low-frequency EMF affects the development of cancer (Boorman et al. 2000; Sommer and Lerchl 2004). The rodents especially mice has been used broadly as animal model for leukaemogenesis. Murine lymphoproliferative disorders are closely similar to that found in human beings. Exposure of rodents to carcinogenic agents was exhibited an association between human carcinogens, and cancer risk (Lagroye et al. 2011).

McCormick et al. (1999) observed a small significant increase in mortality in B6C3F mice that were continuously exposed to pure transient-free 60 Hz low-frequency EMF at 10G (Gauss). The authors found that low-frequency EMF did not induce leukemia, breast cancer, and brain cancer in B6C3F mice (both sexes).

Mandeville et al. (2000) examined the promoter effect of low-frequency EMF using Fisher 344 rats. N-ethyl-N nitrosourea (ENU) was injected prenatally for induction neurogenic tumours in Fisher 344 rats. The offspring were exposed to different EMF intensity ranged from 2 to 2000 mT, 20 h/day, 7 days/weeks for 60 weeks. The results pointed out that EMF did not induce glioma, meningioma and schwannoma indicated that EMF has no promoter effect.

Boorman et al. (2000) exposed a group of F344/N rats to continuous low-frequency 60 Hz EMF (pure, linearly polairized, transient -free) at flux intensity of 2 mG, 2G, and 10G. The authors also exposed another group to intermittent (1 h on/1 h off) EMF (60 Hz, 10 G). The findings showed that no statistical change in mortality percentage, body weight, and rate of benign and malignant tumors in all groups. The occurrence of leukemia, breast cancer, and brain cancer did not statistically increase in the two groups. However, chronic exposure to EMF (20 mG and 2G) has a little effect on cancer development in the male rat. EMF did not exert an effect on oncogenic activity.

The AKR/J mouse model for thymic lymphoblastic lymphoma was used in two following investigations:low-frequency EMF (sinusoidal 50 Hz, 1 and 100 mT), 24 h/day, 7 days/week for 38 weeks (Sommer and Lerchl 2004); low-frequency EMF (sinusoidal 50 Hz, 1000 mT), 7 days/week (Sommer and Lerchl 2006). The findings suggested no evidence that low-frequency EMF induce survival time, hematological parameters, and body weight and lymphoma development. The authors concluded that

exposure to sinusoidal 50 Hz EMF did not induce haematopoietic malignancy event at the high intensity 1000 mT.

Bernard et al. (2008) used WKAH/Hkm male rat for induction B acute lymphoblastic leukemia (ALL) by n-butylnitrosourea (BNU). From the onset of BNU treatment, the rats exposed to low-frequency EMF (50 Hz, 100uT, sinusoidal) for 53 weeks. The positive control was irradiated with gamma ray (4.8G) prior to BUN treatment. No remarkable difference was recorded in parameters of induced leukemia between the positive control group and BUN-treated. However, a considerable decrease in erythroleukaemia and increase in immature leukemia and the most immature ALL was found in rats treated by gamma rays. Exposing the rats to EMF did not induce a significant increase in the percentage of leukemia and type of leukemia between the group treated with BNU and groups treated with EMF and BNU.

Some investigations supported the hypothesis that chronic exposure low-frequency EMF is an important risk factor for tumor development. For example, Mevissen et al. (1993) used DMBA (7, 12-dimethylbenz[a]anthracene) for induction breast cancer in female rats. Female rats were exposed to low-frequency EMF (50 Hz, 50 mT, 24/day) for three successive months with or without DMBA. The results showed that EMF acts as promoter and enhance the development of mammary tumors in DMBA model.

Qi et al. (2015) exposed pregnant C57BL/6NCrj mice to low-frequency EMF (50 Hz, 500mG, 12h/day) and exposed their offspring B6C3F1mice to EMF for 15.5 months. The results showed that significant reduction in the body weight of the EMF-exposed groups compared to the control group. Chronic myelogenous leukemia (7%) was observed in bone marrow of female exposed mice.

Soffritti et al. (2016) studied the carcinogenic effect of synergistic exposure to low-frequency EMF (50 Hz) and gamma radiation in Sprague-Dawley rats. The rats were exposed to EMF (20 and 1000μT) from prenatal life until natural death and gamma radiation (0.1Gy) at single exposure at 6 weeks of age. The results showed that EMF increased heart schwannoma malignant, breast cancer, and lymphomas/leukemias. These data supported the hypothesis that EMF induced cancer in animal model.

It is rationally hypothesized that EMF acts as initiator or co-initiator (promoter) of carcinogenic tumors. Since EMF can alter the DNA configuration which could stimulate the initiation of carcinogenetic processes or can accelerate the development or spreading of already present cancer (Mevissen et al. 1993).

3.3 Effect of EMF on Genetic Material

There is a bulk of data concerning the assessment of low-frequency EMFs on the genetic material in humans and animals. However, their genotoxicity remains controversial in *"in vivo"* and *"in vitro"* models (Phillips et al. 2009). The controversial results are due to the different exposure conditions such as field intensity and field's regularity. Genetic damage of EMF may occur through direct or indirect mechanisms. Direct genetic toxicity may occur by injury to chromosome or damage to DNA repair mechanisms. Indirect genetic damage may arise by various processes such as the generation of free radicals or impairment of radical scavenging mechanisms. The conflicting data have been used different genotoxic endpoints such as sister chromatid exchange (SCE), micronuclei (MN), chromosomal aberrations (CAs), comet assay and DNA adducts at exposure EMF intensities ranging from 1 μT to 10 mT (Ivancsits et al. 2002; Phillips et al. 2009).

According to International Agency for Research on Cancer (IARC), low-frequency EMFs are classified as "possibly carcinogenic" to human (IARC 2002). The main causes for the increase of human cancers are still inadequately understood. However, there are at least two pathways to understand the causation of cancer (Fig. 2). These pathways are not mutually and included: (1) Genotoxic Pathway; (2) Epigenetic Pathway (Vijayalaxmi and Obe 2005).

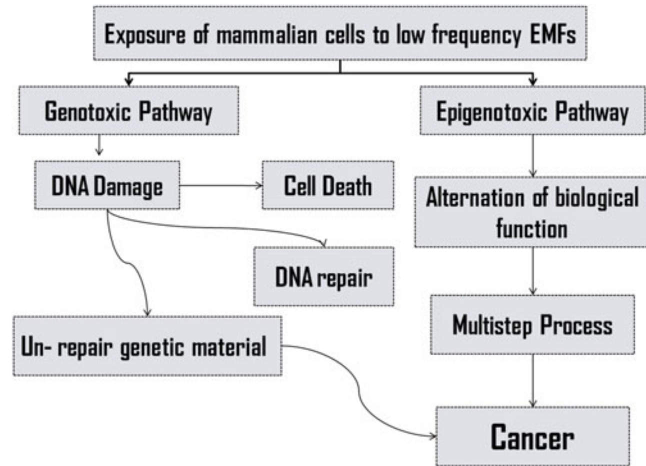


Fig. 2 Genotoxic and Epigenotoxic (non-genotoxic) pathways of carcinogenesis process

A Genetic Pathway
The exogenous gents (physical, chemical) can induce genetic damage in mammalian cells. The damaged cells could go through death or undergo to repair process. The unrepaired cells induced single and double strand breaks in the DNA molecule causing the formation of mutation, micronucleus, sister chromatid exchanges, and chromosomal aberrations. Some of these genetic endpoints can lead to the development of cancer (Phillips et al. 2009; Vijayalaxmi and Obe 2005).

An Epigenetic Pathway
The exogenous agents cannot induce genotoxic effect or cancer by themselves. However, they can contribute to development of carcinogenesis/tumorigenicity by increasing the genotoxic effect of other agents, interfering with the DNA repair process, permitting a cell with DNA lesion to survive and stimulating the cell division causing alteration in normal biological activities of the cell (Phillips et al. 2009; Vijayalaxmi and Obe 2005).

3.3.1 Genotoxic Effect of EMF

Winker et al. (2005) used human diploid fibroblast (ES-1, male, 6 years ago) which initiated from a skin biopsy of a healthy donor. The cells were exposed to intermittent exposure low-frequency EMF (50 Hz, sinusoidal, 1mT, 5 min field-on/10 min field off, for 2–24 h). Variation of exposure of human fibroblasts to EMF from 2 to 24 h revealed a time-dependent increase in the frequency of micronucleus and chromosomal aberrations. The occurrence of micronuclei became significant after 10 h of intermittent exposure and reached a constant level of micronuclei (three times above the control value) after 15 h of exposure. These findings supported the hypothesis that EMF exerts clastogenic activity.

Udroiu et al. (2006) exposed newborn mice and their parents to low-frequency EMF (50 Hz, 650μT) during the intrauterine life (21 days). DNA damage was detected by using micronucleus assay with antikinetochore antibody staining (CREST staining). The data pointed out that low-frequency EMF produced a significant increase in CREST-negative micronuclei (chromosome fragment) and a highly significant increase in CREST-positive micronuclei (whole chromosome) in newborn mice. However, no remarkable increase in micronuclei incidence was observed in their parents exposed to EMF. These data suggested that EMF possess aneugenic properties which may be related to the possible carcinogenesis.

Rageh et al. (2012) exposed newborn rats (10 days after delivery) to low-frequency EMF (50 Hz, 0.5mT, 24 h/day) for successive 30 days. The authors found that a remarkable increase in Olive tail moment in rat brain cells, as well as four-fold increase in the incidence of micronucleus in rat bone marrow cells.

Balamuralikrishnan et al. (2012) found that a remarkable increase in the occurrence of chromosomal aberrations and micronucleus formation in blood lymphocytes of workers occupationally exposed to low-frequency EMF in electric transformer and distribution station. Exposing African green monkey kidney epithelium cells (Vero) to 100 Hz EMF caused a blockage of the cells in S-phase. Also, EMF induced DNA damage as indicated by a remarkable increase of the tail lengths, the quality of DNA in the tail and Olive tail moments (Mihai et al. 2014). As well, 50 Hz EMF at high intensities (2, 3Tm) induced DNA damage in mouse spermatocytes-derived GC-2 cell line detected by alkaline comet assay (Duan et al. 2015).

On the other hand, many studies are rejected the hypothesis that low-frequency EMF may cause genomic instability. For example, Erdal et al. (2007) exposed Wistar male rats to acute (4h for day) and chronic (4h/day for 45days) to days horizontal low-frequency (50Hz, 1mT). The results showed that acute and chronic exposure EMF did not induce a significant increase in the occurrence of chromosomal aberration in rat bone marrow cells.

Furthermore, occupational exposure to low-frequency EMF did not induce chromosomal aberrations, sister chromatid exchange, and micronucleus formation among the workers (Scaringi et al. 2007). For example, Burdak-Rothkamm et al. (2009) exposed human skin fibroblast (VH25) to intermittent low-frequency EMF (50 Hz). The cells were exposed to switching fields (5 min on, 10 min off) for 15 h, with field intensity of 50, 100, 500 and 1000 μT. Neither the alkaline comet DNA assay nor the γH2AX assay could detect significant damage at the DNA-breakage level in VH25 cells. No remarkable increases in chromosome-type aberrations, sister chromatid exchange, and cytokinesis-block assays were observed in VH25 cells. No significant damage at the DNA-breakage level in VH25 cells was detected using alkaline comet DNA assay nor the γH2AX assay.

Zhu et al. (2016) exposed human lens epithelial cells (LECs) to low-frequency EMF (50 Hz, 0.4 mT) for short term (2 h, 6 h), and long term (12 h, 24 h, 48 h). The results demonstrated no DNA damage in alkaline comet assay for short and long term in human LECs. Recently, Ross et al. (2018) found that exposed human mesenchymal stromal cells (HMSC) to extremely low-frequency EMF (5 Hz, 0.4 mT for 20 min/day, three-time/week, for 2 weeks) did not induce cytotoxicity and chromosomal breakage.

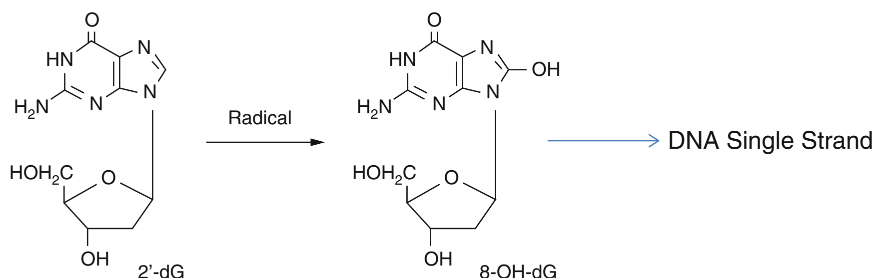
It is rational to hypothesize that genotoxic effects of EMF mediated through indirect mechanisms such as producing of free radical species or disruption of DNA repair pathway. Free radical can interact to DNA molecule (2-Deoxyguanosine, 2dG) forming primarily 8-hydroxy-2′-deoxyguanosine (8-OHdG) adduct that caused single-strand breaks. These strand breaks are usually removed by a specific repair pathway. However, genomic instability could become a site of mutation and the main step to the carcinogenesis process if the DNA damage were extensive sufficient to overcome the repair capacity of the cells (Cavalcanti et al. 2012).

3.3.2 Epigenetic Effect of EMF

Exposing human brain glioma (M054) to low-frequency EMF (100 Hz, 50 or 400 mT) did not induce DNA damage in alkaline comet assay. When the cells were exposed to X-ray (5 Gy) followed by low-frequency EMF (50 or 400 mT), the positive findings were detected using comet assay as indicated by a significant increase in tail moment compared with that for X-rays alone (Miyakoshi et al. 2000).

Nakahara et al. (2002) found that exposing Chinese hamster ovary (CHO) to static EMF alone (up to 10 T) has no genotoxic effect on the cell viability, cell cycle distribution, and formation of micronuclei. By contrast, the CHO cells exposed to EMF followed by X–irradiation (4 G) caused a significant increase in micronuclei formation. Surprisingly, the cells exposed to X-irradiation (1–2 Gy) and EMF did not induce the frequency of micronucleus.

Low-frequency EMF (60 Hz, 0.8 mT) did not cause genetic damage in human lymphocytes. However, co-exposure to benzo(a)pyrene (BP) and EMF provoked a remarkable increase in the frequencies of micronucleus and sister



chromatid exchanges compared to the cells treated with BP alone (Cho and Chung 2003).

Cho et al. (2014) reported that low-frequency EMF (60 Hz, 0.8 mT) boosted the cytotoxic and genotoxic activities of gadolinium (Gd). Coincident exposure to EMF and Gd increased micronucleus, single strand DNA breakage, Olive tail moment, apoptotic cells, and formation of free radical in human lymphocytes compared to gadolinium alone.

Other studies have rejected the hypothesis that co-exposure to EMF and other mutagenic agents may increase genetic damage. For example, Stronati et al. (2004) exposed human blood lymphocytes of five donors for 2 h to 50 Hz low-frequency EMF (1 mT) which generated by the Helmholtz coil system. Negative results were recorded in alkaline single cell electrophoresis assay, micronucleus assay and, chromosomal aberrations in human blood lymphocytes. As well, the synergistic effect between X-ray and EMF has no influence on DNA damage which is one hallmark of malignant cell transformation.

Gadhia et al. (2010) examined genetic damage in blood lymphocytes of electric train engine drivers who occupationally exposed to relatively high EMF intensity. The authors reported that no significant increase in the occurrence of chromosomal aberration and sister chromatid exchange. The co-mutagenic effect showed that exposing blood lymphocytes of electric train engine drivers to mitomycin C (6 ng/ml) have no genotoxic effect on the incidence of chromosomal damage and sister chromatid exchanges. It is rational to hypothesize that, EMF in the presence of initiator (e.g X-ray radiation) act as promoter to stimulate the DNA damage of genetically altered cells, rather than acting as initiator resulting in the proper lesion in DNA molecule (Timmel et al. 1998).

4 Assessment of the Published Literature and Further Research

The majority of original reports that indicated an absence of genotoxic or carcinogenic effect have explained the EMF exposure conditions and experimental protocols in detail. Therefore, the findings could be confirmed by other independent researchers. The findings are not in conflict with the other recognized characteristics of EMF. In other words, the interpretations for the presence of the genotoxic or carcinogenic effect of EMF were not substantiated by experimental data. Considering the "weight of scientific evidence" for scientific studies as suggested by IARC (2002), the preponderance of findings available in the literature review exhibits that EMF exposure by itself is not genotoxic or carcinogenic in mammalian cells. However, research must continue to resolve the controversial data published in the literature.

Many studies have reviewed the occurrence of non-reproducible positive results particularly *"in vitro"* assays (Vijayalaxmi and Obe 2005; Vijayalaxmi and Prihoda 2009). The following potential causes for conflicting findings can be reviewed according to Vijayalaxmi and Obe 2005:

1. The changes in environmental conditions in "*in vitro*" studies resulted in oxidative stress and false positive results. For example, high osmotic conditions, and low pH of media may

induce gene mutation, sister chromatid exchange, chromosomal aberrations, and morphological cell transformation.
2. There have been about 10% incidences of random and non-reproducible positive results in micronucleus assay in *"in vivo"* studies.
3. Data analysis obtained from many different bioassays, without appropriate statistical analysis reflecting the various observations tested could have misrecognized as a "significant effect" as a result of random chance occurrence (statistical deviations).
4. The findings from a well harmonized and multicenter collaborative investigation with adequate statistical analysis can be required the factors that cause these controversial data. The studies of EMF exposure can be conducted in a single laboratory with validated equipment for generation EMF. Many bioassay endpoints (e.g comet assay) and multiple cell lines from different origin (e.g human, mouse) should be examined. It may also be valuable to examine cells with different genetic backgrounds (heterozygous and homozygous mutation).

5 Conclusion

According to above investigations that showed a number of shortcomings and contradictions in findings of these studies, no firm conclusion can be drawn about the effect of EMF on genetic material. However, we cannot simply ignore the supported studies for the hypothesis that EMF induced genetic damage and cancer. Therefore, we need future better controlled investigations using the right and accurate biomarker assays and sufficient number of the individuals, adequate statistical analysis of data.

References

Ahlbom IC, Cardis E, Green A et al (2001) Review of the epidemiologic literature on EMF and health. Environ Health Perspect 109(Suppl 6):911–933. https://doi.org/10.1289/ehp.109-1240626

Balamuralikrishnan B, Balachandar V, Kumar SS et al (2012) Evaluation of chromosomal alteration in electrical workers occupationally exposed to low frequency of electro magnetic field (EMFs) in Coimbatore population, India. Asian Pac J Cancer Prev 13:2961–2966. https://doi.org/10.7314/APJCP.2012.13.6.2961

Bernard N, Alberdi AJ, Tanguy ML et al (2008) Assessing the potential leukemogenic effects of 50 Hz magnetic fields and their harmonics using an animal leukemia model. J Radiat Res 49:565–577. https://doi.org/10.1269/jrr.08019

Boorman GA, Rafferty CN, Ward JM, Sills RC (2000) Leukemia and lymphoma incidence in rodents exposed to low-frequency magnetic fields. Radiat Res 153:627–636. https://doi.org/10.1667/0033-7587(2000)153[0627:LALIIR]2.0.CO;2

Burdak-Rothkamm S, Rothkamm K, Folkard M et al (2009) DNA and chromosomal damage in response to intermittent extremely low-frequency magnetic fields. Mutat Res 672:82–89. https://doi.org/10.1016/j.mrgentox.2008.10.016

Calvente I, Fernandez MF, Villalba J, Olea N, Nunez MI (2010) Exposure to electromagnetic fields (non-ionizing radiation) and its relationship with childhood leukemia: a systematic review. Sci Total Environ 408:3062–3069. https://doi.org/10.1016/j.scitotenv.2010.03.039

Carlberg M, Koppel T, Ahonen M, Hardell L (2018) Case-control study on occupational exposure to extremely low-frequency electromagnetic fields and the association with meningioma. Biomed Res Int 2018:5912394., 6 pages. https://doi.org/10.1155/2018/5912394

Cavalcanti BC, Ferreira JR, Cabral IO et al (2012) Genetic toxicology evaluation of essential oil of Alpinia zerumbet and its chemoprotective effects against H_2O_2-induced DNA damage in cultured human leukocytes. Food Chem Toxicol 50:4051–4061. https://doi.org/10.1016/j.fct.2012.03.038

Cho YH, Chung HW (2003) The effect of extremely low frequency electromagnetic fields (ELF-EMF) on the frequency of micronuclei and sister chromatid exchange in human lymphocytes induced by benzo(a) pyrene. Toxicol Lett 143:37–44. https://doi.org/10.1016/S0378-4274(03)00111-5

Cho S, Lee Y, Lee S et al (2014) Enhanced cytotoxic and genotoxic effects of gadolinium following ELF-EMF irradiation in human lymphocytes. Drug Chem Toxicol 37:440–447. https://doi.org/10.3109/01480545.2013.879662

Draper G, Vincent T, Kroll ME, Swanson J (2005) Childhood cancer in relation to distance from high voltage power lines in England and Wales: a case-control study. BMJ (Clin Res Ed) 330:1290. https://doi.org/10.1136/bmj.330.7503.1290

Duan W, Liu C, Zhang L et al (2015) Comparison of the genotoxic effects induced by 50 Hz extremely low-frequency electromagnetic fields and 1800 MHz

radiofrequency electromagnetic fields in GC-2 cells. Radiat Res 183:305–314. https://doi.org/10.1667/RR13851.1

Erdal N, Gurgul S, Celik A (2007) Cytogenetic effects of extremely low frequency magnetic field on Wistar rat bone marrow. Mutat Res 630:69–77. https://doi.org/10.1016/j.mrgentox.2007.03.001

Fam WZ, Mikhail EL (1996) Lymphoma induced in mice chronically exposed to very strong low-frequency electromagnetic field. Cancer Lett 105:257–269. https://doi.org/10.1016/0304-3835(96)04324-8

Feychting M, Forssen U (2006) Electromagnetic fields and female breast cancer. Cancer Causes Control 17:553–558

Finlay CC et al (2010) International geomagnetic reference field: the eleventh generation. Geophy J Int 183:1216–1230. https://doi.org/10.1111/j.1365-246X.2010.04804.x

Furse C, Christensen DA, Durney CH (2009) Basic introduction to bioelectromagnetics, 2nd edn. CRC Press/Taylor and Francis group, Boca Raton. https://doi.org/10.1201/9781420055436

Gadhia P, Chakraborty S, Pithawala M (2010) Cytogenetic studies on railway engine drivers exposed to extremely low frequency electromagnetic fields (ELF-EMF). Int J Hum Genet 10:263–269. https://doi.org/10.1080/09723757.2010.11886116

Hug K, Grize L, Seidler A, Kaatsch P, Schuz J (2010) Parental occupational exposure to extremely low frequency magnetic fields and childhood cancer: a German case-control study. Am J Epidemiol 171:27–35. https://doi.org/10.1093/aje/kwp339

IARC (2002) Non-ionizing radiation, part 1: static and extremely low-frequency (ELF) electric and magnetic fields. International Agency for Research on Cancer (IARC) monographs on the evaluation of carcinogenic risks to humans. vol 80. IARC Press, Lyon, pp 273–338

IARC (2013) Non-ionizing radiation, part 2: radiofrequency electromagnetic fields. International Agency for Research on Cancer (IARC) working group on the evaluation of carcinogenic risks to humans. IARC Monogr Eval Carcinog Risks Hum 102:1–460. PMC4780878

Irmak MK, Fadillioglu E, Gulec M et al (2002) Effects of electromagnetic radiation from a cellular telephone on the oxidant and antioxidant levels in rabbits. Cell Biochem Funct 20:279–283. https://doi.org/10.1002/cbf.976

Ivancsits S, Diem E, Pilger A et al (2002) Induction of DNA strand breaks by intermittent exposure to extremely-low-frequency electromagnetic fields in human diploid fibroblasts. Mutat Res 519:1–13. https://doi.org/10.1016/S1383-5718(02)00109-2

Jin YB, Choi SH, Lee JS et al (2014) Absence of DNA damage after 60-Hz electromagnetic field exposure combined with ionizing radiation, hydrogen peroxide, or c-Myc overexpression. Radiat Environ Biophys 53:93–101. https://doi.org/10.1007/s00411-013-0506-5

Johansen C, Raaschou Nielsen O et al (2007) Risk for leukaemia and brain and breast cancer among Danish utility workers: a second follow-up. Occup Environ Med 64:782–784. https://doi.org/10.1136/oem.2006.029116

Juutilainen J, Kumlin T (2006) Occupational magnetic field exposure and melatonin: interaction with light-at-night. Bioelectromagnetics 27:423–426. https://doi.org/10.1002/bem.20231

Kaszuba-Zwoinska J, Gremba J, Galdzinska-Calik B et al (2015) Electromagnetic field induced biological effects in humans. Przegl Lek 72:636–641

Kheifets L, Afifi AA, Shimkhada R (2006) Public health impact of extremely low-frequency electromagnetic fields. Environ Health Perspect 114:1532–1537. https://doi.org/10.1289/ehp.8977

Kheifets L, Monroe J, Vergara X, Mezei G, Afifi AA (2008) Occupational electromagnetic fields and leukemia and brain cancer: an update to two meta-analyses. J Occup Environ Med 50:677–688. https://doi.org/10.1097/JOM.0b013e3181757a27

Koeman T, van den Brandt PA, Slottje P et al (2014) Occupational extremely low-frequency magnetic field exposure and selected cancer outcomes in a prospective Dutch cohort. Cancer Causes Control 25:203–214. https://doi.org/10.1007/s10552-013-0322-x

Lagroye I, Percherancier Y, Juutilainen J et al (2011) ELF magnetic fields: animal studies, mechanisms of action. Prog Biophys Mol Biol 107:369–373. https://doi.org/10.1016/j.pbiomolbio.2011.09.003

Lin RS, Dischinger PC, Conde J, Farrell KP (1985) Occupational exposure to electromagnetic fields and the occurrence of brain tumors. An analysis of possible associations. J Occup Med 27:413–419. https://doi.org/10.1023/A:1007487503382

Livermore PW, Hollerbach R, Jackson A (2013) Electromagnetically driven westward drift and inner-core superrotation in Earth's core. Proc Natl Acad Sci U S A 110:15914–15918. https://doi.org/10.1073/pnas.1307825110

Mandeville R, Franco E, Sidrac-Ghali S et al (2000) Evaluation of the potential promoting effect of 60 Hz magnetic fields on N-ethyl-N-nitrosourea induced neurogenic tumors in female F344 rats. Bioelectromagnetics 21:84–93. https://doi.org/10.1002/(SICI)1521-186X(200002)21:2<84::AID-BEM2>3.0.CO;2-B

Marcilio I, Gouveia N, Pereira Filho ML, Kheifets L (2011) Adult mortality from leukemia, brain cancer, amyotrophic lateral sclerosis and magnetic fields from power lines: a case-control study in Brazil. Rev Bras Epidemiol 14:580–588. https://doi.org/10.1590/S1415-790X2011000400005

McCormick DL, Boorman GA, Findlay JC et al (1999) Chronic toxicity/oncogenicity evaluation of 60 Hz (power frequency) magnetic fields in B6C3F1 mice. Toxicol Pathol 27:279–285. https://doi.org/10.1177/019262339902700302

McElroy JA et al (2007) Occupational exposure to electromagnetic field and breast cancer risk in a large, population-based, case-control study in the United States. J Occup Environ Med 49:266–274. https://doi.org/10.1097/JOM.0b013e318032259b

Mevissen M et al (1993) Effects of magnetic fields on mammary tumor development induced by 7,12-dimethylbenz(a)anthracene in rats. Bioelectromagnetics 14:131–143. https://doi.org/10.1002/bem.2250140206

Mihai CT, Rotinberg P, Brinza F, Vochita G (2014) Extremely low-frequency electromagnetic fields cause DNA strand breaks in normal cells. J Environ Health Sci Eng 12:15. https://doi.org/10.1186/2052-336X-12-15

Miyakoshi J, Yoshida M, Shibuya K, Hiraoka M (2000) Exposure to strong magnetic fields at power frequency potentiates X-ray-induced DNA strand breaks. J Radiat Res 41:293–302. https://doi.org/10.1269/jrr.41.293

Nakahara T, Yaguchi H, Yoshida M, Miyakoshi J (2002) Effects of exposure of CHO-K1 cells to a 10-T static magnetic field. Radiology 224:817–822. https://doi.org/10.1148/radiol.2243011300

Ng K-H (2003) Electromagnetic field and our health: non-ionizing radiations–sources, biological effects, emissions and exposures, Proceedings of the International Conference on Non-Ionizing Radiation (ICNIR) at Universiti Tenaga Nasion (UNITEN), Malaysia, pp 1–16

Pedersen C et al (2014) Distance from residence to power line and risk of childhood leukemia: a population-based case-control study in Denmark. Cancer Causes Control 25:171–177. https://doi.org/10.1007/s10552-013-0319-5

Phillips JL, Singh NP, Lai H (2009) Electromagnetic fields and DNA damage. Pathophysiology 16:79–88. https://doi.org/10.1016/j.pathophys.2008.11.005

Qi G, Zuo X, Zhou L et al (2015) Effects of extremely low-frequency electromagnetic fields (ELF-EMF) exposure on B6C3F1 mice. Environ Health Prev Med 20:287–293. https://doi.org/10.1007/s12199-015-0463-5

Rageh MM, El-Gebaly RH, El-Bialy NS (2012) Assessment of genotoxic and cytotoxic hazards in brain and bone marrow cells of newborn rats exposed to extremely low-frequency magnetic field. J Biomed Biotechnol 2012:716023. https://doi.org/10.1155/2012/716023

Randall C, Harvey V, Manney G et al (2005) Stratospheric effects of energetic particle precipitation in 2003–2004. Geophys Res Lett 32:L05802. https://doi.org/10.1029/2004GL022003

Ross CL et al (2018) Evaluation of cytotoxic and genotoxic effects of extremely low-frequency electromagnetic field on mesenchymal stromal cells. Glob Adv Health Med 7:2164956118777472. https://doi.org/10.1177/2164956118777472

Scaringi M, Temperani P et al (2007) Evaluation of the genotoxicity of the extremely low frequency-magnetic fields (ELF-MF) in workers exposed for professional reasons. G Ital Med Lav Ergon 29:420–421

Schuz J (2011) Exposure to extremely low-frequency magnetic fields and the risk of childhood cancer: update of the epidemiological evidence. Prog Biophys Mol Biol 107:339–342. https://doi.org/10.1016/j.pbiomolbio.2011.09.008

Sermage-Faure C, Demoury C, Rudant J et al (2013) Childhood leukaemia close to high-voltage power lines – the Geocap study, 2002-2007. Br J Cancer 108:1899–1906. https://doi.org/10.1038/bjc.2013.128

Soffritti M et al (2016) Life-span exposure to sinusoidal-50 Hz magnetic field and acute low-dose gamma radiation induce carcinogenic effects in Sprague-Dawley rats. Int J Radiat Biol 92:202–214. https://doi.org/10.3109/09553002.2016.1144942

Sommer AM, Lerchl A (2004) The risk of lymphoma in AKR/J mice does not rise with chronic exposure to 50 Hz magnetic fields (1 microT and 100 microT). Radiat Res 162:194–200. https://doi.org/10.1667/RR3219

Sommer AM, Lerchl A (2006) 50 Hz magnetic fields of 1 mT do not promote lymphoma development in AKR/J mice. Radiat Res 165:343–349. https://doi.org/10.1667/RR3513.1

Sorahan T (2012) Cancer incidence in UK electricity generation and transmission workers, 1973-2008. Occup Med (Lond) 62:496–505. https://doi.org/10.1093/occmed/kqs152

Stronati L, Testa A, Villani P et al (2004) Absence of genotoxicity in human blood cells exposed to 50 Hz magnetic fields as assessed by comet assay, chromosome aberration, micronucleus, and sister chromatid exchange analyses. Bioelectromagnetics 25:41–48. https://doi.org/10.1002/bem.10141

Timmel C, Till U, Brocklehurst B et al (1998) Effects of weak magnetic fields on free radical recombination reactions. Mol Phys 95:71–89. https://doi.org/10.1080/00268979809483134

Udroiu I, Cristaldi M, Ieradi LA et al (2006) Clastogenicity and aneuploidy in newborn and adult mice exposed to 50 Hz magnetic fields. Int J Radiat Biol 82:561–567. https://doi.org/10.1080/09553000600876660

Vijayalaxmi, Obe G (2005) Controversial cytogenetic observations in mammalian somatic cells exposed to extremely low frequency electromagnetic radiation: a review and future research recommendations. Bioelectromagnetics 26:412–430. https://doi.org/10.1002/bem.20111

Vijayalaxmi, Prihoda TJ (2009) Genetic damage in mammalian somatic cells exposed to extremely low frequency electro-magnetic fields: a meta-analysis of data from 87 publications (1990-2007). Int J Radiat Biol 85:196–213. https://doi.org/10.1080/09553000902748575

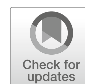
Wertheimer N, Leeper E (1979) Electrical wiring configurations and childhood cancer. Am J Epidemiol 109:273–284. https://doi.org/10.1093/oxfordjournals.aje.a112681

WHO (2007) Extremely low frequency fields, Environmental health criteria monograph no. 238. World Health Organization (WHO), Geneva, p 121

Willett EV, McKinney PA, Fear NT et al (2003) Occupational exposure to electromagnetic fields and acute leukaemia: analysis of a case-control study. Occup Environ Med 60:577–583. https://doi.org/10.1136/oem.60.8.577

Winker R, Ivancsits S, Pilger A, Adlkofer F, Rüdiger HW (2005) Chromosomal damage in human diploid fibroblasts by intermittent exposure to extremely low-frequency electromagnetic fields. Mutat Res 585(1–2):43–49. https://doi.org/10.1016/j.mrgentox.2005.04.013

Zhu K, Lv Y, Cheng Q, Hua J, Zeng Q (2016) Extremely low frequency magnetic fields do not induce DNA damage in human lens epithelial cells In vitro. Anat Rec (Hoboken) 299:688–697. https://doi.org/10.1002/ar.23312



Adv Exp Med Biol - Cell Biology and Translational Medicine (2019) 7: C1
https://doi.org/10.1007/978-3-030-37845-5_442
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Correction to: Application of iPSC to Modelling of Respiratory Diseases

Ben A. Calvert and Amy L. Ryan (Firth)

Correction to:
Chapter 1 in: K. Turksen (ed.), *Cell Biology and Translational Medicine, Volume 7*, Advances in Experimental Medicine and Biology 1237, https://doi.org/10.1007/5584_2019_430

The chapter "Application of iPSC to Modelling of Respiratory Diseases" was previously published non-open access and has now been changed to open access under a CC BY 4.0 license and the copyright holder updated to "The Author(s)". The book has also been updated with these changes.

The updated online version of this chapter can be found at
https://doi.org/10.1007/5584_2019_430

Adv Exp Med Biol - Cell Biology and Translational Medicine (2020) 7: 151–153
https://doi.org/10.1007/978-3-030-37845-5
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