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Kursad Turksen *Editor*

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Editor

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Stem Cells in Regenerative Medicine

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Editor
Kursad Turksen (Retired)
Ottawa Hospital Research Institute
Ottawa, ON, Canada

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Preface

In this next volume in the Cell Biology and Translational Medicine series, we continue to explore the potential utility of stem cells in regenerative medicine. Chapters in this volume cover advances and challenges in applications of particular stem cell populations in a variety of diseases and conditions, and certain governance and policy issues and options.

I remain very grateful to Gonzalo Cordova, the 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 thank you goes to Rathika Ramkumar and Anand Venkatachalam for their 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 insight and effort to capture both the 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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The Future of Stem Cell Research and its Clinical Translation in Canada: Exploring Questions of Governance and Policy Options

Amy Zarzeczny

Abstract

Stem cell research is a promising area of biomedical research with tremendous potential for increasing our understanding of human development and for improving clinical treatment options across a range of serious conditions. However, it has historically also been a complex field, both scientifically and ethically. It raises numerous policy tensions including those related to the acceptability of different forms of research in the field and, more recently, regarding how to respond to the rapidly growing private market for clinical applications that lack broadly accepted forms of evidence of safety and efficacy. Using the Canadian market for unproven stem cell interventions as a case study, this review paper identifies questions of governance and policy options as they relate to the future of stem cell research and its clinical translation in Canada. Key areas of inquiry include the roles and influence of evidence, scientific and clinical imperatives, and public pressure on policy decisions, as well as the role of regulation in managing risks and uncertainty in fast moving fields of biomedicine. Examining these questions in a Canadian context is particularly timely at present given the emerging domestic private market for stem cell-based interventions coupled with scientific developments in the field that are highlighting ambiguities and other challenges with our current regulatory framework.

Keywords

Stem cell · Policy · Ethics · Regulation · Governance · Unproven interventions

1 Introduction

Regulating fast moving fields of science, like stem cell research, is no simple feat. One challenge policy makers face is that it can be difficult to predict with any accuracy both the potential benefits and also the potential risks of new technologies and avenues of research (Butenko and Larouche 2015; Harmon et al. 2013). However, understanding benefits and risks is critically important when it comes to trying to balance diverse and sometimes competing policy priorities. For example, in the stem cell field (as is true in others), there are a number of both pushes and pulls relevant to policy choices including, though certainly not limited to, the desire to stimulate economic growth, create jobs, encourage biomedical developments likely to improve clinical treatment options, respond to

A. Zarzeczny (✉)
Johnson Shoyama Graduate School of Public Policy, University of Regina, Regina, Canada
e-mail: amy.zarzeczny@uregina.ca

pressure from the public, patients or other stakeholder groups, address ethical, moral, and/or other concerns, and be responsive to international pressures. Although not always clearly delineated or directly acknowledged, it is important to be cognizant of these various potential influences on decision-making with respect to science and innovation policy (Phillips and Schmeiser 2017) – including in the context of stem cell research – when identifying and considering policy options.

It can also be difficult for regulators to identify what tools or strategies are the best fit for a given area or issue in order to achieve their desired objectives while limiting negative or unintended effects. In many cases, hard law approaches such as criminalization are ill-suited to regulate fields that are changing quickly. Their inflexible nature can make it difficult to adapt to unanticipated developments in the regulated field, risking legal ambiguity and/or unintended consequences when the law does not keep pace with scientific realities (Ogbogu et al. 2018b). Legal authority is also generally restricted by jurisdiction. Indeed, limited jurisdictional reach for regulators is a growing concern, particularly when considering issues with international implications that include cross-border and internet-based activities, such as markets for stem cell-based interventions (Shalev 2010; Sipp et al. 2017).

In this review paper, I will identify questions of governance and policy options as they relate to the future of stem cell research and its clinical translation in Canada, using the market for unproven stem cell interventions as a case study. To set the stage for this discussion, I will begin by framing the relevant regulatory context and by introducing what I suggest are governance challenges in this space, drawing on current examples from the field of stem cell research to highlight specific issues. I will then use the market for unproven stem cell interventions as a case study to illustrate these governance challenges, and to serve as a foundation for an exploration of future strategies. The paper will conclude with a brief discussion of policy options and governance strategies, with a view to contributing to future research strategies in this area.

2 Stem Cells: Promises, Pitfalls and Policy Challenges

2.1 Context – Regulation and Governance of Stem Cell Research in Canada

Stem cell research is a promising area of biomedical research with tremendous potential for increasing our understanding of human development and for improving clinical treatment options across a range of serious conditions. However, it has historically also been a complex field, both scientifically and ethically. It raises numerous policy tensions including those related to different avenues of stem cell research. For example, early debates about the acceptability – or lack thereof – of research involving the destruction of human embryos and concerns about different kinds of applications such as reproductive cloning, shaped much of the early policy discourse in the field and featured prominently in public forums including the media (Caulfield et al. 2010) and Canadian parliamentary debates (Caulfield and Bubela 2007).

In many ways, the current governance of stem cell research in Canada is a product of those early debates, with a long history of controversy. It is beyond the scope of this paper to provide a fulsome account of that history here (see Cattapan and Snow 2017). The work of the Royal Commission on New Reproductive Technologies, established in 1989, was particularly influential. Its 1993 report, *Proceed with Care – Final Report of the Royal Commission on New Reproductive Technologies* (Baird 1993), called for the regulation of a wide range of activities related to reproductive technologies, including research using human embryos. This report was followed by a number of failed legislative initiatives, ultimately leading to the passing of the *Assisted Human Reproduction Act* (S.C. 2004, c. 2) (AHRA) in 2004. The AHRA was federal legislation that purported to govern aspects of reproductive technologies as well as particular avenues of research. It did so by way of creating two categories of activities – those that were

controlled (relating to activities involved in assisted human reproduction), and those that were prohibited. The prohibited procedures related primarily (though not exclusively) to research activities, and included the following:

> 5 (1) No person shall knowingly
> (a) create a human clone by using any technique, or transplant a human clone into a human being or into any non-human life form or artificial device;
> (b) create an in vitro embryo for any purpose other than creating a human being or improving or providing instruction in assisted reproduction procedures;
> (c) for the purpose of creating a human being, create an embryo from a cell or part of a cell taken from an embryo or foetus or transplant an embryo so created into a human being;
> (d) maintain an embryo outside the body of a female person after the fourteenth day of its development following fertilization or creation, excluding any time during which its development has been suspended;
> (e) for the purpose of creating a human being, perform any procedure or provide, prescribe or administer any thing that would ensure or increase the probability that an embryo will be of a particular sex, or that would identify the sex of an in vitro embryo, except to prevent, diagnose or treat a sex-linked disorder or disease;
> (f) alter the genome of a cell of a human being or in vitro embryo such that the alteration is capable of being transmitted to descendants;
> (g) transplant a sperm, ovum, embryo or foetus of a non-human life form into a human being;
> (h) for the purpose of creating a human being, make use of any human reproductive material or an in vitro embryo that is or was transplanted into a non-human life form;
> (i) create a chimera, or transplant a chimera into either a human being or a non-human life form; or
> (j) create a hybrid for the purpose of reproduction, or transplant a hybrid into either a human being or a non-human life form.

Contravening any of these provisions is associated with significant criminal sanctions, including a fine of up to $500,000 and/or imprisonment for up to 10 years (AHRA, s. 60).

The AHRA was controversial from the beginning, for a number of reasons (Caulfield 2002). For example, some people viewed it as being overly restrictive in terms of the limits it placed on scientific research; others noted its lack of clarity and responsiveness to emerging avenues of research (Ogbogu and Rugg-Gunn 2008; Rugg-Gunn et al. 2009); still others objected to what they saw as an inappropriate intrusion of the federal government into an area of provincial jurisdiction – that of regulation of the practice of medicine. This division of powers issue ultimately came before the Supreme Court of Canada (SCC) in the *Reference re Assisted Human Reproduction Act*, 2010 SCC 61. In this reference case, the Attorney General of Quebec challenged the constitutionality of provisions of the law which it suggested were an attempt to regulate the practice of medicine and research related to assisted reproduction. The SCC determined that the impugned provisions did indeed exceed the legislative authority of the federal government (see Ogbogu 2013). It is important to note for the purpose of this discussion that the impugned provisions related only to the use of assisted human reproductive technologies. The prohibitions outlined above (AHRA, s.5) relating to research using human reproductive materials were not challenged and remain in force. As will be discussed below, they have their own criticisms at present related in part to the evolution of the field.

The AHRA is not the only relevant source of authority with respect to stem cell research and its applications in Canada. To the contrary, there are various actors – both governmental and non-governmental – that have power and responsibility in this area. Federal regulators such as Health Canada play a key role in regulation of advanced medicinal products, including cell-based therapies (Chisholm et al. 2019). Research involving human participants, including clinical experiments and research using human tissues or health information is subject to research ethics oversight. The Tri-Council Policy Statement (TCPS) (CIHR 2014) governs all research funded by any of the Tri-Council agencies in Canada (Canadian Institutes of Health Research (CIHR), the Natural Sciences and Engineering Research Council (NSERC) and the Social Sciences and Humanities Research Council (SSHRC)). It specifically addresses "research involving materials related to human reproduction" – which includes

various avenues of stem cell research – and is intended to compliment (i.e. operate alongside) legal requirements, including those contained within the AHRA. Although the TCPS is not hard law, because of its implications for future funding eligibility for both individual researchers and their institutions, its reach in Canada is broad and powerful.

Professional regulatory bodies also have an important role to play in terms of establishing the parameters of professional and ethical standards, providing guidance to their members, and with respect to the investigation and discipline of members who engage in unprofessional conduct (Zarzeczny 2017). Accordingly, regulated healthcare professionals – such as physicians – must also be concerned with their professional obligations when engaging in both research with human participants and clinical practice. In Canada, physicians have the privilege of self-regulation, which carries an obligation – in some cases enshrined in the empowering legislation – to act in the public interest (Collier 2012). As will be discussed in greater detail below, the value and potential influence of professional regulation in current, but more importantly the future, governance of stem cell research and its clinical applications should not be underestimated.

2.2 Governance Challenges – Examples from the Field

There are a number of current examples one can point to in stem cell research and related fields that highlight different governance challenges associated with emerging areas of biomedicine. For the purpose of this discussion, the term governance will be used to capture "the pattern or framework within which the exercise of power occurs" (Fairburn et al. 2015 at 5) as well as "steering" activities that "influence, shape, regulate or determine outcomes" (Gamble 2000 at 110). Governance provides a useful lens through which to explore tensions in the field of stem cell research because it contemplates the roles of different actors operating in complex contexts, using various policy instruments (Le Galès 2011).

For example, recent debates about how to respond to gene editing technologies such as CRISPR-Cas 9 (clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9), that allow for purportedly faster, more efficient and more accurate changes to the genome, are attracting attention and highlighting the complex balancing of interests engaged by emerging technologies that offer potential advantages (e.g., elimination disease), alongside potential controversies (e.g., eugenics) (Bosley et al. 2015; Sugarman 2015; Isasi et al. 2016). Both research and clinical applications that involve potentially heritable alterations made to human embryos or somatic cells are controversial. Indeed, a 2018 review highlights 61 statements issued over the previous 3 years by the international community addressing related questions (Brokowski 2018) and the World Health Organization has convened an expert panel tasked with developing global standards of governance and oversight (WHO 2018).

Leaving aside some of the bigger questions surrounding the ethics of potential clinical applications of this technology, many of which are discussed in the statements reviewed by Brokowski (2018), in Canada it is currently unclear whether or not some of these avenues of genetic research are even legal. As outlined above, s. 5(1) of the AHRA provides "No person shall knowingly . . .(f) alter the genome of a cell of a human being or in vitro embryo such that the alteration is capable of being transmitted to descendants". However, the meaning of "capable" is debatable in this context and there are different potential interpretations. One interpretation is that genetic alterations to a human embryo are permissible, as long as the embryo is intended for research uses only and will never be implanted (Master and Bedford 2018). Another interpretation is that any alteration that could potentially be passed down to future generations, regardless of whether or not an affected embryo is ever intended to be implanted in a woman, is prohibited by the AHRA (Knoppers et al. 2017;

Health Canada 2014). At present, it is unclear which of these interpretations will prevail which makes it difficult for scientists to identify with certainty where the boundaries of the law lie with respect to their work, which has implications for various avenues of both research and clinical use (Cohen et al. 2019).

There are also debates about how section 5(1)(b) of the AHRA should be interpreted. This section makes it illegal to "create an *in vitro* embryo for any purpose other than creating a human being or improving or providing instruction in assisted reproduction procedures" (AHRA). In recent years, researchers in other countries have been honing techniques for developing structures *in vitro* that share some features with human embryos (Warmflash et al. 2014; Harrison et al. 2017). Referred to by some as synthetic human entities with embryo-like features (SHEEFs) (Aach et al. 2017), these two-dimensional structures are seen by many as a valuable research tool that may help develop understandings of early embryo development and developmental disorders, albeit while raising their own ethical issues (Pera et al. 2015). However, although the similarities between these entities and actual human embryos remain slight, and although there is no suggestion these entities would be viable even if implanted into a womb, at present it is not clear whether or not they would be considered "embryos" under Canadian law and therefore be illegal to create. An "embryo" is defined in the AHRA as "a human organism during the first 56 days of its development following fertilization or creation, excluding any time during which its development has been suspended, and includes any cell derived from an organism that is used for. . .creating a human being" (s.3). This definition does not incorporate viability as a criterion and leaves room for entities created through means other than traditional fertilization of a sperm and egg. Accordingly, it very well may preclude Canadian scientists from creating SHEEFs for research purposes – but this interpretation is far from certain.

These are only two examples of emerging forms of research that currently fall into ambiguous or grey areas in the Canadian legal landscape, but they serve as helpful illustrations of some of the governance challenges outlined above. In particular, they demonstrate the difficulty criminal law can have with keeping pace with scientific developments and the ensuing lack of clarity that can result. In the absence of judicial consideration or interpretative guidance from Health Canada, scientists wishing to work in these areas do so at risk of criminal liability. This uncertainty risks serving as a chill on science, to the potential detriment of the Canadian research community and its stakeholders, which include patients and the public. Concerns about the implications of these areas of legal uncertainty have shaped calls for legal reform, including changes to the AHRA and – potentially – to the governance of this area of research more broadly (Bubela et al. 2019; Ogbogu et al. 2018b; Knoppers et al. 2017; Knoppers et al. 2017b).

Together with a multidisciplinary group of scholars and other experts, I have argued previously for a principled approach to policy making in this area. Specifically, we have suggested that:

> Research policy limits should be *proportional*, with appropriate balancing of risks and benefits, as well as of possible penalties for harm. They should be *guided by evidence*, rather than speculation about hypothetical risks. They should be *consistent*, so that like activities are treated similarly and exceptionalism is avoided. They should be *responsive* rather than static, and amenable to flexible interpretation as circumstances change. They should be *clear* and supported by substantive criteria guiding how to interpret and apply them. Finally, they should be grounded in recognition of the *value of scientific discovery* and the *interests of citizens* in benefiting from science and its applications. (Ogbogu et al. 2018b)

Although likely not without its own challenges, such an approach would respond – at least in part – to common criticisms that law is often largely reactive to scientific developments. Building on the work of Harmon (2016) and others, I would also suggest there is a need to examine and characterize current and past governance decisions in respect to emerging areas of biomedical science, with a view to understanding how we might work towards more coherent, consistent and effective strategies in future.

Regardless of the approach taken, there are salient contextual factors in the policy making context surrounding stem cell research that need to be recognized and accounted for in order for a governance strategy to succeed. Alongside the ethical and legal debates that permeate different aspects of the field, there continues to also be a tremendous amount of interest and expectation surrounding stem cells. Indeed, the attention the field draws has grown to a degree now commonly referred to as "hype" (Caulfield et al. 2016; Kamenova and Caulfield 2015). Although an in-depth discussion of the role and impact of hype is beyond the scope of this paper, it must be acknowledged that it is part of the context in which policy makers operate and make decisions, and that it permeates the public sphere with the potential to shape public expectations about what the field should be delivering in terms of clinical applications.

A related contextual factor is the apparent growing patient-driven push for access to experimental and unproven medical interventions. This trend is perhaps best exemplified in the "Right to Try" movement. Under the umbrella of the Right to Try movement, patients are advocating for early and expanded access regimes that permit access to therapeutic interventions that have not yet been approved. While not without its critics and limitations, this movement has gained considerable traction in the United States (US), where it has been implemented by law at both federal and state levels (Zettler and Greely 2014). Though not limited to stem cell interventions, the Right to Try movement has implications for this sphere as with other unproven and experimental treatments (Servick 2016; Shah and Zettler 2010). More broadly, it is arguably reflective of a growing public interest in, and demand for, access to experimental and unproven medical interventions – many of which are available on growing private, direct-to-consumer markets.

In the section that follows, I will use the market for unproven stem cell-based interventions as a case study to further illustrate the governance challenges introduced above, and to ground a discussion of how we might approach policy choices going forward in fast-moving and complex fields of biomedicine, such as stem cell research.

3 Case Study – The Market for Unproven Stem Cell Interventions

3.1 Overview: A Murky Market

As noted above, stem cells are widely thought to have the potential to improve clinical options for a number of different diseases and conditions. Indeed, there are a growing number of promising avenues of clinical exploration occurring around the world (Li et al. 2014) that may someday improve the lives of many individuals for whom existing treatment options are lacking. However, the clinical translation of stem cell research is a highly complex, and potentially high risk, process. Identifying treatment protocols that are effective and do not cause inappropriate risk to patients takes time and rigorous science (Daley 2017). The potential treatment paths also vary by condition, as do the timelines around when new treatment options may be ready for application in humans. At present, there are only a small number of stem cell-based treatments that are part of a routine standard of care (e.g., bone marrow transplants for leukemia).

However, notwithstanding this relatively early stage of much clinical research into stem cell treatments, a robust private market has developed where a wide range of stem cell-based interventions are advertised directly to patients. This market has been documented for some time, with the first studies analyzing the content of private market provider websites published a decade ago (Lau et al. 2008; Regenberg et al. 2009). Operating on a direct-to-consumer basis, where products and services are sold directly to individuals without the need for a medical referral, this market relies heavily on online marketing and the use of websites to promote products and services, and as an entry contact point for prospective patients (Connolly et al. 2014). The early research on this market found a strong clinic presence in jurisdictions including China, India and Mexico (Lau et al. 2008; Regenberg et al. 2009). More recent work in the field has revealed a remarkable expansion of this market into the

US, Australia and Japan (Turner and Knoepfler 2016; Berger et al. 2016; McLean et al. 2015; Fujita et al. 2016; Munsie et al. 2017).

Gathering robust data on the market for unproven stem cell-based interventions has proven to be a considerable challenge, and researchers have had to engage in a range of creative strategies to gather information. What is known about this market has been drawn from systematic analyses of clinic websites (Connolly et al. 2014; Ogbogu et al. 2013), studies of media reports (Zarzeczny et al. 2010) and patient blogs (Rachul 2011), interviews with patients and their supporters (Petersen et al. 2013), interviews with healthcare providers (Levine and Wolf 2012), and studies of social media (Robbillard et al. 2015; Kamenova et al. 2014), among other approaches. In many cases, clinics offering unproven stem cell interventions on the private market purport to treat a dubiously broad range of conditions (e.g., from aging to orthopedic injury to degenerative diseases such as ALS), often with a lack of specificity and/or a 'one size fits all' treatment approach (Berger et al. 2016). Patients are generally required to pay the often considerable costs associated with these treatments out of pocket, because they are not covered by public or private health insurance (McLean et al. 2015; Turner 2018). For example, in one informal poll of over 500 individuals, participants most often reported having paid between $2500 and $7500 USD (Knoepfler 2018). Patients are increasingly turning to crowdfunding as a means to support their interest in pursuing these interventions (Snyder et al. 2018).

It is important to be clear that not all private market providers can or should necessarily be painted with the same brush. Indeed, as is discussed in more detail below, practices vary and some may be bona fide forms of clinical innovation (Lindvall and Hyun 2009; Sleeboom-Faulkner 2016). However, in many cases the lack of transparency and clarity regarding the nature of the services provided in private clinics offering stem cell-related interventions makes it difficult to ascertain how they fit within current regulatory and practice standards (Zarzeczny et al. 2018). Notwithstanding the challenges with drawing firm lines between different categories of providers/practices, there are a number of general concerns associated with different activities occurring within this market.

In many cases, traditional forms of medically accepted evidence of safety and efficacy are lacking (Lau et al. 2008). There is often limited to no information available about quality control, purification procedures, cell processing mechanisms or facility standards. There also often appears to be minimal patient follow-up by the clinics providing the treatments, which is particularly concerning when patients later require care (sometimes urgently) as a result of adverse events (Bauer et al. 2018). There is no mandatory reporting system (unlike with clinical trials, for example) for adverse event reporting, which limits the ability of these activities to advance scientific knowledge in the field. The overwhelmingly positive portrayals of the clinic offerings on their websites also calls into question whether the standards of informed consent are being met (Lau et al. 2008; Ogbogu et al. 2013). Although valuable work is being done to develop professional standards for informed consent in the context of stem cell therapies (Sugarman et al. 2019; ISSCR 2019), enforcement seems likely to prove challenging among private market providers.

Finally, one of the most important concerns surrounding the wide range of allegedly stem cell based interventions offered in the private market concerns physical risks to patients. Reports of tumours, lesions, infections and vision loss, among other issues, associated with unproven stem cell interventions of one form or another (e.g., Kuriyan et al. 2017; Saraf et al. 2017; Thirabanjasak et al. 2010; Bauer et al. 2018) highlight that the risks of stem cell related interventions are not insignificant. There are different kinds and degrees of risk, depending on the types of cells used, what has been done to the cells (i.e. whether and how they have been manipulated/processed) and how they are administered. Unfortunately, these important distinctions often appear to be lost in many of the products and services currently advertised on the private market (Zarzeczny et al. 2018).

Indeed, in addition to the data limitations noted above, as I have argued elsewhere with colleagues, there are policy challenges stemming from terminology tensions and a lack of clarity in this space (Zarzeczny et al. 2018). As the market grows and diversifies it is becoming increasingly difficult to draw firm boundaries between practices of responsible medical innovation and inappropriate administration of ineffective and/or potentially harmful interventions. This murkiness exists at various levels in the current marketplace; including (though not limited to): what kinds of cells are being used; what processing procedures are used (and whether they are used consistently); what training and experience the providers have; what standard of care applies; where the appropriate line falls between research and clinical practice; what patients are told about these treatments, including whether they have the necessary information about both known risks, and regarding what is currently unknown/uncertain to provide truly informed consent, among many other key issues. This lack of clarity presents a considerable policy challenge, particularly if one accepts that regulation requires a clear understanding of what exactly is being regulated, and how existing laws and policies will apply – all of which is increasingly difficult in this dynamic and rapidly growing area.

3.2 An Unfolding Story: Stem Cells for Sale in Canada

Until fairly recently, there was no evidence to suggest the presence of a significant market for unproven stem cell-based interventions available in Canada. Canadians participated in international markets by seeking these interventions in other jurisdictions, which raised questions about what pre-procedure and post-procedure care obligations Canadian physicians have when working with patients who are interested in pursuing these interventions, and/or who return from stem cell treatment elsewhere requiring some form of follow-up care (emergent and otherwise) (Zarzeczny and Clark 2014). However, this situation is changing quickly and the market is growing in Canada. Media stories have drawn attention to Canadian involvement in the market for unproven stem cell interventions (Crowe 2017; Blackwell 2017), including via links to American providers that have come under scrutiny by the United States Food and Drug Association (FDA) (Crowe 2018).

The expansion of the Canadian market noted in these media reports has also been documented in recent research. In their 2016 study, Berger et al. noted 6 clinics in Canada (Berger et al. 2016). In a review of online offerings published in 2017, Chisholm et al. found 11 clinics advertising cell therapies in Canada (Chisholm et al. 2017). Following a study in 2017, Ogbogu et al. report on 15 clinics offering some form of unproven stem cell intervention in Canada, with representation from Ontario, Saskatchewan, Alberta, British Columbia, Quebec and Nova Scotia (Ogbogu et al. 2018a). Another study conducted between 2017 and 2018 identified 30 Canadian companies marketing stem cell treatments at 43 different clinic locations (Turner 2018). The Canadian market, as captured by these various reports, is currently small in comparison to other jurisdictions such as the US. However, the Canadian market appears to be growing quickly and if the US is any indication, is likely to continue to spread and diversify if left unchecked. As is discussed below, Health Canada has recently engaged directly with this issue from a regulatory perspective but how that activity will impact the market's long term trajectory remains to be seen.

3.3 Policy Options – Opportunities & Challenges for Canada

Notwithstanding the various concerns and risks outlined above, the market for unproven stem cell interventions has continued to expand and diversify in countries around the world for over a decade. This expansion is arguably reflective of regulatory failure and/or insufficient governance – perhaps related at least in part to the types of governance challenges noted earlier (Sipp 2011). Its more recent spread into Canada presents an

opportunity for decision-makers at various levels in this country to be proactive and exercise leadership by pursuing a deliberate and coordinated governance strategy in order to curtail problematic practices, while protecting the long term health of this promising field. In this section, I will address several specific strategies that could –with appropriate implementation and coordination – provide a solid foundation for an effective governance regime.

As is true of stem cell research and its clinical applications more generally, the market for unproven stem cell-based interventions arguably falls under a distributed governance regime. Health Canada is the federal body with responsibility over pharmaceuticals, medical devices and therapeutic products pursuant to the *Food and Drugs Act* (R.S.C., 1985, c. F-27) and its regulations. It plays an important role in protecting the safety of the public through this regulatory mandate. Although jurisdiction over health-related areas is a complex topic in Canadian law due to our Constitutional framework, it is sufficient for the purpose of this discussion to acknowledge that provincial and territorial ministries of health also have important responsibilities related to the administration of healthcare within their jurisdictions, including regulation of healthcare providers, healthcare facilities and healthcare insurance (Klein 2017). In addition, as identified earlier, regulated healthcare professions are also subject to the authority of their regulating bodies. For example, the practice of medicine is a self-regulating profession in Canada governed by provincial colleges of physicians and surgeons. In general, the colleges have responsibilities for setting educational requirements and standards of practice as well as over licensing conditions and disciplinary processes (Zarzeczny 2017). Accordingly, when it comes to the provision of stem-cell related interventions, whether as established standard of care treatments or unproven alternatives, there are various potential forms of oversight over the facilities where treatment is provided, over the professionals providing the intervention, and over the products themselves.

The distributed nature of governance of this market leads to various possible routes when it comes to policy options for responding to the concerns it raises, each of which offers different merits and challenges (Caulfield and Murdoch 2019). Although it is beyond the scope of this paper to engage in a fulsome analysis of these options, identifying some of the most promising alternatives is an important first step towards developing a robust strategy. At the state level, countries have responded in different ways to this burgeoning market, and there are various examples of attempts to rein in concerning practices. By way of early examples, German authorities pursued a particularly high-profile clinic following the death of a child in the context of an unproven stem cell intervention and ultimately forced the clinic to close (Vogel 2011). China, an early world leader as a destination for stem cell 'tourists' given its large and initially unregulated market, has been working on tightening regulations for the last several years – though questions remain regarding their implementation and enforcement (Rosemann and Sleeboom-Faulkner 2015). More recently, Australia has been taking an increasingly proactive approach to update its regulatory regimes, in an apparent effort to resolve ambiguities and provide clarity around how different kinds of stem cell-based interventions are to be approached in research and clinical contexts (e.g., Australian Government 2017). The FDA has also responded to the rapid growth of the market in the US (Turner and Knoepfler 2016) in a fairly public manner. For example, it provided guidance regarding how relevant regulations are to be interpreted and applied to the kinds of services provided in the growing private market (FDA 2017b, c). It was also took enforcement action including clinic inspections and warning letters for non-compliance (FDA 2017a, d, 2018a), and via the department of justice seeking permanent injunctions against two stem cell clinics (FDA 2018b).

Looking to these international examples is helpful in terms of informing a discussion about how decision makers in Canada might respond to the concerning and particularly risky aspects of

the market growing within our boarders. However, it will also be important that any such response account for relevant factors specific to Canada including – though not limited to – our constitutional division of powers, how our health care providers are regulated, the organization and administration of our health care systems including the largely public nature of their financing, and our existing legal frameworks. With these considerations in view, the following three policy options may have particular value, especially if approached in a deliberate and coordinated manner: (i) regulatory clarification and enforcement from Health Canada; (ii) rigorous enforcement of existing truth in advertising and consumer protection legislation, and (iii) maximization of the power of professional regulation (Caulfield and Murdoch 2019).

Health Canada did not initially engage in this issue as publicly as its American counterpart and analyses of existing regulations, including more specifically those relating to how minimally manipulated autologous cell therapies for homologous use are regulated, suggested there are ambiguities that require clarification so as to prevent private market offerings taking advantage of regulatory grey areas (Chisholm et al. 2017). In the spring of 2019, Health Canada issued a policy position paper clarifying regulatory questions about autologous cell therapies in Canada relating to their risks, how they fit within existing federal product safety rules, and therapy development activities (Health Canada 2019). More specifically, Health Canada clarified that all cell therapies are considered "drugs" for the purpose of regulation and thus are subject to regulation pursuant to the *Food and Drugs Act*. The Government of Canada issued a related public safety advisory (Government of Canada 2019), and there have been subsequent reports of enforcement activities by Health Canada against individual clinics (Crowe 2019). This situation is evolving rapidly and at the time of writing, it remains to been seen what impact this regulatory clarification and accompanying enforcement efforts will have on the future of the market for unproven stem cell-based interventions in Canada.

Canada also has strong consumer protection and truth in advertising standards in place. In Canada, business conduct is regulated primarily via the *Competition Act* (R.S.C. 1985, c. C-34), a federal statute that includes both civil and criminal provisions that deal with false or misleading representations and deceptive marketing practices. Although there are recognized enforcement challenges in this area, consumer protection legislation has considerable potential to be used to restrict false or misleading claims being made to consumers regarding private market offerings of stem cell-based interventions (Ogbogu 2016; von Tigerstrom 2017). Indeed, many of the claims made on clinic websites, including those found in the studies referenced above, are likely to run afoul of Canada's *Competition Act* provisions (Murdoch et al. 2018). The *Competition Act* is enforced by the Competition Bureau, which has a wide range of enforcement options at its disposal including not only both criminal and civil law routes, but also education and other soft-law approaches (Competition Bureau 2019). Although we have yet to see robust enforcement action by the Competition Bureau against problematic stem cell-related marketing in Canada, at least a public level, the availability of this diverse set of responses is a regulatory strength.

Professional regulation is another potentially powerful though as-yet underutilized tool for addressing particularly concerning elements of this market (Zarzeczny et al. 2014). Physicians have been found to play a prominent role as providers of unproven stem cell based interventions (Murdoch et al. 2018; Ogbogu et al. 2018a). Accordingly, professional regulatory bodies – including the colleges of physicians and surgeons in Canada – could play a central role in curtailing problematic practices by their members and, in so doing, restrict the unchecked spread of potentially high risk and/or deceptive practices (Zarzeczny et al. 2014; Murdoch et al. 2019). There are a few examples of initial activity in this realm. For example, the College of Physicians and Surgeons of Alberta recently issued Standards and Guidelines for Stem Cell Regenerative Therapy (CPSA 2018). However, arguably considerably more could be

done to provide clarity to physicians across the country regarding acceptable standards of practice with respect to stem cell-related interventions and with regard to enforcement activities to address unprofessional conduct.

The courts may offer another potential avenue for redress when individuals suffer harm in relation to the provision of unproven stem cell-based interventions. For example, a patient who is injured by a stem cell-based intervention may have an action in negligence against the provider of the intervention and/or their employer (through vicarious liability). Individuals who suffer financial loss may also have claims related to fraud or other heads of liability (Horner et al. 2018; Caulfield and Murdoch 2019). There has been limited litigation in this area to-date and one significant concern with civil law actions, particularly as a remedy for medical harms, is the burden (financial, emotional and otherwise) they often place on individual plaintiffs who may already be in a vulnerable position. Nonetheless, the remedies available through civil litigation and the role it can have in deterrence give it a place worth considering within the broader governance matrix.

There are also an ever-growing collection of information-based responses led by diverse stakeholder groups including scientific organizations, patient advocacy groups, policy researchers and others (e.g., ISSCR 2016; Master and Caulfield 2014). These efforts typically focus on supporting patients (or their substitute decision-makers) in making informed decisions based on accurate and balanced information. Although they arguably have considerable value, the limitations of communication strategies that rely largely on information deficit models are well-recognized; nonetheless, they remain persistent in science communication, likely for various reasons (Simmis et al. 2016). Encouragingly, there is valuable work being done in numerous health-related fields to enhance understanding of different ways to engage and empower patients, including via online mechanisms that could inform future communication strategies (Fagotto et al. 2019). With the proliferation of misinformation online and the use of forums such as YouTube as a marketing strategy for unproven stem cell-based interventions (Hawke et al. 2019), it is increasingly important for efforts seeking to provide patients and the public with accurate and balanced information to explore new modes of engaging their audience, such as the creative use of narratives (Caulfield et al. 2019).

As this brief review of a small sample of options highlights, addressing policy challenges in the field of stem cell research – including the private market for unproven stem cell-based interventions – does not necessarily require dramatic regulatory change. Clarifying and enforcing our existing laws and regulatory structures is arguably a logical first step to responding to the concerns associated with the Canadian market for unproven stem cell-based interventions (Zarzeczny et al. 2018; Caulfield and Murdoch 2019). Doing so will not necessarily require new policies or regulation (although these may ultimately be useful), but rather as a starting point could focus on bringing renewed vigour to those that are already in place. This strategy may or may not provide a complete answer, but we will arguably not be able to reliably identify problematic gaps and ambiguities until we give full effect to existing controls. The market in Canada is in its early stages, meaning we have an opportunity at present to 'get it right' from a regulatory perspective, ideally before too much harm is done – whether to individual patients or to the reputation of the field of stem cell research and regenerative medicine. Indeed, there is a risk that the public may come to lose trust and hope in this field of research if ineffective and potentially harmful interventions are permitted to proliferate in advance of the science (Cossu et al. 2018).

4 Implications & Future Agendas

As the case of the market for unproven stem cell-based interventions demonstrates, there are clear and present governance challenges when it comes to arriving at effective policy solutions that address complex issues in the multi-faceted field of stem cell-related research and its clinical applications.

Although some of the issues raised in the case discussed in this paper are specific to the particular area of stem cell-based interventions (e.g., interpretive questions regarding how current regulations apply to autologous cell therapies), many are shared with other avenues of regenerative medicine and more broadly with different areas of biomedical innovation.

For example, when fields develop quickly and/or unpredictably, regulation can struggle both with particulars such as clear and unambiguous definitions and with larger questions about regulatory fit. Legitimate excitement about the potential of cutting-edge research can also easily lead to hype and unrealistic expectations from the public, which may underpin demand for early access to a technology – even though it may not be ready for routine use outside of a research context. Questions about what level and type of evidence of safety and efficacy should be required before access to it is permitted, facilitated (e.g. via public funding), or both, can also be contentious.

Going forward, there is a strong imperative to consider what the growing patient-driven demand for access to experimental and unproven medical interventions means for health system governance and regulation in Canada, and what an effective, consistent and coordinated approach to regulation might look like. The fast-moving nature of these fields may benefit from anticipatory governance strategies (Quay 2010; Barben et al. 2008), taking into account the value of a principled and responsive approach that is proportional, guided by evidence, clear and consistent (Ogbogu et al. 2018b). Notwithstanding the numerous limitations associated with efforts to predict where medical innovations are headed, some form of legal foresighting may prove useful in efforts to support law's relevance and role in shaping science and innovation (Laurie et al. 2012).

In addition to understanding biomedical science and its potential, other important areas of inquiry will need to include the roles and influence of evidence (in different forms), scientific and clinical imperatives and of public pressure on policy decisions. The appropriate role of regulation in managing risks as well as scientific and clinical uncertainty in fast moving fields of biomedicine is a closely related issue that also bears focused consideration. It is important to stress that law is but one tool in a broader governance framework. Indeed, we sometimes hear competing allegations that law is failing to keep pace with scientific advancements, or that it is leaping ahead of science and imposing unnecessary or ill-fitting restrictions on its development. Establishing an appropriate balance between these two extremes can be a difficult challenge, particularly given the different and sometimes competing objectives that inform the policy making process including, but not limited to, protection of the public, promotion of various agendas – political, moral, religious, economic – and the desire to encourage innovation in science and health technology.

These challenges underscore the importance of scientists and policy makers working together, and of exploring how key stakeholders – including the public – can have a meaningful voice in the policy process. They further highlight the value of both hard and soft regulatory strategies (Harmon et al. 2013) and of arriving at governance strategies that facilitate deliberate coordination between them. This agenda is far from simple, but is important to the larger goal of facilitating biomedical innovation while managing and mitigating its risks in a manner suited to the Canadian context.

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Future Cell and Gene Therapy for Osteoarthritis (OA): Potential for Using Mammalian Protein Production Platforms, Irradiated and Transfected Protein Packaging Cell Lines for Over-Production of Therapeutic Proteins and Growth Factors

Ali Mobasheri

Abstract

In this paper I provide a personal perspective on future prospects for cell and gene therapy for osteoarthritis (OA) and how mammalian protein production platforms, virally transfected and irradiated protein packaging cell lines may be used as "cellular factories" for over-production of therapeutic proteins and growth factors, particularly in the context of intra-articular regenerative therapies. I will also speculate on future opportunities and challenges in this area of research and how new innovations in biotechnology will impact on the field of cell and gene therapy for OA, related osteoarticular disorders and the broader discipline of regenerative medicine for musculoskeletal disorders. Mammalian protein production platforms are likely to have a significant impact on synovial joint diseases that are amenable to cell and gene therapy using therapeutic proteins and growth factors. Future cell and gene therapy for OA will need to re-consider the current strategies that employ primary, aged and senescent cells with feeble regenerative properties and seriously consider the use of mammalian protein production platforms.

Keywords

Cell therapy · CHO · Gene therapy · GP-293 · Growth factors · HEK-293 · Mammalian protein production platforms · Osteoarthritis (OA) · Transfected protein packaging cell lines · Transforming Growth Factor-β (TGF-β) · Growth differentiation factor 5 (GDF5) · TissueGene-C (TG-C)

A. Mobasheri (✉)
Department of Regenerative Medicine, State Research Institute Centre for Innovative Medicine, Vilnius, Lithuania

Research Unit of Medical Imaging, Physics and Technology, Faculty of Medicine, University of Oulu, Oulu, Finland

Centre for Sport, Exercise and Osteoarthritis Research Versus Arthritis, Queen's Medical Centre, Nottingham, UK

Sheik Salem Bin Mahfouz Scientific Chair for Treatment of Osteoarthritis with Stem Cells, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia
e-mail: ali.mobasheri@imcentras.lt; ali.mobasheri@oulu.fi

Abbreviations

ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
BMI	body mass index
BMP-7	bone morphogenic protein
CHO	Chinese hamster ovary
DMOAD	disease-modifying osteoarthritis drug
ECM	extracellular matrix
EULAR	European League Against Rheumatism
FDA	Food and Drug Administration
FGF-18	fibroblast growth factor 18
GDF-5	growth differentiation factor 5
GM-CSF	granulocyte macrophage-colony stimulating factor
GP-293	HEK 293-based retroviral packaging cell line
HEK-293	human embryonic kidney 293 cell line
IH	intermittent hydrarthrosis
IL-1 β	interleukin 1 β
IL-6	interleukin 6
iNOS	inducible nitric oxide synthase
JSW	joint space width
MCB	Master Cell Bank
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
MSC	mesenchymal stem cell
NO	nitric oxide
NSAIDs	non-steroidal anti-inflammatory drugs
OA	osteoarthritis
OARSI	Osteoarthritis Research Society International
PGE_2	prostaglandin E_2
PRP	platelet rich plasma
R&D	research and development
RA	rheumatoid arthritis
RMDs	rheumatic and musculoskeletal diseases
ROS	reactive oxygen species
SASP	senescence-associated secretory phenotype
SV-40	simian virus 40
TG-C	TissueGene-C
TGF-β	transforming growth factor β
TNF-α	tumour necrosis factor α

1 Introduction

According to the European League Against Rheumatism (EULAR) there are more than 200 different forms of rheumatic and musculoskeletal diseases (RMDs). These diseases most commonly affect synovial joints. Osteoarthritis (OA) is the most common form of joint disorder with degenerative and inflammatory components (Martel-Pelletier et al. 2016). As a disease of aging, it is accountable for more disability in the elderly population than all other musculoskeletal diseases altogether across the developed and developing countries (Christensen et al. 2009). Recent information provided by the Osteoarthritis Research Society International (OARSI) suggests that OA is a serious disease, affecting 240 million people worldwide and is twice as likely to affect women than men over the age of 50. It is estimated that a "tsunami" of new OA cases will hit countries with a much larger ageing population in the developed world by the year 2050, when 130 million people will be suffering from OA worldwide and 40 million will be severely disabled.

Despite the growing prevalence and burden of OA there is no cure for this disease (Mobasheri 2013). In the absence of a cure, the only way to reduce the societal burden attributed to OA is to change gears and shift the approach to management, and focus on developing long-term strategies for prevention. The main treatments for OA include lifestyle measures, such as maintaining a healthy weight and exercising regularly, anti-inflammatory medication to reduce inflammation and relieve pain and supportive therapies to help make everyday activities easier. Surgery may be considered to repair, strengthen or replace components of damaged joints. However, when all other treatment options fail, joint arthroplasty is the final solution.

OA is also a cause of significant morbidity. It limits daily life by reducing movement so that 25% of people affected cannot engage in normal daily routines and activities. It affects the professional and personal lives of the people affected and has adverse impacts on their partners, families, friends, careers and society as a whole. By reducing mobility OA significantly increases the risk of cardiovascular disease (Kim et al. 2016), diabetes (Louati et al. 2015) and hypertension (Courties et al. 2015; Calders and Van Ginckel 2017).[1] OA contributes to depression and anxiety in many patients (Sharma et al. 2016) and is a major cause of sleep disturbance (Parmelee et al. 2015).

Signs and symptoms of OA include joint pain, stiffness, tenderness, loss of flexibility, crepitus and a grating sensation as the joint articulates. The affected joints hurt during or after movement.[2] There may be inflammatory episodes and flares that lead to soft tissue inflammation around the joint and to joint effusion and swelling. The accumulation of excess fluid in the synovial space is known as intermittent hydrarthrosis (IH), although this is less common in OA compared to rheumatoid arthritis (RA). In later stages of the disease bone spurs known as osteophytes can form around the affected joint and these are seen on plain radiographs.

2 The Hallmarks of Osteoarthritis

All major diseases are characterised by "hallmarks", which summarise the key biological alterations that occur in that disease. For example, cancer comprises six biological capabilities that are gradually acquired during the multistep development of human tumors (Hanahan and Weinberg 2000; Hanahan and Weinberg 2011). In the case of OA, there are many similarities with the hallmarks of aging (López-Otín et al. 2013). The hallmarks of aging include genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication. Many of the hallmarks of aging are also seen in OA. However, for the sake of simplicity, the gradual loss and degradation of articular cartilage is the major hallmarks of OA (Mobasheri and Batt 2016; Tonge et al. 2014). This feature is particularly important as is a clinical measure of disease progression when loss of articular cartilage is measured on plain radiographs over time as a reduction in joint space width (JSW). For decades we have used radiography as the "gold standard" to diagnose OA and assess disease progression over time in routine clinical practice and in clinical trials (Hunter et al. 2015). However, by the time the disease has been diagnosed using plain radiographs, it has already progressed significantly and there are suggestions that plain X-ray radiography should no longer be considered a surrogate outcome measure for longitudinal assessment of cartilage in clinical trials of new drugs and combination treatments for knee OA (Guermazi et al. 2011). However, cartilage thickness remains a key parameter that can determine the efficacy of new regenerative treatments and this will be discussed later in this article. In obese and overweight OA patients cartilage degrades even further and this is thought to occur through a combination of biomechanical mechanisms due to the excess weight and the pro-inflammatory cytokines (adipokines) and leptin produced by white adipose tissue (Francisco et al. 2018, 2019; Scotece and Mobasheri 2015).

3 Molecular Alterations in OA

The precise sequence of molecular events involved in the pathogenesis of OA are not clear. There is evidence that there are multiple phenotypes of OA with underlying molecular endotypes and these are likely to vary between individuals (van Spil et al. 2019; Mobasheri et al. 2019). Therefore, OA is no longer regarded as a

[1] https://www.arthritis.org/living-with-arthritis/comorbidities/heart-disease/osteoarthritis-ups-cvd-risk.php

[2] https://www.mayoclinic.org/diseases-conditions/osteoarthritis/symptoms-causes/syc-20351925

homogeneous disease. It is a heterogeneous disease that can be stratified into different subsets and subgroups (Driban et al. 2010), some of which may overlap in older patients with multiple co-morbidities, although all the different pathways lead to cartilage erosion and loss of joint function. In terms of disease initiation, it is thought that there is a long and asymptomatic "molecular phase", which is followed many years later by radiographic changes and the appearance of symptoms (Kraus et al. 2015). In addition to aging, obesity, gender and genetics, inciting risk factors may include previous joint trauma or repetitive injuries or the presence of metabolic and endocrine disease (Fig. 1).

There are biomechanical (Englund 2010), inflammatory (Berenbaum 2013) and metabolic (Mobasheri et al. 2017) factors that have been shown to play key roles in the initiation and progression of the disease. We now know that chondrocytes are not simply passive participants and bystanders in disease progression. Chondrocytes become progressively inflammatory and activated in OA. The increased pro-catabolic and pro-inflammatory factors in OA reduce anabolic activity, alter cellular metabolism, and disturb the delicate balance between extracellular matrix (ECM) synthesis and degradation (Loeser 2011). Other joint tissues can contribute to the loss of homeostasis and metabolic regulation in the joint as well, since OA also involves the synovial membrane (Siebuhr et al. 2016; Rahmati et al. 2016), subchondral bone (Mahjoub et al. 2012) and peri-articular soft tissues (Goldring and Goldring 2007). Figure 2 summarises the major molecular players involved in the alterations that occur in the OA joint.

Synovitis appears to be a very common feature in both the early and late phases of OA (Scanzello and Goldring 2012), with infiltrating macrophages, T cells and mast cells (de Lange-Brokaar et al. 2012). Synovitis and the innate inflammatory network (Liu-Bryan 2013) expectedly play a key role in OA; pro-inflammatory cytokines are most frequently found in the inflamed synovium (de Lange-Brokaar et al. 2012). Catabolic and pro-inflammatory mediators such as cytokines, reactive oxygen species (ROS), nitric oxide (NO), prostaglandin E_2 (PGE_2) and neuropeptides from the inflamed synovium, all affect chondrocyte metabolism and matrix turnover in the cartilage (Sutton et al. 2009). Synovitis leads to excess production of proteolytic enzymes responsible for cartilage breakdown (Sellam and Berenbaum 2010). On its turn, cartilage matrix catabolism releases molecules that perpetuate synovial inflammation, creating a vicious and self-perpetuating cycle (Sellam and Berenbaum 2010). Inflammatory mediators from chondrocytes and synoviocytes also drive oxidative stress and inflict joint damage by releasing ROS (Poulet and Beier 2016). Once activated by stress such as pro-inflammatory cytokines,

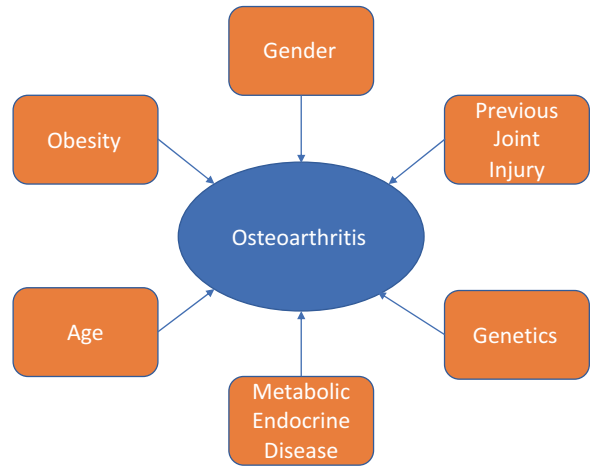

Fig. 1 Risk factors for osteoarthritis (OA)

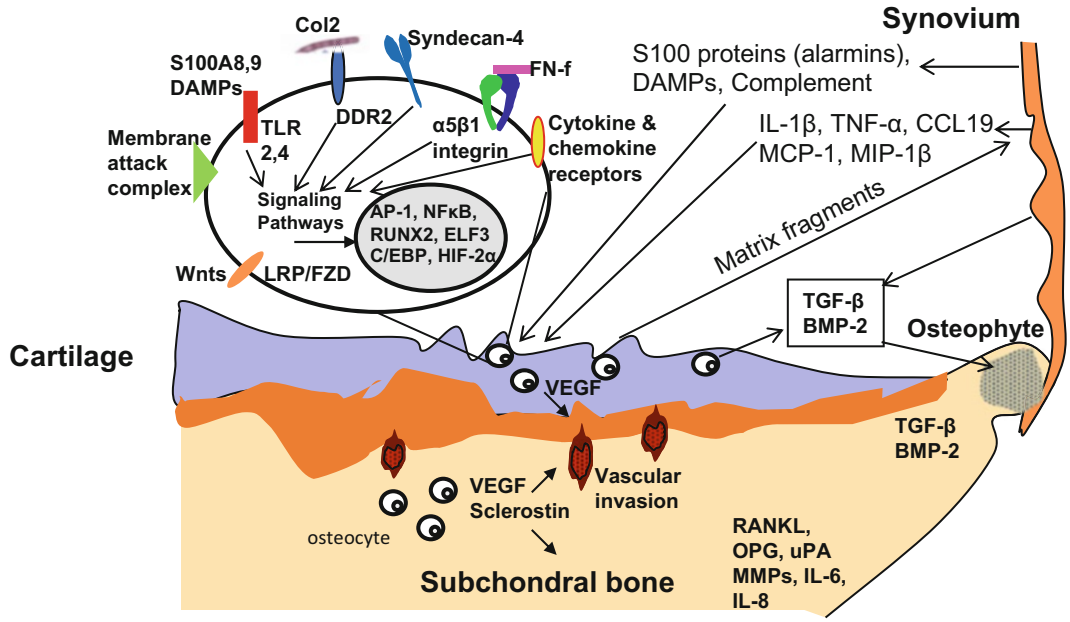

Fig. 2 Chondral, subchondral and synovial alterations in OA. Reproduced with permission from Mobasheri et al., (Chapter 3 – Cartilage and Chondrocytes, Kelley & Firestein's Textbook of Rheumatology 11th Edition, in press)

prostaglandins and ROS, the normally quiescent articular chondrocytes become activated and undergo a phenotypic shift through a phenomenon recently described as "chondrosenescence" and the development of a senescence-associated secretory phenotype (SASP) (Salminen et al. 2012) leading to further disruption of homeostasis and metabolism in cartilage (Mobasheri et al. 2015).

As mentioned earlier, at the present time, there is no cure for OA. The existing treatments are unsatisfactory, and only address the symptoms (Zhang et al. 2016).[3] There are no approved drugs that prevent OA and there are no approved drugs that impede the progression of the disease.[4] In many cases of advanced OA joint surgery and replacement is the only viable solution when joint function has been irreversibly and permanently compromised (Katz et al. 2010). Arthroplasty can replace the knee or the hip, but it cannot restore the original function of the joint (Crawford and Murray 1997). OARSI treatment guidelines propose that all OA patients should receive up-to-date information and education to enable them to self-manage their disease to some extent (Zhang et al. 2008). All OA patients are advised to manage their weight and lose weight if they have a high body mass index (BMI), remain active and exercise regularly (Bliddal et al. 2014). All OA patients are advised to avoid a sedentary lifestyle because joints need to move and articulate. Clinical evidence suggests that some OA patients will benefit from drugs, but the currently existing drugs (non-steroidal anti-inflammatory drugs, NSAIDs) only address the symptoms of pain and inflammation, and they do this unsatisfactorily, with small effect sizes and significant side effects and cardiovascular, gastrointestinal, renal and hepatobiliary risks (Matthews and Hunter 2011). Many OA patients remain dissatisfied with the currently approved pharmacological interventions.[5]

[3] https://www.arthritisresearchuk.org/arthritis-information/arthritis-today-magazine/156-spring-2012/osteoarthritis.aspx

[4] https://www.bones.nih.gov/health-info/bone/osteoporosis/conditions-behaviors/osteoporosis-arthritis

[5] https://www.ncbi.nlm.nih.gov/books/NBK333051/

There is emerging evidence that some OA patients benefit from joint injections, including intra-articular steroids and viscosupplementation. However, numerous and repeated steroid injections are not recommended. Furthermore, viscosupplementation does not work well for every patient especially in advanced cases, where significant joint degradation has already occurred and there is bone-on-bone contact upon weightbearing and joint mobilization (Martel-Pelletier et al. 2016). Many pharmaceutical companies that were active in the area of OA drug development in the 1990's exited this challenging disease area in the 2000's because they had exclusively focused on developing oral drugs for inhibiting matrix degrading enzymes, but these targets were too downstream and failed to inhibit key upstream catabolic pathways and disease driving switches. The reduced Research and Development (R&D) efficiency of pharmaceutical companies has made it necessary for them to realign their R&D concepts and strategies (Schuhmacher et al. 2016). The outcomes of the clinical trials of matrix metalloproteases and cathepsins were poor, because of adverse events and fibrotic events. The pharmaceutical companies involved learned a very important lesson: targeting end-stage catabolic mediators is unlikely to work as a strategy for curing OA.

So why is disease modification in OA so challenging? Why are existing treatments so futile? Why has progress in OA drug development been so slow compared to other arthritic and rheumatic diseases? The answers to these questions are not so straightforward. The problem is that patients desire effective pain relief but the drug companies are more ambitious, hoping for treatments that address symptoms as well as improving structure. Unfortunately, there are no such magic bullets in the modern drug development arena. We know that the development of symptom modifying OA drug is now exploding with a plethora of pain pathways being pursued and multiple candidates are in advanced stages of clinical development. However, structure modification in OA remains extremely complex with significant development challenges (Hellio Le Graverand-Gastineau 2009). To make progress, we do need to revisit some basic concepts in physiology and pathophysiology. We know that the balance between anabolic and catabolic activity is disturbed in OA and normal physiological turnover of joint tissues in perturbed (Fig. 3). New drugs must address the imbalance between catabolic and anabolic activity in order to halt disease progression. New treatments must have the capacity to positively influence cartilage metabolism.

The pharma companies that are still engaged in this challenging arena have learned important and useful lessons from the failures of previously defeated players. Drug companies that remain engaged with the OA challenge have accepted that targeting inflammation and catabolic activity is not enough. Greater efforts must be made to stimulate anabolic activity and this is one of the main reasons why current therapeutic strategies are focusing on the development of cell and gene therapy and recombinant anabolic growth factors. There is significant ongoing effort in this area, especially focusing on the improvement of autologous chondrocyte transplantation techniques, the use of stem cells and the application of chondrocyte and stem cell-derived growth factors in preclinical and translational models of OA. For example, emerging regenerative therapies that target OA joint tissues include injectable biological such as mesenchymal stem cells (MSCs) (Mobasheri et al. 2009, 2014; Richardson et al. 2016), platelet rich plasma (PRP), growth differentiation factor 5 (GDF5) (Enochson et al. 2014), bone morphogenic protein 7 (BMP-7) (Chubinskaya et al. 2007; Funck-Brentano et al. 2014), fibroblast growth factor 18 (FGF-18) (Gigout et al. 2017; Yu and Hunter 2016; Lohmander et al. 2014) or injectable small molecules and drugs such as WNT signalling pathway inhibitors (Lories et al. 2013; Stampella et al. 2018) or even revisiting the classical approaches to inhibiting extracellular matrix degradation – by targeting catabolic enzymes using matrix metalloproteinase (MMP) inhibitors and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) inhibitors (Yang et al. 2017; Murphy 2017) (Fig. 4).

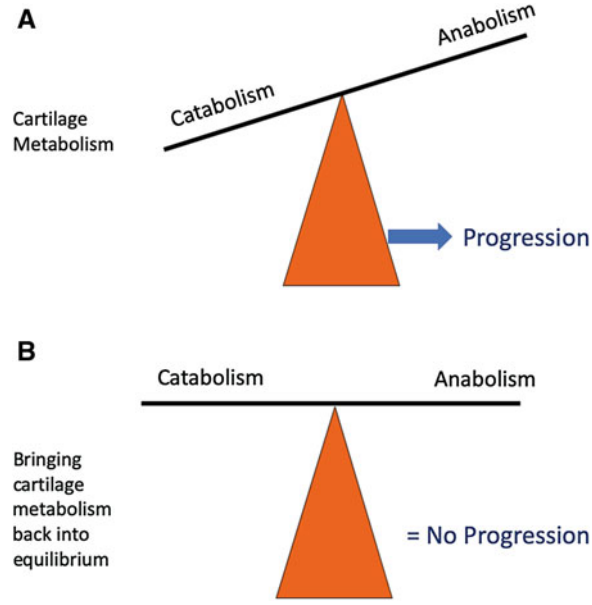

Fig. 3 (**a**) Loss of the delicate physiological balance between anabolism and catabolism in OA leads to impaired cartilage metabolism and disease progression. (**b**) Effective drugs can bring cartilage metabolism back into equilibrium and stop disease progression

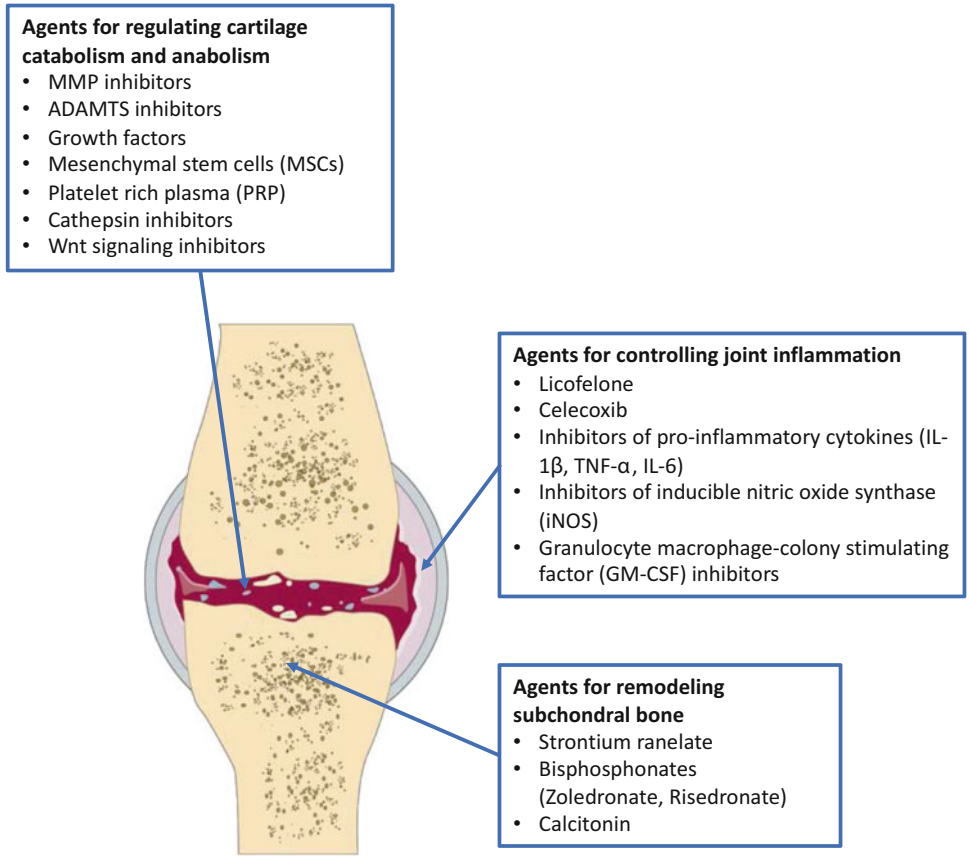

Fig. 4 Therapeutic strategies for targeting synovial joint structure in OA. (Reproduced with permission from Huang et al. 2017)

One crucial quantum leap in the development of new therapies for OA has been the realisation and acceptance that the most ideal treatments must target the affected joint through injection rather than the oral route. Another important advance has come from the field of rheumatology and the treatment of RA: multiple joint injections may be needed and a single joint injection is unlikely to work, even if we have a magic bullet. However, many of the key researchers in this field are still stuck in silos and have yet to accept that biological therapy, whether using autologous chondrocytes and stem cells cannot be achieved with primary tissue-derived native cells. Despite progress and advancements in MSC biology and the introduction of various bioactive scaffolds and growth factors in preclinical studies, current clinical trials are still at very early stages with preliminary aims to evaluate safety, feasibility and efficacy (Lee and Wang 2017) and this is where we must focus our efforts. Clinical trials of stem cell therapies and MSCs in particular have yet to demonstrate efficacy and while we anxiously wait for outcomes of several ongoing stem cell trials in OA, there are a number of companies that have innovated by focusing efforts on developing treatments using mammalian protein production platforms, including transformed and modified cells, as well as immortalized cells that were originally developed as research tools and protein packaging cell lines for over-production of target proteins. It is evident that the field of biotechnology has already offered us powerful and versatile tools for the over-production of therapeutic proteins but since many OA researchers are still stuck in silos and rarely speak to scientists in other fast-moving disciplines, such as biotechnology, we are unaware of the expanded and enhanced toolbox that is at our disposal. We need to break down these disciplinary barriers, open-up our minds and accept that new therapeutic innovations in OA are likely to come from other biological and biomedical disciplines, including biotechnology, protein engineering and immunology.

4 Mammalian Protein Production Platforms

Mammalian protein production platforms are indispensable cell factories that are used for large-scale production of antibodies and therapeutic proteins (Zucchelli et al. 2016). Expression of antibodies and proteins in mammalian cells is a key technology that is vitally important for many functional studies on human and higher eukaryotic genes. Mammalian cell expression systems allow the production of proteins, especially of those of clinical relevance and human origin (Aricescu and Owens 2013). Over the last few decades these platforms have evolved and had a profound impact in many areas of basic and applied research, and an increasing number of biological drugs are now recombinant mammalian proteins made using these tools (Bandaranayake and Almo 2014). Recombinant proteins and a vast array of antibodies are now produced in mammalian cell lines instead of bacterial expression systems to ensure that proper protein folding and post-translational modifications, which are essential for full biological activity, are properly introduced in a eukaryotic and "mammalian" context. Mammalian cell expression systems are the dominant tools for producing complex biotherapeutic proteins (Estes and Melville 2014). The most commonly used mammalian cell lines found in the research and industrial therapeutic protein production settings are Chinese hamster ovary cells (CHO) (Omasa et al. 2010) and human embryonic kidney 293 cells (HEK-293) (Dyson 2016).

Various mammalian and non-mammalian expression systems are also being used for protein and glycoprotein production and recent cellular engineering strategies have been developed to increase protein and glycoprotein productivity (Lalonde and Durocher 2017). "Omics" technologies are continually being used to improve cellular expression systems and enhance such platforms for therapeutic protein production. Figure 5 summarises the expression systems used for protein and glycoprotein production by industry.

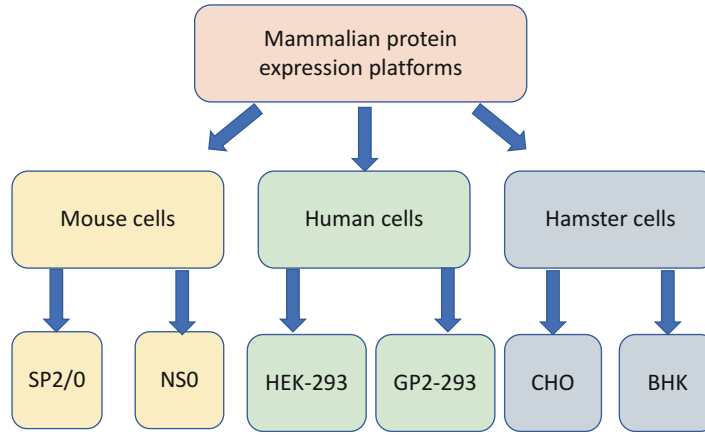

Fig. 5 Expression systems used for protein and glycoprotein production by biopharmaceutical industries. (Adapted from Lalonde and Durocher 2017)

Transient expression systems in mammalian cells have also become the method of choice for producing large quantities of antibodies (Vink et al. 2014). By using a combination of simian virus 40 (SV40) large T antigen, p21 and p27, it has been possible to scale-up expression level of antibodies, from 0.1 ml up to 1200 ml in bioreactors (Vink et al. 2014). The ability to scale-up allows biotechnology companies to produce sufficient quantities of therapeutic antibodies and proteins for tests in preclinical studies and early phase clinical trials.

5 Platforms for Over Production of Recombinant Growth Factors

If a pharmaceutical company or a biotechnology company aims to make large quantities of a growth factor capable of stimulating cartilage matrix synthesis, it might be useful to have access to mammalian cell models that truly mimic chondrocytes, with phenotypic chondrocytic properties. There are several chondrocyte-like transformed cell-lines developed by Dr. Mary Goldring. However, none of these cells have been used in such a context. At present, there are no cell-lines derived from cartilage or other joint tissues that are truly comparable to CHO or HEK-293 cells. Therefore, other cellular models must be employed, including CHO, HEK-293 cells and their derivatives such as GP2-293. These are immortalized cell lines that function as "cellular factories" for over-production of proteins. GP2-293 cells are specialised protein packaging cells. These cells are specialised transfection models, protein packaging tools and "cellular factories" for over-production of target human proteins and are promising candidates for over-producing therapeutic proteins and growth factors that native primary cells (i.e. chondrocytes) or stem cells (i.e. MSCs) cannot produce in sufficiently large quantities, either in the short-term or in the long-term. Although these cells cannot be used in their immortalized form for the development of clinically relevant cell therapies for OA, they can be irradiated to obliterate their proliferation capacity so that they remain protein packaging cellular factories, but without the ability to proliferate. Elimination of their proliferation through irradiation makes the use of such cells feasible in cellular therapies for OA, especially if the cells are to be injected into the closed micro-environment of the synovial joint, isolated from the circulatory system. Irradiated cells will retain their capacity for protein over-production, but they cannot divide and proliferate, which means that they will die shortly after being injected into the joint.

6 Case Study: Production of Transforming Growth Factor β1 (TGF-β1) by GP2-293 in the Kolon TissueGene Cell and Gene Therapy Product TG-C

In November 2018 Kolon TissueGene, Inc. based in Rockville, MD, announced that it has treated its first patient in the pivotal Phase III clinical trials of TG-C (the product is known as TG-C in the United States of America and as Invossa in South Korea), a revolutionary cell and gene therapy for the treatment of knee OA. TG-C is a unique first-in-class cell and gene therapy targeting knee OA through a single intra-articular injection of joint-derived chondrocytes, irradiated GP2-293 cells and, most importantly, the biological growth factors that they overproduce to possibly promote anabolic repair and regeneration in the diseased joint" as a future possibility in the treatment for OA (Fig. 6).

The first dosing in November 2018 followed the lifting of a clinical hold by the United States Food and Drug Administration (FDA) in July 2018. The phase III trials for US approval of TG-C were expected to recruit over a thousand patients in approximately 60 clinical sites across the United States. This ambitious trial was hoping to assess pain and physical function endpoints as well as imaging outcomes, including magnetic resonance imaging (MRI), radiographs and wet biochemical markers in patients receiving TG-C treatment.

Clinical trials of this approach have demonstrated some degree of pain relief, increased mobility, as well as indicators of reduced OA progression and improvements in joint structure, function and reduced stiffness. The allogeneic (off-the-shelf) drug consisting of cells and biological growth factors could potentially provide an alternative to traditional pharmacological OA treatments, which are largely unsatisfactory, with numerous undesired side-effects.

The ultimate aim is to achieve symptom and structure modification to the extent that the knee joint is preserved and surgery is delayed, for a substantial period of time. Knee arthroplasty is not for everyone, and is risky for some patients and costly for healthcare providers.

Thus far, with the exception of weight loss and exercise for overweight and obese individuals with OA, there are no effective therapies that can halt or delay the progression of OA and minimize the need for multiple surgical interventions.

7 DMOAD Status for TG-C

A Phase II clinical trial conducted by Kolon TissueGene in the United States demonstrated a

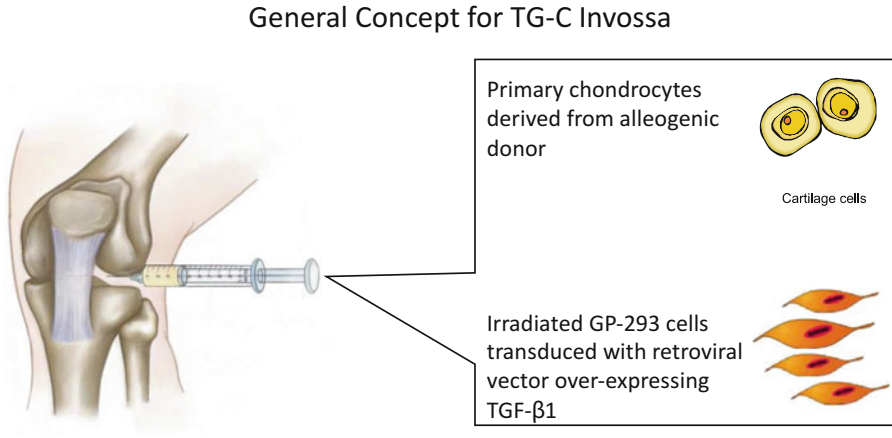

Fig. 6 The intra-articular injection concept for TG-C Invossa, a novel cell and gene therapy targeting knee OA through a single intra-articular injection of joint-derived primary chondrocytes, irradiated GP2-293 that produce TGF-β1, the biological growth factor that promotes anabolic repair and regeneration

two-year improvement of pain and function. The company then sought to continue supporting these efforts through its recently initiated national Phase III clinical trial across the United States. In addition, Kolon TissueGene has designed a trial to seek a disease-modifying osteoarthritis drug (DMOAD) designation for TG-C from the FDA, potentially making TG-C the first cell and gene therapy to receive such an elevated DMOAD status.

8 Use of GP2-293 Cells in TG-C

The human GP2-293 cell line is one of the key components of TG-C. These cells carry out the vital function of over-producing the crucially important growth factor TGF- β1. The GP2-293 cells have been used throughout the whole developmental process from the first production of the Master Cell Bank (MCB) to the next step, which is the development of the working cell bank and the final product formulation. As mentioned earlier, GP2-293 is a HEK 293-based retroviral packaging cell line used for large-scale protein production. It is a cellular platform for over-production of therapeutically relevant human proteins. This is the first time that such a human protein production platform has been employed in the context of OA treatment and cartilage regeneration. They are transformed with adenovirus type 5 DNA and the cells were engineered to express the MoMuLV Gag and Pol proteins. Effectively these cells are a protein producing tool and "cellular factory". Native patient derived chondrocytes simply do not have the capacity to over-produce TGF-β1 in sufficiently high quantities for cellular therapy and regenerative applications.

9 Safety of GP2-293 Cells in TG-C

Transduced and irradiated GP2-293 cells may be transformed cells but since they have lost their capacity for proliferation, they cannot proliferate. Therefore, the GP2-293 cells in TG-C cannot survive and proliferate in the joint. These cells will simply carry out their transient function as radiation inactivated transfection models, protein packaging tools and "cellular factories" for over-production of therapeutic TGF-β1. Therefore, the cells cannot survive for more than a very short period after being injected into the joint. The Korean Food and Drug Administration and the Ministry of Health in South Korea have stated they are not concerned about the safety of Invossa, the Korean version of TG-C, noting that cells no longer survive 44 days after administration. Furthermore, no drug-related side effects were identified from those subject to clinical trials.[6] After the cells carry out their TGF-β1 over-production duties, they will die and their remains will be cleared by joint resident inflammatory macrophages through the process of phagocytosis (Fig. 7).

The scientific basis for the use of mammalian cell transfection models is clear in the development of TG-C. There is a well-established literature on the use of HEK-293 cells as a transfection model and cell culture model for protein production. The efficacy and safety of HEK-293 cells and their derivatives in regenerative medicine has not been extensively reviewed but the prospects for future use of transfection tools in regenerative medicine and cell therapy is very positive, especially since native and untransformed cells do not have the appropriate regenerative capacity.

10 Summary and Conclusions

Cell and gene therapy for OA is a thriving and promising area of research and clinical development. This is a technologically challenging area but thanks to the innovations coming from the field of biotechnology and advances in mammalian protein production platforms, transfected and irradiated protein packaging cell systems new therapeutics can be developed and tested for OA. These mammalian platforms may be used as "cellular factories" for over-production of

[6] http://www.businesskorea.co.kr/news/articleView.html?idxno=32318

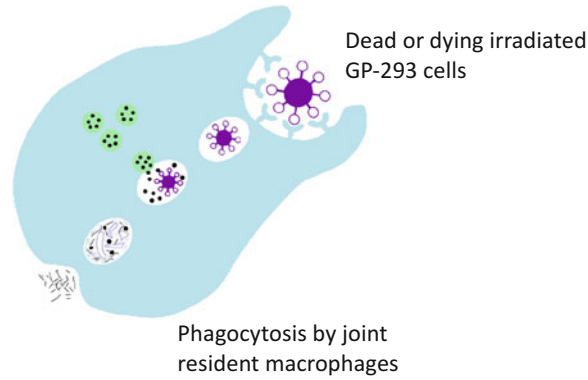

Fig. 7 Phagocytosis and destruction of dead GP2-293 or their cellular debris by joint resident macrophages

therapeutic proteins and growth factors, especially for intra-articular regenerative therapies. I would speculate that in the near future we will see many new innovations in this area, as we have seen for other disease areas using similar biotechnology tools.

The painful fact is that native and primary cells are unlikely to have the capacity for producing sufficient quantities of growth factors for stimulating cartilage repair. Mammalian cells such as HEK-293 and their derivative GP2-293 counterparts have the capacity to transiently produce high quantities of many therapeutic growth factors in addition to TGF-β1. If these cells are irradiated, such as the GP2-293 in the TG-C product, they will gradually die off in the joint within a few days of performing their protein over-production duties. So what happens to these cells after they have died? The dead cells and their debris will be cleared and destroyed by joint macrophages.

I speculate that all future cellular and gene therapy will require packaging cell lines and the field of cell and gene therapy for OA will benefit from a reality check regarding the poor regenerative capacity of native and patient derived cells, which are unlikely to work without a helping hand from protein packaging mammalian cells. Hopefully some of the future packaging cell tools will be derived from chondrocytes and bone for application in cartilage and bone regeneration. However, in the meantime, we must continue to use and refine existing cellular models while we develop new cartilage-derived protein packaging cells. Innovations in biotechnology will further propel new therapeutic concepts and impact positively on the field of cell and gene therapy for OA, related osteoarticular disorders and the broader discipline of regenerative medicine for musculoskeletal disorders. Mammalian protein production platforms are likely to have a significant and positive impact on synovial joint diseases that are amenable to cell and gene therapy using therapeutic proteins and growth factors.

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The Horizon of Gene Therapy in Modern Medicine: Advances and Challenges

Babak Arjmand, Bagher Larijani, Motahareh Sheikh Hosseini, Moloud Payab, Kambiz Gilany, Parisa Goodarzi, Peyvand Parhizkar Roudsari, Mobina Amanollahi Baharvand, and Negin sadat Hoseini Mohammadi

Abstract

Gene therapy as a novel study in molecular medicine will have a significant impact on human health in the near future. In recent years, the scope of gene therapy has been developed and is now beginning to revolutionize therapeutic approaches. Accordingly, many types of diseases are now being studied and treated in clinical trials through various gene delivery vectors. The emergence of recombinant DNA technology which provides the possibility of fetal genetic screening and genetic counseling is a good case in point. Therefore, gene therapy advances are being applied to correct inherited genetic disorders such as hemophilia, cystic fibrosis, and

B. Arjmand (✉)
Cell Therapy and Regenerative Medicine Research Center, Endocrinology and Metabolism Molecular-Cellular Sciences Institute, Tehran University of Medical Sciences, Tehran, Iran

Metabolomics and Genomics Research Center, Endocrinology and Metabolism Molecular-Cellular Sciences Institute, Tehran University of Medical Sciences, Tehran, Iran
e-mail: barjmand@sina.tums.ac.ir

B. Larijani
Endocrinology and Metabolism Research Center, Endocrinology and Metabolism Clinical Sciences Institute, Tehran University of Medical sciences, Tehran, Iran
e-mail: emrc@tums.ac.ir

M. Sheikh Hosseini and P. Parhizkar Roudsari
Metabolomics and Genomics Research Center, Endocrinology and Metabolism Molecular-Cellular Sciences Institute, Tehran University of Medical Sciences, Tehran, Iran
e-mail: mt_sh_hosseini@yahoo.com; Peyvand.parhizkar@yahoo.com

M. Payab
Obesity and Eating Habits Research Center, Endocrinology and Metabolism Molecular-Cellular Sciences Institute, Tehran University of Medical Sciences, Tehran, Iran
e-mail: Moloudpayab@gmail.com

K. Gilany
Reproductive Immunology Research Center, Avicenna Research Institute, ACECR, Tehran, Iran

Integrative Oncology Department, Breast Cancer Research Center, Motamed Cancer Institute, ACECR, Tehran, Iran
e-mail: k.gilany@ari.ir

P. Goodarzi
Brain and Spinal Cord Injury Research Center, Neuroscience Institute, Tehran University of Medical Sciences, Tehran, Iran
e-mail: pr_goodarzi@yahoo.com

M. Amanollahi Baharvand and N. s. Hoseini Mohammadi
Cell Therapy and Regenerative Medicine Research Center, Endocrinology and Metabolism Molecular-Cellular Sciences Institute, Tehran University of Medical Sciences, Tehran, Iran
e-mail: cameliaa1999@gmail.com; Hsynyngyn1@gmail.com

familial hypercholesterolemia as well as acquired diseases like cancer, AIDS, Alzheimer's disease, Parkinson's disease, and infectious diseases like HIV. As a result, gene therapy approaches have the ability to help the vast majority of newborns with different diseases. Since these ongoing treatments and clinical trials are being developed, many more barriers and challenges have been created. In order to continue this positive growth, these challenges need to be recognized and addressed. Accordingly, safety, efficiency and also risks and benefits of gene therapy trials for each disease should be considered. As a result, sustained manufacturing of the therapeutic gene product without any harmful side effects is the least requirement for gene therapy. Herein, different aspects of gene therapy, an overview of the progress, and also the prospects for the future have been discussed for the successful practice of gene therapy.

Keywords

Biosafety · Challenges · Ethics · Genes · Genetic vectors · Genetic diseases · GMP facilities · Therapeutic uses

Abbreviations

BMT	Bone Marrow Transplant
G-CSF	Granulocyte-Colony Stimulating Factor
HCT	Hematopoietic Cell Transplantation
HSC	Hematopoietic Stem Cell
HSCT	Hematopoietic Stem Cell Transplantation
CRISPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats associated protein 9
RNAi	RNA interference
PIDs	Primary Immune Deficiencies
SCID	Severe Combined Immunodeficiency
SCID-X1	X-linked Severe Combined Immunodeficiency
ADA-SCID	Adenosine Deaminase deficient Severe Combined Immunodeficiency
CGD	Chronic Granulomatous Disease
WAS	Wiskott – Aldrich Syndrome
WASP	Wiskott – Aldrich syndrome Protein
ALD	Adrenoleukodystrophy
MLD	Metachromatic Leukodystrophy
$gp91^{phox}$	Nicotinamide Adenine Dinucleotide Phosphate Oxidase 2
ABCD1	ATP Binding Cassette Subfamily D Member 1
ARSA	Arylsulfatase A
$IL2Ry_C$	Interleukin 2 Receptor
CCR5	C-C Chemokine Receptor type 5
SB-transposon	Sleeping Beauty (SB) transposon
Anti-CD19 CAR	Anti-CD19 Chimeric Antigen Receptor
SIN-lentiviral	Self-Inactivating (SIN) lentiviral vector
LDL receptor	Low-Density Lipoprotein Receptor
P53 (TP53)	Tumor Protein P53
LPLD	Lipoprotein Lipase Deficiency
SERCA2a	Sarcoplasmic/Endoplasmic Reticulum Ca^{2+}-ATPase
RPE65	Retinal Pigment Epithelium-specific 65
GAD	Glutamate Decarboxylase
FIX	Factor IX Padua
ASPA	Aspartoacylase
LPL	Lipoprotein lipase
CNS	Central Nervous System
SMA	Spinal Muscular Atrophy
LCA	Leber Congenital Amaurosis
LCA2	LCA type 2
SCA	Sickle Cell Anaemia
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Regulator
DSB	Double Stranded Break
HDR	Homology Directed Repair

NHEJ	Non-Homologous End Joining
MN	Meganuclease
ZFNs	Zinc-Finger Nucleases
ZFPs	Zinc Finger Proteins
TALE	Transcription Activator Like Effector
TALENs	Transcription Activator-Like Effector Nucleases
PAM	Protospacer Adjacent Motif
HBB	Hemoglobin subunit Beta
HPV	Human Papillomaviruses
HIV	Human Immunodeficiency Virus
HBV	Hepatitis B virus
ALL	Acute Lymphoblastic Leukemia
GGE	Germline Gene Editing
DDR	DNA Damage Response
ICL	Interstrand Crosslink
BER	Base Excision Repair
NER	Nucleotide Excision Repair
TC-NER	Transcription-Coupled NER
GG-NER	Global Genome NER
MMR	Mismatch Repair
HR	Homologous Recombination
ROS	Reactive Oxygen Species
NO	Nitric Oxide
UV	Ultraviolet light
CPDs	Cyclobutane Pyrimidine Dimers
PAH	Polycyclic Aromatic Hydrocarbon
AGTs	O6-Alkylguanine-DNA Alkyltransferases
MSH	MutS Homolog
MLH/PMS	MutL Homolog/ Premenstrual Syndrome
TLS	Translesion Synthesis
AEP	Archaeo-Eukaryotic Primase
alt-EJ	alternative End Joining
SSA	Single-Strand Annealing
TGE	Transient Gene Expression
R-proteins	Recombinant Proteins
HEK	Human Embryonic Kidney
CHO	Chinese Hamster Ovary
CMV	Cytomegalovirus
HCMV	Human Cytomegalovirus
EBNA-1	Epstein–Barr virus Nuclear Antigen-1
VLPs	Virus-Like Particles
BEVS	Baculovirus Expression Vector System
PEI	Polyethylenimine
ds	double-stranded
ss	single-stranded
AAV	Adeno Associated Viruses
rAAV	recombinant Adeno Associated Virus
LV	Lentiviral
IBCs	Institutional Biosafety Committees
NIH	National Institutes of Health
RAC	Recombinant DNA Advisory Committee
BSL	Biosafety Level
CBER	Center for Biologics Evaluation and Research
cGMP	current Good Manufacturing Practices
IND	Investigational New Drug
GLP	Good Laboratory Practice
RODAC	Replicate Organism Detection and Counting
DMD	Duchenne Muscular Dystrophy
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
CFR	Code of Federal Regulations
IGF1	Insulin-like Growth Factor 1
HGF gene	Hepatocyte Growth Factor gene
Reg3g	Regenerating islet-derived protein 3 gamma gene
G6Pase	Glucose-6-Phosphatase
SAEs	Serious Adverse Events
OTC	Ornithine Transcarbamylase
OTCD	Ornithine Transcarbamylase Deficiency
ATMPs	Advanced therapy medicinal products
GTMP	Gene Therapy Medicinal Products
sCTMPs	somatic Cell Therapy Medicinal Products
TEPs	Tissue-Engineered Products
MMA	Marketing Authorization Application
CAT	Committee for Advanced Therapies
FDA	Food and Drug Administration
IND	Investigational New Drug
OCTGT	Office of Cellular, Tissue and Gene Therapy
CHMP	Committee for Medicinal Products for Human Use

1 Introduction

Gene therapy is the science of making specific changes in human genome in order to improve it or reach therapeutic effects in gene-related diseases (Amer 2014; Goncalves and Paiva 2017). These changes include replacing and editing mutated genes or even introducing a normal copy of genes to cells to bring back the normal function of proteins (Steffin et al. 2019).

1.1 The Historical Overview of Gene Therapy

Before gene therapy was existed or recognized, many experiments had been carried out and much effort had been done in order to establish and improve this field of science to such a level it has reached now (Wirth et al. 2013). Here some critical milestones in gene therapy's way to progress will be mentioned: After Frederick Griffith's experiment led to discovering the transformation principle (Griffith 1928) and also after its confirmation by Dawson and Sia (Sia and Dawson 1931), McCarty and Avery demonstrated the correlation between the transformation phenomena and DNA (Avery et al. 1944). Another important discovery that took place after transformation's introduction was "transduction"(Zinder and Lederberg 1952), which was a major step towards the emergence of gene therapy. In 1962, Waclaw Szybalski was the first person to prove that if a normal gene of interest has expressed from a foreign source to a mutated form of cell, the function of that gene would be improved and this change would be inherited by the next generation of the target cell (Szybalska and Szybalski 1962). Besides, in 1961, Howard Temin proved that genetic mutations can be inherited stably because of virus infections and after that in 1968, Rogers et al. conducted a successful virus-mediated gene transfer experiment and later in 1973 they conducted the first direct human gene therapy trial which was not convincing, unfortunately. Finally, in 1989, Steven A Rosenberg performed the first approved human gene therapy that brought encouraging results (Fig. 1) (Wirth et al. 2013).

1.2 Types of Gene Therapy

Gene therapy is categorized in two major groups: germ line gene therapy (genes are introduced into sperm or egg cells and is not widely applied because of ethical issues) and somatic gene therapy (introducing genes of interest into patients or somatic cells) (Steffin et al. 2019). Somatic gene therapy is experimented in two primary categories: in vivo and ex vivo. Ex vivo gene therapy is based on the collecting cells from a donor, transducing cells with target gene and finally introducing cells to patient body (Fig. 2) (Kaufmann et al. 2013), while in vivo gene therapy is summed up in direct introduction of functional and normal genes to patient cells by different types of vectors (vectors are DNA molecules used to carry genetic materials into cells of interest and are divided into two main groups; viral vectors which include abroad spectrum of vectors (Lundstrom 2018) and non-viral vectors (Ramamoorth and Narvekar 2015)). Some of the important target diseases treated by EX vivo and in vivo gene therapy with their target cells, transgenes, and vectors can be seen in Tables 1 and 2 (Kaufmann et al. 2013; Thorne et al. 2018).

So far gene therapy has come a long way and made a lot of advances. Therefore, it demonstrated a proof of concept for many diseases including hereditary diseases such as inherited immunodeficiencies, neurologic disorders, lysosomal storage, hematologic diseases and acquired diseases like cancer. There will be still a lot to do in this field in the future (Steffin et al. 2019).

2 Current Trends in Gene Therapy

Providing more advanced information about molecular biology of human diseases and

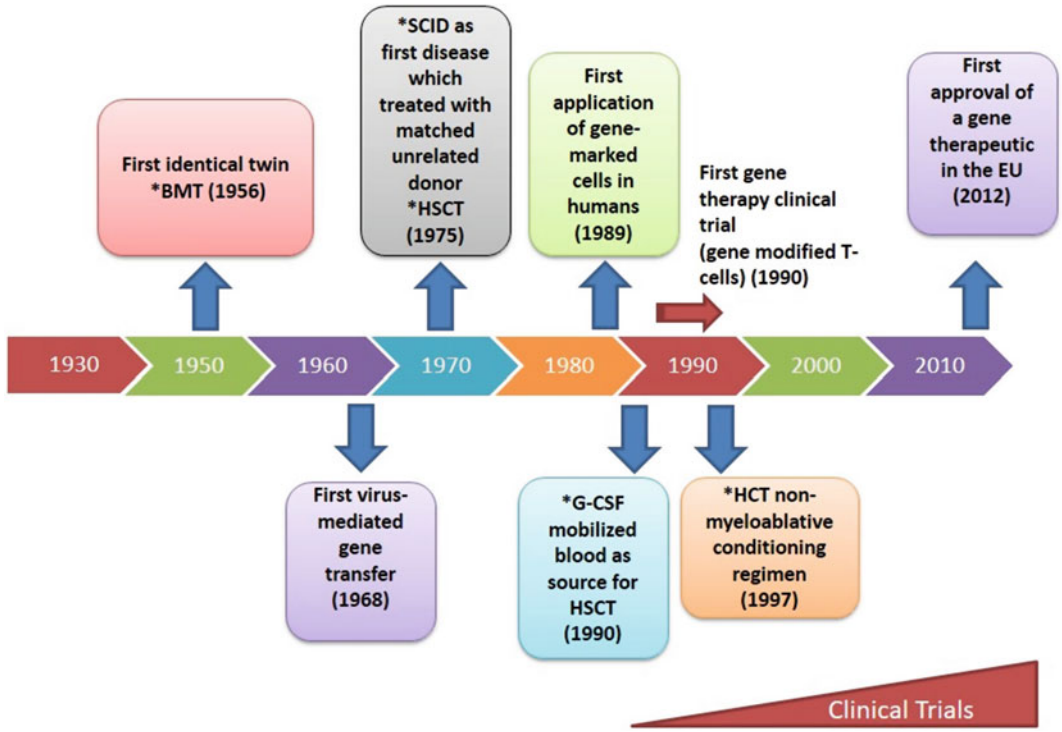

Fig. 1 History of gene therapy, important milestones, and gene therapy for monogeneic disorders using haematopoietic cells
The first bone marrow transplant (BMT) was performed by Dr E. Donnall Thomas in 1956. In this milestone bone marrow received from the healthy identical twins, and given to the other, who had leukaemia. In 1968 the first virus mediated gene transfer was utilized. Then in 1975, SCID was first disease which treated with matched unrelated donor *Hematopoietic Stem Cell Transplantation* (Morgan et al. 2017). In 1989 the first application of gene-marked cells in humans has done. In 1990, the first gene therapy clinical trial has performed and also Granulocyte-Colony Stimulating Factor mobilized blood as source for *Hematopoietic Stem Cell Transplantation. In 1997* Hematopoietic cell transplantation (HCT) with non-myeloablative conditioning regimen has performed. Finally in 2012, the first gene therapeutic in the EU has approved (Wirth et al. 2013; Wirth et al. 2013).
∗**BMT:** Bone Marrow Transplant
∗**SCID:** Severe Combined Immunodeficiency
∗**G-CSF:** Granulocyte-Colony Stimulating Factor
∗**HSCT:** Hematopoietic Stem Cell Transplantation
∗**HCT:** Hematopoietic cell transplantation

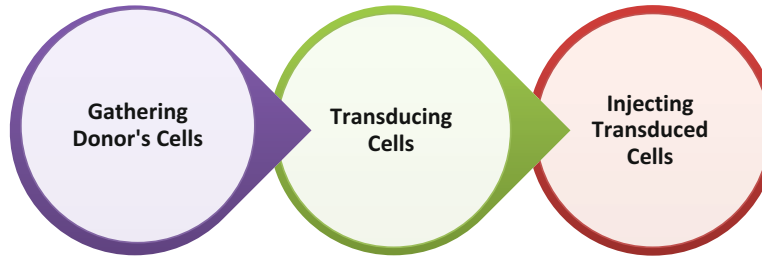

Fig. 2 Ex vivo gene therapy main steps; Firstly, patient cells are isolated from the body, secondly these cells genetically modified outside the body, and then reintroduced into the patient as an autologous transplant (Thorne et al. 2018)

dramatic progression of gene transfer techniques resulted in practical approaches to human gene therapy and also novel techniques being developed at an increasing rate (Amer 2014). The current trends of gene therapy have been promising and over the past several decades the knowledge gained in this field provides so much hope for the future. In this regard, gene therapy

Table 1 Some potential target diseases with target cells, transgenes, and vectors which treated by EX vivo gene therapy (Kaufmann et al. 2013)

Diseases	Target cells	Gene	Vector
∗ADA-SCID	T-lymphocytes	∗ADA	Gammaretroviral
ADA-SCID	∗HSC	ADA	Gammaretroviral
∗SCID-X1	–	∗IL2Ry$_C$	Lentiviral
∗X-CGD	–	∗*gp91^{phox}*	
∗X-ALD	–	∗ABCD1	∗SIN-lentiviral
∗MLD	HSC	∗ARSA	SIN-lentiviral
∗HIV	HSC	∗ZFNs targeting ∗CCR5 (knock out)	Adenoviral
∗WAS	HSC	∗WASP	(±SIN design)
B-Thalassaemia	HSC	B-Globin	SIN-lentiviral
Familial hypercholesterinemia	Hepatocytes	∗LDL receptor	Gammaretroviral
B-cell malignancies	T-lymphocytes	∗Anti-CD19 CAR	SIN-lentiviral∗*SB-transposon*
Epidermolysis bullosa	Keratinicytes	Laminin 5 *β3*	Gammaretroviral

∗ *gp91^{phox}*: Nicotinamide Adenine Dinucleotide Phosphate Oxidase 2
∗ADA-SCID: Adenosine Deaminase deficient Severe Combined Immunodeficiency
∗SCID-X1: X-linked Severe Combined Immunodeficiency
∗X-CGD: X linked Chronic Granulomatous Disease
∗X-ALD: X-linked Adrenoleukodystrophy
∗HIV: Human Immunodeficiency Virus
∗WAS: Wiskott – Aldrich Syndrome
∗*HSC: Hematopoietic Stem Cell*
∗ADA: Adenosine Deaminase
∗ABCD1: ATP Binding Cassette Subfamily D Member 1
∗*ARSA*: Arylsulfatase A
∗IL2Ry$_C$: Interleukin 2 Receptor
∗ZFNs: Zinc-Finger Nucleases
∗CCR5: C-C Chemokine Receptor type 5
∗*WASP*: Wiskott – Aldrich syndrome Protein
∗LDL receptor*:* Low-Density Lipoprotein *Receptor*
∗Anti-CD19 CAR*: Anti-CD19* Chimeric Antigen Receptor
∗SIN-lentiviral: *Self-Inactivating (SIN)* lentiviral vector
∗*SB-transposon: Sleeping Beauty* (*SB*) *transposon*
∗*MLD*: Metachromatic Leukodystrophy

enables a successful process of extensive medical interventions in the genetic context and infectious diseases, which is now closer to reality with the success of using recombinant adeno-associated virus (rAAV) vectors in clinical trials, novel vector engineering, recent discoveries of miRNAs, and CRISPR/Cas9 (*Clustered regularly interspaced short palindromic repeats* associated protein 9) (Wang and Gao 2014). Moreover, the impact of these factors on the treatment of several diseases is significant and undeniable and it seems likely that certain genetic diseases will be approachable with CRISPR/Cas9 and RNA interference (RNAi) – based therapies that were not approachable with prior methods (Cho et al. 2013; Mali et al. 2013; Nelson et al. 2016). For instance, remarkable effects of rAAV vector-mediated gene therapy have proven in certain conditions. Using rAAV viral vectors for recessive monogenic disorders demonstrates the importance of this claim and highlights its promising effect clearly. Hemophilia B, spinal muscular atrophy (SMA) (gene therapy for SMA is in development), alpha 1 antitrypsin, and leber congenital amaurosis (LCA) are notable examples of clinical efficacy with rAAV (Nelson

Table 2 Some potential target diseases with target cells, transgenes, and vectors which treated by in vivo gene therapy (Kaufmann et al. 2013)

Diseases	Target cells	Gene	Vector
Head and neck squamous cell carcinoma	Intratumoular	∗P53	Adenovirus
∗OTCD	Systemic/portal vein	∗OTC	Adenovirus
∗LPLD	Intramuscular	∗LPL	∗AAV1
∗LCA	Subretinal	∗RPE65	AAV2
Parkinson's disease	Intracerebral	∗GAD	AAV2
Haemophilia B	Intramuscular and Systemic/portal vein	∗FIX	AAV2
Canavan disease	Intracerebral	∗ASPA	AAV2
Heart failure	Coronary artery infusion	∗SERCA2a	AAV1

∗SERCA2a: Sarcoplasmic/Endoplasmic Reticulum Ca^{2+}-ATPase
∗P53 (TP53): Tumor Protein P53
∗OTC: Ornithine Transcarbamylase
∗OTCD: Ornithine Transcarbamylase Deficiency
∗AAV: Adeno Associated Viruses
∗*LPLD*: Lipoprotein Lipase Deficiency
∗*LPL*: *Lipoprotein Lipase*
∗LCA: Leber Congenital Amaurosis
∗RPE65: Retinal Pigment Epithelium-specific 65
∗GAD: Glutamate Decarboxylase
∗*FIX*: Factor IX Padua
∗*ASPA*: Aspartoacylase

et al. 2016). Furthermore, with the help of RNAi discovery, the focus of gene therapy shifts from gene augmentation to down-regulation of gene expression for diseases which are based on toxic gain of function. Transthyretin-mediated amyloidosis and complement-mediated diseases are the examples of using small RNAi for therapeutic application (Chakraborti and Lewis 2016). CRISPR/Cas9 seems to target specific regions of the human genome to achieve a therapeutic effect by enabling a permanent and definitive germ-line correction of a genetic disorder (Flotte 2015; Ishii 2015). Other diseases which targeted by the current trends of gene therapy are primary immune deficiencies (PIDs). Despite their low prevalence, PIDs are considered as serious life-threatening diseases. X-linked severe combined immunodeficiency (SCID-X1), adenosine deaminase deficient severe combined immunodeficiency (ADA-SCID), chronic granulomatous disease (CGD), and Wiskott-Aldrich syndrome (WAS), are the prime examples of PIDs. On the other hand, the monogenic hemoglobin disorders, sickle cell anemia (SCA), and β-thalassemia account for the majority cause of morbidity and early mortality all over the world. Although available therapies ultimately lead to improve the quality of life and longevity, there is no definitive long-term treatment. Accordingly, certain therapeutic approaches, such as *bone marrow transplant* (BMT), have certain risks and requirements such as the need for more marrow donors. Therefore, these hematological diseases provide a vast field for gene therapy interventions. As an example, hemophilia is a hematological disorder caused by mutations in the X-linked gene encoding coagulation factor VIII (hemophilia A) or IX (hemophilia B). Today, using both small and large animals with hemophilia, in vivo gene transfer to the liver using adeno associated virus (AAV) vectors form one of the most efficient and promising protocols. On the other side, it seems that the treatment of neurological disorders with conventional pharmacological drugs is less than expected because of the complexity of the central nervous system (CNS) structure and biological barriers such as the blood brain barrier. Despite the remarkable success of gene therapy in overcoming some of these limitations, it still faces with complexities such as substantial barriers to delivery of the vector, targeting specific cell types within the CNS, and obtaining sufficient levels of gene expression within a therapeutic window (Frederickson and Brenner

2013). For instance, in the field of ocular disease, the effect of gene therapy interventions is undeniable. Accordingly, one of the prominent and highly effective interventions that can be seen in the recent trends of gene therapy is the application of AAV vectors for ocular gene transfer. Therefore, these vectors have been widely applied in gene therapy protocols of several retinal diseases, including inherited forms of blindness which does not seem to have certain treatment. LCA type 2 (LCA2) is the first retinal hereditary disease that has shown positive promising result with this type of gene therapy and provides hope for the future of gene therapy interventions (Bainbridge et al. 2008). Another gene therapy recent intervention is to direct delivery of cystic fibrosis transmembrane regulator (CFTR) gene into respiratory tract epithelium cells as the target tissue which is a novel method for cystic fibrosis treatment. Recently, researchers found that gene therapy is also promising in the treatment of diabetic polyneuropathy. The progress in genomics and human genetics over the past two decades has shown that cancer is caused by anomalies in the somatic cells of the host genome and that was the beginning of many cancer researchers activities to use treatments based on genetic manipulation and modification to treat cancer and find a potential cure for these diseases (Thorne et al. 2018; Pranke et al. 2019). However, the current efficiency of gene editing may be subtherapeutic for certain diseases, problems like genotoxicity, as one of the most threatening post-treatment complication and concern, cannot be ignored. Therefore, these gene-editing tools need more refinement before they can be effectively and safely applied in the clinic. Although clinical studies and practices that control the current trends of gene therapy, circumventing some of the therapeutic constraints, still require extensive knowledge and advanced therapeutic equipment (Thorne et al. 2018).

3 Classification of New Gene Therapy Platforms

3.1 Gene Editing

Gene editing which is progressing rapidly has made such a great ability for alteration of the genome and it has developed the possibility for direct editing like gene addition, gene ablation, and gene correction (Dunbar et al. 2018). Moreover, gene editing can be used in therapeutic approaches for accessing to a new generation of drugs and correction of some mutations in human genome which can be harmful (Gupta and Shukla 2017).

The tools for manipulating genome in gene editing have developed for about 10 years ago. Because manipulating genome for gene editing has long-lasting effects, we should aware that unintended alterations in genome can lead to permanent consequences (Maeder and Gersbach 2016). Therefore, it is important to address safety for each new therapy in early phase (Lux and Scharenberg 2017). Although studies have shown great promise for the gene-editing role in modifying T cells, still the safety of this alteration is mentioned as an important concern. However,

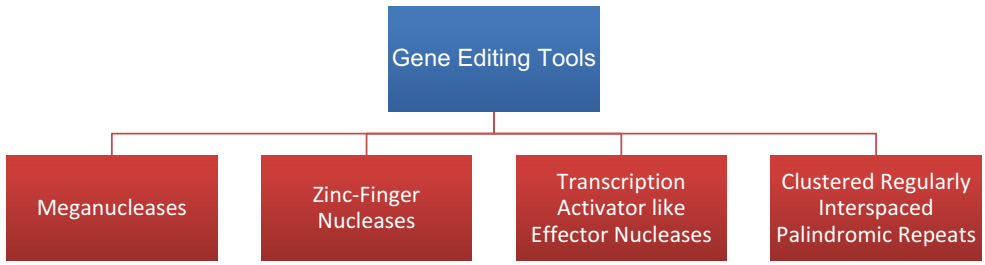

Fig. 3 Major groups developing for manipulating DNA including; meganucleases (MGs), zinc-finger nucleases (ZFNs), transcription activator like effector nucleases (TALENs), and Clustered regularly interspaced palindromic repeats (CRISPR/cas9) (Gupta and Shukla 2017)

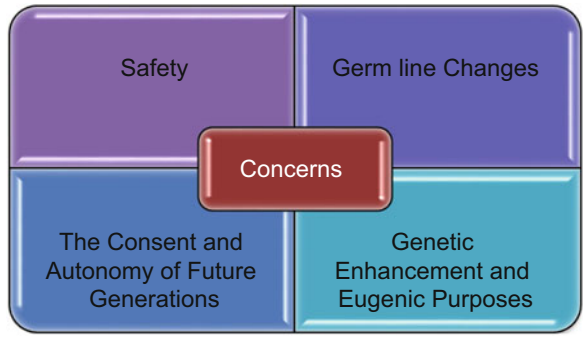

Fig. 4 Some of the arguments against germ line gene editing: The most important concern is misusing of GGE for human enhancement and eugenic purposes which insert genes that would not occur naturally. Another concern is that GGE has the potential to change the germ line which has negative effects for future generations. The third concern is safety which can lead to off-target mutations. Finally, ethical problems introduced by changing the germ line and affect the next generation without their consent (Gyngell et al. 2017)

there is a new generation of nuclease for inducing targeted sequence now, there are some challenges in minimizing off-target events yet (Zhang et al. 2017). Tools that have developed for gene editing (which mentioned in Fig. 3), can make nuclease-induced double stranded break (DSB) (Gupta and Shukla 2017) which is a type of DNA that it's both strands are cleaved (Zhang, Zhang et al. 2019). The next mechanism after that is repairing damaged DNA. Accordingly, homology directed repair (HDR) is one of the mechanisms that only be used when there is a homologous piece of DNA (Pardo et al. 2009; Gaj et al. 2013). The other mechanism for repairing is non-homologous end joining (NHEJ) that does not need a homologous piece of DNA to ligate directly (Moore and Haber 1996).

Despite the positive effects of germ line gene editing (GGE), there are some arguments against GGE (Fig. 4).

Concerning safety is one of the major arguments against GGE which mostly regarded as off-target mutations. They can lead to cancers and other pathologies development and it can be harmful to future generations. The second concern is about the consequences of unintended mutations. GGE could be used to increase genes which are beneficial and useful in one generation but maybe the same genes could be harmful to generations in the future. Another argument is misusing GGE as a tool for human enhancement and eugenic purposes which can be universally problematic because it could have wide effect on social conflict. In other words, it could promote human beings to supranormal levels. Despite all of these concerns, the moral permission and desire for GGE are more considerable than those arguments against GGE. But regarding bans is important, because it makes us more aware of human development (Gyngell et al. 2017).

3.1.1 Meganucleases

One of the first methods for gene editing is meganucleases (MN) which are known for their large recognition site and low toxicity for mammalian cells (Zhang, Zhang et al. 2019) so they provide high specificity. Homing endonuclease which represents meganucleases, generally encoded by introns or inteins. MNs are 5 major families which LAGLIDADG has known as the largest one. The other ones are GIY-YIG, HNH, His-Cys box, and PD-(D/E) XK. I-CreI is a member of the LAGLIDADG family which function as a homodimer and recognizes and cleaves a 22 bp pseudo-palindromic target (Silva et al. 2011).

3.1.2 Hybrid Nucleases

The other tools are hybrid nucleases like zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) which are restriction enzymes (Gupta and Shukla 2017). ZFN is the fusions of DNA binding domain of zinc finger proteins (ZFPs) to the

DNA-cleavage domain of FokI restriction enzyme (Chandrasegaran 2017). ZFN consists of 30 amino acids which form two anti-parallel b-sheets and an alpha-helix. Three to six ZFs are used to make ZFP that fused to cleavage domain and eventually recognize a 9–18-bp target site (Chandrasegaran 2017; Gupta and Shukla 2017). Researchers believe that ZFN technology can be useful for treating HIV (Human Immunodeficiency Virus) particularly (Nishitsuji et al. 2015). The competition between HDR and NHEJ pathways for repairing is one of the major limitations for ZFN. Zinc finger nickases which engineered from ZFN, induce site-specific nicks and it is the way to reduce the amount of unwanted NHEJ mutations (Ramirez et al. 2012). Like ZFN, TALENs are the fusing form of the DNA binding domain and DNA-cleavage domain of FokI endonuclease; but DNA binding domain is derived from transcription activator like effector (TALE) proteins. TALENs can be used more widely; because TALE proteins can be engineered easier than ZFN proteins. TALE proteins are a tandem repeat of 33–35 amino acids that each of them recognize specific base of DNA and thus it makes TALENs high specificity (Ramirez et al. 2012; Gupta and Shukla 2017). As a result, genome editing's therapeutic aspect has been developed widely. Accordingly, ZFN and TALENs can modify the underlying causes of disease. For instance, in the severe combined immunodeficiency (SCID), hemophilia B and sickle-cell disease which are x-linked diseases, ZFN can modify the mutations directly. ZFN is also have used in modifying the Parkinson's mutations (Goodarzi et al. 2015). ZFN can disable co receptor C-C chemokine receptor type 5 (CCR5) in primary stem cells and hematopoietic stem cells (HSC) that results in HIV resistance (Gaj et al. 2013).

3.1.3 CRISPR/Cas9

The last genome engineering tool is CRISPR/Cas9 and its founding has wide effect on gene editing. CRISPR/Cas9 mechanism can be used in different ways; for instance, to treat genetic

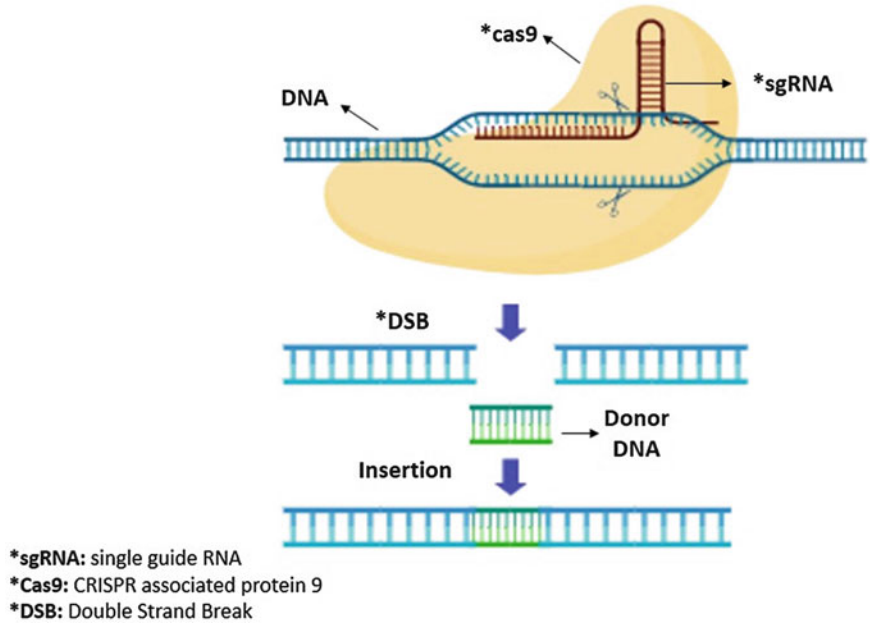

Fig. 5 The mechanism of CRISPR/cas9 gene editing. CRISPR/cas9 gene editing needs a single guide RNA (sgRNA) which directs the Cas9 endonuclease to a target region of the genomic DNA and creates a double-strand break (DSB). A transgenic DNA can be created by adding a donor DNA in trans. In the absence of donor DNA, DSB will be repaired by host cells and cause insertion or deletion. Therefore, it disrupts the open reading frame of a gene (Sander and Joung 2014)

disorders; because it can turn-on and turn-off genes very quickly and in a short time. It has been known that this technology can help to repair defective DNA in mice and it can also repair defects in human embryos. CRISPR/Cas9 consists of a guide RNA which cleaved to target gene and Cas9 an endonuclease that cuts both strands of DNA and, therefore, resulted in modifications (Fig. 5) (Sander and Joung 2014). Guide RNA has 18–20 nucleotides which two or five of them should lay in the 3′ end of that for the purpose of cutting genome. It is named the protospacer adjacent motif (PAM). After these phases, the process of repairing will happen in two ways that have mentioned before (Mali et al. 2013). Moreover, cystic fibrosis (CF), duchenne muscular dystrophy (DMD), haemoglobinopathies, and HIV are some of the diseases that this technology has considerable influence on them. CRISPR/Cas9 technology can be used for monogenic disorders more than polygenic ones. For example, DMD is an x-linked disease in which there is a mutation in dystrophin gene. The CRISPR editing in the germline can be used to modify this mutation in mosaic offspring. CRISPR/Cas9 also has been used to treat cataract in a mouse germline. Mutations in the human hemoglobin beta gene (HBB) can lead to Beta thalassemia. One research group combined CRISPR/Cas9 system with the piggyback transposon and this resulted in the correction of HBB mutations (Maeder and Gersbach 2016). SCA and Tyrosinemia are other diseases in which CRISPR/Cas9 plays an important role to treat them. Furthermore, CRISPR/Cas9 function against viral diseases is also known by scientists recently. CRISPR/Cas9 has been used against Epstein – Barr virus which causes Hodgkin's disease and Burkitt's lymphoma and patients with Burkitt's lymphoma showed lower viral load (like the way of the apoptosis pathway) after using the CRISPR/Cas9 technology. Human papillomaviruses (HPV), Hepatitis B virus (HBV), and HIV are other viral diseases that researchers have proven the positive effects of CRISPR/Cas9 technology in their therapies (Khan et al. 2018).

Besides the positive uses of the CRISPR/Cas9, there are some limitations yet (Li et al. 2015). One of the issues is the way to deliver gene editing in correct cells especially if we have therapeutic purposes and the process is to deliver in vivo. Due to safety aims, special vectors are needed which can affect the efficacy of the transduction. The other concern for this technology is the possibility of the off-targets on the genes except for the targeted ones (Wang et al. 2017). Furthermore, there are ethical boundaries around CRISPR/ Cas9 technology; because all the modifications that are applied in the genome, are permanent and it is clear that oppose and unwanted modifications have long-term consequences (Redman et al. 2016).

3.2 Gene Repair Mechanisms

Living organisms are always at the risk of numerous defects in their genome that may reduce their health quality and make them susceptible to some types of disorders. DNA repair, damage tolerance, cell cycle checkpoints, and cell death pathways play a pivotal role to reduce the consequences of some types of diseases like cancer and also aging. Therefore, repairing mechanisms protect genome in the special pathways. But if errors happen in those processes and deviate the mechanisms from the correct pathway, different types of cancer will occur. DNA damage response (DDR) pathways stimulate the reaction of cells to start the repairing pathways. DNA repair mechanisms include; direct chemical reversal, interstrand crosslink (ICL) repair, base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination (HR), NHEJ, and translesion synthesis (Fig. 6). Endogenous and exogenous DNA damages are two main groups for the classification of the damages which endogenous ones play a more considerable role in damaging (Chatterjee and Walker 2017). One of the endogenous damages is the creation of the Abasic or AP sites (apurinic/apyrimidinic sites) which are being made when the N-glycosidic bond (between DNA base and

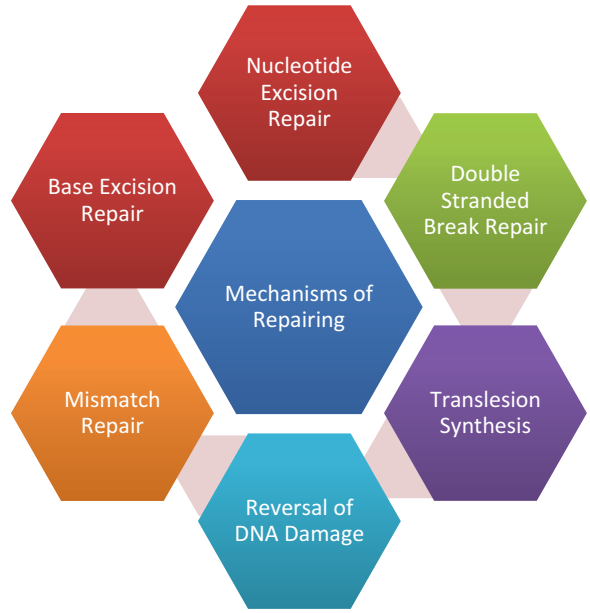

Fig. 6 Some of the genome repair mechanisms. These mechanisms recognize and correct damages which occur in DNA molecules. DNA repair can be classified into a set of mechanisms including; nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR),reversal of DNA damage, translesion synthesis, and double-stranded breaking (DSB) (Chatterjee and Walker 2017)

deoxyribose) hydrolyzes spontaneously. Also, AP sites can be created in the process of BER. Approximately 10,000 sites are created per cell per day. Another method that hydrolysis has its effect on that is deamination of DNA bases which the results are the formation of uracil, hypoxanthine, and xanthine. Moreover, reactive molecules have their own effects on genome damage. They are created during the normal metabolism of human body. Reactive oxygen species (ROS) are one of them which can affect base modification, deoxyribose oxidation, single or double-strand breakage, and DNA-protein cross-links. Nitric oxide (NO) as a type of reactive molecules and also alkylation are other types of DNA damage. Errors in physiological DNA processing reactions like DNA repair processes and replication can also lead to DNA damage (Dexheimer 2013).

Besides these endogenous DNA damages, there are also exogenous sources that can affect genome. Physical stresses are one of them. For example, ultraviolet light (UV) from the sun is one of the physical stresses that lead to creation of DNA lesions. Primarily cyclobutane pyrimidine dimers (CPDs) and pyrimidine-6,4-pyrimidinone photoproducts (6,4PPs) are photoproducts that affect genome. Therefore, they can inhibit DNA replication and transcription (Dexheimer 2013; Roy 2017). Ionizing radiation is mainly used for therapeutic purposes and its power to control malignancies mostly depends on DNA damage. It induces the DNA damage response and generates DSBs that are a lethal form of DNA damage. HR and NHEJ pathways mostly repair them (Santivasi and Xia 2014), but it is still harmful because of its creation of DNA lesions and also indirect damage of ROS production (Dexheimer 2013). As a result, chemical agents like Alkylating agents, Aromatic amines, Polycyclic aromatic hydrocarbon (PAH), toxins and environmental stresses like heat or cold, hypoxia, and oxidative stress play an important role in DNA damage (Chatterjee and Walker 2017).

As it was mentioned, endogenous and exogenous sources can affect genome. Therefore, they resulted in generating cytotoxic products which can cause senescence or cell death. Mutagens can lead to creation of DNA lesions that cause a deficiency in replication and transcription. There are different pathways for repairing such damages to protect genome such as reversal of DNA Damage or direct DNA-repair system which can reverse covalent DNA products simply. There are three mechanisms for this pathway: photolyases, O6-alkylguanine-DNA

alkyltransferases (AGTs) and the AlkB family dioxygenases. UV radiation can generate two DNA products which photolyases are specific for both of them and can reverse these UV light-induced photo lesions. These two lesions are the CPDs and the pyrimidine pyrimidones (6–4) photoproducts (6–4 PPs). Furthermore, AGTs reverse O-alkylated DNA damage and AlkB family dioxygenases reverse N-alkylated base adducts (Yi and He 2013).

BER is another mechanism in which DNA damage could be corrected. These damages consist of oxidation, deamination, and alkylation that impair DNA helix structure. However, exogenous sources have their remarkable role in generating these damages, but the source of most of these damages is spontaneous decay of DNA. BER is started with DNA glycosylase which can recognize and remove the damaged base. Indeed, the bond between deoxyribose and a modified or mismatched DNA base cleaves by glycosylase. There are at least 11 different DNA glycosylase and depending on type of lesion, BER is initiated by one of them (Krokan and Bjoras 2013). NER is the main pathway used for removing a broad spectrum of bulky DNA lesions which are created by endogenous or exogenous sources. 2 sub-pathways are known for that: Transcription-Coupled NER (TC-NER) and Global Genome NER (GG-NER). These two sub-pathways are different in the initial step that is recognition of DNA damage. According to their names, GG-NER recognizes DNA lesions throughout the genome, however, TC-NER only recognizes those that are located in the transcribed strand of active genes. In addition to the recognition step, there are 3 other steps for both of these sub-pathways: DNA helix unwinding, creation of dual incisions flanking the DNA lesion site, excision the lesion site, and eventually the processes of DNA repair synthesis and ligation (Melis et al. 2013). MMR consists of MutS homologs (MSH) and MutL homologs/ *Premenstrual Syndrome* (MLH/PMS). It has a major role in post replication repair pathway and has three steps: first recognizing the impaired bases, then excision the damage-containing strand that results in a gap and finally, the gap is filled with resynthesize DNA (Dexheimer 2013). Damaged and impaired DNA lesions can cause errors in replication and transcription. Replication fork arrest and cell death are other consequences of unrepaired DNA lesion. Accordingly, there are two ways to protect cell from replication fork stalling. In the first one which is error-free, the sister's undamaged chromatin is used as a template to synthesize recombination-dependent DNA and the second one is using translesion synthesis (TLS) DNA polymerases (pols) that can replicate opposite and past DNA lesions. This process that causes mutagens, is error-prone. There are 17 human DNA polymerases that are divided into A, B, X, Y, and AEP archaeo-eukaryotic primase superfamily) families. A and X polymerases families play an important role in BER and NHEJ in which contributes to mutagenesis in DNA repair. TLS polymerases are also needed for ICL repair. DNA polymerase/primase PrimPol (AEP superfamily) has two important roles: one of them is bypassing DNA lesion and second important one is de novo DNA synthesis that could re-start the replication of downstream of a stalled fork. In addition to DNA repair role of translesion synthesis and their contribution to mutagenesis in tumors, now their lesion bypass activity may lead to an increase in the diversity of the immunoglobulin gene during hypermutation and also they can help to overcome secondary DNA structures during DNA copying (Zhao and Washington 2017). ICLs are cytotoxic lesions in which two bases from complementary strands are linked and this is because of crosslinking agents. Accordingly, the Fanconi anemia (FA) is a human genetic disease in which ICL repair is defective and FA proteins recognize those toxic legions. There are 21 groups of FA proteins that are known for ICL sensitivity suppression. When forks collide with an ICL, the pathway of ICLs repair is consisting of these steps: initiation through excision ICLs, then forming a double-stranded DNA break that must be repaired. Therefore, programmed fork collapse which is dependent on FA proteins is a point of difference between ICLs repair and other DNA damage repairing methods (Zhang and Walter 2014; Chatterjee and Walker 2017). DSBs are

cytotoxic lesions that are the most hazardous types of DNA damage and because they can cause cell death and genome instability, repairing of DSBs is critical. There are two main pathways for repairing this lesion: HR and NHEJ. In addition, alternative end joining (alt-EJ) and single-strand annealing (SSA) which have been recently known play important role in alternative error-prone DSB repair pathways (Ceccaldi et al. 2016).

3.3 Transient Gene Expression

Recognizing new proteins for doing clinical trials has an important and wide effect on developing biology processes. Transient gene expression (TGE) provides the fast and cost-effective materials that are needed in preclinical researches and it lasts 2–10 days. Those products could be used in the way of developing drugs or in screening processes in a short time, but a classic way for expression lasts for about 1 month. The technology to creating the recombinant proteins (R-proteins) has been mentioned as an important procedure. These R-proteins are mostly complex and for having sufficient activity they must pass the post-translation modifications. These modifications could be done by mammalian cells which are the major host for R-proteins expression and this process is done by insertion of the recombinant gene to the host's genome. But this is costly and long-lasting procedure and TGE technology is appropriate for our need to generate R-proteins in high quality. Some proteins that have been expressed by TGE are secreted membrane, and intracellular proteins (Baldi et al. 2007). Because TGE technology does not need special tools, it can be done in almost every cell culture laboratory. Changing the interested gene placed in plasmid DNA can lead to expression of the several proteins that are a candidate for special purposes. Due to toxicity causes, TGE also can be set up when the host cell line cannot be used for expression. Function of TGE can cover a very small scale to large-volume cultures (Gutierrez-Granados et al. 2018).TGE is widely used at large-scale with human embryonic kidney (HEK) 293 suspension cells to produce recombinant proteins in large amounts. These cells grow in serum-free media. Also, Chinese hamster ovary (CHO) cells, insect cell lines (Sf9 and others), HKB11, CAP-T cells, and baby hamster kidney are known as other cell lines (L'Abbe et al. 2018). The HEK293 cells have shown higher efficiency in TGE process than CHO cells (Lee et al. 2017). Serum-free media and polyethylenimine (PEI) are two reagents that allow transient transfection of HEK 293 (L'Abbe et al. 2018). Non-viral and viral vectors have been known for transferring genes into cells (Baldi et al. 2007). Large amounts of plasmid DNA are needed for transient transcription of cells. Sequence and size set the vectors stability and smaller plasmids provide more efficient transfection into nucleus. The human Cytomegalovirus (CMV) immediate early promoter is widely used in transient transcription. Vectors need some important elements which support the producing of recombinant protein and increase product yield. These elements are consist of the recombinant protein's coding sequence, stop transcription signal and polyadenylation. These elements stabilize recombinant transcripts. Vectors mostly have ultra-high copy origin of pUC19 and so it's provided the facilitated production of plasmid DNA in E. coli in large amounts (Jäger et al. 2015). Viral episomal vectors have developed special ways for TGE. Epstein–Barr virus nuclear antigen-1 (EBNA-1) which can cause latent infection in lymphocytes has a special role in the expression of transgenes. HEK293 EBNA-1 (HEK293E) cells have a higher expression level than HEK293 cells. Generally, EBNA-1 amplification could improve the ability to produce drugs in the base of HEK293 cells (Lee et al. 2017). Episomal replication of plasmids which contain OriP and SV40ori origins of replication is allowed by HEK293-EBNA and HEK293-T (expressing SV40 large T antigen) that leads to the production of many proteins; such as small peptides and monoclonal antibodies. Also, viral vectors and virus-like particles (VLPs) can be produced. CHO cells that grow in suspension in serum-free are another platform that is chosen for the transient

expression of proteins. Post-translational modifications can be done by these cells and are known by their transient expression of proteins in different scales. Insect cells have also become a platform that play role in the expression of proteins, VLPs and also gene therapy vectors by the baculovirus expression vector system (BEVS) (Gutierrez-Granados et al. 2018).

In transient transfection process, it is more important to find and choose the suitable cell culture than in stable cell lines. Cell culture conditions must have the transfection adaptability to all the stages of transient transfection. Calcium, phosphate and other ingredients interact with the plasmid DNA complex (Jäger et al. 2015). CHO-S and HEK 293 are transfected successfully with PEI. This procedure is faster, cost-effective and widely used. PEI is a cationic polymer that condenses DNA molecules into positively charged particle and link to anionic cell surfaces; then the cell endocytosis the complex of DNA: PEI is and Consequently, the DNA molecule released into the cytoplasm (Longo et al. 2013). The first method for transient transfection of suspension cells was calcium phosphate-mediated transfection which is a principle for a large scale. CaPi-mediated transfection has a role in transient transfection in high efficiency that is about 40–60% and also it isn't expensive. In spite of its advantages, there are some issues for CaPi transfection. Sometimes more amount of serum is needed for CaPi transfection because serum-free low-calcium medium formulations are not suitable for it. It's due to the need for critical concentration of calcium. Besides, this methodology is appropriate for transfecting adherent cell lines especially. Also, it is not efficient for the transfection of CHO cells. Moreover, CaPi transfection has a limitation for large-scale applications. This is because of the short time of incubation which is needed for the formation of the CaPi precipitates and so on large scales can lead to delays. Because of these limitations, PEI is more widely used than CaPi as transfection reagent (Gutierrez-Granados et al. 2018). CCL12 is a chemokine that is necessary for recruiting the immunosuppressive cells to inflammation areas and also it's a necessary factor for survival of CXCR4+ cancer cells. Reduction of CCL12 can lead to a decrease in the tumor. Anti-CXCL12 methodologies are followed with off-target toxicities; so the transient expression of a CXCL12 trap is important. in the study, delivery of plasmid DNA was done by lipid calcium phosphate nanoparticle and an engineered CXCL12 protein trap encoded to the hepatocytes nucleus of liver and the decrease in liver metastasis from breast and colon was reported (Goodwin et al. 2016). Electroporation is another method which used in low-scale transfection with high efficiency and also some years ago large-scale electroporation, was done and it was successful. Another technology for gene delivery is using cationic lipids (Gutierrez-Granados et al. 2018). It has known that lipids in diverse forms are well-suited for DNA-transfection and the use of lipofection was first reported by Felgner et al. (1999) liposomes are generated by a combination of those lipids with plasmid DNA and they are endocytosed into cells. Different lipid-based reagents have been known and they have a high dependence on the cell line which used in transfection. This method is costly and it causes the limitation on large scales for producing a transient recombinant protein (Jäger et al. 2015). Most of studies on epithelial cells need the transient expression of exogenous proteins in polarized epithelial cells, but obtaining transient gene expression in those cell cultures has difficulties that lead to major limitation. According to luciferase assay and FACScan analysis, PEI/DNA/adenovirus system plays an efficient role in transfection and it lasts shorter than stable cell lines. But its limitation is the difficulty in obtaining transfection in higher efficacy in fully polarized epithelial monolayers (Bischof et al. 1999). Moreover, TGE needs a high amount of plasmid DNA. Mostly the amount of about 1–1.25 lg plasmid DNA per ml culture is used by protocols. Despite, the identification of many advantages for TGE, there are also some limitations and challenges for it (Baldi et al. 2007).

4 Manufacturing and Development of Gene Therapy Products

4.1 Good Manufacturing Practice Facilities for Gene Therapy

The Center for Biologics Evaluation and Research (CBER) have developed a regulatory policy that more particularly concentrated on issues associated with somatic cell and gene therapies (Grilley and Gee 2003). Accordingly, this center legislated laws which essentially resulted in the decision that vectors must be produced under current good manufacturing practices (cGMP) (Larijani et al. 2012). Based on these regulations drugs should be manufactured under a controlled and auditable process that cause a safe and effective product. Subsequently, they were extended to cover blood products. Now they cover cell and gene therapy products manufactured under investigational new drug (IND) regulations. Therefore, cGMP regulations should be familiar to pharmaceutical companies, blood banks and academic cell and gene therapy studies (Burger 2000; Larijani et al. 2012). Moreover, there should be some regulations that addressed before the production of a vector. In particular, the data which supports the utility and functionality of a proposed clinical vector must be generated under good laboratory practices (GLP) (Grilley and Gee 2003).

4.2 cGMP

Vectors considered as pharmaceuticals and based on this, cGMP regulations were published in Title 21 Part 210 and 211 of the Code of Federal Regulations (CFR). Gene therapy IND holders should consider the comprehensive information on the quality assurance and control procedures, manufacturing practices and history, adverse event reporting, etc. for clinical vector manufacturer. As a result, when this information analyzed, a clearer set of regulations and expectations will appear (Grilley and Gee 2003).

4.3 cGMP Facilities

cGMP manufacturing of vectors requires applying a controlled environment. Therefore, most facilities produce in class 100 biological safety cabinets (BSCs) in a class 10,000 facility (Thorne et al. 2018). There should be a comprehensive documentation of production procedures to ensure that these specifications are being met and this is often in the form of environmental monitoring. It is important to ensure facility and equipment are effective against the particular adventitious agents. Some SOP details include (Swindle 2018):

- Measuring particle and viable counts with air samplers for monitoring of the production room for air quality.
- Using touch plates for monitoring personnel gowns and gloves.
- Using air samplers, fallout plates and Replicate Organism Detection and Counting (RODAC) plates for monitoring the production area within the BSC, before, during and after manufacturing.

When a new vector manufacturing is started, there should be procedures to ensure that facility has been decontaminated completely to prevent cross-contamination with the vector which is manufactured in the same area (Swindle 2018; Thorne et al. 2018).

4.4 Equipment and Reagents

One of the areas which require special attention is the selection of manufacturing equipment and reagents. Clinically approved and infusion grades wherever possible should be achieved for reagents. Otherwise, the highest purity nonclinical equivalent must be used and before using, require extra safety and purity testing (Grilley and Gee 2003). It is advisable to develop formal specifications for raw materials and to obtain certificates of analysis from the producers for each batch or lot used. Until all specifications

are met, the item should be held in quarantine and also manufacturers must be audited if possible. Due to exceeding the budget of most academic cGMP facilities, an audit questionnaire can be developed and sent to all vendors (Larijani et al. 2012). For encoding reagent and equipment identity, lot numbers, calibration dates, receipt and expiration dates, etc. a barcoding system can be used. Then, these barcodes can be scanned at the time of production to provide a listing of all items used during production (Grilley and Gee 2003).

5 Vectors Used in Gene Therapy

Gene therapy delivery systems classified into two main categories including (Ginn et al. 2018);

1. Recombinant viral systems
2. Non-viral physicochemical approaches

In gene therapy trials, different vectors and delivery techniques have been used. Viral vectors are the most popular approaches and have applied in approximately two- thirds of the trials. On the other hand, non-viral vectors are becoming increasingly common nowadays (Nayerossadat et al. 2012). The merits of non-viral system approaches include (Ramamoorth and Narvekar 2015);

- Ease of chemical characterization
- Simplicity of production
- Reproducibility of production
- Larger packaging capacity
- Reduced biosafety concerns

Although the effects of non-viral gene delivery systems are often transient and are relatively inefficient compared to recombinant viral systems, they are being advanced due to improvements to nucleic acid durability and potency and also advances in the technology of lipid and polymer delivery systems (Ginn et al. 2018).

5.1 Viral Vectors

There are a broad range of viral vectors including delivery vectors which are developed for temporary short-term and permanent long-term expression. Furthermore, both DNA and RNA viruses are used for representing viral vectors with double-stranded (ds) or single-stranded (ss) genomes (Lukashev and Zamyatnin Jr. 2016; Lundstrom 2018). Retroviral vectors used in the first gene therapy trial and also for long-term gene therapy applications. Appling retroviral vectors in gene therapy trials have been declined in recent years (Robbins and Ghivizzani 1998). In 2004, 28% of trials used retroviral vectors while this trend declined to only 17.4% in 2018. On the other hand, lentiviral vectors have become a more favorable profile in recent years. In 2012 only 2.9% of trials applied lentiviral vectors however, in 2018 it increases to 7.3% (David and Doherty 2017; Ginn et al. 2018). Additionally, use of AAV in gene therapy followed a similar trend which in 2012 was 4.9% and increased to 7.6% in 2018. Although the most common vectors applied in gene therapy trials are adenoviruses which were 20.5%, it has decreased since 2012 and remained on 23.3% of all trials (Ginn et al. 2018). In comparison with retroviruses, adenoviruses can carry a larger DNA payload up to 35 kb but they cannot accommodate certain genes which are essential for clinical applications due to their small capacity (David and Doherty 2017). Adenoviral vectors have three main advantages; (1) they can achieve a high capability of transduction (2) high levels of gene expression (3) the ability to transducing non-dividing cells. Other viruses used in vectors are not widely popular including (Lundstrom 2018);

- *Vaccinia Virus*: used in 4.9% of all trials
- *Herpes Simplex Virus*: used in 3.6% of all trials
- *Poxvirus*: used in 2.7% of all trials

60 trials have used a combination of two vectors which included poxvirus and vaccinia

Table 3 Some important viral vectors used in gene therapy (Lukashev and Zamyatnin Jr. 2016, Lundstrom 2018)

Viruses	Genome	Capacity	Integration	Host Range	Expression	Number of Trials
Retroviruses	ssRNA	8 kb	Random integration	Transduces only dividing cells	Long-term expression	438 (18.76%)
Lentiviruses	ssRNA	8 kb	Low cytotoxicity, integration	Broad host range	Long-term expression	134 (5.73%)
AAV	ssDNA	<4 kb	Chromosomal integration	Relatively broad host range	Slow expression onset	155 (6.63%)
			Immune response			
Adenoviruses	dsDNA	<7.5 kb	Strong immunogenicity	Broad host range	Transient expression	517 (22.14%)
Herpes Simplex Viruses	dsDNA	>30 kb	Latent infection	Broad host range	Long-term expression	84 (3.59%)
Poxvirus	dsDNA	>30 kb	Large inserts	Broad host range	Transient expression	103 (4.41%)
			Replication-competent vectors			

virus (37 trials) and adenovirus (23 trials). Adenovirus was in combination with vaccinia Ankara virus used in 11 trials, vaccinia virus used in eight trials, retrovirus used in three trials, and Sendai virus used in 1 trial. Main groups of viral vectors are indicated in Table 3 (Lukashev and Zamyatnin Jr. 2016, Lundstrom 2018).

5.2 Non-viral Vectors

Two disadvantages of viral vectors used in gene therapy were; safety concerns and small capacity for therapeutic DNA that resulted in synthesis vectors which are not based on viral systems (Yin et al. 2014). The simplest gene delivery system which is applied in non-viral vectors often uses plasmid DNA. When naked DNA or plasmid DNA has been injected into certain tissues (especially muscles) directly, significant levels of gene expression produced that are lower than ones obtained in viral vectors (Ramamoorth and Narvekar 2015). More importantly, when naked DNA delivery combined with in vivo electroporation and also used clinically in advanced trials which treated cervical dysplasia can be improved dramatically. In recent years, the popularity of naked DNA has been remained constant between years 2004 and 2018 which was 16.5% in 2018, 18.3% in 2012, 18% in 2007, and 14% in 2004. Additionally, naked DNA is the most common non-viral system used in 4.5% of all clinical trials when followed by lipofection involving cationic lipid/DNA complexes. Moreover, a small number of trials use a range of modified bacteria or brewer's yeast strains which were applied in 47 and nine trials respectively.

6 Gene Therapy and Biosafety

Ethical considerations of gene therapy are often a secondary issue among researchers. Integration of proviral genome is one of these concerns which faced in gene therapy in recent years (Swindle 2018). The risk of pathologies caused by the changes in gene expression resulted after the integration of proviral genome (Gonin et al. 2005; Swindle 2018). Gene delivery systems can express high levels of nucleic acids and proteins. Almost all delivery systems can infect practically any cell type. Although, gene transfer vectors intended to meet patient's satisfaction, they can be dangerous to clinical or laboratory personnel (Doi and Takeuchi 2015). One of these harmful approaches is a strong autoimmune response which can be induced on accidental exposure to a vector which expresses a high level of normal human protein. If these proteins expressed in one organ, these can be useful to a patient while expression elsewhere may be harmful (Swindle

2018). On the other hand, the preparation of vector can be harmful too and resulted in unforeseen health effects such as inflammatory reactions, presence of replication-competent virus contaminants, and insertional mutagenesis (Fang and Zhu 2013). Moreover, unexpected exposure to vector preparations may be toxic to laboratory and clinical investigators (Fang and Zhu 2013; Swindle 2018). For this reason, Institutional Biosafety Committees (IBCs) have established to recognize possible risks related to recombinant DNA studies, design methods for reducing the possibility of exposure, and developing mechanisms for responding to accidental exposures (Pergament 2016; Swindle 2018). More than 25 years ago the biosafety committee system has originated and a group of scientists, physicians, ethicists, and members of the general public has been gathered by the National Institutes of Health (NIH) to establish a mechanism for regulating the laboratory and applying recombinant DNA technology. The group's name was Recombinant DNA Advisory Committee (RAC) (O'Reilly et al. 2015; Adelman et al. 2018). The result of the discussion out of this meeting was establishing a system that has continued to this day formulating two main principles. The first one was to determine the proper procedures and containment methods for laboratory experiments by giving primary responsibilities to IBCs. RAC retained control over certain especially hazardous experiments while, other laboratory studies which used recombinant DNA were under the regulatory control of local committees (O'Reilly et al. 2015; Swindle 2018). The second principle was assessing risks related to recombinant DNA experiment. These experiments are classified into four risk groups according to the RACs scheme. Setting procedures and laboratory containment should be associated with risk groups. These sets are named biosafety levels 1 to4. For example, working with Risk Group 3 agents would usually be performed in Biosafety Level 3 (BSL3) facilities which follow BSL3 procedures. Accordingly, human gene transfer vectors and methods are inevitably placed in the two least dangerous risk groups (Risk groups 1 and 2) (Swindle 2018). On the other hand, most modern vectors are limited or incapable of their replication in vivo and therefore they classified as nonpathogenic risk group 1 agents. However, due to unknown effects of accidental exposure and potential for the production of modern vectors (replication-competent vectors), many Biosafety Committees require applying BSL2 containment and procedures when preparing these vectors. There are effective preventive or treatment measures which are used in risk group 2 studies. Risk group 2 agents are transmitted mostly by physical contact. BSL2 procedures are designed to offer staff preventive measures such as vaccinations, to prevent physical contact with cultures, and to establish techniques for treating staff that have been exposed to the agent (Swindle 2018). Biosafety Committees often consider clinical gene transfer protocols and concentrate on the potential risk to study subjects. Accordingly, the Committee usually cooperates with local Institutional Review Boards. They review informed consent statements for technical precision and make risk-benefit assessments. All human gene transfer protocols must be registered with the NIH and approved by an IBC (Adelman et al. 2018).

7 Gene Therapy for Several Diseases

In recent years, a variety of inherited genetic diseases and more importantly cancer have improved by gene therapy approaches. Here some of the important diseases that can be treated by gene therapy and also some diseases which recently targeted have mentioned. The number of gene therapy clinical trials in different diseases can be seen in Table 4 (Hanna et al. 2017). Accordingly, there are three types of disorders which can be treated by gene therapy; (1) monogenic disorders, (2) polygenic disorders, and (3) infectious diseases (Kaufmann et al. 2013; Wang and Gao 2014).

Table 4 Number of gene therapy clinical trials in different diseases (Hanna et al. 2017)

Phases of Clinical Trials → / Diseases Targeted by Gene Therapy ↓	Phase I	Phase I/II	Phase II	Phase II/III	Phase III	Phase IV	Single subject	Total
Cancer diseases	886 (65.97%)	273 (57.59%)	271 (67.41%)	12 (52.17%)	57 (66.28%)	2 (100%)	3 (60.00%)	1504 (64.41%)
Cardiovascular diseases	76 (5.65%)	34 (7.17%)	50 (12.44%)	7 (30.43%)	10 (11.63%)	–	–	177 (7.58%)
Gene marking	42 (3.12%)	5 (1.06%)	3 (0.75%)	–	–	–	–	50 (2.14%)
Infectious diseases	106 (7.89%)	44 (9.28%)	22 (5.47%)	–	6 (6.98%)	–	–	178 (7.62%)
Inflammatory diseases	9 (0.68%)	–	5 (1.24%)	–	–	–	–	14 (0.60%)
Monogenic diseases	128 (9.54%)	78 (16.45%)	13 (3.23%)	4 (17.40%)	6 (6.98%)	–	2 (40.00%)	231 (9.90%)
Neurological diseases	16 (1.19%)	15 (3.16%)	12 (2.98%)	–	–	–	–	43 (1.84%)
Ocular diseases	14 (1.04%)	10 (2.11%)	8 (1.99%)	–	1 (1.16%)	–	–	33 (1.41%)
Healthy volunteers	41 (3.05%)	2 (0.43%)	8 (1.99%)	–	2 (2.32%)	–	–	53 (2.27%)
Others	25 (1.86%)	13 (2.74%)	10 (2.49%)	–	4 (4.65%)	–	–	52 (2.22%)
Total	1343 (57.51%)	474 (20.29%)	402 (17.22%)	23 (0.98%)	86 (3.68%)	2 (0.08%)	5 (0.21%)	2335 (100.00%)

7.1 Monogenic Disorders

These kinds of disorders are the result of a mutation in one gene which occurs in all cells of the body. Some examples of monogenic diseases are including; ADA-SCID, DMD, CF, hemophilia, obesity, Huntington disease, Parkinson's disease, hypercholesterolemia, CGD, fanconi anemia, and sickle cell anemia (Wang and Gao 2014) in which CF, hemophilia, and obesity as some important monogenic disorders have been mentioned here.

7.1.1 Cystic Fibrosis

CF is a recessive disorder caused by gene mutations (Griesenbach et al. 2016). It has the highest prevalence among genetic diseases in white population (Griesenbach et al. 2015). CF has several manifestations including respiratory problems like chronic pulmonary infection (which is the leading cause of death in CF patients), endocrine and gastrointestinal comorbidities (Castellani and Assael 2017). Since the most mortality rate of CF is for pulmonary disease the efforts to cure the disease are mostly concentrated on lungs (Armstrong et al. 2014). Gene therapy has some advantages in comparison to other drugs; for example, it will solve the root of the problem rather than symptoms and can be used for all CF patients, not just a specific class (Griesenbach et al. 2015). One of the important genes that play a key role in CF gene therapy is CFTR gene that is expressed to lungs (Griesenbach et al. 2016). The best target cells to express CFTR gene to, are epithelial cells (Griesenbach et al. 2015) (in fact airway epithelial cells which CFTR is expressed normally in (Prickett and Jain 2013)). This gene is transferred by viral and non-viral vectors, preferably viral vectors because of their less induction of body immune system and consequently their more efficiency (Griesenbach et al. 2016).

7.1.2 Hemophilia

Hemophilia is an X-linked recessive disorder characterized by bleeding (Rogers and Herzog 2015), consisting of two major categories: hemophilia A (caused by *F8* gene mutation; resulted in coagulation factor VIII deficiency (Doshi and Arruda 2018), which is more prevalent) and hemophilia B (caused by *F9* gene mutation; resulted in coagulation factor IX deficiency (Doshi and Arruda 2018)). Since hemophilia is a monogenic disorder, it can be an ideal candidate for gene therapy (Doshi and Arruda 2018). There are two approaches we follow in gene therapy for hemophilia; the first way is to directly administer the vector which carries our target genes in vivo, the second approach is ex vivo transduced cells and transplantation, the first way is mainly used in current clinical studies. As discussed above *F8* gene mutation is responsible for hemophilia A; now if we aim to package the normal gene into a vector in order to express it to cell genome, we have to separate the B domain of it to make it smaller. Most of the recent gene therapies for hemophilia expressed the gene to liver hepatocytes where the coagulation factors are generated (Ohmori 2018). Vectors used in hemophilia gene therapy are mostly viral ones; AAV vectors are often used in in vivo gene administration, while lentiviral (LV) vectors are commonly used in ex vivo gene transduction trials (Fig. 7) (Ohmori 2018). Accordingly, AAV vectors are

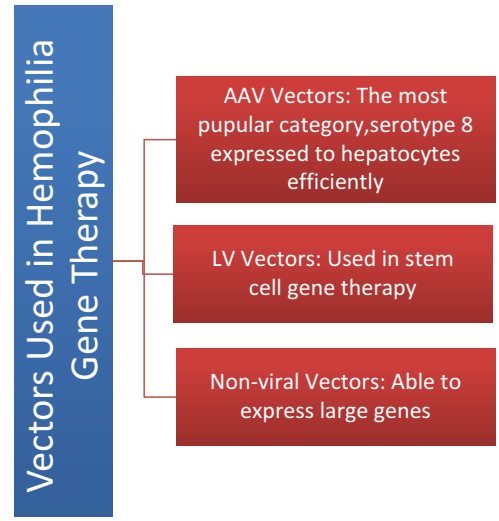

Fig. 7 Vectors associated with gene therapy in hemophilia: AAV, LV and Non-viral vectors. Using vectors in gene therapy can improve the ability to achieve therapeutic factor levels in hemophilia (Ohmori 2018)

the most popular vectors in clinical trials for hemophilia gene therapy. Two important reasons in which why they are preferred over other types include; (1) because of several serotypes in order to use for specific purposes (for example if serotype 8 used in hepatocytes, high gene expression and consequently higher efficacy would have seen), (2) AAV vectors neither trigger immune system nor have pathological effects (Ohmori 2018). Due to the integration of LV vectors into the DNA of the transduced cells, they can lead to permanent gene expression, so that they are used in stem cell gene therapy (Ohmori 2018). This category is suitable for expressing large genes like full-length F8, but there is a problem with this method that it can be invasive for being used in clinical trials (Ohmori 2018).

7.1.3 Obesity

Obesity is a very remarkable risk factor for immune dysfunction, heart disease, hypertension, arthritis, neurodegenerative diseases, and certain types of cancer. Therefore, the risk of mortality increased as a result of obesity (Jimenez et al. 2018; Payab et al. 2018). Due to some undesirable side effects of conventional treatment for obesity, there is an essential need for novel and well-tolerated techniques for the large and heterogeneous population of obese patients (Payab et al. 2018). Therefore, gene therapy approaches are a promising therapeutic factor in the treatment of obesity (Sahu et al. 2018). There are several genes which are responsible for applying in gene therapy to treating and preventing obesity. Pro-protein convertase subtilisin/kexin 1 (PCSK1), pro-opiomelanocortin (POMC), and melanocortin 4 receptor (MC4R) genes are some good examples of gene therapy approaches in obesity. Also, genetic polymorphisms of some adrenergic receptors like β1-adrenergic receptor (ADRB1) and β3-adrenergic receptor (ADRB3) play crucial roles in regulating energy expenditure, and genetic polymorphisms of these receptors have been related to obesity and type 2 diabetes (Gao and Liu 2014). Scientists have proved the potential of FGF21 gene therapy to treat obesity recently (Jimenez et al. 2018).

7.2 Polygenic Disorders

In polygenic disorders multiple genes involved. These kinds of diseases may be dependent on environmental factors and lifestyle (Raghavan and Tosto 2017). Some examples of polygenic disorders are; Heart disease, Cancer, Diabetes, Schizophrenia, and Alzheimer's disease. Herein, diabetes and cancer as important polygenic disorders have been discussed.

7.2.1 Diabetes

- ***Diabetes type 1:*** Type 1 diabetes mellitus (T1DM) is an autoimmune polygenic (mostly affected by HLA region on chromosome 6) with a destructive effect on pancreatic beta cells insulin secreting function. Currently, the most common cure for this disorder is insulin injection multiple times per day. Despite all the progress made in diabetes type 1 treatment until now, there are still many problems associated with present treatment (Atkinson et al. 2014). One of the most important methods which gene therapy has given attention to is overexpression of genes. Since the reason behind the occurrence of diabetes type 1 is underexpression of some specific genes, overexpression of them can be a good choice for gene therapy, for example insulin-like growth factor 1 (IGF1), Regenerating islet-derived protein 3 gamma gene (Reg3g), glucose-6-phosphatase (G6Pase) gene, Klotho gene, and hepatocyte growth factor gene (HGF gene) (Chellappan et al. 2018).
- ***Diabetes type 2:*** Gene therapy for diabetes mainly focuses on T1DM but as type 2 diabetes mellitus (T2DM) has a strong genetic predisposition, several genes have been evaluated for its gene therapy. There are several new therapeutic targets which have been determined. Accordingly, at least 75 independent genetic loci for T2DM have identified (Stafeev et al.

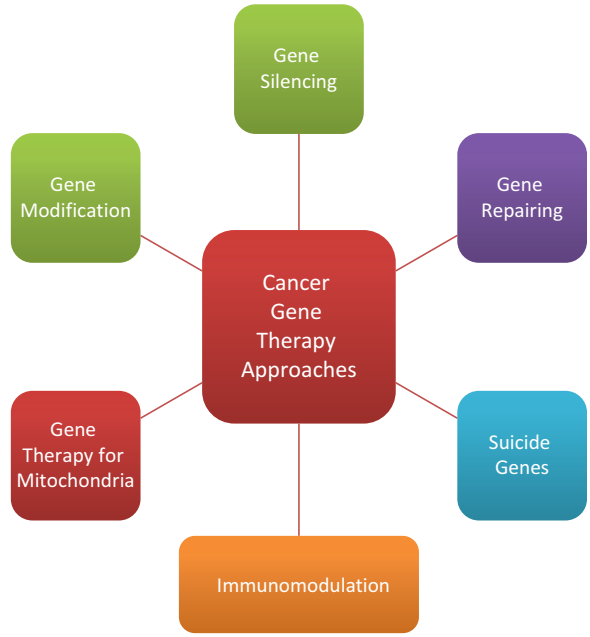

Fig. 8 A few numbers of cancer gene therapy approaches including gene silencing, gene repairing, suicide genes, immunomodulation, gene modification, and gene therapy approaches for mitochondria. These approaches can control biological results of genomic changes in cancer cells (Barar and Omidi 2012; Amer 2014)

2019). For instance, three novel mutations in gene *KCNJ11* are related to the development of autosomal dominant and early-onset T2DM. Genetic loci may have much stronger effects on drug response in comparison with their limited effects on disease development. There are a large number of genetic polymorphisms which affect the oral antidiabetic drugs response. Another good example of genes with potential for T2DM gene therapy is nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3). Inhibition of gene NLRP3 attenuates inflammation, protects pancreatic β-cells from apoptosis, and prevents T2DM development in mice. As a result, Target genes are involved in the onset, development, and deterioration of T2DM (Stafeev et al. 2019).

7.2.2 Cancer

Cancer as a major issue for global health is being responsible for 9.6 million deaths in 2018 has been experimented widely in gene therapy trials from the beginning. As we know genome changes cause cells to increase rapidly and lead to cancer; now what gene therapy does in this matter is to cure cancer by inserting specific genes into tumor cells genome. Gene therapy has made much progress in the last two decades (Amer 2014) and may be preferred over conventional therapies because of having only local effects instead of causing systemic side effects. Some examples of cancer targeted with gene therapy are acute lymphoblastic leukemia (*ALL*), brain, lung, breast, pancreatic, liver, colorectal, prostate, bladder, head and neck, skin, ovarian, and renal cancer (Wirth and Ylä-Herttuala 2014). As mentioned before genes must be inserted into the genome; there are several methods to transfer them into tumor cells, divided into four major categories consisting of physical, chemical, bacterial and viral-mediated gene transfer. Several types of viruses are widely used in cancer gene therapy including adenoviruses, ADA, herpes simplex viruses, lentiviruses, and retroviruses (Amer 2014). After transgenes are integrated into the target cells genome they aim to fight tumors by a few

approaches categorized into several groups including suicide genes (transgenes which induce cancer cells apoptosis), gene silencing, modification and repairing, gene therapy for mitochondria (transferring genes into cell mitochondria) and finally immunomodulation (Fig. 8) (Amer 2014).

Ovarian cancer as an example of cancer gene therapy was the seventh most common cancer in women in 2018 and has a high mortality rate due to its late-stage diagnosis (Ayen et al. 2018). Common treatments for ovarian cancer are debulking surgery, neoadjuvant therapy, and chemotherapeutic agents, intraperitoneal chemotherapy, and vitamin D (Stewart et al. 2019). Late diagnosis has decreased mentioned approaches efficacy; thus, gene therapy is being experimented in clinical trials. Here are some methods used in ovarian cancer gene therapy: antiangiogenic gene therapy, tumor suppressor gene therapy, cofactor inhibition strategies, suicide gene therapy, and oncolytic therapy (Ayen et al. 2018).

7.3 Infectious Diseases

Gene therapy as an alternative treatment for a wide range of infectious diseases such as HIV is in progress (Rogers and Cannon 2017).

7.3.1 HIV

Several gene therapies approach for treating HIV are under research. A leading strategy involves genetically modifying immune system cells to resist HIV infection. The aim of this strategy is preventing the virus from being able to cause disease even if the latent reservoir is not reduced or eliminated (Dubé et al. 2019). Conventional approaches of gene therapy for HIV are based on engineering HIV target cells which are not allowed to viral replication. Therefore, the development of gene-modified HIV target cells has been limited in patients. As a result, there are some alternative genetic strategies which concentrate on generating gene-modified producer cells that secrete antiviral proteins (AVPs). The secreted AVPs intervene with HIV entry. Therefore, they increase the protection against infection to unmodified HIV target cells. Furthermore, hematopoietic and non-hematopoietic cell lineages can perform as producer cells due to

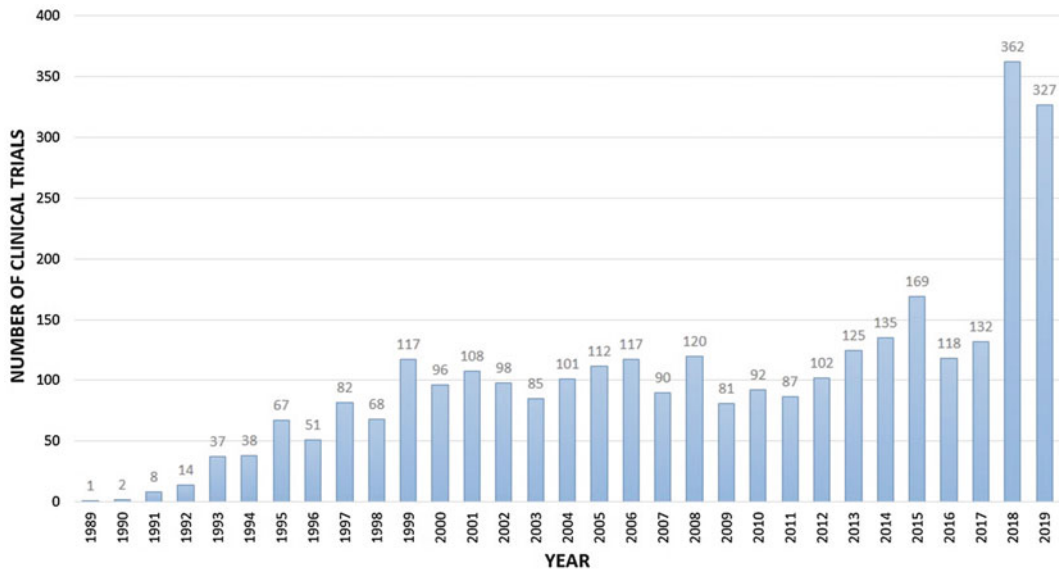

Fig. 9 Gene therapy development. Number of gene therapy clinical trials per year from 1989 to 2019 (Hanna et al. 2017, Dunbar et al. 2018, Ginn et al. 2018)

their potential to secret AVPs. Therefore, the secretion of AVPs from non-hematopoietic cells opens new windows of gene therapy for HIV prevention (Falkenhagen and Joshi 2018).

8 Risks and Benefits of Gene Therapy

As mentioned before, therapeutic efficacy following gene therapy has now been reported for several inherited diseases including SCID-X1, ADA-SCID, CGD, LCA, adrenoleukodystrophy (ALD), β-thalassemia, and hemophilia B as well as leukemia and HIV. Although gene therapy has demonstrated the difficulties of assessing risks, given the intrinsic uncertainty of trial outcome, recent clinical trials have shown the increasing promise of gene therapy. The number of gene therapy clinical trials development per year from 1989 to 2019 has illustrated in Fig. 9 (Hanna et al. 2017; Dunbar et al. 2018; Ginn et al. 2018). Only in recent years, this promise has begun to be realized. The delay in the transition from promising early laboratory results to clinical advantages, emphasizes the uncertainties and challenges faced in the clinical translation of complex therapeutic products. These challenges continue to pose obstacles for gene therapy researches and are expected to face other emerging research fields, including research in stem cell therapy and nanomedicines. One of the inescapable aspects of clinical research is risk that is increasingly relevant to the gene therapy field as the imperative for clinical trial activity grows. In recent years, extensively reported the appearance of serious adverse events (SAEs) in gene therapy studies, including trials for ornithine transcarbamylase, deficiency (OTCD), SCID-X1, and rheumatoid arthritis, has heightened fear in public perceptions of gene therapy (Deakin et al. 2009). Although the strategy behind the SCID-X1 gene therapy was successful, applying the integrating vectors to transduce hematopoietic progenitors that consequently experience extensive replicative expansion in vivo also takes the risk of insertional mutagenesis (Epstein 1996). The Declaration of Helsinki confirms this ambiguity in requiring a risk assessment to be based on "predictable risks and burdens". The future of gene therapy is moving towards establishing a balance between the benefits and risks of its procedure (2017).

9 Challenges and Limitations of Gene Therapy

Although there are still many significant technical challenges to be overcome, recent advances in modern medicine and the molecular and cellular biology of gene transfer have made it possible that gene therapy will soon begin to play an increasingly important role in clinical practice (Goncalves and Paiva 2017). Accordingly, gene therapy will not be limited to the management of monogenic disorders but will have applications across many other fields of medicine, especially the treatment of cancer and infectious disease. Gene delivery and activation, disrupting important genes in target cells commercial validity financial limitations are some of its frustrating concerns (Kozarsky and Wilson 1995). Therapeutic gene transfer for treating certain human diseases, so-called somatic gene therapy, has had some limited success (Shim et al. 2017). Previous studies which have done in animals predicted that retrovirus-mediated gene transfer resulted in a potential risk of insertional oncogenesis, although it is unlikely. Retroviruses can up-regulate cellular genes over long distances (more than 10 kb). Considering the appearance of more than 100 proto-oncogenes in the human genome, oncogene dysregulation may happen in about 0.1–1% of all retroviral gene-transfer events. The major challenges have been delivery of DNA to the target cells and duration of expression (Baum et al. 2003). Therefore, gene therapy still needs more clarifying preclinical studies to bypass the constraints and challenges ahead. On the other hand, some of the important challenges which gene therapy medicinal products (GTMP) are faced with include; manufacturing hurdles, safety risks, limited efficacy, and ethical conflicts. During the development process, it is essential to take steps for overcoming these challenges.

Clinical uses for approved and investigational GTMPs and also reviewing critical challenges present by the structure of advanced therapy medicinal products (ATMPs) (Carvalho et al. 2017).

9.1 Advanced Therapy Medicinal Products Regulation

ATMPs include; GTMPs, somatic cell therapy medicinal products (sCTMPs) and tissue-engineered products (TEPs), and combined ATMPs (Salmikangas et al. 2015).

The ATMP regulations tasks are;

- Presenting a clear definition of ATMPs
- Outlining the marketing authorization requirements and procedures
- Focusing specifically on safety, efficacy and risk management
- Describing post-authorization obligations

9.2 Marketing Authorization Application for ATMPs

Marketing Authorization Application (MAA) for ATMPs should obey a compulsory centralized procedure. The advantages of centralized review include (Salmikangas et al. 2015);

- Overcoming the lack of expertise in this area
- Ensuring a high level of scientific assessment by a specialized committee
- Improving market access for these innovative therapies

As the primary review is performed by the committee for advanced therapies (CAT), the scientific assessment of ATMPs is slightly different in comparison to other medicinal products. CAT is an independent specialist committee which its main responsibility is to review MAA for ATMPs and publish a draft opinion for the committee for medicinal products for human use (*CHMP*) to make a recommendation to the European Commission which has the final authority to grant marketing authorization (Carvalho et al. 2017). Another major task of the CAT in addition to the reviewing applications for marketing authorization is to encourage the development of new ATMPs. Several regulatory strategies which CAT plays a key role in them and support ATMP development are the Innovative Task Force, the priority Medicines scheme, the ATMP Certification, the ATMP Classification, and the Scientific Advice. Finally, the CAT should also scientifically assist in the elaboration of any documents related to the achievement of the ATMP regulation purposes. For obtaining Marketing Authorization Approval of a gene or cell therapy product, depending on the evaluating regulatory body, different steps and requirements may be needed. For example, in the US, gene and cell therapy are considered biologic therapies. Within the Food and Drug Administration (FDA), these products' primary oversight falls under the Office of Cellular, Tissue and Gene Therapy (OCTGT) which is a division of the Center for Biologics Evaluation and Research. The status and Phases of gene therapy clinical trials are shown in Table 5 (Hanna et al. 2017). At first, an investigational new drug (IND) Application is required for the investigational use of a biologic product. It aims to support clinical use of the investigational product based on quality and non-clinical data. To market a biologic drug product, FDA needs sponsors to hold an approved biologics license application (BLA). Timelines for evaluation range from 10 to 12 months from filing, depending on the pathway under which the BLA is reviewed. Same as the European Medicines Agency (EMA), the FDA has several actions to support the development of Gene and Cell therapies including (Carvalho et al. 2017);

- Breakthrough Therapy designation
- Fast Track designation
- Priority Review designation
- Accelerated Approval

Table 5 Phases and status of gene therapy clinical trials (Hanna et al. 2017)

	Open	Closed	Withdrawn	On clinical hold	Conditional approval	Canceled	Under review	Submission not completed	Total
Phase I	970 (57.81%)	337 (58.10%)	12 (42.86%)	3 (42.86%)	9 (60.00%)	–	10 (66.67%)	2 (33.33%)	1343 (57.52%)
Phase I/II	327 (19.49%)	129 (22.24%)	8 (28.57%)	3 (42.86%)	1 (6.67%)	1 (16.67%)	2 (33.33%)	3 (20.00%)	474 (20.30%)
Phase II	288 (17.16%)	96 (16.55%)	8 (28.57%)	1 (14.28%)	5 (33.33%)	1 (16.67%)	1 (6.67%)	2 (33.33%)	402 (17.21%)
Phase II/III	15 (0.89%)	7 (1.21%)	–	–	–	1 (16.67%)	–	–	23 (0.99%)
Phase III	1(16.67%)	11 (1.90%)	–	–	–	2 (33.33%)	71 (4.23%)	1(6.67%)	86(3.68%)
Phase IV	2 (0.12%)	–	–	–	–	–	–	–	2 (0.09%)
Single subject	5 (0.30%)	–	–	–	–	–	–	–	5 (0.21%)
Total	1678 (71.86%)	580 (24.83%)	28 (1.20%)	7 (0.30%)	15 (0.64%)	6 (0.26%)	15 (0.64%)	6 (0.26%)	2335 (100.00%)

10 Ethical Considerations

Gene therapy cause several ethical issues including (Karpati and Lochmüller 1997);

- Gene therapy is a costly treatment modality. Investigators question how the health care system can afford the costs required for implementing and developing gene therapy without compromising the quality of standard health care even in the developed countries (Karpati and Lochmüller 1997). On the other hand, others claimed that the cost-effectiveness of gene therapy for certain devastating diseases such as DMD (Chamberlain and Chamberlain 2017) or cystic fibrosis (Guggino and Cebotaru 2017), etc. can be easily proven.
- As somatic cell gene therapy for genetic diseases does not correct the genetic defect in germ cells but at the same time raises longevity and reproductive capacity of the affected individuals which resulted in increasing the defective gene pool (Karpati and Lochmüller 1997).
- Resistance to gene therapy research has been established in the areas which represent a heavy intervention in the course of nature because it targets the most basic aspect of life. Gene therapy is managed by the same set of rules that apply to reproductive technologies in some countries. This could represent an obstacle to gene therapy research (Goncalves and Paiva 2017).

11 Conclusion and Future Perspectives

Despite the existence of critical side effects observed in the initial clinical trials, gene therapy is being developed to fulfill the early promises that are made recently. Already most of the risks related to gene therapy are addressed or occupied center stage for current researches (Goncalves and Paiva 2017). Recent advances in gene therapy raise hope for many patients who are suffering from rare diseases and also many other common illnesses (Arjmand et al. 2017). Gene therapy is no longer an alternative therapy for patients whose conventional therapy was not applicable to them but In the near future, it could become also the first-line treatment for a broad range of diseases (Dunbar et al. 2018). Accordingly, the recent advances and therapeutic successes which have seen in many treated patients have motivated pharmaceutical companies to support gene therapy developments. In recent years because of the technical and ethical issues, germ line gene therapy is not being considered (Goncalves and Paiva 2017; Ma et al. 2017). Therefore, the scientific community has decided to try somatic cell gene therapy which has some advantages for individuals and also cannot be passed on to the next generation (Bank 1996). The biotechnology industry, financial sector, scientific community, and patients and their families had high expectations from gene therapy up until recently and they predicted prompt therapeutic approaches for important diseases. This resulted in the increasing number of research publications, growing of funds and capital invested and rising in the number of patents, and also industry-academic partnerships (Burnight et al. 2014). When the ongoing process turned out to be slower than initially expected the general attitudes became not only less enthusiastic but also completely pessimistic and even hostile about the prospects of gene therapy. In reality, gene therapy cannot be considered a failure by any evidence although, it's potential may have been overstated, particularly in relation to the timetable of its successful implementation (Ma et al. 2017). Accordingly, several examples of clinical successes and gene therapy strategies are being more important and the main point in this regard is the long-term safety of gene transfer techniques. Therefore, a 15-year follow-up for those registered in gene therapy trials has been mandated by regulatory agencies. Because of the power and variety of gene transfer procedures, nowadays there are few severe diseases in which gene transfer methods are not being developed. For instance, recombinant proteins and monoclonal antibodies are recent development of new therapeutic approaches (Anguela and High

2019). As a result, further progress in gene transfer technology can lead to having useful therapeutic approaches in modern molecular medicine.

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Developments in Artificial Platelet and Erythroid Transfusion Products

Neslihan Meric, Gulen Guney Esken, Merve Uslu, and Fatih Kocabas

Abstract

Platelet and blood transfusions have vital importance to the lives of many patients. Platelet transfusions are a life-saving intervention by reducing risk of bleeding in thrombocytopenic patients. Due to the short shelf life of platelets and their limited availability, researchers have developed various platelet transfusion production technologies. Understanding the cellular and biophysical mechanisms of platelet release is particularly important for development of platelet transfusion products (PTPs) and to translate them to clinical applications in patients requiring platelet infusion. Similarly, due to donor dependence and increased clinical need of blood transfusions, studies on the erythroid transfusion products (ETPs) have recently gained momentum. This led to development of ETP technologies involving differentiation of stem cells to fully functional erythrocytes *in vitro*. During megakaryopoiesis or erythropoiesis, various stimulatory factors, growth factors, transcription factors, and biophysical conditions have been shown to play a crucial role in the formation final blood products. Thus, understanding of the *in vivo* mechanisms of platelet release and erythrocyte maturation is particularly important for mimicking these conditions *in vitro*. This review focuses on latest and up-to-date information about the innovations in PTP and ETP technologies. We also discuss some of the recent fundamental findings that have changed our understanding of *in vivo* platelet release and blood formation.

N. Meric (✉)
Regenerative Biology Research Laboratory, Department of Genetics and Bioengineering, Faculty of Engineering, Yeditepe University, Istanbul, Turkey

Graduate School of Natural and Applied Sciences, Yeditepe University, Istanbul, Turkey

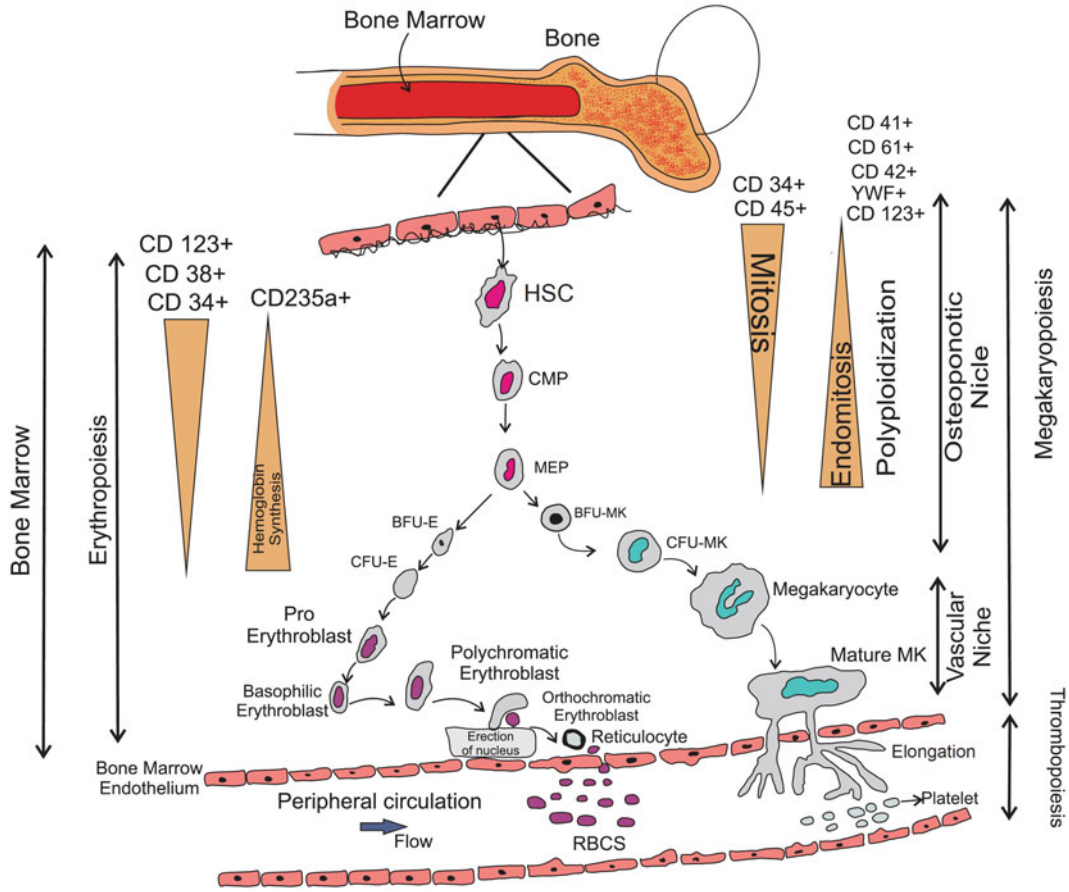
Bone Marrow Transplantation Center, Anadolu Medical Center, Kocaeli, Turkey
e-mail: neslihan.meric@std.yeditepe.edu.tr

G. Guney Esken
Regenerative Biology Research Laboratory, Department of Genetics and Bioengineering, Faculty of Engineering, Yeditepe University, Istanbul, Turkey

M. Uslu and F. Kocabas (✉)
Regenerative Biology Research Laboratory, Department of Genetics and Bioengineering, Faculty of Engineering, Yeditepe University, Istanbul, Turkey

Graduate School of Natural and Applied Sciences, Yeditepe University, Istanbul, Turkey
e-mail: Fatih.Kocabas@yeditepe.edu.tr

Graphical Abstract



Human bone marrow acts as a source of cells required for erythropoiesis and megakaryopoeiesis. Understanding of molecular mechanism and physiology of these vital and curitial events allowed us to mimic these conditions *ex vivo* and to develop artificial platelet and erythroid transfusion production technologies.

Keywords

Erythroid transfusion products · Erythropoiesis · ETPs · Hematopoietic stem cells · Megakaryocytopoiesis · Megakaryopoeiesis · Platelet transfusion products

Abbreviations

ABI	Aurora B Inhibitor
ADSC	Adipose Derived Stem Cells
BFU-MK	Burst Forming Unit Megakaryocyte
BM	Bone Marrow
CFU-GEMM	Colony Forming Unit – granulocyte, erythrocyte, monocyte, megakaryocyte

CFU-Mk	Colony Forming Unit Megakaryocyte
CMP	Common Myeloid Progenitor
CMPs	Common Myeloid Progenitors
DMS	Demarcation Membrane System
ECM	Extracellular Matrix
ESC	Embryonic Stem Cell
ES-sacs	Embryonic Stem Cell–Derived Sacs
ETPs	Erythroid Transfusion Products
FLI1	Friend Leukemia Integration 1
GATA1	GATA binding protein 1
GPV	Glycoprotein V
HDFs	Human Dermal Fibroblasts
hESCs	Human Embryonic Stem Cells
hiPSCs	Human Induced Pluripotent Stem Cells
HPC	Hematopoietic Progenitor Cells
HSC	Hematopoietic Stem Cell
hTERT	Human telomerase reverse transcriptase
IL-1α	Interleukin 1 alpha
MAPK	Mitogen Activated Protein Kinase
Meis1	Myeloid ectopic viral integration site 1
MEP	Megakaryocyte Erythroid Progenitor
Mk	Megakaryocyte
MKP	Megakaryocyte Progenitor
Mks	Megakaryocytes
NF-E2	Nuclear Factor Erythroid 2
PB	Peripheral Blood
PDGF	Platelet Derived Growth Factor
PI	Phosphatidylinositol
PTPs	Platelet Transfusion Products
RBCs	Red Blood Cells
RRI	Rho Rock inhibitor
RUNX1	Runt related transcription factor 1
SCF	Stem Cell Factor
SDF-1	Stromal-Derived Factor-1
SI	Src Inhibitor
STAT1	Signal Transducer and Activator of Transcription 1
TAL-1	T-cell Acute Lymphocytic Leukemia Protein 1
TGF-β	Transforming Growth Factor-Beta
TPO	Thrombopoietin
UCB	Umbilical Cord Blood
VEGF	Vascular Endothelial Growth Factor
vWF	von Willebrand Factor

1 Introduction

Platelets are anucleated cytoplasmic discs derived from megacaryocytes that circulate in the blood. They play an important role in hemostasis, immunology, inflammation and cancer (Golebiewska and Poole 2015; Semple et al. 2011; Klinger and Jelkmann 2002; Franco et al. 2015). They involve in wound healing and angiogenesis through the delivery of stored growth factors, such as platelet-derived growth factor (PDGF), *basic fibroblast growth factor* (bFGF), vascular endothelial growth factor (VEGF), platelet factor-4, transforming growth factor-beta 1 (TGF-**β1**), thrombospondin-1 to sites of injury (Huang and Cantor 2009) Thrombopoeitin is produced in the liver at constant rate and controls circulating platelets rate by stimulating bone marrow to produce megakaryocyte. Platelets circulate in the blood for 7–10 days and count is between 140,000 and 440,000/mcl. A number of diseases are associated with platelet deficiency including thrombocytopenia, thrombocythemia, idiopathic thrombocytopenic purpura, myelodysplastic syndromes, chemotherapy-induced thrombocytopenia, aplastic anemia, human immunodeficiency virus infection, and major cardiac surgery (Thon and Italiano 2010). The platelet requirement in the clinic of these patients is increasing day by day. Increasing numbers of donors are needed for platelet transfusions (Ono-Uruga et al. 2016). Donor-dependent platelet availability can be challenging and transfusions can cause issues. In addition, platelets have short life spans of 7–10 days (Lu et al. 2011), donor-dependent platelet supply is limited (Ono-Uruga et al. 2016), and long-term storage of platelets is difficult (Handigund and Cho 2015). Bacterial or viral contamination and the other transfusion-transmitted infections and immunological reactions due to Human leukocyte or platelet antigensare some of the risks that need to be

considered (Ono-Uruga et al. 2016; Cho 2015; Pineault and Boisjoli 2015). Human leukocyte or platelet antigens are also problem for donor-dependent platelet transfusion (Pavenski et al. 2013).

Red blood cells (RBCs) are the most common type of cell found in the blood. RBCs or erythrocytes contain a special protein called hemoglobin, oxygen-binding protein, Which transports oxygen from the lungs to the tissues then returns carbon dioxide from the tissues to the lungs so it can be exhaled. A drastic reduction in the number of blood cells in the body can be associated with thalassemia syndromes, inherited bone marrow failure as well as in the anemia of chronic disease (Zivot et al. 2018). It is important that the infection tests, crossmatch and blood group were determined before transfusion. The need for rare blood groups and the urgency of blood transfusion in emergency cases reveal the challenges of blood transfusion (Sharma et al. 2011).

For all these reasons, studies on the production of non-donor-independent artificial blood elements *in vitro* have been a source of hope for many patients. In this review, current strategies for the development of artificial platelet and erythroid transfusion products will be discussed.

2 Developments in Artificial Platelet Transfusion Production Technologies

2.1 Megakaryopoiesis

The process of megakaryocyte maturation and differentiation in bone marrow is called megakaryopoiesis (Deutsch and Tomer 2013; Smith and Murphy 2014). Hematopoetic stem cells within the bone marrow can differentiate into Megakaryocytes (Mks). Mks are scarce, polyploid, big bone marrow cells able to produce platelets (Huang and Cantor 2009). Mks in the bone marrow are very rare population of cells, which only makes 0.02–0.05% of total nucleated cells (Li and Kuter 2001). Mouse Mks constitute 0.1–0.5% of bone marrow cell (Corash et al. 1989). Mks are associated with bone marrow sinusoidal endothelium, extending cytoplasmic protrusions into the sinusoids to produce platelets.

Megakaryopoiesis process and platelet production is complex and has multiple stages. Firstly, HSC gives rise to the early common myeloid progenitors (CMPs) that can be quantified as the multi-lineage (granulocyte, erythrocyte, Mks and monocyte) colony-forming unit (CFU-GEMM) (Deutsch and Tomer 2006). Erythroid and Mks lineages arise from a common Mk-erythroid progenitor (MEP) derived from the early CMPs (Fig. 1). MEP can develop into the highly proliferative, early MK burst-forming unit (BFU-MK), or the more mature smaller CFU-MK, with the help of cytokines and chemokines, which both express the CD34 antigen (Briddell et al. 1989). On the other hand, MEP can give rise to early and late erythroid progenitors, the BFU-E and CFU-E (Schulze and Shivdasani 2004). The proliferating diploid megakaryoblasts (MKP) lose their capacity to divide, but keep their ability for DNA replication and cytoplasmic maturation (Deutsch and Tomer 2006). This ability of MKP is called *endomitosis* or *endoreduplication*. During endomitosis stage, mature Mks are produced with a ploidy number of $N \geq 8$. Mature Mks build up a unique membrane complex called the Demarcation Membrane System (DMS), as well as various types of granules, including lysosomes, dense granules, and α-granules (Nurhayati et al. 2016; Takayama and Eto 2012). Mature Mks produce long cytoplasmic extensions proplatelets, which release platelets from their tips (Fig. 1).

Megakaryopoeisis process occurs in osteoblastic/endosteal and vascular/endothelial niches of bone marrow (BM). In osteoblastic niche, $CD41^{+}CD34^{+}$ cells arise from HSC and proliferate and differentiate into platelet-producing mature Mks (Machlus and Italiano 2013; Panuganti et al. 2013; Lorenzo et al. 2008). Mature Mks migrate from the osteoblastic niche to the sinusoidal blood vessels of BM, where they extend proplatelets in order to release growing platelets directly into the blood stream (Avecilla et al. 2004). During the megakaryopoiesis

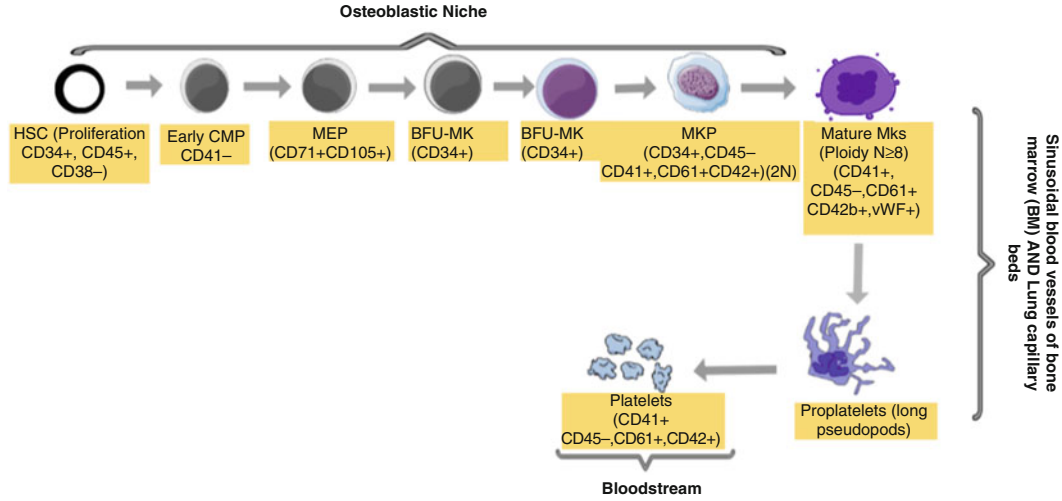


Fig. 1 Overview of cellular differentiation and maturation steps of megakaryopoiesis. CD34 + CD45+ HSC differenciate into CD41 + CD34+ MK in osteoblastic niche. Mature MK migrate to the sinusoidal blood vessels of BM and lung capillary beds where produce platelets into the blood stream. *HSC* Hematopoeitic stem cells, *CMP* Common myeloid progenitor, *Mk* Megakaryocyte, *MKP* Megakaryocyte progenitor, *MEP* Mk-erythroid progenitor, *BFU-MK* Burst forming unit-MK, *CFU-Mk* colony forming unit- Mk

process, before release platelets, mature Mks undergoes endomitosis, granule formation, proplatelet formation, and terminal platelet formation (Machlus and Italiano 2013; Panuganti et al. 2013; Lorenzo et al. 2008). Megakaryopoeisis and thrombopoeisis are very dynamic and efficient system, resulting in the generation of 10^{11} platelets per day 5 (Lambert et al. 2013). Every megakaryocyte can release approximatively 2000 and 5,000 platelets per day. The degree of polyploidization correlates with overall platelet production (Mattia et al. 2002; Ferrer-Marin et al. 2010).

Megakaryocyte differentiation is regulated positively or negatively by transcription factors and cytokines. Various molecular and cellular mechanisms regulate megakaryopoiesis process.

2.2 Stimulating Factors in Platelet Formation

Megakaryopoiesis require cytokines, chemokines and cellular interactions between HSC and bone marrow stromal cells (Avecilla et al. 2004). Megakaryocyte maturity and the level of polyploidization play important roles in the number of platelets released per cell (Mattia et al. 2002) (Fig. 2). The BM microenvironment has a unique protein composition crucial for platelet production. Extracellular matrix (ECM) components are also major factor for regulation of platelet production (Malara et al. 2011; Aguilar et al. 2016; Abbonante et al. 2017). Sinusoidal endothelial cells secrete thrombopoietin (TPO), VEGF and Interleukin 1 alpha (IL-1α) that are important regulator of megakaryopoiesis (Avecilla et al. 2004; Gars and Rafii 2012; Nishimura et al. 2015; Wang and Zheng 2016).

Cytokine TPO has pivotal role in the megakaryocyte development, regulation and maturation. Liver as a TPO producing organ generate TPO at steady level and this available level of TPO is controlled by circulating platelets, which carry on TPO receptors. Therefore, this feedback mechanism driven by TPO, provide the regulation and maintance of the platelets produced by bone marrow megakaryocytes (Yu and Cantor 2012; Lebois et al. 2016; Huang et al. 2016; Bertino et al. 2003; Kuter 2002).

c-Mpl is the surface receptor for TPO. Both megakaryocytes and HSCs have a common

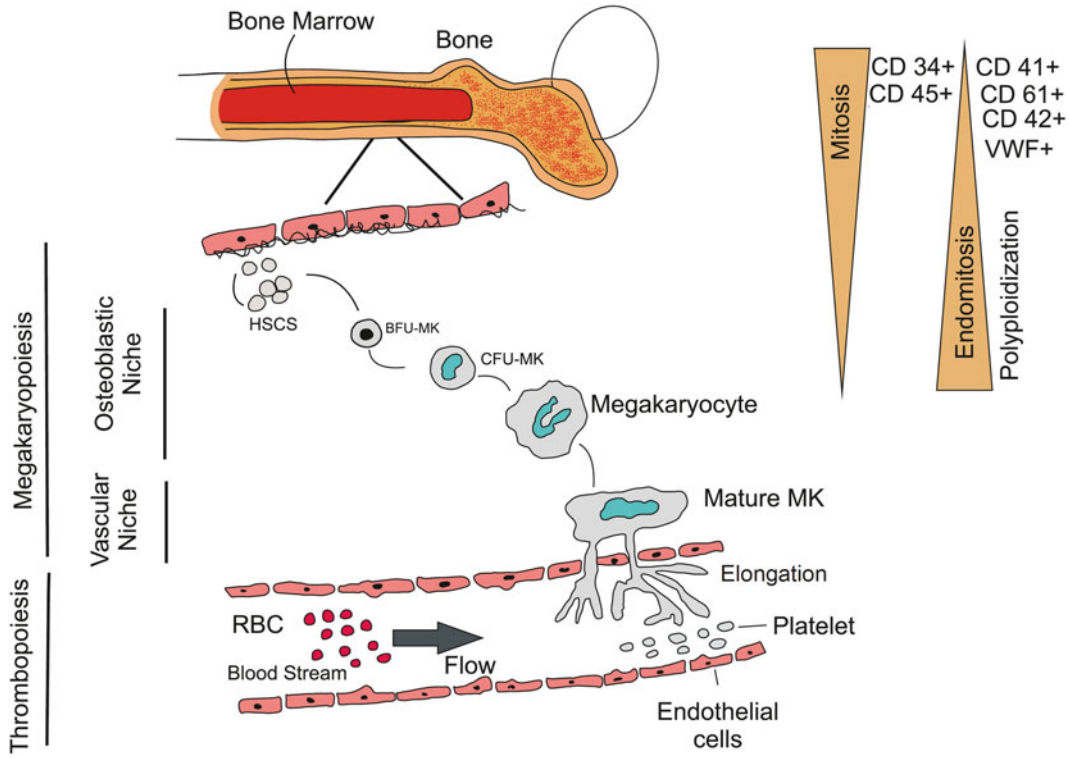


Fig. 2 Platelet release formation journey. Megacaryocyte are derived from HSC, differenciate and proliferate in the bone marrow niches. During proliferation megakaryoblasts lose their mitosis capacity, but keep endomitosis ability. Various stimulating factor regulates megakaryopoiesis process. Mature Mks undergoes endomitosis, granule formation, proplatelet formation, and terminal platelet formation. Thrombopoiesis occurs in vascular system where mature Mks build up the DMS and produce long cytoplasmic extensions proplatelets, which release platelets from their tips in the blood stream. *HSC* Hematopoeitic stem cells, *Mk* Megakaryocyte, *BFU-MK* Burst forming unit-MK, *CFU-Mk* Colony forming unit-Mk, *RBC* red blood cell

feature for the expression c-Mpl receptor on their cell surface. The importance of TPO receptor expression in HSC compartment has been revealed by bone marrow transplant studies taking advantage of the knock-out strategy. Mice lacking TPO receptor c-Mpl displayed deficiencies in long-term repopulating activity in bone marrow (Huang and Cantor 2009). Upon binding TPO to c-Mpl receptor, several downstream signaling pathways are activated including JAK/STAT, phosphatidylinositol (PI) 3-kinase-Akt, ERK1/ERK2 and mitogen-activated protein kinase (MAPK). TPO alone is able to trigger the differentiation of megakaryocyte as well as the HSCs maturation. However, it is not suitable for the production of wide range of megakaryocyte because of their quite low mitotic activity. Besides TPO activated pathways, there are also non-TPO pathways that effect megakaryopoiesis and thrombopoiesis. For instance; Notch signaling, stromal-derived factor-1 (SDF-1)/CXCR4, integrin signaling, src family kinase and platelet factor 4/low-density lipoprotein receptor-related protein 1, have been recently demonstrated both *in vitro* and *in vivo* (Pineault and Boisjoli 2015; Yu and Cantor 2012; Drachman et al. 1999; Gurney et al. 1995; Morita et al. 1996; Pallard et al. 1995; Rouyez et al. 1997; Sasaki et al. 1995; Sattler et al. 1995).

SDF-1 chemokine and its receptor CXCR4 have critical role in the trafficking and homing of hematopoietic precursor cells in the BM. Since

megakoryotic lineages express SDF-1 receptor CXCR4, SDF-1 provides megakaryopoiesis as well as the migration of megakaryocytes progenitors and mature megakaryocytes adhesion to endothelium (Wang et al. 1998). It is also stated that megakaryocyte-active chemokine such as SDF-1 and FGF-4 supports the relocation of progenitors inside the bone marrow microenvironment which is needed for maturation of megakaryocytes and thrombopoiesis (Avecilla et al. 2004; Broxmeyer 2001). Several reports showed that megakaryocytes as HSC-derived bone marrow niche cells modulate HSC quiescence by means of CXCL4 secretion (Wang et al. 1998) or TGF-β-SMAD signaling. However, it is also stated that FGF1 produced by megakaryocytes induces the expansion of HSC under stress condition (Bruns et al. 2014; Zhao et al. 2014).

Besides, Guo et al. demonstrated that c-Myc protein affects the fate of megakaryocyte-erythrocyte progenitors by controlling these progenitors differentiation ability. Mice lacking c-Myc showed increased megakaryocytopoiesis, lower polyploidy and blocked erythrocyte differentiation. Thus, the severe thrombocytosis-anemia-leukopenia syndrome was observed in mice from c-Myc (−/−) (Takayama et al. 2010; Guo et al. 2009). Another study showed that the transient expression of c-Myc plays an important role in both megakaryocyte polyploidization and platelet formation from iPSC (Takayama et al. 2010; Guo et al. 2009).

Since the megakaryocyte-derived platelets are generated from proplatelets through cytoplasmic extension, it is thoughout that the fragmentation of proplatet takes place when they come cross with biomechanical circulatory forces. Shear stress is one of the biomechanical forces known to enhance the formation of proplatet and platelet production inside the sinusoid vessels in bone marrow as well as the blood circulation. Once mature megakaryocytes expose to high shear stress on von Willebrand Factor (vWF), which is adhesive blood glycoprotein, platelet production is assisted through GPlb receptor and this is dependend on microtubules organization. However, ex vivo generation of megakaryocytes in static culture conditions shown to have a low rate of proplatelet formation when they are exposed to shear stress (Dunois-Larde et al. 2009).

Stimulating factors such as interleukin IL-3, IL-6, IL-9, IL-11 and stem cell factor (SCF) are important in megakaryopoiesis (Nurhayati et al. 2016). TPO, high-dose SCF, IL-3, IL-9, and IL-11 treatment are seem to be the favorable compination that supported Mk enlargement. While IL-3 is robustly increase total cell count and IL-9 supports expression of CD41 and CD42b. High-dose of SCF (100 ng/mL) induces Mk production and ploidy (Panuganti et al. 2013).

Several small molecules are shown to enhance Mks production and ploidy (Huang et al. 2016). Src kinase inhibitor (SU6656), Rho-associated kinase inhibitor (Y27632), Aurora B kinase inhibitor (AZD1152) can enhance functional platelet release *in vivo* (Jarocha et al. 2018). During the megakaryocyte polyploidization, endomitosis could be blocked by cytokinesis inhibitors. For this purpose, Zou et al. used four small molecules; Rho-Rock inhibitor (RRI, Y27632), nicotinamide (NIC, vitamin B3), Src inhibitor (SI, Su6656), and Aurora B kinase inhibitor (ABI, ZM447439) as cytokinesis inhibitors in their study. They found that SI (Src inhibitor) and ABI (Aurora B inhibitor) could increase significantly the percentage of polyploidy both in leukemic cells and primary mononuclear cells (Zou et al. 2017). Although ABI induces cell death and apoptosis, it could be used as Mks differentiation supplement. They also found that combination of RRI (Rho-Rock inhibitor) and SI could increase the expression of CD61 and enhance the polyploidy level without a statistically significant cell apoptosis. These indicated that RRI and SI were the suggested small molecule combination for Mk polyploidization (Zou et al. 2017).

Transcription factors such as GATA binding protein 1 (GATA1), RUNt-related transcription factor 1 (RUNX1), Friend leukemia integration 1 (FLI1), T-cell acute lymphocytic leukemia

protein 1 (TAL-1) have crucial role in Mk lineage commitment (Goldfarb 2009; Tijssen and Ghevaert 2013; Moreau et al. 2016). GATA1 plays a fundamental role in megakaryocyte lineage and regulation of polyploidization through its downstream effector stromal-derived factor-1 (STAT1). In addition, RUNX1 and nuclear factor erythroid 2 (NF-E2) genes are critical for terminal megakaryocyte differentiation (Bluteau et al. 2009; Italiano et al. 2007). In their study, Matsubara et al. suggested that p45NF-E2 has a crucial role in the generation of Mks and platelets from OP9 cells (Matsubara et al. 2013).

Myeloid ectopic viral integration site 1 homolog (Meis1) has been implicated as an important regulator of hPSC early hematopoietic differentiation. It was reported that Meis1 deletion disrupts the generation of megakaryocytes *in vivo* (Azcoitia et al. 2005; Carramolino et al. 2010; González-Lázaro et al. 2014; Hisa et al. 2004). Embryonic stem cell (ESC) derived hematopoietic cells study suggested that Meis1 represses erythroid development at the MEP stage for the benefit of megakaryocyte development (Cai et al. 2012).Miller et al. also showed that, Meis1 play important role in the erythroid and megakaryocytic compartments (Miller et al. 2016). In parallel with these studies, Wang et al. demostrated that Meis1 is vital for DMS development and polyploidization during megakaryocyte maturation (Wang et al. 2018).

Studies suggested that microenvironment in the maintanance of HSC pool and their differentiation to megakaryocyte are crucial. Mostafa et al. demonstrated that differential effects of pO2 on Mks progenitor enlargement, differentiation and maturation (Mostafa et al. 2000).Yang et el. also showed that higher pH supports Mk-cell differentiation, maturation, and apoptosis (Yang et al. 2002). Proulx et al. demostrated that cord blood CD34+ cell cultures incubated under mild hyperthermia (i.e. 39 °C) had accelerated and increased megakaryocyte differentiation and maturation over those maintained at 37 °C. Their study showed that mild hyperthermia had little impact on polyploidization and that transient early incubation at 39 °C is sufficient to increase megakaryocyte differentiation (Proulx et al. 2004). In addition, mild hyperthermia could accelerate and enhance Mk differentiation (Pineault et al. 2008). This was not because of cell-secreted factors but could be mediated by the enhanced expression of Mk transcription factors.

2.3 Surface Markers of Mk Differentiation and Platelet Formation

Mk lineage and maturation can be associated with many flow cytometric markers such as CD41 (GPIIb/IIIa), CD42b (GPIb), CD42a, PAC-1, CD62P, vWF, glycoprotein V (GPV) and Annexin V (Nurhayati et al. 2016; Jarocha et al. 2018). While expression of CD34 is gradually decrease during megakaryopoiesis, expression of CD41 (GPIIb/IIIa) increase in megakaryoblast stage. CD41 is a considerable marker for Mks (Nurhayati et al. 2016). In addition, Mori et al. identified CD71 + CD105+ (erythroid progenitor marker) unipotent erythroid progenitors within MEP, confirming that the majority of human MEPs (about 85%) are committed to the erythroid lineage (Mori et al. 2015). Specific markers of Mk maturation are GPV, vWF and CD42b (GPIb) (Nurhayati et al. 2016).

3 Sources of Artificial Platelet Production *In Vitro*

Since the first report by Choi et al., many researchers also have demonstrated human megakaryocytes and platelets could be generated *in vitro* from CD34+ peripheral blood progenitor cell as well as from umbilical cord blood (UCB) (Choi et al. 1995; Tao et al. 1999; Avanzi et al. 2012; Pineault et al. 2013), fetal liver (Ma et al. 2000), peripheral blood (PB) (De Bruyn et al. 2005), human embryonic stem cells (hESCs) (Gaur et al. 2006; Takayama et al. 2008), human induced pluripotent stem cells (hiPSCs) (Gekas and Graf 2010; Takahashi et al. 2007), or BM (Wang and Zheng 2016; Guerriero et al. 1995;

van den Oudenrijn et al. 2000). Although platelet products can be obtained by using these sources, obtained platelet product is not sufficient for clinical use. All these strategies are only proof of principle that platelets may in fact be generated *ex vivo* from stem cells (Avanzi and Mitchell 2014).

In vitro platelet studies have gained momentum in recent years. In these studies, researchers utilized umbilical cord bood, human induced pluripotent stem cells, adipose–derived stem cells (ADSC), human embryonic stem cell, and human CD34+ cells as starting cells for platelet production.

3.1 UCB as Starting Source of Cells for Platelet Production

Despite their similar membrane phenotype, UCB-Mks indicated reduced polyploidization and platelet number compared to PB-Mks. In addition, UCB-Mk has a low 8 N percentage compared to PB-Mks (Mattia et al. 2002). Matsunaga et al. cultured 500 UCB CD34+ cells on telomerase gene-transduced human stromal cells (hTERT stroma) in serum-free medium supplemented with SCF, Flt-3/Flk-2 ligand (FL), and TPO. With a three-stage culture system, they have demostrated that UCB-platelets have similar morphological features with PB-platelets (Matsunaga et al. 2006). De Bruyn et al. cultured UCB with TPO, FL, IL-6, and IL-11 and they obtained a suitable number of immature Mk progenitor cells expressing both CD34 and CD41 antigens (De Bruyn et al. 2005). Studies have shown that UCB-Mks are significantly smaller than those CD34+ PB and BM (Ignatz et al. 2007).

3.2 Use of hiPSCs in Platelet Production

In recent years, because of their availability and low ethical concerns, iPSCs are accepted as the most chosen cell source for *in vitro* platelet generation. A number of research groups have investigated that immortalized iPSC-Mks could be platelet source. Takayama et al. successfully generate platelets from hiPSC clones derived from human dermal fibroblasts (HDFs). The in vitro and in vivo functionality of these platelets had shown in the NOG (nod-scid/IL-2 γc-null) mouse thrombocytopenia model (Takayama et al. 2010; Nakamura et al. 2014). Takayama and colleagues co-cultured undifferentiated hESCs or hiPSCs on either mesenchymal C3H10T1/2 cells or OP-9 stromal cells that support differentiation of hematopoietic niche cells that contains hematopoietic progenitor cells (HPC), which are named "embryonic stem cell-derived sacs" (ES-sacs). They have developed *in vitro* culture system for differentiation of HPC into mature megakaryocytes with the ability to deliver platelets (Takayama and Eto 2012). Nakamura et al. have demonstrated lentivirus-based gene dosage manipulation can be used to immortalize iPSC-megakaryocyte lines. These lines could be grown for extended months and retained megakaryocyte maturation potential when the transgenes Bmi-1 and c-Myc were switched off. Maturing megakaryocytes released platelet with normal function *in vitro* assays and could participate formation of thrombus in a murine model (Nakamura et al. 2014). Feng et al. could managed to generate universal platelets from hiPSCs in serum and animal feeders free conditions (Feng et al. 2014). Other studies supported this view and showed that hiPSCs are potentially source of Mks, which can produce human platelets look alike peripheral blood (Moreau et al. 2016; Nakamura et al. 2014; Feng et al. 2014). The main benefit of hiPSCs will be the possibility to produce patient specific hiPSCs or genetically engineered cells lacking HLA antigens able to generate platelets. In this manner, when recipients are transfused with such transfusion products, they could escape immune response (Hod and Schwartz 2008). For this purpose, Figueiredo and colleagues recently reported that the *ex vivo* production of low expression of human leucocyte antigen (HLA) platelets from adult HSCs resulting in low-immunogenic response. These platelet could be used preferentially in alloimmunized patients or in patients

required frequent platelet transfusions to reduce platelet transfusion refractoriness due to alloimmunization (Figueiredo et al. 2010). On the other hand, few studies showed that iPSC-Mks are tend to be smaller, and have lower ploidy, and release fewer platelets with a short half-life when infused into mice (Bluteau et al. 2013; Potts et al. 2014).

In another study human dermal fibroblasts were transdifferentiated within about 17 days into Mks by the overexpression of *p45NF-E2, Maf G* and *Maf K* genes in the induction medium (Ono et al. 2012). CD41+ polyploid iMks were morphologically similar to BM-Mks and had ability to produce CD41+ platelet-like particles after infusion into immunodeficient mice. Tail-vein blood samples were collected from recipient mice before and after iMks infusion. The rate of human CD41 positive platelet increased in a time-dependent manner.

IPSC-platelet technologies still have several bariers to overcome such as need for robost HSC expansion and output of low number of megakaryocyte progenitors. Thus, several research groups have investigated the molecular method to improve development of iPSC- HSCs.

3.3 ADSC for Platelet Production

Adipose tissues containing abundant of multipotent preadipocytes are able to differentiate to adipocytes, osteoblasts and chondrocytes. Matsubara and her colleagues have been conducted the pioneer study on the generation of megakaryocytes and platelets from human adipocyte precursor cells in vitro (Matsubara et al. 2009). Ono-Uruga et al. showed that adipose tissue-derived stromal cells (ASCs) are able to induce megakaryocytes and platelets differentiation through endogeneous TPO secretion. Thus, ADSCs could be utilized as an alternative cell source for the platelets production and further clinical application (Ono-Uruga et al. 2016).

3.4 hESCs as a Source of Platelets

hESCs are appealing alternative source to achieve megakaryocytes maturation and platelets generation due to their promising well-known features being able to differentiate into all hematopoietic lineages for studying normal hematopoiesis, thrombopoiesis as well as disease conditions.

Eto et al. obtained sufficient amount of large, polyploidy and functional Mks from ES cells when cocultured with OP9 stromal cells suppelemented with TPO, IL-6, and IL-11 cytokines (Eto et al. 2002). Gaur et al. developed a trackable system benefited from genetic approaches to study megakaryocytopoiesis and the function of integrin. This system included coculturing with OP9 stromal cells to differentiate ESC to megakaryocytes. hESC-Mks were able to produce platelet-like particles and sharing similar features in ultrastructure, morphology, and functionality in comparision to blood platelets (Gaur et al. 2006). Additionaly, Takayama et al. established a unique method for culturing cells within the ES-sacs, a structure that provided a suitable microenvironment for hematopoietic progenitor differentiation. In these ES-sacs, hESC cultured with OP-9 cells or C3H01T1/2 stromal cells and further supported with exogeneous VEGF. It is shown that sufficient numbers of mature megakaryocytes could be obtained inside this ES-sacs and they have ability to release platelets which demostrate the activation of integrin alpha IIb beta 3 and extension against to ADP and thrombin (Takayama et al. 2008). Large scale of functional megakaryocyte and platelet production from hESC has been also studied by Lu et al. (Lu et al. 2011). In this study, morphological properties and structural features of hESC-platelets showed similarity with normal blood platelets after analysis with the differential-interference contrast and electron microscopy. Upon thrombin stimulation, hESC-platelets showed functional characteristics such as

microaggregation, enhanced clot formation/retraction, lamellipodia and flopodia formation as well as the linkage between them. hESC derived platelets takes part in the thrombi progression in mice carrying laser-induced vascular injury, which is similarly observed in human blood platelets. These findings indicated that hESC-platelets could be utilized for the platelet transfusion (Lu et al. 2011).

3.5 Human Hematopoietic CD34+ Cells as a Starting Material for Platelet Production

Since megakaryocytes are HSC derived bone marrow niche cells, HSCs are the most widely used cell types for *in vitro* platelet production. Several studies reported the feasible methods to generate functional megakaryocytes and platelets from hematopoietic stem and progenitor cells.

Huang N et al. have isolated human $CD34^+$ cells from CB or BM samples in vitro cultured using StemSpan SFEM media containing cytokines and tested the TGF-β pathway 616,452 inhibitor on the polyploidization and megakaryocyte maturation. It is shown that this small molecule is able to increase polyploidization greater as 64 N. Both CB and adult mobilized PB megakaryocytes size and ploidy were enhanced when treated with this small molecule in a dose-dependent manner. Although attenuated maturation of Mks derived from CB post treatment, platelets production was observed post small molecule treatment in mice transplanting CB CD34+ cells (Huang et al. 2016).

Mattia G et al. compared the ability of CB and PB derived human CD34+ cells to generate Mk and platalets. While CB derived Mks decreased polyploidization and the number of platelet in comparision to PB derived Mks, they both share similar membrane phenotype. Besides, enhanced polyploidization and high number of released platelets were determined compared to CB-Mks (Mattia et al. 2002).

Although they are originated from HSC and other benefits being natural sources, there ara some limitation of platelet production from CD34+ progenitor/stem cells in a large scale such as donor dependency, found in a small quantity and limited capacity of proliferation (Baigger et al. 2017) (Table 1).

4 Recent Breakthroughs in the Biogenesis of Platelet Production

4.1 Megakaryocyte Polyploidization and Associated Platelet Formation

Polyploidy can be occur in plant and animal cells, as a result of mitotic cycle remaining in different levels or doubling of chromosome sets (Winkelmann et al. 1987). Importance of megakaryocyte maturity and the polyploidization (endomitosis) has been shown in the number of platelets released per cell. It is known that megakaryocytes form up to 128 N polyploidy in healthy bone marrow compated to 16 N ploidy *in vitro* cell culture systems. Most of cells stay at the 2 N and 4 N stage after tissue culture (Mattia et al. 2002). Beside cultured megakaryocytes have lower ploidy they are also smaller and less granular than primary megakaryocytes (Takayama et al. 2010). Different strategies have been tested to increase ploidy of *in vitro* grown megakaryocytes.

Mattia G et al. found that there is a relationship between different levels of platelet release and different ploidy levels of megakaryocytes (Mattia et al. 2002). In their study CB-Mks showed reduced polyploidization and platelet number compared with PB-Mks, but they have same membrane phenotype. Most CB-Mks showed a 2 N DNA content (∼80%) and only 2.6% had

Table 1 Megakaryocyte differentiation using different protocols

Cell Source	Serum	Culture Components	Mk/platelet yield	References
UCB CD34+ HSCs	No	StemSpan SFEM, FBS, Pen Strep, SCF, TPO, and Flt-3 ligand, SCF, TPO, IL-3, and 616,452	Greater ploidy	Huang et al. (2016)
UCB CD34+ HSCs Coculture with hTERT stroma	No	SCF, Flt-3/Flk-2 ligand (FL), IL-11 TPO	$1{,}26x10^{11}$–$1{,}68 \times 10^{11}$ platelets	Matsunaga et al. (2006)
UCB CD34+ HSCs	No	TPO, FL, SCF, IL-3, IL-6, IL-11. Growth factors (T, FT, ST, SFT, S3FT, 611 T, F611 T) were added once at the start of the culture.	9.9 ± 1.9 UCB (8 N)	De Bruyn et al. (2005)
iPSCs	No	MK maturation medium (MK-M), STEM Span-ACF + TPO, SCF, IL-6, IL-9 and heparin	2.06×10^9 (MKPs)	Feng et al. (2014)
hESCs	Yes	DMEM high glucose, L-glutamine, penicillin/streptomycin, FBS	$5–20 \times 10^3$	Gaur et al. (2006)
Co-culture with OP9			$1–4 \times 10^4$ (h TPO)	
hESC-MA09 cell lines	No	Stemline II supplemented with TPO, SCF and IL-11	6×10^8	Lu et al. (2011)
CD34+ HSCs	No	StemSpan SFEM, TPO, IL3, IL6, SCF, FMS-related tyrosine kinase 3	1.0×10^6	Ivetic et al. (2016)
CD34+ CD38lo BM HSCs	No	Serum-free X-vivo 10 medium, IL-3, IL-6, SCF, and TPO	5.2 ± 0.2% ≥ 8 N	Shim et al. (2004)
MPB HSCs (Mobilized Peripheral Blood)	No	Serum-free medium, SCF, FL, IL3, IL6, IL11, and TPO	11.4 ± 1.4 MPB (8 N)	De Bruyn et al. (2005)
hADSCs	No	Mk lineage induction (MKLI) medium + human rTPO	62,000 ± 8,400 MKs from 4×10^5 CD71+	Ono-Uruga et al. (2016)
(3 T3-L1 and OP9)				
Adipose tissue	Yes	1)Preadipocyte growth medium-bullet kit	2×10^6 ± 2,500 and 15×10^4 ± 270 Mks and platelets	Matsubara et al. (2009)
		2)TPO media		
		3)DMEM, L-glutamine, Pen/Strep, bovine serum albumin, LDL cholesterol, iron-saturated transferrin, insulin, mercaptoethanol, nucleotide, dNTP, and TPO		

8 N, however 40% of the PB cells had 8 N or more. Platelets were substantially released in PB culture from day 12; at day 14 the CB-Mks were able to release platelets although at a reduced level (∼35%), correlating with their reduced size (Mattia et al. 2002).

During polyploidization of Mks, D-type cyclins are found to be important regulators of G1/S progression (Sherr and Roberts 1999). Sun et al. reported that transgenic mice overexpressing cyclin D1 in Mks displayed higher ploidy than did control mice (Sun et al. 2001). Several researchers used small molecules to promote megakaryocyte polyploidization (Zhang et al. 2004; Lannutti et al. 2005; Giammona et al. 2006). Studies show that hESC-Mks or iPSC- Mks have lower ploidy than in bone marrow, hESC-Mk has maximum ploidy of 126 N and iPSC-Mk has 16 N (Takayama et al. 2010; Giammona et al. 2009). Avanzi et al. found that megakaryocytes treated with a myosin inhibitor both presented a higher level of polyploidization and a higher number of proplatelet formation released in culture (Avanzi and Mitchell 2014).

4.2 Lung as a Major Organ for Platelet Formation

Platelets play key roles in hemoostasis, thrombosis and immune system. There have been recent

and major discoveries on the formation and maturation of platelets in various tissues and organs, however, the exact mechanism has not been completely understood yet. Megakaryocytes are produced in the bone marrow where they are released as proplatelet. Beside this, megakaryocytes were largely located in the lung in a number of early studies (Davis et al. 1997; Levine et al. 1993) but their role and relation to lung microenvironment were unknown. The contribution of lungs to platelet biogenesis were thought to be substantial with approximately half of total platelet production or ten million platelets per hour (Alexander et al. 1996). It was also estimated that each megakaryocyte reaching the lungs produces about 10^4 platelets. Recent studies provided major findings and in vivo quantifiacations which were supporting that the lung may have a major role in platelet formation.

To this end, researchers utilized the ex vivo cultured murine megakaryocytes and infused them into recipient mice. These infused megakaryocytes were shown to locate at pulmonary vasculate, which is the trapped site where they release platalets. They also found that increased platelet numbers approximately 100-fold in recipient mice post infusing ex vivo cultured megakaryocytes. While these released platalets in recipient mice showed slightly comparable half-life to infused platalets, other functional properties such as surface marker expression, size seemed to normal characteristic (Fuentes et al. 2010). In parallel to these, one study conducted on the lung-damaged rats revealed that the level of circulating platelets decreased by this injury. Therefore, this highlihts the pivotal role of lung in the Mk maintance and platelet formation (Machlus and Italiano 2013).

One of the most intriguint studies on the lung as a site for platelet formation in recent years was that of Lefrançais et al. (2017). This study demonstrated the lung as a fundamental site of platelet release from megakaryocytes in mice by video microscopy technology. In addition, Looney (2018) used lung intravital microscopy with fluorescently labeled mouse strains, he observed platelets released from megakaryocytes primarily in lung circulation (Looney and Headley 2018; Looney 2018). In addition, peripheral blood platelet count and bone marrow hematopoietic progenitor regeneration were observed by single lung transplantation to animals lacking thrombocytopenic and hematopoietic progenitors. These studies also showed that lung is reservoir for hematopoietic progenitors. All together, lung appeared as one of the key modulator and terminal production site for platelets.

4.3 Developments in Microfluidic Chips and Bioreactors Designed for Platelet Production

Recently, developments in microfluidic chip and bioreactor desing have gained popularity in the human platelet production. A number of research groups have used different materials to design effective bioreactors and microfluidic systems like platelet on-a-chip.

These systems are based on the mimicking the physiology of bone marrow niche by using bioengineered and biocompatible materials in order to generate platelets in a culture condition. Upon coating stem/progenitor cells within biomaterial, functional Mks make an extension to form proplatelet within perfused culture medium which mimicks the blood flow. The release of platelet in a culture condition can be mediated by flow rate using controlled electronic pump. These systems can be design not only implementing shear stress but also stiffness, surface topography, coculture and extracellular matrix component. Various microfluidic models combined with different biomaterials have been implemented to improve the ex vivo platelet production. This includes but not limited to microfluidic device consisting of micropillars coated with VWF (Blin et al. 2016), polydimethylsiloxane (PDM) bioreactor for platelet on-a-chip system (Thon et al. 2014), bioengineered 3D silk films on PDM (Di Buduo et al. 2015) and bioreactor containing PDM within 2D flow system to yield Mks from hESC or iPCs (Nakagawa et al. 2013).

Sullenbarger et al. developed a woven polyester fabric suited to a 3-dimensional continuous-perfusion bioreactor. With this bioreactor,

they collected platelets for over 30 day of culture (Sullenbarger et al. 2009) and resulted in 300 functional platelets released per starting CD34+ cell after 30 day of culture (Lasky and Sullenbarger 2011). Thon et al. was designed a biomimetic microfluidic bioreactor whice had microchannel size, ECM composition, BM stiffness, endothelial cell contact, and hemodynamic vascular shear stress within a single platform device (Thon et al. 2014). They were isolated hiPSC-Mks and were replaced in designed bioreactor. hiPSC-Mks began producing proplatelets at 6 h post-isolation and reached maximal proplatelet production at 18 h in static culture. After 15 day of culture, Mks with 20–60 mm of diameters were generated, which were ultrastructurally indistinguishable from human blood platelets by electron microscopy. By the end of this study, they were managed to produce functional human platelets for infusion (Thon et al. 2014).

Blin et al. developed microfluidic bioreactor containing wide array of vWF-coated micropillars to produce platelets from cultured megakaryocytes. These micropillars act as anchors on megakaryocytes, letting them to remain trapped in the bioreactor and exposed to hydrodynamic shear. Together effect of anchoring and shear caused the elongation of megakaryocytes and lastly they broke into platelets and proplatelets. Microfluidic device could produce large amount of platelets and their biological characterisation were functional (Blin et al. 2016).

Di Buduo et al. engineered a silk sponge containing 3D structure model closely mimicking the "spongy" marrow in bone and further combined with ECM components and fludic shear rate. This bioreactor setup, which is taking advantages of BM modeling maintained the Mk function and platelet production inside perfused vascular tube lumen (Di Buduo et al. 2015). Due to the functional characteristic of silk protein including weak immunogenicity, non-toxicity and low thrombogenicity, it is favorable to be a substitute of blood vessel. When silk protein was integrated with characteristic ECM constituents, Mks changed their characteristic behavior according to recognition of these proteins. Therefore, silk provides suitable system in order to reconstitute all BM properties that yield the generation of platelet (Di Buduo et al. 2017).

The bone marrow possesses highly dynamic structure harboring different cell types such as hematopoietic stem cells, fibroblast, osteoblast, osteoclast, adipose cells, endothelial cells so on so forth. This heteregenous cell populations in the niche give rise to the special connection and mechanism between bone and blood formation (Morrison and Scadden 2014). It is also indicated in several studies that the important role of vascular microenvironment components in contact with other cell types for the regulation of hematopoiesis (Arai et al. 2004; Butler et al. 2010; Ding et al. 2012; Doan et al. 2013; Kunisaki et al. 2013; Wang and Wagers 2011). In addition to bone marrow endothelial cells constructing the blood vessels network, it contributes the hematopoietic stem cells maintance (Wang and Wagers 2011; Gori et al. 2015; Itkin et al. 2016) as well as the thrombopoiesis. Kotha et al. developed 3D structure mimicking human vascular microenvironment (VME) in order to determine the function of marrow vasculature in the hematopoietic and thrombopoietic mechanistic. It is reported that thrombopoietic VME is constructed with microvessels which contains collagen matrix by seeding differentiated megakaryocytes from HSPCs in culture condition. This system successfully supplies the megakaryocytes migration into the wall of vessel, maturation as well as the platelets release into lumen. This interaction and recent findings correspond to the behavior of megakaryocytes in vivo (Kotha et al. 2018). Furthermore, Nakagawa et al. designed artificial blood vessels mimicking bone marrow in vivo. In this designed biomimetric system, flow culture system is structured with two flows coming from different directions. This provides favourable shear stress and pressure condition to be enforced

on Mks to generate plateletes in vitro from pluripotent stem cells. Moreover, this bioreactor sytem allows real time monitoring of the Mks that are derived from pluripotent stem cells during the time of cell culture (Nakagawa et al. 2013).

5 Artificial Erythroid Transfusion Product Technologies

5.1 Erythropoiesis

The one of the most common cells found in an adult individual are erythrocytes. Human blood contains ~5 × 10^6 erythrocytes per microliter (normal range 4.7 × 10^6–6.1 × 10^6 for males and 4.2 × 10^6–5.4 × 10^6 for females); erythrocytes have an average life span of 120 days. Erythrocytes provide oxygen distribution in our body and are essential in all stages of our life (embryonic, fetal, neonatal, adolescent, and adult). The earliest production of erythroid cells is observed in the yolk sac (Belaoussoff et al. 1998). These early erythroid cells are nucleated and mesodermal origin. Mesodermal cells migrate to yolk sac and have close relationships with endodermal cells. Very early erythropoiesis has been initiated as a result of the interaction of these two cell layers. Erythropoiesis is the name of a process in which red blood cells are produced (Malik et al. 2013; Suzuki et al. 2015). Erythropoiesis covert HSCs eventually into mature red blood cell. Many stages such as cell proliferation, apoptosis, autophagy, and cell differentiation take place in a balanced way during the erythrocyte formation. During erythropoiesis CMPs turn into MEPs. It is rapidly divide in to burst forming unit erythroid (BFU-E), which is known as immature erythroid-restricted progenitors. More mature erythroid progenitors, colony forming unit erythroid (CFU-E) and proerythroblasts (ProE), erythroblasts (EB), reticulocytes (Retic), and mature RBCs are formed respectively (Fig. 3).

5.2 Erythropoiesis Stimulating Factors

Several technologies have been developed for the formation and growth of erythroblasts in liquid

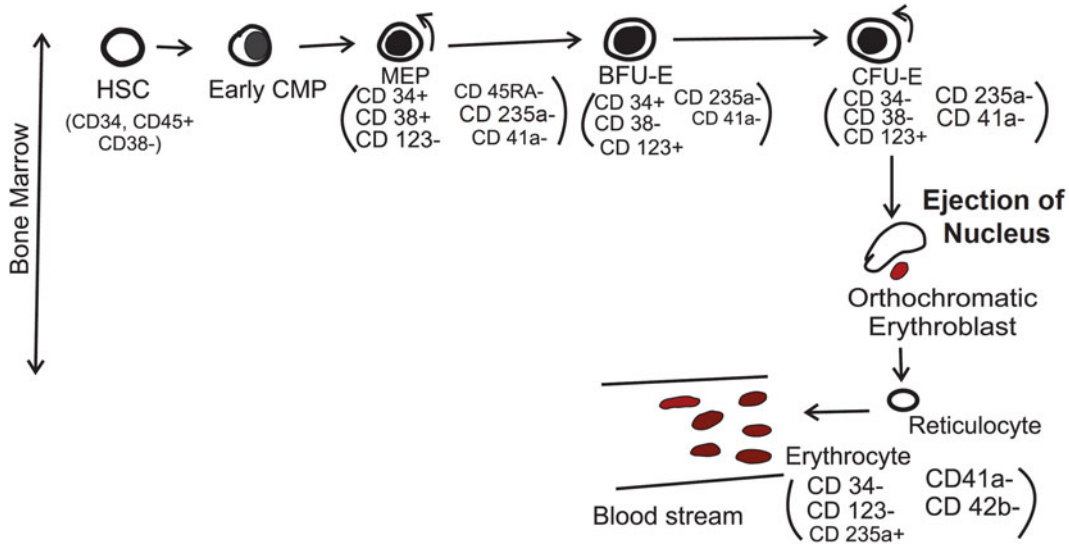


Fig. 3 Overview of biological phases of erythropoiesis. During Eryhtropoeisis HSC differentiate in a mature erythrocyte. Common myeloid progenitors (CMP) differentiate into megakaryocyte erythroid progenitor (MEP), burst forming unit erythroid (BFU-E), colony forming unit erythroid (CFU-E), erythroblasts (EB), reticulocytes and mature erythrocyte

Table 2 Summary of ETP development studies

Cell source	Components	Approach	Analysis/results	References
CD34 + HSCs are	SCF, Flt3L, Tpo, IL3, and HDAC inhibitors VPA, TSA and SAHA	HDAC inhibitors were used for differentiation of erythroid progenitors	NOD/SCID mouse model used to show functionality of erythrocytes	Chaurasia et al. (2011)
CD34 + HSCs	SCF, EPO, IL3, VEGF, IGF-II	Different growth factors and cytokines used for stimulation of enucleation	*In vitro* studies without feeder cells. Enucleation was shown	Miharada et al. (2006)
CD34 + HSCs	Transferin, insulin, hydrokortison, SCF, IL3, EPO	Differentiation to erythrocytes by growth factors and cytokines	Large scale, 100% terminal and functional differentiation to erythrocytes	Giarratana et al. (2005)
CD34 + HSCs	Transferin, ferric sulfate, ferric nitrate, insulin, lipids, hydrocortison, SCF, IGF-I	Differentiation to erythrocytes by growth factors and cytokines	*Ex vivo* human erythrocyte production on a large scale it was performed. *In vitro* and *in vivo* verifications were performed	Neildez-Nguyen et al. (2002)
CD34 + HSCs	SCF, IL3, EPO, Transferin, Insulin, vitamin C, ferrous nitrate, cholesterol,	Both bone marrow and cord blood erythroid transfusion products were developed from HSCs	It has been shown that there are functional erythrocyte differentiation using morphological, phenotypic, and biochemical analysis methods	Jin et al. (2014)
CD34 + HSCs	Transferin, ferric sulfate, ferric nitrate, insulin, SCF, IL3, EPO, hydrokortison	They demonstrated that red blood cell production can be performed *in vitro* using peripheral HSCs	*In vitro* flow cytometry analysis, cell cycle erythrocyte differentiation with analyzes are shown	Boehm et al. (2009)

culture media. The culture conditions used in these techniques varies but stem cell factor (SCF), erythropoietin (Epo), dexamethasone (Dex), and transferrin are commonly present, usually supplemented by insulin or IGF-1 (Table 2) (Dzierzak and Philipsen 2013). The stem cells in the bone marrow are transformed into BFU-E and CFU-E cells, then into the pro-erythroblasts under the influence of cytokines such as IL3, SCF and IGF1. With the help of *Epo*, they are transformed into basophilic erythroid cells. Epo plays an important role in the differentiation of polychromatic and orthochromatic erythroid cells. Transcription factors like PU.1, GATA2, TAL2, GFI-1B, STATs are effective in transforming them into reticulocyte cells. In addition, signal transduction factors such as EpoR, Jak2, and GAB1/2 also are differentially expressed in these stages and play an important role in the formation of mature erythrocyte. Epo, is a hormone that plays a very major role in erythropoiesis (Malik et al. 2013). Epo glycoprotein of 34 kDa interacts with the Epo receptor and regulates the growth, differentiation and viability of erythroid progenitors relative to the low oxygen content. Epo is mainly produced by a hypoxia-induced mechanism from Epo-producing renal cells in the kidney. Therefore, Epo production in any chronic kidney disease decreases and causes complications. In such cases, the recombinant human Epo protein may be used in the treatment of anemia resulting from Epo deficiency. Epo injections reduce the need for blood transfusions and improve the quality of life of patients. However, it was observed that anti-Epo antibodies were formed after continuous and high doses of recombinant Epo injections (Janda et al. 2010). Therefore, it has been predicted that strategies should be developed in the treatment of different anemias. The effect of Epo protein is achieved by binding to Epo receptor (EpoR). Epo knockout mice with EpoR knockout mice show the same anemia phenotype and cause embryonic death with advanced anemia. The two EpoR proteins undergo a conformational change and activate JAK2, and signal

molecules SHP1, SHP2, Grb2, and STAT5 (Suzuki et al. 2015; Ravasi et al. 2010). STAT5 is found to be required during fetal development to for evelevated rate of erythropoietis (Socolovsky et al. 1999).

5.3 Surface Markers in Erythropoiesis

Various cell surface markers have been utilized in the characterization of erythropoiesis. Xi et al. demonstrated the erythroid cells derived from CB HSCs by analyzing the different set of surface marker, which are CD3, CD4, CD8 CD13, CD14, CD19, CD33, CD34, CD38, CD41, CD45, CD71, CD117 and glyccophorin A expression using flow cytometry. CB HSCs cultured over 50 days in a condition supporting erythroid differentiation. During this period, CD71+, CD117+ and GPA+ cell populations were 95%, 90% and 15% at the day of 21, respectively. In addition, cell surface antigens belonging to myelomonocytic, megakaryocytic and lymphatic lienages showed quite low expression level such as 1% for CD14, 2.4% for CD41, 3% for CD4, 2.5% CD8, 0.7% for CD34 and 0.5% for CD3 (Xi et al. 2013).

Some studies have shown that CD71 and Ter119 dual staining work in separating erythroid cells (Suzuki et al. 2015). For example, CD71 proeryroblasts and early basophilic erythroblasts were found to have high expression and decreased expression of cells as erythroid differentiation was observed. In addition, Ter119 is expressed in terminally differentiated erythroblasts. $CD71^{high}Ter119^{high}$ cells (basophilic erythroblasts), $CD71^{high}Ter119^{low}$ cells (proeryroblasts), and $CD71^{low}Ter119^{high}$ cells (orthochromatic erythroblasts) was characterized at different times with flow cytometry (day 1, 4. day, 7th day, 14th day, 21st day) (Suzuki et al. 2015). Proeryroblasts were also characterized by $TER119^{+}CD44^{high}FSC^{high}$. In addition, $TER119^{+}CD44^{low}$ (reticulocytes, erythrocytes) and $TER119^{+}CD44^{high}FSC^{low}$ (erythroblasts) markers were also used.

6 The Importance of HSCs as Erythrocyte Source for *In Vitro* Artifical ETP Development

HSCs are characterized by self-renewal and ability to turn into all types of blood cells. HSCs are responsible for producing billions of mature blood cell lines daily throughout their adult life. HSCs are responsible for producing billions of mature blood cell lines daily throughout their adult life. While these cells form the basis of bone marrow transplantation, they are also promising for ETP studies. The success of HSC transplantation and transfusion products containing artificial erythroid progenitor depends on the presence of human leukocyte antigen (HLA) compatible donors and the ability to reproduce sufficient number of cells *in vitro* for a donor. Even if there are HLA-compatible donors, the lack of the number of HSCs obtained often reduces the success rate of erythroid progenitor *in vitro*. Therefore, an alternative method such as HSCs *in vitro* duplication should be developed to obtain a sufficient number of erythroid transfusion products (Nishino et al. 2011; Zheng et al. 2011; Maung and Horwitz 2019; Sniecinski and Seghatchian 2018; Schuster et al. 2012). It is possible to recognize and isolate human erythroid cells and HSCs surface antigens by flow cytometry. In addition, they can be treated *in vitro* with cytokines such as TPO, FL3, and SCF (Zheng et al. 2011). However, when erythroid growth factors are used, the use of small molecules targeting HSC silence factors is not common. In the studies conducted for the *in vitro* HSC replication, various problems have been encountered due to the loss of self-renewal ability in HSCs, increased differentiation, limited knowledge about HSC regulators (Choi and Harley 2016; Csaszar et al. 2013; Walasek et al. 2012; Knaan-Shanzer et al. 2008). Cytokines such as TPO, FL3, IL3, IL6, IL11, and SCF have been shown to amplify HSCs. Cytokines stimulate dormant (in G_0 phase) long-term HSCs to enter the cell cycle. It is believed that they are doing this by increasing self-renewal factors or by

suppressing cell cycle inhibitors. Interestingly, some inhibitory factors such as p38 have been shown to be increased and activated during *in vitro* replication. To this end, it was shown that use of p38 inhibitor increases the proliferation of HSC *in vitro* (Zou et al. 2012). In addition, Chaurasia et al. (2011) used HDAC inhibitors in combination with cytokine and valproic acid, and demonstrated that CD34 + cells could be expanded *in vitro* and turn into ETPs (Chaurasia et al. 2011; Broxmeyer 2014). Other inhibitors such as StemRegenin 1 (AhR antagonist), Garcinol (non-specific HAT inhibitor), Nicotinamide (SIRT1 inhibitor), and c-Myc inhibitor 10,074-G5 (Aksoz et al. 2018) are also shown to be effective for the growth of human and mouse HSCs (reviewed in (Yucel and Kocabas 2018)). In summary, studies involving loss of function of HSC quiescence genes showed a clear link between normal hematopoiesis, *ex vivo* and *in vivo* HSC proliferation.

7 Conclusions

Approaches to *ex vivo* thrombocyte production are promising technologies to be adapted to clinical practice. Low number of platelets obtained from stem cells, difficulties encountered during maturation, shelf life of platelets are the most important difficulties encountered in ex vivo PTP development. The demostration of lung as a major platelet releasing organ and on-going development of microfluidic chips and bioreactors designed for PTP technologies are the most important advanvements recently in the field. ETP studies have gained importance in recent years due to the increasing need for blood in the clinic and a limited number of healthy donors. The difficulties in PTP and ETP technologies have been partially overcome in the last decade. Making these artificial PTP and ETP technologies accesible to clinical practice will be a source of hope for patients in need of repeated platelets and blood transfusions.

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Advancing Mesenchymal Stem Cell Therapy with CRISPR/Cas9 for Clinical Trial Studies

Ali Golchin, Forough Shams, and Farshid Karami

Abstract

Currently, regenerative medicine and cellular-based therapy have been in the center of attention worldwide in advanced medical technology. Mesenchymal stem cell (MSC) as a suitable stem cell source for cell-based therapy has been shown to be safe and effective in multiple clinical trial studies (CTSs) of several diseases. Despite the advantages, MSC needs more investigation to enhance its therapeutic application. The CRISPR/Cas system is a novel technique for editing of genes that is being explored as a means to improve MSCs therapeutic usage. In this study, we review the recent studies that explore CRISPR potency in gene engineering of MSCs, which have great relevance in MSC-based therapies. However, CRISPR/Cas technology make possible specific targeting of loci in target genes, but next-generation MSC-based therapies to achieve extensive clinical application need dedicated efforts.

Keyword

Cell therapy · CRISPR · Gene editing · Mesenchymal stem cell

Abbreviations

CRISPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats-associated-9
crRNAs	CRISPR RNAs
CTSs	Clinical Trial Studies
DSBs	Double-Strand Breaks
hESC	human Embryonic Stem Cell
iPS	Induced Pluripotent Stem Cells
IVF	*in vitro* Fertilization
MSC	Mesenchymal Stem Cell
PAM	Proto-spacer Adjacent Motif
SCNT	Somatic-cell Nuclear Transfer
TALEN	Transcription Activator-like Effector Nucleases
tracrRNAs	Trans-activating crRNAs
ZNFs	Zinc-Finger nucleases

A. Golchin (✉)
Department of Biochemistry, School of Medicine, Urmia University of Medical Sciences, Urmia, Iran

Department of Tissue Engineering and Applied Cell Sciences, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
e-mail: agolchin.vet10@yahoo.com

F. Shams
Department of Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

F. Karami
Department of Tissue Engineering and Applied Cell Sciences, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

1 Introduction

Cellular therapy has been defined as using healthy and effective cells for therapeutic purposes. However, is placed cell and tissue-based therapy together to introduce a new field of medicine that is called regenerative medicine. Cell and tissue-based therapies involve the transplantation of cells, tissues or their products developed for the purpose of repairing and/or restoring the function of diseased or dysfunctional cells or tissues. Therefore, there are different types of cells that are the candidate for using in cell therapy approach. These different types of cells can be categorized into three main groups include in stem cells, somatic cells, and genetically engineered cells (Golchin and Farahany 2019). As the introduction of regenerative medicine, the unique characteristics and potency of various source of stem cells have drawn a great deal of attention with many promises in the field of cell-tissue based therapy (Golchin et al. 2019). Among different source of stem cells, mesenchymal stem cells (MSCs) due to their suitable features and accessibility have been more commonly used in cell-based therapy research and clinical applications (Golchin et al. 2018a).

Gene therapy, that provides an innovative treatment option, is defined as introducing genetic material into living cells to compensate for abnormal genes or to express a beneficial protein for treating or preventing of certain diseases (Kohlscheen et al. 2017; Golchin and Farahany 2019). Transfer of gene-corrected auto/allogeneic stem cells in some patients has emerged as a new therapeutic approach. As mentioned, MSCs are primordial, unspecialized and undifferentiated cells containing the potential of self-renewal through continuous cell division and differentiation into various other types of cells We discussed the MSCs underlying advantages and limitations and reviewed the genetically engineering guideline for clinical MSC therapy to improve their therapeutic efficacy in a separate study (Golchin et al. 2018b). In order to overcome the technical challenge of MSCs for therapeutic applications, gene engineering provides several gene editing systems include meganucleases, zinc-finger nucleases (ZNFs) system, Transcription activator-like effector nucleases (TALEN) system and clustered regularly interspaced short palindromic repeats (CRISPR)-associated-9 (CRISPR/Cas9 system). In this study, we focused on CRISPR/Cas9-engineered MSCs (Fig. 1) as a new and effective tool for developing cell-based therapy.

2 Clinical Advantages of MSCs in Regenerative Medicine

Mesenchymal stem cells (MSCs) are used in many types of research because of self-renewing and multipotent adult stem cells of mesodermal origin with a considerable potency to differentiate into several cell types like chondrocytes, adipocytes, osteoblasts, and other cell types (Ardeshirylajimi et al. 2014, 2017). As mesenchymal stem cells (MSCs) reside are placed mainly within the stromal portion of bone marrow, have multiple differentiation potentials under appropriate conditions (Shen et al. 2018). The International Society for Cellular Therapy (ISCT), as a global society with a shared vision to translate cellular therapy into safe and effective therapies, listed the minimum criteria and markers of MSCs that include (Dominici et al. 2006):

1. Plastic-adherent cells isolated from different tissues in the standard culture conditions;
2. Specific surface antigen (Ag) expression: positive expression for CD105, CD73, CD90, and negative for markers including CD45, CD34, CD14 or CD11b, CD79α, CD19, and HLA-DR;
3. In vitro differentiation into three cell types including osteoblasts, adipocytes, and chondrocytes.

There are several special advantages for MSCs in comparison other stem cells, for instance, lack of their ethical issue, easily accessible and isolated from different tissues (such as bone marrow (Friedenstein et al. 1987), adipose tissues

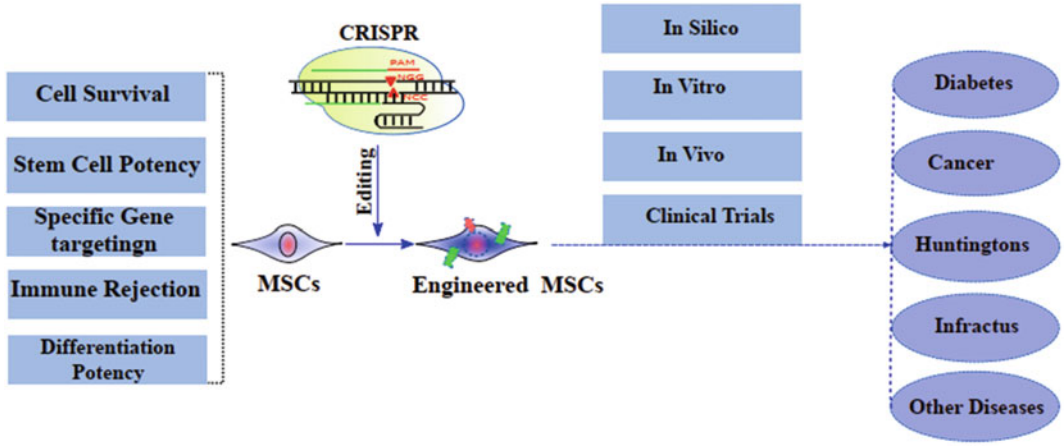

Fig. 1 Schematic illustration of cooperation between MSCs and CRISPR for improving MSC properties in stem cell-based therapy field

(Zuk et al. 2002), umbilical cord (McElreavey et al. 1991) and etc), suitable differentiation potential (differentiated to adipocytes, osteoblasts, chondrocytes, myoblasts, and etc. (Chamberlain et al. 2007)), good proliferation rate, and safety for clinical application (Golchin et al. 2018a). MSCs can migrate to the injury sites and carry out immune regulation, site-specific differentiation, support hematopoiesis (Ullah et al. 2015). Therefore, they are perfect candida ting cells in widely applied in experimental and clinical researches and gene engineering for regeneration of bone, heart, cartilage, central nervous, skin and so on that possessing a great application landscape in the field of tissue repair (Reiser et al. 2005).

As mentioned, MSCs are present in several tissues such as liver, skin, bone marrow, dental pulp, brain, adipose tissue and skeletal muscle and are associated in processes like immunosuppression and have an ability to migrate towards sites of tumors and inflammation zones. Hence, owing to their differentiation capabilities, easily isolation, and immunomodulatory features, the therapeutic potential of mesenchymal stem cells (MSCs) has been determined in many pre-clinical and clinical settings (Zhang et al. 2017). All of these reasons and most importantly high self-renewal potential makes them a great candidate for delivering genes and restituting organ systems function (Shen et al. 2018).

Alongside advantages of MSCs, there are several limitations that decrease the efficacy of therapeutic properties of MSCs. For instance, the low potency in biological (*in vivo*) condition comparison in vitro condition (Samsonraj et al. 2015), the low homing rate in the target site, insufficient expression of some factors and low cell viability after transplantation (Golchin et al. 2018b). Forasmuch as gene therapy and gene-engineering allow the addition of new functions to cells, this opportunity is provided to enhance MSCs features and applications.

3 Gene-Engineering for Stem Cell Therapy

In recent years, the appearance of varied genome-editing technologies has provided the ability to economically and rapidly introduce sequence-specific modifications into the genomes of a wide range of cell types for biologist and researchers (Gaj et al. 2016). For this purpose, different methods such as physical and chemical non-viral methods and viral vector-based methods are used to introduce target genes to MSCs.

- Non-viral method: Physical (Electroporation, microinjection, plasmid-injection, Ballistic injection) and Chemical (Liposome-based methods, calcium phosphate, DEAE dextran, protein-based methods)
- Viral methods: RNA virus (Retrovirus, HIV (lentivirus) and DNA virus (Adenovirus, Adeno-associated virus (AAV), Herpes simplex virus)

Genome editing with programmable nucleases has opened a new way for various applications from basic research in disease model via animal and cellular models to regenerative medicine and clinical trial studies (Barrangou et al. 2007). A series of studies showed that genome editing could greatly stimulate by targeted DNA double-strand breaks (DSBs). Till now for genome editing, four major classes of adjustable DNA-binding proteins have been engineered based on site-specific DNA DSBs: meganucleases or homing endonucleases obtained from microbial mobile genetic components, zinc finger (ZF) nucleases based on eukaryotic transcription factors, transcription activator-like effectors (TALEs) from Xanthomonas bacteria, and the RNA-guided DNA endonuclease Cas9 from the type II bacterial adaptive immune system CRISPR that found recently (Cong et al. 2013). Genome editing based on nuclease systems can be classified into two groups via their mode of DNA identification-TALEN, ZFN, and meganucleases attain specific DNA binding by protein-DNA interactions. While Cas9 is targeted to particular DNA sequences by a short RNA guide molecule and after that its targeting DNA, protein-DNA interactions that have an important role (Bayes-Genis et al. 2005). The modification of MSCs properties is necessary to fully use their potential. Gene-engineering with novel techniques to induce gene expression in a correct and considerable manner is particularly attractive for stem cell-based therapy purpose.

4 Meganucleases

The LAGLIDADG family is the largest class of homing endonucleases, which contains the well-characterized and generally used I-CreI and I-SceI enzymes. They are the smallest class of engineered nucleases with large (>14 bp) recognition sites that help them potentially amenable to all standard gene delivery methods (Bitinaite et al. 1998). Although many studies suggest using meganucleases in genome editing, an important problem was reported about cleavage domains of endonucleases and the DNA-binding that are difficult to separate. For solving this limitation, chimeric proteins consist of ZFs, meganucleases and TALEs have been engineered to generate novel monomeric enzymes. Formation of DSB by these enzymes results in a 3′ overhang that can be more recombinogenic for HDR than 5′ overhang generated by FokI cleavage and this is one of the meganuclease technology advantages. So, multiple meganuclease monomers could be wrapped into single viral vectors to make multiple DSBs simultaneously (Bitinaite et al. 1998).

5 Zinc Finger

Zinc finger (ZF) proteins are the large class of transcription factors and the Cys2-His2 zinc finger domain is one of the most current DNA-binding domains encoded in the human genome. By detection the independent function of the DNA-binding domain and the cleavage domain of the FokI restriction endonuclease, the zinc finger nuclease (ZFN) technology was made. As the FokI nuclease acting as a dimer, using two ZFNs binding opposite strands of DNA are needed for induction of a DSB. Since ZFN-induced DSBs were used to modify the genome through either NHEJ or HDR, this technology has been applied to modify genes in a pluripotent stem and human somatic cells

successfully (Sebastiano et al. 2011). One of the great concern connected with the use of ZFNs for genome editing is off-target mutations (Koo et al. 2015).

6 TALEN

The development of TALEN system is associated with the study of the Xanthomonas genus bacteria that secrete effector proteins (transcription activator-like effectors) via capable of DNA binding and activating the expression of their target genes by mimicking the eukaryotic transcription factors (Hockemeyer et al. 2011). Like ZFNs, TALENs consist of individual modules targeting 3 or 1 nucleotides (nt) of DNA, respectively. Also like ZFNs, TALENs are modular in form and dimerization of TALEN proteins is mediated by the FokI cleavage domain, which cuts within a 12- to 19-bp spacer sequence that detaches each TALE binding site. The DNA-binding domain was indicated to contain monomers; each of them binds one nucleotide in the target nucleotide sequence. Monomers are tandem repeats of 34 amino acid residues, two of which are highly variable located at 12 and 13 positions, and they are responsible for the diagnosis of a particular nucleotide (Nemudryi et al. 2014). Thymidine is the target DNA molecule that affects the binding efficiency and locates before the5'-end of a sequence bound by a TALE monomer. A half-repeat is the last tandem repeat that binds a nucleotide at the 3′-end of the diagnosis site consists only of 20 amino acid residues. There are two distinct advantages for TALENs compared with ZFNs in genome editing I: they have been reported to indicate ameliorated specificity and decreased toxicity compared to some ZFNs, because of their increased affinity for target DNA.II: There is no selection or directed evolution for engineering TALE arrays, so reducing the amount of time and experience that needed to collect a functional nuclease (Maeder and Gersbach 2016). The absence of obvious correspondence between meganuclease protein residues and their target DNA sequence caused that meganucleases have not been adopted as a genome engineering platform. On the other hand, ZF domains, because of interference between neighbor modules when gathered into a larger array, exhibit context-dependent binding priority. Identically, although TALE DNA-binding monomers are for the most part modular, they can travail from context-dependent specificity and their repetitive sequences provide a construction of novel TALE arrays labor intensive and expensive (Hsu et al. 2014).

Multiple strategies have been developed to account for these limitations one of them was CRISPR nuclease Cas9. Because the Cas9 protein is constant and can be retargeted to new DNA sequences easily by changing a small portion of the sequence of an accompanying RNA guide that base-pairs with target DNA straightly. Also, an important potential of Cas9 is its ability to demonstrate multiple DSBs in the same cell via expression of separate guide RNAs (Cong et al. 2013).

7 CRISPR/Cas Nucleases

CRISPR (clustered regularly interspaced short palindromic repeat)-Cas RNA-guided nucleases are derived from an adaptive immune system that progressed in bacteria for preventing assault of viruses, plasmids and exogenous genetic elements (EGEs) that incorporate with Cas proteins. After a decades, scientists could illuminate a mechanism that short sequences of invading nucleic acids were consolidated into CRISPR loci (Maeder and Gersbach 2016). Then these sequences transcribe and process three main components to cleave foreign nucleic acids CRISPR RNAs (crRNAs), trans-activating crRNAs (tracrRNAs) and CRISPR associated (Cas9) endonuclease. crRNA sequences are complementary to exogenous genetic elements and acting as a target site-specific sequences which will be cleaved by the Cas9 endonucleases. The tracrRNA has homology regions and acts as a link between the variable crRNAs and Cas9. For simplifying laboratory applications the crRNA and tracrRNA have been composed into a single chimeric RNA sequence named short guide RNA

(sgRNA) (Albitar et al. 2018). In order to ensure DNA diagnosis and cleavage, six CRISPR systems according to different mechanisms have been identified. These systems are divided into two classes: Class 1(types I, III, and IV), and Class 2 (types II, V, and VI). Class 2 systems due to their simplicity were appealing for genome engineering and only type II that obtained from Streptococcus pyogenes,has been used for RNA-guided engineered nucleases (Koonin et al. 2017). The effector protein of type II CRISPR-Cas systems is Cas9 and this multi-task protein has been engineered into a key tool for genome editing. The guide RNA (sgRNA) manages the CRISPR associated protein Cas9 duo to present a sequence specific DNA cleavage by double-strand breaks (DSBs) in the target DNA. NGG motif or proto-spacer adjacent motif (PAM) is a short-conserved sequence that is required for introducing a break. Hence, CRISPR/Cas9 by utilizing sgRNA with Cas9 nuclease can recognize a variable 20-nucleotide target sequence adjacent to a 5'-NGG-3' PAM and introduces a DSB in the target DNA three base pairs upstream of the PAM sequence (Li et al. 2018a, b). Since the induced DSB is a lethal happening for cells, these cells need a mechanism for DNA repair. These mechanisms consist of the homology-direct repair (HDR) pathways and the non-homologous end joining (NHEJ). The HDR pathway of DNA damage repair includes a precise strand-exchange process based on existing homologous DNA formats, which contain homology to sequences flanking the DSB demonstrated by homology arms (He et al. 2016). The mechanism of NHEJ repair consists in joining of the free DNA ends via a homology independent and mechanistically flexible process, which often produces random small deletions or insertions (Albitar et al. 2018).

8 Advantages of CRISPR

The CRISPR/Cas9 genome-editing system proposes several advantages over transcription activator like effector nuclease (TALEN) and the zinc-finger nucleases (ZFNs) in adult stem cells (ASCs) and human pluripotent stem cells (PSCs). First of all, CRISPR/Cas9 is more economical because there is little related cost for plasmid-mediated CRISPR/Cas9. Second, as the fastest existing genome-editing technique, because this system can typically be performed in 2 weeks. Third, CRISPR/Cas9 is more user-friendly than TALEN and ZNF (Zhang et al. 2017). Fourth, the capability of Cas9 to display multiple DSBs in the same cell via expression of separate guide RNAs is a potential advantage. At last, CRISPR/Cas9 displays a higher editing efficiency than TALEN and ZNF in human stem cells. CRISPR can target multiple loci simultaneously in the genome with high efficiency and without remarkably increasing the required dose. As XL et al. demonstrated treatment with a BCL inhibitor ABT-263 further improves HDR efficiency by 70% and knockout (KO) efficiency by 40% via CRISPR-Cas9 in human pluripotent stem cells. The increased efficiency of genome editing is ascribed to higher expressions of Cas9 and sgRNA in surviving cells after electroporation (Li et al. 2018a, b). Table 1 demonstrates comparison of different programmable nuclease platforms. However, CRISPR/Cas9 technology is one of the great promises as a means to produce biological products and especially therapeutic cellular products.

9 Application of CRISPR/Cas9 in Mesenchymal Stem Cell Studies

CRISPR/Cas9-based gene manipulation including in gene knock-in, gene knockout, gene activation or interference, and other chromosome-related usages, has been widely employed in stem cell research and specially MSC research (Table 2; Shen et al. 2018).

One of the main limitations of cell therapy is the immune rejection of transplanted cells. Due to no or low expression of MHC class II proteins, MSCs prevent allogeneic rejection. However, studies don't refuse the role of MHC class 1 in immune rejection completely (Fukami et al. 2009; Ayala García et al. 2012). The result of a study

Table 1 A brief summary of comparison of different programmable nuclease platforms

Option	Meganucleases	ZFN	TALEN	CRISPR
Nuclease	I-CreI, I-SceI	*FokI*	*FokI*	Cas
DNA-binding section	Protein	Protein	Protein	RNA
Target site size [bp]	14–40	18–36	30–40	22
Binding and cleavage domains	Non-modular	Modular	Modular	Non-modular
Design availability	More complex	More complex	Complex	Simple
Cytotoxicity	–	Variable to high	Low	Low
Ease of multiplexing	Low	Low	Low	High
Targeting constraints	Targeting novel sequences frequently results in low efficiency	Difficult to target non-G-rich sequences	5′ targeted base must be a T for each TALEN monomer	Targeted sequence must precede a PAM

Table 2 Summary of CRISPR gene engineered-MSC studies

The source of mesenchymal stem cells	Gene	Outcome	Reference
Human MSCs that don't secrete CCL2	Monocyte chemoattractant protein-1 (MCP-1/CCL2)	The CRISPR-Cas 9 approach was proved to be successful in damaging the CCL2 gene in MSCs better than the shRNA approach	Técnico et al. (2015)
Rabbit bone marrow mesenchymal stem cells (BMSC)	PTEN gene	The PTEN-Knocking-out (PTEN-KO) strain showed an increased proliferation capability but decreased multi-directional differentiation potential	Shen et al. (2018)
Mouse bone marrow stromal stem cells (mBMSCs)	SV40T into a safe harboring site at Rosa26 locus	CRISPR/Cas9 HDR-mediated immortalization of BMSCs can be more effectively reversed than that of retrovirus-mediated random integrations	Hu et al. (2017)
UE7T-13 (JCRB) cells were used as MSCs	PPARG, CEBPA, and KLF5	Endogenous activation of adipogenic genes through the dCas9-based transcription system, and achieved efficient induction of different types of adipocyte-like cells from MSCs	Furuhata et al. (2017)
H1 cell line & hMSCs (Lonza PT-2501)	EWSR1–WT1	Model the EWSR1–WT1 translocation associated with the rare DSRCT (Desmoplastic small-round-cell tumor) using both immortalized and non-immortalized human mesenchymal stem cells	Vanoli et al. (2017)
Mesenchymal stem cells derived from human bone marrow (BM-MSCs)	Promotor of ectodysplasin (EDA)	After transfection with sgRNA-guided dCas9-E, the BM-MSCs acquired significantly higher transcription and expression of EDA by doxycycline (Dox) induction	Sun et al. (2018)
Human adipose mesenchymal stem cells (hAMSCs)	Thymidine kinase2 (TK2)	The therapeutic capacity of the new CRISPR/Cas9-engineered hAMSCs was equivalent to that of therapeutic hAMSCs generated by transduction with a lentiviral vector	Meca-Cortés et al. (2017)

(continued)

Table 2 (continued)

The source of mesenchymal stem cells	Gene	Outcome	Reference
Human mesenchymal stem cells	Exon of the five R-SMAD genes	MSC stably expressing CRISPR/Cas9 exhibit Normal differentiation Characteristics efficient targeting of genes using CRISPR/Cas9, leading to strongly decreased protein expression in total cell populations, is feasible without clonal election	van den Akker et al. (2016)
Primary human mesenchymal stem cells	β2-microglobulin (B2M)	Electroporation method can deliver CRISPR/Cas9 RNP components without or without single stranded DNA oligonucleotide (ssODNs) at ribonucleoprotein (RNP) remarkably high efficiency into various human stem cells and primary cells that are hard-to-transfect	Xu et al. (2018)
Human Mesenchymal stem cells	First intron of the PPP1R12C gene	The test in MSCs was not finished.	Carpenter et al. (2015)
Bone marrow-derived MSCs	Notch1 or COX2	MSCs was reprogram host macrophage differentiation towards an anti-inflammatory M2 phenotype via a Notch/COX2/PGE2-dependent manner.	Li et al. (2016)
Immortalized MSC cell line (hTERT MSCs)	Runx2 and Sox9	Genome editing of Runx2 did not appear to absolve osteogenic potential in the hTERT MSCs and targeting of Sox9 via the CRISPR/Cas9 technology demonstrated an apparent increase in adipogenesis.	Carstairs (2017)
Human bone marrow (hBM)-MSCs	Platelet derived growth factor B (PDGF-B)	Accelerated wound healing kinetics in wounds treated with PDGFB-hBM-MSCs compared to wounds in the other treatment groups as early as day 13 after wounding, and this significant difference in healing rate persisted through 23 days post-wounding	Kosaric et al. (2017)

has reported that hMSC with B2M (the light chain of MHC class I molecule (Chen et al. 2017)) knockdown by CRISPR/Cas9 is a suitable and useful stem cell source to treat myocardial infarction without inducing immune rejection (Li et al. 2018b). Another study has reported that by knocking-out β2-microglobulin (B2M) in primary hMSCs can be utilized to increase the gene ablation rate in cells relevant to clinical applications (Xu et al. 2018).

Both of viral and non-viral vectors could be used in CRISPR/Cas9-engineered MSCs (Meca-Cortés et al. 2017; Xu et al. 2018). Meca-Cortés et al. report that the therapeutic capacity of the electroporation as a transfection method for CRISPR/Cas9-engineered hAMSCs is equivalent to that of therapeutic hAMSCs generated by introduction of the same therapeutic gene by transduction with a lentivirus vector (Meca-Cortés et al. 2017).

In recent years, the use of MSCs in both gene and cell therapies especially as vehicles has accelerated. For example, MSCs can be used as vehicles to deliver anti-tumor agents and drugs to tumor sites. Almeida demonstrated the genetic edition of MSCs to be vehicles for drug delivery of azurin into target sites due to their migration potential towards tumors and unique immunomodulation. The primary steps of this strategy were the designing and testing of gRNAs to produce DSBs in a genomic safe harbor, and the design of a donor pattern that causes the interpolation of the azurin gene that encodes this protein into safe locus via CRISPR/Cas9 (Filipa and Almeida

2017). According to azurin properties as an anticancer protein and the tropism ability of the MSCs towards tumor sites, the formulated strategy of this work was to test the possibility of steadily incorporating a gene coding for azurin within the genome of MSCs. In this study, the Cas9 guides were tested in MSCs and HEK293T cells and selecting one guide for CRISPR/Cas9 technology in order to cleavage the selected safe harbor AAVS1 locus in the intron 1 of PPP1R12C gene was done. After the design of the guides, these were tested in HEK293T cells displaying that the Guide 3 was also the best considering its cleavage efficiency observed in the agarose gel. The best guide was Guide 3 because of its good score and the zero exonic off-targets was tested in the second cell line (MSCs). The producers of azurin in MSCs was not done in this step because of problems in designing the guides RNA and surveying the off-targets while these items were done successfully. Hence, the next steps are the ligations between azurin gene AAVS1 locus to produce a donor template capable of repairing the DSB by using MSCs in future experiments (Filipa and Almeida 2017).

Considering the importance of mesenchymal stem cells (MSCs) for curing type 1 diabetes (T1D), Gerace et al. suggested utilization of clustered regularly interspaced short palindromic repeat (CRISPR) for performing the improved clinical trial design for the future success of T1D MSC derived therapies. Although islet or pancreas transplantation is the only cure for people with type 1 diabetes (T1D), MSCs have been employed either natively or transdifferentiated into insulin-producing cells (IPCs) as a second treatment (Gerace et al. 2017). As some researches showing the ability of MSCs to differentiate into insulin-producing cells (IPCs) via ex vivo chemical induction or different gene therapy procedures describes them as ideal candidates for cell transplantation. Gerace and colleague displayed the success of MSC-derived therapies in pre-clinical models and reflected the failure of the translation of these studies into the clinical setting. Hence, the limitations of common clinical trials of MSCs for the treatment of T1D suggested the novel clustered regularly interspaced short palindromic repeat (CRISPR) gene-editing technology for ameliorating the clinical trial plan as strategies to translate pre-clinical success to the clinical setting (Gerace et al. 2017).

Another study done by Shen et al. was about gene editing of PTEN in MSCs and its changes in differentiation and proliferation in vivo (Shen et al. 2018). As the tumor suppressor, PTEN is associated with lineage determination, motility, the regulation of cell proliferation, apoptosis and adhesion. Mutation or loss of PTEN has existed in several human cancers and diverse hereditary disorders. Since PTEN was recognized to increase MSCs migration ability, this study clarifies the role of PTEN in the in vivo proliferation and differentiation via a gene-editing approach. They used CRISPR/Cas9 to knockout the PTEN gene in MSCs and obtained the PTEN-KO BMSCs from rabbit. Results illustrated that rabbit BMSCs are agreeable to accurate genetic manipulations. By using this technology for PTEN knockout cells, increased proliferation capability and decreased osteogenic and adipogenic differentiation ability was shown compared with the WT. These results display when BMSCs using as the seed cells for tissue engineering, indicated a low expression of PTEN, the findings suggest a spoiled differentiation and tissue repair function (Shen et al. 2018). Recently, a study was done by van den Akker and colleague about CRISPR/Cas9 technique for inactivation of genes in hMSC (van den Akker et al. 2016). They determined the possibility of generating knockout cell populations from human mesenchymal stem cells, without sub-cloning of cells. As transforming Growth Factor (TGF-ß) signaling is important for chondrogenic differentiation of MSC and the conservation of the articular chondrocyte phenotype, CRISPR guide RNAs were designed to target the second coding exon of the five R-SMAD genes and cloned into a lentiviral Cas9 expression system. The efficiency of CRISPR was evaluated by using surveyor nuclease assay on MSC and HEK293 cells. The surveyor nuclease assay displayed a higher percentage of genomic modification. This targeting strategy reduced SMAD protein

expression in HEK293T by 90% and MSC was expected to be more unprotected to CRISPR/Cas9 genome engineering or high viral loads. Hence, primary findings determine that MSC expressing CRISPR/Cas9 steadily show normal differentiation characteristics (van den Akker et al. 2016). All these researches are in the early stages and required more time for finding more acceptable results and using them in clinical trials.

10 Conclusion and Outlook

The rise of powerful, effective and cost-effective methods for the genetic manipulation of cells is opening up wide prospects for cell-based therapy. CRISPR/Cas has emerged as future technologies due to the rapidity and specificity of gene delivery using gene-editing techniques. MSCs as an accessible and suitable source of stem cell confirmed by extensive research. According to preclinical and clinical trial studies, MSCs considered nearby to approved clinical therapeutics (Golchin et al. 2018a). On the other hand, the ability to modify a cell's DNA with precision and achieve of gene-engineered cells in biomedicine, enabled by methods based on CRISPR, has paved the way for a degree of appropriate cell customization for clinical application. Therefore, combining the stemness potential of MSCs with CRISPR/Cas9 technology has made to an interesting field that made an accessible tool to clinical application. Despite different research for using mesenchymal stem cells (MSCs) via (CRISPR) gene-editing has been registered only one in clinical trials in this regard. Currently, a clinical trial is registered in clinicaltrial.gov that combine the self-renewal potential of MSCs with CRISPR/Cas9 technology that will be developed an epigenome editing approach as a therapeutic strategy to rescue the activity of MLL4 (51). It is expected that the number of clinical trials in this regards enhances. However, CRISPR/Cas technology enables specific targeting of loci in target genes, but next-generation stem cell-based therapies to achieve widespread application need dedicated efforts.

Author Contributions A.G. conceptualized the outline and contents of the article. All authors contributed to writing and editing of the manuscript.

Conflict of Interest The authors confirm that this article content has no conflict of interest.

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Mesenchymal Stem Cells in Asthma

Tunc Akkoc

Abstract

Asthma is one of the worldwide respiratory health problem that affect children and adult. Current treatment strategies such as conventional and allergen immunotherapy still fall behind. Mesenchymal stem cells (MSCs) have wide regenerative capacity and immunoregulatory activity with their wide range of secretions and contact dependent manner. In this review, we focus on the current treatment strategies for asthma and MSCs as a new therapeutic tool.

Keywords

Asthma · İmmunoregulation · İmmunotherapy · Mesenchymal stem cells

Abbreviations

MSCs	Mesenchymal Stem Cells
AIT	Allergen immunotherapy
GVHD	Graft versus host disease
SCIT	Subcutaneous immunotherapy
SLIT	Sublingual immunotherapy
TGF-β	Transforming growth factor beta
SARSs	Systemic adverse reactions
HLA	Human leukocyte antigen
MHC	Major histocompatibility complex
IDO	Indoleamine 2,3-dioxygenase
NO	Nitric oxide
EAMG	Experimental autoimmune myasthenia gravis

T. Akkoc (✉)
Faculty of Medicine, Department of Pediatric Allergy and Immunology, Marmara University, Istanbul, Turkey
e-mail: tuncakkoc@gmail.com

1 Introduction

Stem cells have special concern due to their capability to help in regenerative medicine. Broadly they are classified due to their potency as pluripotent (embryonic and inducible) and multipotent (mesenchymal). Among these, mesenchymal stem cells gain much attention because of their postnatal origin, differentiation capacity, lack of immune activity, and safety for the host. Stem cells have important immunomodulatory effect on autoimmune and allergic diseases.

Asthma is a chronic inflammatory disease and its prevalence has significantly increased with the western life style. Th2 dominant immune response is mainly responsible for pathogenesis. Besides conventional therapies with short- and long-acting beta agonists, inhaled low dose corticosteroids and other anti-IgE/anti-leukotriene therapies, there is an immunotherapy approach with sensitized allergens. Recent studies obviously showed the immunoregulatory effects of Mesenchymal Stem Cells (MSCs) in various autoimmune and atopic disorders.

This review outlines the mechanism of the immunotherapy and immunomodulatory effect of MSCs in allergic disease. Also, the implications of recent studies on immunoregulatory aspects of MSC and dysregulated immune systems are discussed.

2 Asthma and the Immune System

2.1 Overview

Asthma is a major chronic, non-communicable health problem affecting children and adults (Asamoah et al. 2017). Its prevalence has increased in urbanized regions particularly with westernized lifestyle and it affects more than 300 million people around the world (Papi et al. 2018). It is characterized by dense airway inflammation leading to reversible airflow limitation and lung tissue remodeling (Papadopoulos et al. 2012). Its basic diagnostic symptoms include dyspnea, cough, wheezing and chest tightness (Becker and Abrams 2017).

Asthma is a Th2 skewed disease with increased number of $CD4^{+}$T cells which produce IL-4 and IL-5 that lead to production of allergen specific IgE and eosinophils, respectively (Brightling et al. 2002). Eosinophilic inflammation is credited to be a substantial contributor to the histopathological inflammatory changes in asthmatic patients (Brightling et al. 2002; Tan et al. 2016). Chronic airway inflammation leads to airway remodeling characterized by basement membrane thickening, goblet cell hyperplasia, smooth muscle proliferation, peribronchial and perivascular inflammatory cell infiltration and mucus plug formation (Akkoc et al. 2001).

Currently there is no radical cure for asthma, but conventional therapeutic approaches control the disease symptomatically. Stepwise pharmacological treatment involves short-acting beta-2 agonist, long acting beta-2 agonists and inhaled low dose corticosteroids alone or with combination depending on the severity of asthma (Parsons et al. 2013; Cates and Karner 2013). Besides these, theophylline, anti-leukotrienes, anticholinergics, and anti-IgE antibodies (Omalizumab) can be used to control severe asthma (Asamoah et al. 2017; Mirra et al. 2018). All of these are symptom relief treatment approaches and do not educate the immune system. Allergen immunotherapy (AIT) is an alternative to regulate the immune response and an effective treatment strategy for allergic diseases (Berings et al. 2017).

2.2 Allergic Disease and the Immune System

The immune system easily differentiates self and non-self and screens harmful pathogens with immune cells in a complex interactive network. Immune tolerance is a well-organized immune mechanism that protects individuals from cancer, autoimmunity, viable pregnancy, graft-versus-host disease (GVHD), rejection of transplanted organs asthma and allergies (Fuchs et al. 2017; Werfel et al. 2016; Chinthrajah et al. 2016). Allergic disease can be classified as allergic rhinitis, allergic asthma, atopic eczema, food allergy and anaphylaxis (Akdis and Akdis 2009; Galli and Tsai 2012; Akdis 2012).

Allergic asthma is basically defined as a type 2 immune response-associated disease. The type 2 dominant immune response is characterized by increased number of CD4 + Th2 cells, IL-4-high and IL-5-high immune activity with enhanced allergen specific IgE, eosinophil and mast cells. This leads to airway hyperresponsiveness with increased airway eosinophilia. Consequently, eosinophilic inflammation responsible for pathophysiological changes causes remodeling seen in asthmatics (Eiwegger and Akdis 2011) (Fig. 1).

2.3 Allergen Immunotherapy

Allergen immunotherapy seems like the only therapeutic approach for allergic conditions and respiratory allergies. This kind of therapy involves delivery of sensitized allergens to patients with gradually increasing amounts of dose for subcutaneous immunotherapy (SCIT),

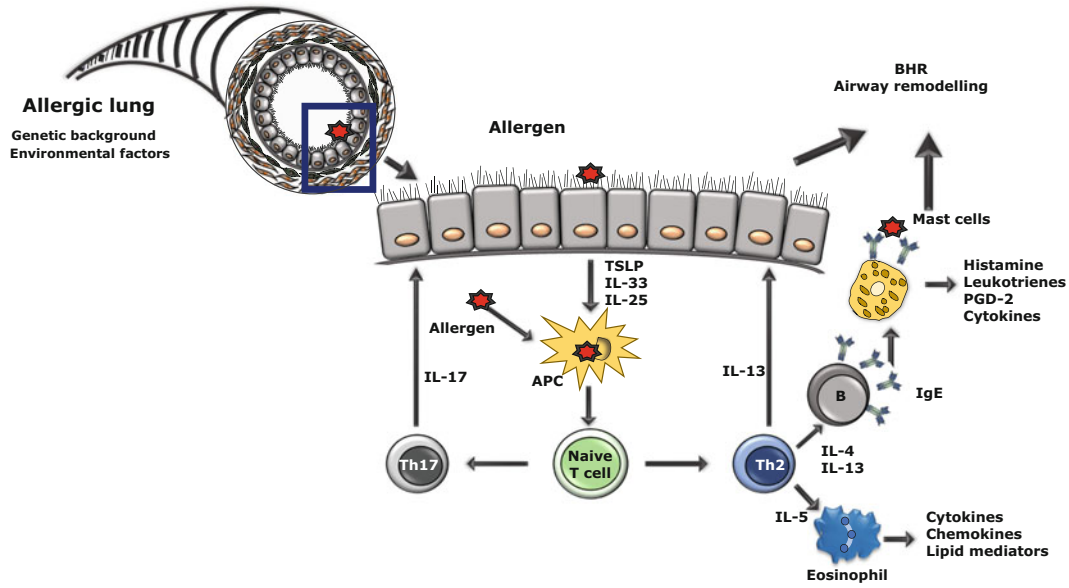

Fig. 1 Pathogenesis of asthma. BHR: Bronchial Hyperreactivity, PDG-2: Prostaglandin E-2, APC: Antigen Presenting Cells, TSLP: Thymic stromal lymphopoietin

sublingual immunotherapy (SLIT), or oral immunotherapy. Regarding this approach, the allergen content, administration route, and duration of administration are important for the safety of therapy and immunoregulatory stimulation (Matricardi et al. 2019). The underlying mechanism of AIT involves shifting the T cell immune response from Th2 to regulatory type. T regulatory cells remain at the center of AIT with their anti-inflammatory cytokine production nature like interleukin 10 (IL-10) and transforming growth factor-beta (TGF-β). Among these IL-10 is responsible for downregulation of allergen specific immunoglobulin E (IgE) antibody production and upregulation of immunoglobulin G4 (IgG4) which are called blocking antibodies (Frew 2010). Also, Treg cells act on mast cells, eosinophils and T cells to reduce the release of proinflammatory cytokines. Further AIT prevents localization and functions of mast cells, basophils and eosinophils in the local sensitized tissues, such as bronchial mucosa (Cox et al. 2011a; Moote et al. 2018) (Fig. 2).

Beside AITs safe clinical outcomes due to the nature of the antigen as natural allergens, some systemic adverse reactions (SARs) are monitored during therapy (Cox et al. 2011b).

3 Mesenchymal Stem Cells

3.1 Overview

MSCs are non-specialized multipotent cells that have self-renewal and differentiation capacity into diverse cell types such as adipose, chondrocyte and osteocyte (Al-Nbaheen et al. 2013). The International Society of Cellular Therapy (ISCT) stated the minimum criteria for MSCs as plastic-adherent in standard culture, expressing special cell surface markers such as CD73, CD90 and CD105 while negative for CD14, CD34, CD45, CD19, CD11b, CD79a, and HLA-DR, and differentiation capability into adipocyte, osteocyte, or chondrocytes *in vitro* (Dominici et al. 2006).

Various sources of MSCs are described as including adipose tissue, umbilical cord blood, Wharton's jelly, the placenta, bone marrow and dental tissue (Sueblinvong et al. 2008; Gronthos et al. 2000). Because of minor ethical apprehensions, ease of attaining them in tissue

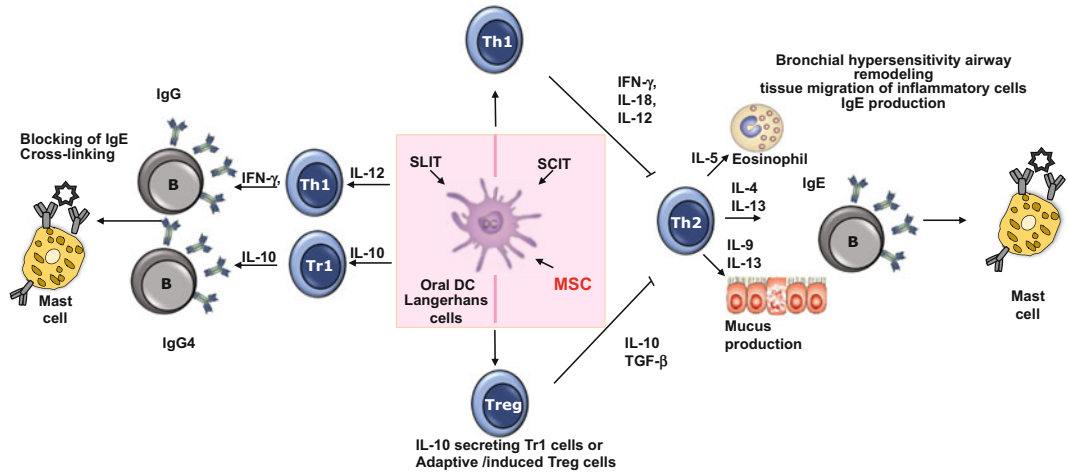

Fig. 2 Mechanism of allergen specific immunotherapy. *SCIT* Subcutaneous Immunotherapy, *SLIT* Sublingual Immunotherapy, *MSC* Mesenchymal Stem Cell, *DC* Dendritic cell

and isolation, suppression of inflammation and role in immunomodulation, they are a promising therapeutic approach for several autoimmune disorders (Ogulur et al. 2014a; Yu et al. 2010).

3.2 Immunomodulation and MSC

The immune system is a communicating network of cells and molecules with the competency to protect the host from a broad range of pathogens, while distinguishing self and non-self-tolerance. Once the immune system loses its capability to discriminate self-cells and tissues from others, immune-tolerance-related diseases arise such as cancer, autoimmunity and allergy (Fuchs et al. 2017; Palomares et al. 2014).

MSCs are specialized cells that regulate the immune system and control inflammatory disease-related immune reactions. Mainly MSCs express low- to moderate-intensity levels of human leukocyte antigen (HLA) and major histocompatibility complex class I (MHC-I) while they lack MHC-II and costimulatory molecules such as CD80, CD86, CD40 and CD40L (Le Blanc and Ringden 2007; De Miguel et al. 2012).

Conversation and regulation of the immune system is provided by cytokines. T cell subsets are signatures with different tendencies toward cytokine secretions. Regarding their cytokine profile, they control or direct the immune system to autoimmunity or allergic disease. Inflammation is also modulated via cytokines and mainly pro-inflammatory cytokines (TNF-α, IL-1-α, IL-6) increase in that state.

MSCs have a high capacity to modulate immune responses. Basically, they suppress pathologic T cell proliferation and regulate the balance of Th1 (autoimmunity related)/Th2(allergic disease related). In addition to this they modulate the T regulatory cells (Tregs), regulate antibody secretion profile of B cells and antigen presentation of dendritic cells (Le Blanc et al. 2003; Saldanha-Araujo et al. 2011). The innate and adaptive immune system is also regulated by MSCs originating other immunomodulatory molecules such as interleukin-10 (IL-10), TGF-**β**, indoleamine 2,3-dioxygenase (IDO), and nitric oxide (NO) (Del Fattore et al. 2015; Kyurkchiev et al. 2014; Castro-Manrreza 2016).

Our previous studies indicate the immunoregulatory properties of MSCs from different sources on different dysregulated-immune system related diseases (Ulusoy et al. 2015; Duman et al. 2019; Cerman et al. 2016). One of these showed human dental follicle MSC treatment for a MuSK-associated experimental autoimmune myasthenia gravis (EAMG) model led to the outcome of downregulated anti-MUSK IgG1, IgG2 and IgG3 and prevention of IgG3 and Complement

3 (C3) deposition into the neuromuscular junction (NMJ) *in vivo*. This regulation was established by suppression of proinflammatory IL-6 and IL-12. These immunoregulatory outcomes also improved clinical grades in the model (Ulusoy et al. 2015). Recent studies also showed that rat bone marrow derived MSCs suppress inflammation by downregulating TNF-α, fibrosis and enhancing NK cells in rat liver in a rat hepatic fibrosis model. Also, IFN-γ, TNF-α and IL-1α are downregulated and IL-10 levels are upregulated in the periphery (Duman et al. 2019).

4 MSCs and Asthma

4.1 Overview

Asthma is characterized by chronic airway inflammation related to dominant Th2 immune response and infiltration of eosinophils and mast cells within small airways (Lai et al. 2009). Several therapeutic approaches are designed to suppress allergic inflammation in acute and chronic murine models. Among them most promising was an immunotherapy approach with an allergen which upregulates Treg cell and downregulates Th2 type immune response in acute and chronic asthma models (Akkoc et al. 2008a, b, 2010, 2011; Eifan et al. 2010; Keles et al. 2011; Townley et al. 2004; Yazi et al. 2008). Allergen-specific immunotherapy is a basic and approved treatment option for allergic diseases. Also, it was shown previously that adjuvants such as *Mycobacterium vaccae* induce T reg cells while reversing Th2 immune deviation in murine models (Akkoc et al. 2018).

Recent studies address MSC's as an encouraging therapeutic approach for curing allergic and autoimmune disorders. Studies were carried out on murine models and in-vitro human studies.

4.2 Murine Models

Close models for type-I allergic disease are important to reflect bronchial asthma that resembles the main features of the human allergic disorder. Depending the severity of disease, acute and chronic models were developed. The acute asthma model is characterized by high levels of allergen-specific IgE production, bronchial hypersensitive reactions to allergens and methacholine provocation test, and cellular infiltrate in proximal airways (Herz et al. 2004). Changes are more dramatic in chronic asthma models. Airway remodeling is mostly seen in chronic models with goblet cell hyperplasia, thickening of smooth, muscle and basement membrane. These dramatic changes also reach the distal small airways (Akkoc et al. 2001). Recent meta-analyses of MSC transplantation in asthmatic models have collected studies (Zhang and He 2019). Really various ways of administrating (intravenous and intratracheal) MSC's successfully downregulates airway inflammation and airway remodeling in acute and chronic asthma models. Our results demonstrated that allogeneic pluripotent stem cells also control allergic acute inflammation, reverse airway cell infiltration in proximal airways, downregulate eosinophil accumulation in Broncho alveolar lavage and allergen specific IgE levels in serum. Further while IL-10 levels increased, IL-4 levels were downregulated in lung cell suspensions (Ogulur et al. 2014b). These results successfully reveal the therapeutic ability of stem cells in acute and chronic asthma models.

4.3 Experimental Human Studies

Stem cell application to patients needs time regarding ethical issues and some unknown side effects that may be seen during long term follow up. Allogeneic MSC's were successfully used without serious adverse reactions. Also encouraging results are seen in those studies.

Importantly the immunomodulatory properties of MSCs are accredited for allergic disease and autoimmune disorders. The most expected results for MSC in allergic disease are downregulation of Th2 type cytokines, allergen specific T cell proliferation and detrimental memory T cells, and upregulation of Treg cells and naive T cells. Genç et al. compared the *in vitro*

immunomodulatory effect of dental follicle MSC (DF-MSC) on mononuclear cells of asthmatic patients and patients that completed 3 years of allergen specific immunotherapy (Genc et al. 2018). DF-MSCs properly suppressed proliferative responses of CD4+ T cells, IL-4 and GATA-3 expression. The outstanding results of this study are downregulation of effector and effector memory CD4+ T cells, and downregulation of IDO and costimulatory pathway in antigen presentation as in immunotherapy group. Another study revealed that IFN-γ pre-treated DF-MSCs enhanced Treg cells andIL10 levels (Genc et al. 2019). Both studies showed the immunomodulatory effect of DF-MSCs on Derp-1^{+} allergic polymorphonuclear cell of patients *in vitro*.

5 Conclusion and Perspective

Recent studies of MSC administration in murine models of asthma provide valuable information concerning the safe application *in vivo* with lack of immunogenicity and adverse effects. MSC applications may be intravenous, intranasal or intratracheal. The source of MSCs is an important manner. In murine models of asthma, MSCs are derived from bone marrow, adipose tissue or umbilical cord. All sources provided safe results with *in vivo* experiments.

In humans, recent developments in relation to the clinical and cellular/molecular mechanisms of AIT aim at enhancing clinical and immunologic tolerance, decreasing side effects, and increasing efficacy. Hypoallergenic recombinant allergen and allergoid vaccines and use of probiotics, vitamins and biological agent supplements to support AIT are expected to enhance efficacy. Many novel developments in molecular mechanisms that affect early desensitization, T- and B- cell tolerance, specific antibody regulation, and induction of IgG4 and several key molecules that can act as biomarkers are continuously being developed. As new technologies and novel strategies emerge, we are in need of more research into the mechanisms, biomarker discovery, and disease phenotyping for AIT. There will always be take-home messages for other immune tolerance-related conditions, such as autoimmunity, organ transplantation, chronic infections, cancer, and recurrent abortion.

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Oocyte Aging: The Role of Cellular and Environmental Factors and Impact on Female Fertility

Toka A. Ahmed, Sara M. Ahmed, Zaynab El-Gammal, Shaimaa Shouman, Ashrakat Ahmed, Ragaa Mansour, and Nagwa El-Badri

Abstract

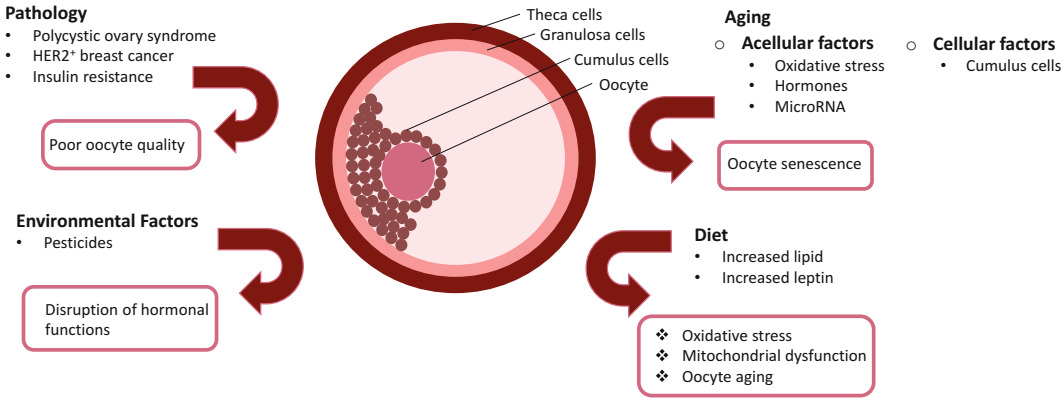
Female aging is one of the most important factors that impacts human reproduction. With aging, there is a natural decline in female fertility. The decrease in fertility is slow and steady in women aged 30–35 years; however, this decline is accelerated after the age of 35 due to decreases in the ovarian reserve and oocyte quality. Human oocyte aging is affected by different environmental factors, such as dietary habits and lifestyle. The ovarian microenvironment contributes to oocyte aging and longevity. The immediate oocyte microenvironment consists of the surrounding cells. Crosstalk between the oocyte and microenvironment is mediated by direct contact with surrounding cells, the extracellular matrix, and signalling molecules, including hormones, growth factors, and metabolic products. In this review, we highlight the different microenvironmental factors that accelerate human oocyte aging and decrease oocyte function. The ovarian microenvironment and the stress that is induced by environmental pollutants and a poor diet, along with other factors, impact oocyte quality and function and contribute to accelerated oocyte aging and diseases of infertility.

Authors Toka A. Ahmed and Sara M. Ahmed have equally contributed to this chapter.

T. A. Ahmed, S. M. Ahmed, Z. El-Gammal, S. Shouman, A. Ahmed, and N. El-Badri (✉)
Center of excellence for stem cells and Regenerative Medicine, Zewail City of Science and Technology, Giza, Egypt
e-mail: nelbadri@zewailcity.edu.eg

R. Mansour
The Egyptian IVF-ET Center, Cairo, Egypt

Graphical Abstract



Keywords

Aging and longevity · Human · Microenvironment · Oocytes

Abbreviations

AGEs	Advanced glycation end products
AKT	Protein kinase B
BCL2	B-cell lymphoma-2
CaMKII	Calmodulin-dependent protein kinase II
CAT	Catalase
CCs	Cumulus cells
cGMP	Cyclic guanosine monophosphate
COC	Cumulus-oocyte complex
COIII	Cytochrome oxidase subunit 3
CoQ10	Coenzyme Q10
Cx-43	Connexin 43
EGF	Epidermal growth factor
EGFR	EGF receptor
FADD	Fas-Associated protein with a Death Domain
FAS	Free α-subunit
FasL	Fas/Fas ligand
FoxO	Forkhead box O
FSH	Follicle-stimulating hormone
GCs	Granulosa cells
GnRH	Gonadotropin-releasing hormone
GSSPx	Glutathione peroxidase
GST	Glutathione S transferase
GTP	Guanosine triphosphate
HMGA2	High-mobility group AT-hook 2
HPG axis	Hypothalamic-pituitary-gonadal axis
IKBKG	Inhibitor nuclear factor kappa B kinase subunit gamma
IR	Insulin resistance
IVF	In vitro fertilization
LH	Luteinising hormone
LINE-1	Long interspersed element
MAPKs	Mitogen-activated protein kinases
MII	Meiotic metaphase II
MnSOD	Mitochondrial SOD
MPF	Maturation-promoting factor
mtDNA	Mitochondrial DNA
NAC	N-acetyl-L-cysteine
NAD+	Nicotinamide adenine dinucleotide
NF-κB	Nuclear factor kappa B
ORFs	Open reading frame
PCOS	Polycystic ovary syndrome
PDE3A	Phosphodiesterase 3A
PGC-1 α	Proliferator-activated receptor coactivator-1α
PGCs	Primordial germ cells
PI3K	Phosphatidylinositol 3-kinase
PTEN	Phosphatase and tensin homolog
RAB5B	Ras-related protein Rab-5B

RAGE	Receptor for advanced glycation end products
ROS	Reactive oxygen species
SDHA	Subunit A of succinate dehydrogenase
sFasL	Soluble fasl
SIRT1	Silent information regulator-1
SOD1	Superoxide dismutase

1 Introduction

The development of oocytes from primordial germ cells (PGCs) is a tightly regulated process. The female fetal ovary begins to display follicular atresia as early as 28 weeks of gestation, and follicular atresia continues throughout adult life. Of the one to two million oocytes in the ovary of a new-born baby, only 300–400 oocytes reach the ovulation stage (Oktem and Oktay 2008). After ovulation, mammalian oocytes are arrested at the meiotic metaphase II (MII) stage. If not fertilized in time, the ovulated oocytes undergo a time-dependent aging process (Yanagimachi and Chang 1961; Whittingham and Siracusa 1978). Aged oocytes are associated with a significant reduction in embryonic development and maturation, especially following *in vitro* fertilization (Whittingham and Siracusa 1978; Iwamoto et al. 2005; Wu et al. 2007). The factors that impact oocyte longevity and aging are poorly understood. Why oocytes age and how the surrounding cells keep them alive while contributing to their aging is a fundamental biological question that requires substantial research. In this review, we identify the microenvironmental factors that contribute to oocyte aging and impair their fecundity. Identifying these factors and the underlying mechanisms of oocyte sustenance is fundamental to understanding oocyte biology and embryological development and could provide new insight into assisted reproductive technology.

2 Oocyte Development and Maturation

2.1 Structure

Ovarian tissue consists of two compartments: the cortex and the medulla. The medulla is the central compartment and is composed of connective tissue, fibrous tissue, and blood vessels. The cortex is the peripheral compartment and contains ovarian follicles at different stages of maturation. Each follicle consists of an oocyte surrounded by a layer of follicular cells. As the oocyte matures, additional layers of follicular cells are formed, and the cells in these layers are called granulosa cells (Grabowski and Tortora 2000).

2.2 Oogenesis

At 7 weeks of gestation, oogenesis begins in the female embryo from PGCs. At 20 weeks of gestation, each fetal oogonium in the ovary becomes a primary oocyte and remains arrested in prophase I of meiosis until puberty (Oktem and Oktay 2008; Oktem and Urman 2010; Mamsen et al. 2011). At 28 weeks of gestation, follicular atresia begins, and thus, there are only one million oocytes present in the ovary at birth (Oktem and Oktay 2008). Atresia continues throughout adult life so that only 300–400 oocytes reach the ovulation stage (Oktem and Oktay 2008). Directly before ovulation, luteinising hormone (LH) induces the continuation of the first meiotic division of the oocyte, resulting in two cells: the secondary oocyte, which is large and has most of the cytoplasm, and the polar body, which is small (de Haan et al. 2010). The secondary oocyte continues meiosis II and becomes arrested in the metaphase stage, which is completed after fertilization (de Haan et al. 2010).

2.3 Folliculogenesis

Starting from 15 weeks of gestation, the primordial follicles develop and mature within the ovaries until the oocyte reaches prophase I (Gougeon 1986). Primordial follicles mature to become primary, secondary, pre-antral, antral, and then pre-ovulatory "Graafian" follicles (Gougeon 1986). This process is known as folliculogenesis. At puberty, gonadotropins secreted by the pituitary gland promote the development of a set of antral follicles into Graafian follicles. Follicle-stimulating hormone (FSH) is produced during the early stage of antral follicle maturation, while both FSH and LH are produced during the late stage. Under the influence of these hormones, the follicles enlarge and develop their antra that contain the follicular fluid (Gougeon 1986). One of these sets of selected follicles becomes dominant, and the rest undergo follicular atresia (Johnson and Everitt 2000).

3 Risk Factors Affecting Oocyte Longevity

3.1 Aging

3.1.1 Cumulus Cells and Aging

Cumulus cells (CCs) are functional cells that originate from undifferentiated granulosa cells (GCs). CCs have highly specialized cytoplasmic projections that pass through the zona pellucida to form gap junctions with the oocyte and surround them to form the cumulus-oocyte complex (COC) (Albertini et al. 2001). The COC has a vital role in the development of healthy embryos since it provides the essential nutrients for oocyte maturation through different paracrine signalling pathways (Mehlmann 2005). Moreover, after ovulation, CCs remain loosely attached to the oocytes to support their journey to be released from the ovary.

Animal studies have shown the importance of CCs in the maturation of rodent oocytes *in vitro*. Oocytes co-cultured with CCs are able to produce a healthy mature foetus, whereas the absence of CCs in *ex vivo* culture conditions leads to a low success rate for oocyte maturation (Vanderhyden and Armstrong 1989). CCs play a dynamic role in regulating the process of oocyte aging and longevity (Perez et al. 2005) through many pathways, most notably the activation of Fas/Fas ligand (FasL) (Ju et al. 1995; Dhein et al. 1995; Matsumura et al. 1998; Poulaki et al. 2001) and the production of ceramide (Kujjo and Perez 2012).

Fas/FasL Pathway

CCs contribute to oocyte aging via activation of the Fas/FasL pathway, a major pathway involved in inducing the death of different cell types (Ju et al. 1995; Dhein et al. 1995; Matsumura et al. 1998; Poulaki et al. 2001). Mediated by metalloproteinases, FasL cleavage releases the soluble form of FasL (sFasL) (Kayagaki et al. 1995; Tanaka et al. 1995; Mitsiades et al. 1998). sFasL increases reactive oxygen species (ROS) levels through an NADPH oxidase-dependent mechanism. This increase in ROS activates Fas in the oocytes, which in turn activates the cytochrome c and phospholipase C-γ pathway and triggers Ca^{2+} secretion from the cytoplasmic reticulum. This Ca^{2+} release in turn activates caspase-3 and calcium/calmodulin-dependent protein kinase II (CaMKII). Activated caspase-3 accelerates further Ca^{2+} production, leading to the activation of more caspase-3 and to oocyte fragmentation (Zhu et al. 2016). Activated CaMKII inactivates maturation-promoting factor (MPF) and causes cyclin B degradation, resulting in increased oocyte susceptibility to apoptosis. Alternatively, upon the binding of FasL to Fas, Fas-Associated protein with a Death Domain (FADD) activates caspase-8, activating apoptosis (Itoh et al. 1991).

Aging oocytes have been found to be surrounded by CCs that secrete sFasL. sFasL secretion is thus believed to be the mechanism by which CCs accelerate oocyte aging and decrease oocyte development and maturation potential (Zhu et al. 2015a). This hypothesis is supported by the finding of the lack of oocyte aging when functional FasL is not present (Zhu et al. 2015b).

The expression of the FasL inhibitor B-cell lymphoma-2 (BCL2) is significantly upregulated in CCs associated with mature oocytes but not in those associated with immature oocytes (Filali et al. 2009). This suggests that BCL2 expression is strongly related to oocyte quality and potential for maturation; however, the mechanism of this association has yet to be investigated.

Ceramide Level and Mitochondrial Activity

When oocytes isolated from aged female mice were cultured with their surrounding CCs, there was a dramatic increase in the oocyte death rate compared to oocytes isolated from young mice (Fujino et al. 1996). Subsequent experiments showed that this age-related oocyte death rate was highly dependent on the presence of oocyte-surrounding CCs, as oocytes harvested from aged females and cultured without their surrounding CCs did not exhibit this high death rate (Perez and Tilly 1997). These experiments confirmed the postulation that CC-derived factor(s) are transported into the oocyte and activate the death programme in aged oocytes (Perez and Tilly 1997). Chemicals such as ceramide, a bioactive lipid produced by CCs and transported into oocytes, could be responsible for enhancing the age-related elevation in oocyte apoptosis (Perez et al. 2005; Kujjo and Perez 2012). Ceramide is translocated from CCs into their neighbouring oocyte via gap junctions, and its release induces apoptosis. This study showed that during aging, apoptosis is accelerated in female oocytes and that this process requires regular oocyte-CC communication. The higher apoptotic rate of the aged oocytes was correlated with higher oocyte sensitivity to increased cytosolic ceramide levels and overexpression of both bax mRNA and Bax protein. Other experimental studies have shown that during aging, the ceramide content of mitochondria decreases, resulting in subsequent structural and functional mitochondrial alterations and effects on oocyte quality (Kujjo and Perez 2012).

During early embryogenesis, mitochondria are the most prominent oocyte organelles, and the mitochondria of the embryo are almost exclusively derived from oocytes (Dumollard et al. 2007; Eichenlaub-Ritter et al. 2011; Van Blerkom 2004, 2011; Van Blerkom et al. 2006). Because of their essential role in cellular energy production and the regulation of cell death, mitochondria control the life and death decisions of most cell types, including oocytes (Danial and Korsmeyer 2004; Perez et al. 2000; Wang 2001). Furthermore, mitochondria are responsible for chromosome segregation and normal spindle formation (Eichenlaub-Ritter et al. 2004). Because of their small sizes and simple internal structures, oocyte mitochondria have been described as morphologically primitive or immature (Dumollard et al. 2007). However, mitochondrial dysfunction is involved in general body aging, (Ames et al. 1995; Sastre et al. 2002) as well as the aging of female reproductive tissues (Ruman et al. 2003; Gougeon 2005; Janny and Menezo 1996; Ottolenghi 2004; Tarlatzis and Zepiridis 2003; Kirkwood 1998). During aging, disruptions in intracellular ceramide synthesis and transport cause abnormal mitochondrial ceramide levels. This ceramide imbalance negatively impacts the functionality of the oocyte mitochondria and the oocyte quality.

3.1.2 Oxidative Stress and Aging

Oxidative stress is considered one of the most critical mechanisms underlying cellular aging (Tatone et al. 2008a; Salmon et al. 2010). Oxidative stress occurs when the production of ROS and the scavenging effects of antioxidants become imbalanced. This imbalance results in the accumulation of ROS that are produced during normal metabolism. Oxidative stress in the ovary leads to follicular atresia and to a reduction in the number and quality of oocytes (Tatone et al. 2008a). This accounts for defects in oocyte maturation and fertilization and for age-associated decreases in fertility (Agarwal et al. 2005). Oxidative stress also enhances telomere shortening and chromosomal segregation disorders, resulting in defects in meiosis, fertilization, embryo development and, ultimately, infertility (Richter and von Zglinicki 2007).

Aging is associated with increased levels of ovarian advanced glycation end products (AGEs), which are responsible for the generation of

increased levels of ROS (Tatone et al. 2008b; Yin et al. 2012). This is mediated by binding to certain receptors called receptor for advanced glycation end products (RAGE) that induce the activation of NAD(P)H oxidase, mitogen-activated protein kinases (MAPKs), and the transcription factor nuclear factor kappa B (NF-κB) (Lander et al. 1997; Brownlee 2001). The follicular fluid of older cows has been reported to have higher levels of AGEs than that of their younger counterparts (Takeo et al. 2017). Higher levels of AGEs in older cows are associated with fertilization defects. Another study shows that the expression of AGE precursor, detoxifying methylglyoxal, is reduced in older female mice compared to their younger counterparts (Tatone et al. 2010). These data reflect the important role of AGEs in ROS production in older ovarian tissue (Tatone et al. 2008b).

The mature oocyte is a large cell with a high number of mitochondria and large amounts of mtDNA (Monnot et al. 2013). Oocytes can be inactive for years, and during this period, they are continually exposed to oxidative stress leading to mitochondrial DNA (mtDNA) mutations (Kitagawa et al. 1993). Moreover, since ovarian tissues undergo slow turnover, mitochondrial-related defects are highly expected. In the oocytes of older women, there is a high risk of a 4977-bp deletion that affects a subset of genes involved in mitochondrial function and the activity of its enzymes, such as ATP synthases 6 and 8, cytochrome oxidase subunit 3 (COIII), and NADH (Fragouli and Wells 2015). Additionally, the mtDNA quantity of older women suffering from diminished ovarian reserve is higher than that of younger women with normal ovarian reserve (Boucret et al. 2015). There seems to be a difference between older and younger oocytes regarding mitochondrial function and the expression of oxidative stress genes (Hamatani et al. 2004). For example, subunit A of succinate dehydrogenase (SDHA), which is involved in energy-generating pathways, is highly expressed in younger oocytes (Hamatani et al. 2004). This may explain the decrease in ATP production in older oocytes. Additionally, oxidative stress-related genes such as superoxide dismutase (Sod1) and thioredoxin family (Txn1 and Apacd) are downregulated in older oocytes (Hamatani et al. 2004), and the heat shock response and ubiquitin–proteasome pathway are inhibited (Matsui et al. 1996). This leads to the accumulation of damaged proteins, decreased expression of Hsp7 family genes (Hspa4, Hspa8 and Hsp70), and decreased expression of heat shock genes of the ubiquitin–proteasome pathway, such as Hip2, Ubc, Ube1c, Ube2a, Ube2e3, Ube2g1, Pama6, Pamb1, Psmb4, Psmc2, Psmc3, Psmd12, Siah2 and Anapc4, in older oocytes (Hamatani et al. 2004).

Furthermore, there is structural damage in the mitochondria of GCs of older oocytes that is similar to that of oocytes exposed to hypoxia (Amicarelli et al. 1999). This may be explained by the oxidative stress caused by the inadequate blood supply to the theca of mature follicles and the increases in metabolism and oxygen demand by the mature oocyte.

Antioxidants that are present in the follicular fluid, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSSPx), have ROS scavenging actions and protect oocytes against ROS-mediated damage (Carbone et al. 2003). Glutathione S transferase (GST) also acts as a detoxifying agent against ROS by products. This antioxidant defence is greatly affected by ovarian aging.

In older women, the levels of GST and CAT in the follicular fluid decrease (Carbone et al. 2003). The CAT/SOD ratio and GSSPx/SOD ratio in the follicular fluid also decrease, reflecting the decrease in ROS scavenging efficiency with aging (Carbone et al. 2003). This is accompanied by downregulation of the activity of antioxidants, namely, the Cu/Zn SOD, MnSOD, and CAT genes in GCs. The roles of these antioxidants are to scavenge the superoxide anions and hydrogen peroxide released during the synthesis of steroid hormones.

As nicotinamide adenine dinucleotide (NAD^+)-dependent histone deacetylases (Morris 2013; Calabrese et al. 2010), sirtuins control the acetylation of histone and non-histone factors, (Huang et al. 2007) thereby controlling the process of aging. For instance, silent information regulator-1 (SIRT1) catalyses the de-acetylation

of the forkhead box O (FoxO) gene promotor, which is critical for the cellular stress response (Brunet et al. 2004). This in turn upregulates key antioxidant enzymes, such as CAT, mitochondrial SOD (MnSOD), and peroxiredoxin, thereby regulating the cellular redox status (He et al. 2010; Kao et al. 2010; Hasegawa et al. 2008; Hori et al. 2013). Additionally, SIRT1 deacetylates the promoter region of the proliferator-activated receptor coactivator-1α (PGC-1α), enhancing its expression. PGC-1α activates genes involved in antioxidative protection (such as glutathione peroxidase, CAT, and MnSOD), mitochondrial biogenesis and metabolic function (Nemoto et al. 2005; Liang and Ward 2006; Gerhart-Hines et al. 2007). Moreover, SIRT1 inhibits nuclear factor β (NF-B), which is a critical inducer of the inflammatory response mediated by oxidative stress. This, in turn, decreases ROS levels and inflammation (Yeung et al. 2004; Kauppinen et al. 2013). However, the levels of SIRT3, a critical regulator of mitochondrial function, decrease with the age of CCs and GCs. This consequently impairs follicular metabolism (Lombard et al. 2011; Pacella-Ince et al. 2014) and decreases the generation of certain hormones, such as progesterone, in gonadal cells (Li et al. 2017).

3.1.3 MicroRNAs and Oocyte Aging

Maternal age is associated with the compromised function of CCs (Tatone and Amicarelli 2013), resulting in epigenetic modifications and altered miRNA functions in aged oocytes (Ge et al. 2015). miRNAs are small noncoding RNAs that bind to target messenger RNAs (mRNAs) to inhibit their expression. mRNAs exist either freely or enclosed in vesicles (exosomes) in the human follicular fluid to enable CCs and the adjacent oocytes to regulate oocyte DNA methylation (da Silveira et al. 2012; Sang et al. 2013a; Assou et al. 2013).

Aging affects the follicular environment, including its protein composition (Pacella et al. 2012; McReynolds et al. 2012). Since miRNAs are responsible for the regulation of protein expression, experimental studies show a correlation between their function and aging oocytes. Aging is associated with increased levels **of miR-190b**, which targets Exostosin-1 (EXT1). EXT1 is a glycosyltransferase that is required for the biosynthesis of heparan sulfate, which regulates the pattern and intensity of the response to oocytes during COC expansion and oocyte maturation (Watson et al. 2012). Via this mechanism, miR-190b contributes to the deregulation of follicle morphogenesis and abnormal glucose metabolism in the follicles of older women (Pacella et al. 2012).

Aging is also associated with decreased levels of **miR-21-5p**; miR-21-5p targets several genes that are important in the p53 pathway, which plays a crucial role in the aging process (Collado et al. 2007). Moreover, higher levels of **miR-134** are observed in older women, which indicates decreased expression of BCL2 and inhibition of the apoptosis inhibitor nuclear factor kappa B kinase subunit gamma (IKBKG). Aged oocytes have lower expression of **miR-132**, which inhibits Sirtuin-1 (SIRT1) expression. This is achieved through increased antioxidant superoxide dismutase 2 (sod2) gene expression and a concomitant decrease in intracellular ROS in response to oxidative stress.

3.1.4 Hormonal Control and Aging

There are differences in the transcriptome profiles of the oocytes of young and older women (Grøndahl et al. 2010). The functions of gonadotropin-releasing hormone (GnRH), FSH, and LH are compromised by the aging process (Santoro et al. 1998). GnRH is a master hormone that is secreted by the hypothalamus and regulates the release of gonadotropins (FSH and LH) from the anterior pituitary gland. LH stimulates theca cells to produce androstenedione, while FSH stimulates the conversion of the theca-derived androstenedione into oestradiol, in addition to the synthesis of inhibin, by the GCs in the small antral follicles (Barbieri 2014). Under physiological conditions, a negative feedback loop of gonadotropin secretion is triggered by gonadal inhibin to allow appropriate follicle growth and development (Luisi et al. 2005).

In animal models, disruption of the hypothalamic-pituitary regulatory axis is

associated with reproductive aging (Wise et al. 1997). It was demonstrated that destabilization of the neuroendocrine signals by the aged brain is responsible for the accelerated rate of follicular loss and early menopausal transition (Wise et al. 1997). When FSH receptors in female mice are knocked out at different ages, haplo-insufficiency (−/+) of FSH receptors in seven-month-old females is associated with accelerated oocyte death. Interestingly, none of the one-year-old (+/−) females produced viable offspring (Danilovich and Sairam 2002).

In humans, the effect of aging on the hypothalamic-pituitary reproductive axis is more controversial. The depletion of the follicle number at menopause leads to cessation of negative feedback inhibition and increased levels of serum FSH and LH (MacNaughton et al. 1992). However, other studies have demonstrated a decline in gonadotropin levels, especially LH, in postmenopausal women, most likely due to changes in hypothalamic GnRH stimulation (Scaglia et al. 1976). These findings are supported by another study, in which a dynamic correlation between aging and a decline in the GnRH pulse was frequently observed and in which this correlation was independent of gonadal feedback (Hall et al. 2000). Reduced levels of serum gonadotropin free α-subunit (FAS) and LH, as neuroendocrine markers of plausible GnRH secretion, were reported in a group of postmenopausal women compared with their younger peers. These results confirmed the role of aging in hypothalamus function, and while this role is independent of gonadal function, it may represent an impetus for reproductive senescence (Hall et al. 2000).

3.2 Diet

3.2.1 The Role of Oxidative Stress

Antioxidants play a critical role in maintaining ovarian function and fertility (Lim and Luderer 2011). Supplementation of antioxidants decreases ROS-mediated oocyte damage and preserves the quality of aging oocytes and follicles. This was shown *in vitro* following the application of antioxidant *N*-acetyl-L-cysteine (NAC) to oocyte culture (Liu et al. 2012). The underlying mechanism was shown to be mediated by a reduction in telomere shortening, telomere fusion, DNA damage and chromosomal instability in oocytes. Long-term melatonin treatment ameliorates ovarian mitochondrial oxidative damage (Song et al. 2016). This protection of the ovarian tissue from aging is achieved by decreasing mitochondrial ROS generation, inhibiting apoptosis, suppressing the collapse of mitochondrial membrane potential and maintaining respiratory chain complex activities. Notably, melatonin is an endogenously generated indoleamine that plays a significant role in preventing the aging-related impairment of redox status through its antioxidants and ROS scavenging actions (Manda et al. 2007). Melatonin administration and vitamin E treatment thus lead to the improvement of the fertilization rate in patients undergoing *in vitro* fertilization (IVF) and embryo transfer (Tamura et al. 2008).

Decreased caloric intake has been shown to be associated with decreases in oocyte ROS in older females (Barja 2002, 2004). Caloric restriction for more than 6 months in 12-month-old mice resulted in reduced aneuploidy, meiotic spindle defects, and mitochondrial dysfunction compared to non-calorie-restricted controls (Selesniemi et al. 2011). This was explained by the decreased expression of proliferator-activated receptor γ coactivator-1α (PGC-1α), a critical regulator of mitochondrial respiration. Thus, caloric restriction may preserve oocyte quality in older females. The levels of coenzyme Q10 (CoQ_{10}) production, an important regulator of the electron transport chain, were shown to be reduced in aged oocytes. This was associated with decreased ATP production and increased spindle abnormities, leading to infertility (Ben-Meir et al. 2015).

3.2.2 Diet and Hormonal Control

As obesity is an evolving worldwide epidemic according the WHO report (Organization, W.H and W.H.O.M.o.S.A. Unit 2014), weight control has become an important contributing factor to preserving fertility. Obese women have difficulty conceiving, even at a younger age (Jensen et al. 1999). Obesity is associated with anovulation,

polycystic ovarian syndrome and pregnancy complications, such as miscarriage, gestational diabetes, preeclampsia, (Jungheim and Moley 2010) and poor IVF outcomes (Shah et al. 2011). However, limited research is available on the underlying pathophysiological mechanisms of many of these complications. Recent studies related obesity to alterations in the ovarian follicular microenvironment (Robker et al. 2009; Metwally et al. 2007). Intra-follicular insulin, triglyceride and lipoprotein receptors, as well as inflammatory markers such as C reactive protein, were shown to be significantly elevated in obese women compared to women of normal weight (Robker et al. 2009). Furthermore, high leptin levels have been reported in the blood and follicular fluid of obese women (Metwally et al. 2007). Leptin is an adipocyte-derived hormone that has a stimulatory effect on the hypothalamic-pituitary-gonadal axis (HPG axis), in addition to its role in energy homeostasis (Garcia-Galiano et al. 2014). During leptin resistance, excess leptin inhibits insulin-induced ovarian steroidogenesis by acting on the receptors of theca and GCs. Furthermore, Leptin inhibits LH-stimulated oestradiol production by GCs (Moschos et al. 2002). The reproduction rate of female rats that have dietary-induced obesity and hyperleptinaemia is decreased by 60% (Tortoriello et al. 2004). In humans, hyperlipidaemia in obese women leads to oxidative stress in the endoplasmic reticulum as well as to the production of ROS, resulting in mitochondrial dysfunctions and aging (Robker et al. 2011).

3.3 Diseases Associated with Oocyte Aging

3.3.1 Polycystic Ovary Syndrome (PCOS)

PCOS is a common endocrine disorder that is frequently encountered in women during their reproductive years (Trikudanathan 2015). It has heterogeneous phenotypic characteristics that include oligo-ovulation or anovulation, clinical and/or biochemical signs of hyper-androgenism, polycystic ovaries, metabolic syndrome, and infertility (Trikudanathan 2015). Hypermethylation of long interspersed element (LINE-1) DNA in CCs correlates with oocyte maturation and PCOS pathophysiology. LINEs are a group of genetic elements that produce RNA and that transcribe in the antisense direction of pre-mRNA. Thus, LINEs limit mRNA levels and control the expression of genes containing LINE regulatory sequences (Sukapan et al. 2014; Yooyongsatit et al. 2015; Wanichnopparat et al. 2013; Aporntewan et al. 2011). LINE-1 has two open reading frames (ORFs) that encode ORF1P and ORF2P, which are essential proteins for its re-integration into the genome. ORF1P is implicated in oocyte meiotic maturation. Hypermethylation of LINE-1 decreases ORF1P expression, which in turn decreases CDC2 and CYCLINB1, which are components of maturation-promoting factors. These factors are regulators of the G2/M transition (Stanford et al. 2003). Additionally, the decrease in ORF1P triggers and upregulates γH2AX, an indicator of the DNA damage response.

miRNA Expression and PCOS

PCOS is associated with decreases in the expression of miRNA-132 and -320 in the follicular fluid. miRNA-132 downregulates the phosphatase and tensin homolog (PTEN) gene (Santonocito et al. 2014), which in turn activates protein kinase B (AKT) and switches on the phosphatidylinositol 3-kinase (PI3K) signalling pathway. This pathway is involved in follicular maturation. Additionally, miRNA-132 and miRNA-320 target the expression of candidate PCOS genes, namely, high-mobility group AT-hook 2 (HMGA2) and Ras-related protein Rab-5B (RAB5B) (Sang et al. 2013b). This sheds light on the role of miRNAs in the aetiology of PCOS.

A reduction in miR-29a-3p is also evident in follicular fluid from PCOS patients. This microRNA targets PTEN (Tumaneng et al. 2012), thereby causing increased cell growth. The decreased expression of miR-29a-3p is thus accompanied by arrested follicle growth and follicular development in PCOS patients (Sørensen et al. 2016). Furthermore, PCOS is accompanied by increases in miR-224, miR-378, and miR-383 expression (Sang et al. 2013b). These miRNAs

regulate aromatase expression during follicle development (Yin et al. 2012; Zhao et al. 2011; Xu et al. 2011). Along with follicular cell activity, aromatase expression is a hallmark of PCOS.

3.3.2 HER2$^+$ Breast Cancer

HER2$^+$ breast cancer is associated with the overexpression of epidermal growth factor (EGF) (Lee et al. 2015). Upon the binding of EGF to EGF receptor (EGFR), Ras (a small GTPase) swaps its GDP for a GTP molecule and becomes activated. Activated Ras activates MAPKs. This pathway is known as the Ras-MAPK pathway. This leads to the phosphorylation of the gap junction protein Connexin 43 (Cx-43) and a decrease in gap junction permeability, resulting in reduced NPR2 activity. NPR2 is a guanylate cyclase that catalyses the conversion of intracellular guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). Reduced cGMP in oocytes activates phosphodiesterase 3A, cGMP-inhibited (PDE3A), which in turn degrades cAMP and leads to meiosis resumption. Specific miRNAs have been shown to function within these pathways and regulate the processes of follicular development and meiotic resumption. The mRNAs that regulate these processes include miR-29a, miR-99a, miR-100, miR-132, miR-212, miR-214, miR-218, miR-508-3p, and miR-654-3p, which are upregulated in follicular fluid compared to plasma.

3.3.3 Insulin Resistance

Insulin is an evolutionarily conserved protein hormone that regulates diverse biological functions such as glucose homeostasis, cellular growth, aging, fertility and reproduction (Sliwowska et al. 2014; Tatar et al. 2003). Many studies have demonstrated the gonadotropic action of insulin on the ovary via specific signalling pathways that interact with FSH and LH during oogenesis and folliculogenesis (Sliwowska et al. 2014; Dupont and Scaramuzzi 2016). Moreover, insulin has been used as a supplement for *in vitro* culture at the early stage of human follicles. It was reported that insulin plays a survival role whereby the number of atretic follicles decreases and the number of healthy viable oocytes increases in culture (Louhio et al. 2000).

Insulin resistance (IR) and the resulting hyperinsulinaemia are well-recognized characteristics of polycystic ovary syndrome (PCOS) that lead to ovulatory dysfunction and infertility in women (Dale et al. 1998). Infertility has been attributed to hyperinsulinaemia, which suppresses sex hormone-binding globulin synthesis in the liver, rather than to peripheral IR (Nestler 1997). Sex hormone-binding globulin is a glycoprotein composed of two 373-amino-acid subunits that transport sex steroids, such as testosterone, to target tissues (Wallace et al. 2013). Hyperinsulinaemia thus promotes high levels of free testosterone in obese women with PCOS, remarkably affecting oocyte quality (Nestler 1997).

In female mice, IR has been found to stimulate oxidative phosphorylation in the mitochondria, where ROS is formed and antagonized by antioxidants (Boirie 2003). However, in IR, an imbalance between oxidants and antioxidants is observed and results in impaired mitochondrial function in the germinal vesicle and MII oocytes of insulin-resistant mice. A previous report indicated that apoptosis of germinal vesicle oocytes occurred at an early stage and that atretic and poor-quality MII oocytes were obtained from these mice.

3.4 Environmental Pollutants and Oocyte Aging

Pesticides and Oocyte Aging

The exposure of women to pesticides on a daily basis and a lack of precautions is considered an important occult cause of fertility problems. Pesticides of different types and variable levels of toxicity have been shown to interfere with female sex hormones and cause dysregulation of the ovarian cycle (Farr et al. 2004). The exposure of women to pesticides occurs in daily life in the form of consuming pesticide-laced fruits and vegetables and contaminated drinking water, the use of household and gardening supplies, and the

use of some cosmetic and cleaning substances, such as dog shampoos. Pesticide-induced disruption of hormonal functions in women has been shown to be associated with disruptions in the menstrual cycle, fertility reduction, conception failure, stillbirths, spontaneous abortions, and developmental defects (Schettler et al. 1997; Razi et al. 2016; Bretveld et al. 2006). Pesticides, such as lindane, atrazine and mancozeb, have toxic hormonal properties, leading to delayed ovulation and menstrual cycle disruptions in animal models (Chadwick et al. 1988; Ashby et al. 2002; van Birgelen et al. 1999). Atrazine was specifically shown to decrease LH concentrations, leading to anovulation (Ashby et al. 2002). Another pesticide, polychlorinated biphenyl 126, has been shown to be associated with alterations in oocyte and blastocyst maturation and follicle destruction (Younglai et al. 2005). Cases of infertility have been reported in several communities due to the use of the pesticide dichlorodiphenyltrichloroethane, which is an endocrine disruptor (Attaran and Maharaj 2000).

4 Conclusion

The aim of this review is to shed light on the risk factors for oocyte aging with the aim of developing new therapeutics that enhance the quality of oocytes and hence the quality of female life. For the sake of maintaining oocyte quality, a healthy lifestyle is crucial. Low caloric intake, caloric restriction, and weight control are very important for inhibiting ROS production in the oocytes of older females and decreasing meiotic spindle shape defects to maintain female fertility. Furthermore, ovarian stimulation regimens have been shown to correct the oocyte microenvironment *in vivo* and improve oocyte quality. Thus, the use of GnRH agonists and antioxidants is beneficial for maintaining oocyte quality. Additionally, avoiding exposure to pesticides and stress maintains female hormonal functions and healthy ovulation. However, we cannot neglect the effects of certain diseases, such as PCOS, $HER2^{+}$ breast cancer, and IR, on oocyte longevity. Moreover, neighbouring cell interactions with oocytes play a pivotal role in maintaining oocyte quality and longevity.

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Targeting Cancer Metabolism and Cell Cycle by Plant-Derived Compounds

Esra Aydemir Çoban, Didem Tecimel, Fikrettin Şahin, and Ayşen Aslı Hızlı Deniz

Abstract

Cancer has an increasing death rate over the world population without discriminating between the industrial and developing countries. Complexity of cancer such as ability of cancer cells to develop resistance to drugs or differential behavior of sub-types and different responses from the patients indicate the continuous need for research and development of new anticancer drugs, new formulations of drug combinations and treatment strategies. Not too surprisingly nature itself, is often the largest territorial reservoir as a source for this type of research and development. Speaking of plant variety, more than 1000 plants have already been identified to produce agents with anticancer activities. In this review, a panel of plant derived anti-cancer agents will be reiterated in terms of their mechanism of action in treatment of disease.

Keywords

Cancer · Chemotherapy · Flavonoids · Plants

E. A. Çoban (✉), D. Tecimel, and F. Şahin
Department of Bioengineering and Genetics, Yeditepe University Faculty of Medicine, Istanbul, Turkey
e-mail: esra.aydemir@yeditepe.edu.tr

A. A. H. Deniz
Health Institutes of Turkey, Turkish Cancer Institute, Istanbul, Turkey

Abbreviations

ASPE	Algal sulfated polysaccharide extract
ATP	Adenosine triphosphate
B-myb	Myb-related protein B
CDK4	Cyclin-dependent kinase 4
CDK6	Cyclin-dependent kinase 6
COX-2	Cyclooxygenase-2
CREBP	cAMP-response element-binding protein
CYP1B1	Cytochrome P450 Family 1 Subfamily B Member 1
E2F	Elongation 2 Factor
FASN	Fatty Acid Synthase
FDA	Food and Drug Administration
GLS2	Glutaminase 2
GLUD1/2	Glutamate Dehydrogenase 1/2
HIF1α	Hypoxia-inducible factor 1-alpha
MAPK	Mitogen-activated protein kinase
MCL1	Myeloid cell leukemia 1
mTOR	mammalian target of rapamycin
Myc	Myelocytomatosis
NFKB	Nuclear factor kappa-light-chain-enhancer of activated B
PI3K/AKT	phosphoinositide-3-kinase/Protein kinase B
pRB	protein Retinoblastoma
TCA	Tricarboxylic acid cycle
TNFα	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

1 Introduction

According to World Health Organization (WHO) reports in 2015 cancer is the second major death cause accounting for 8.8 million of deaths all over the world. In general terms, it is considered as a disease that can affect any epithelial and mesenchymal tissue in human body resulting in malignant tumors or neoplasms of these tissue types (Torre et al. 2015). Based on the model developed by Robert Weinberg and Douglas Hanahan, six basic differences are proposed in their seminal article 'The Hallmarks of Cancer' to distinguish a healthy cell from a cancerous cell. Evading apoptosis, lack of need for growth signals due to self-sufficiency, insensitivity to anti-growth signals, tissue invasion & metastasis, sustained angiogenesis, and limitless replicative potential constitute the core criteria of discerning a normal cell from a cancer cell apart (Hanahan and Weinberg 2000).

There are new approaches to treat cancer such as gene therapy and immunotherapy which are promising -yet- demanding in cost and require further studies. Although, the standard and current treatment relies on chemotherapy, it creates serious side effects caused by the cytotoxic effects of the anti-cancer agents on healthy cells. Eighty percent of the world population depends on the plant based systems to meet their general health care needs. Hence, medicinal plants have been suggested as a valuable resource to look for better anti-cancer drugs for almost centuries. The natural agents such as terpenoids, lipids, proteins etc. come either from microbial, plant or animal sources. There are FDA approved drugs including Dactomycin, Bleomycin that come from the microbial origin whereas Taxol, Teniposide and its related derivatives are examples of plant-derived anticancer compounds (Mukherjee et al. 2001).

Downmodulation of cell cycle and other signaling pathways related to cancer progression, tumorigenicity, impediment of metastasis, induction of apoptosis, necrosis, necroptosis or suppression of autophagy through inducing relevant changes in gene expression are among the known types of mechanism-of-action for these natural anti-cancer agents. Moreover, plant-based chemotherapeutic agents can affect major pathways that control carbohydrate, amino acid, and fatty acid metabolism. In this review, our current understanding of the therapeutic mechanisms for a short panel of plant derived agents are summarized.

1.1 Reprogramming the Cancer Cell Metabolism

1.1.1 Glucose Metabolism: A Flavonoid Effect

In some cancer types the demand for proliferation is propelled by deregulation of anabolic pathways that produce macromolecules -most likely- through reprogramming of those pathways of the core carbon metabolism through induction of PI3K/AKT and mTOR that is central for the control of nutrient-uptake. Altered metabolic pathways might affect -not only- the biosynthesis of macromolecules, but also bioprocessing of amino acid and fatty acid metabolism (DeBerardinis and Chandel 2016). Therefore, enzymes controlling and regulating the key biochemical steps in the reaction cascades of complex metabolic networks have been envisioned as promising targets to correct or block the activity of dysregulated metabolic pathways in cancer cells. Potentially, with this approach, a consequential deregulation in the activity of "*undruggable*" downstream (oncogenic) targets, such as transcription factor Myc and HIF1-α, oncogenic activity for which are widely recognized, can be blocked (DeBerardinis and Chandel 2016).

The earliest evidence that metabolism is involved in cancer progression was discovered by Otto Warburg who observed that cancer cells do not behave as normal cells in terms of glucose utilization. When the oxygen levels are high, normal cells direct the glucose to the mitochondria for oxidative phosphorylation, whereas cancer cells show increased uptake of glucose regardless of oxygen levels (Cairns et al. 2011). Hence, blocking the glucose transporters to repress glucose uptake has emerged as a promising strategy. The levels of these transporters are elevated in a variety of

cancer cell types suggesting their suppression as an efficient therapeutic approach, however, due to their expression in multiple isoforms their targeting selectively has been challenging (Adekola et al. 2012). Another strategy is to use a glucose analogue that is trapped inside the cells in the form of 2-deoxyglucose-6-phosphate, which cannot be metabolized further, resulting in lethality induced by energy crisis (Aft et al. 2002). In addition to glucose transporters, glycolytic enzymes are also tempting -however- challenging targets due to the existence of their multiple isoforms (Tseng et al. 2018; Yu et al. 2017). Therefore, a strategy based on enhanced drug specificity unique for each isoform should be developed. A research study conducted by Jae B. Park suggests that flavonoids which are the polyphenolic compounds found in fruits and vegetables can block the glucose uptake in myelocytic U937 cells. Flavonoids are focus point of many studies due to their anti-oxidative, anti-inflammatory, anti-mutagenic, and anti-carcinogenic properties. Most importantly this properties are coupled with their ability to alter cellular enzyme function. For the plant itself, they are responsible for the color and aroma of flowers, support pollination, and also protect plants from biotic and abiotic stresses. They are classified under 6 groups; flavones, flavonol, isoflavone, anthocyanidin, flavanone, and flavan-3-ol (Cheong et al. 2012; Panche et al. 2016; Park 1999) (Fig. 1).

In their study Park et al., used apigenin as a flavone, fisetin, myricetin, quercetin as flavanols;, naringenin as a flavonone, hesperetin as a isoflavone; and genistein, daidain, and anthocyanidin as cyanidings. The results revealed that all flavonoids inhibited the glucose uptake in U937 cells in a dose-dependent-manner with varying efficacy (Cheong et al. 2012; Panche et al. 2016; Park 1999).

1.1.2 Glutamine Metabolism: Contribution of Honokiol from Magnolia Officinalis

Nonetheless, these early studies with a focus on glucose metabolism unraveled that both amino acids and fatty acids also provide building blocks for the growth and proliferation for cancer cells when reprogrammed. In blood, glutamine is the most abundant amino acid and it is the major source of nitrogen for the synthesis of nucleotides, amino acids, and glutathione. Glutamine is utilized in cells that have highly proliferative characteristics as a carbon source for the TCA cycle. Particularly, cancer cells growing under hypoxic conditions display an increased dependency for glutaminolysis since the α- ketoglutarate that is derived from glutamine can undergo reduction via carboxylation and produce citrate and lipids (Altman et al. 2016). Alterations in amino acid metabolism can promote changes in the levels of oncogenic factors such as NF-kB, MYC, and CREBP. Like in the case of carbohydrate metabolism, regulatory enzymes of amino acid metabolism (i.e. GLUD 1/2, GLS2) can be potential targets for regulating the levels of oncogenic factors (Amelio et al. 2014; Cluntun et al. 2017; Wise and Thompson 2010). For instance, glutamine uptake and degradation is controlled by c-MYC in which, the released Myc oncogene often produces ample MYC protein, which in turn triggers related genes that play a role in biogenesis of ribosome and mitochondria, glucose and glutamine metabolism. Abundant Myc production leads to the biosynthesis of lipids and nucleotides allowing rapid generation of aminoacids to endure increased tumor cell number. Therefore, c-MYC might be a good target in a novel therapeutic approach (Cluntun et al. 2017; Bott et al. 2015; Gao et al. 2009). A study on prostate cancer by Eun-Ryeong Hahm and his colleagues shows that honokiol from *Magnolia officinalis* decreases both cytoplasmic and nuclear levels of c-Myc protein levels while downregulating mRNA expression. Honokiol is a small polyphenol. It has been used as an important agent in disease treatment for over the centuries in the traditional eastern homeopathic medicines. By intermolecular interactions such as hydrogen bonding it can interact with the cellular membrane. Since it can pass through the blood-brain barrier and blood-cerebrospinal fluid barrier it will be optimally available during the therapy. Honokiol does not just exert an effect on c-MYC,

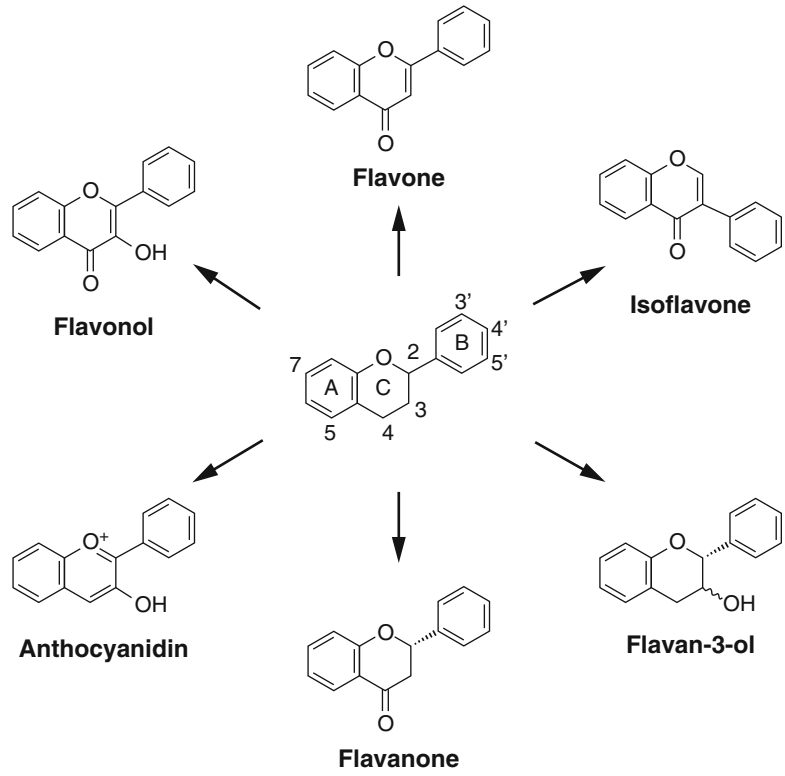

Fig. 1 Sub-classes of flavonoids and their structures. The basic structure of flavonoids composed of benzene and benzopyran groups

but it can also induce caspase-dependent apoptosis via TRAIL pathway while reducing phosphorylation levels of mitogen activated protein kinase (MAPK) which is considered as an oncogenic signal for several types of human cancer. It can also target pro-apoptotic factors and inhibit survival factors such as vascular endothelial growth factor (VEGF), induced myeloid leukemia cell differentiation protein (MCL1), and cyclooxygen ase 2 (COX-2) by regulating nuclear factor kappa B pathway (NFkB) (Cheong et al. 2012; Bai et al. 2003; Hahm et al. 2016).

1.1.3 Fatty Acid Metabolism: Green Tea Polyphenol, Epigallocatechin-3-Gallate Inhibits FASN Activity

The high energy content of lipids is used for cellular processes. Especially, fatty acid metabolism is tightly linked to both glucose and glutamine metabolism since they provide the necessary substrates (i.e acetyl-coA) for the synthesis of fatty acids in addition to stimulating the proliferation and growth of the cells. These findings allude to a new perspective in which cancer can be a disease that manifests itself as a compilation of faulty metabolic processes. In support of this view, cancer tissues show very high rates of fatty acid synthesis compared to healthy tissues. The most common strategy regarding this mechanism is the use of small molecules that inhibit the key lipogenic enzymes such as fatty acid synthase (FASN), Acetyl-CoA carboxylase, and ATP- citrate lyase (Currie et al. 2013; Saavedra-Garcia et al. 2018). Especially, FASN is frequently reported to be upregulated in most types of cancer including prostate, ovarian, breast cancers. Furthermore, it is associated -not only- with elevated proliferation, but also resistance to anti-cancer drugs (Flavin et al. 2010; Vriens et al. 2019). Moreover, similar set of deregulatory changes in the expression of lipogenic genes have also been reported in cancer stem cells. Although underlying molecular links remain unclear it is tempting to suggest their survival

and switching to a proliferative state can be dependent on the activities of these enzymes such as FASN (Kuo and Ann 2018). Inhibition of FASN is the most betaken therapeutic strategy while studying the lipid metabolism contribution to cancer progression. A study on prostate cancer cell line LNCaP, concluded that natural polyphenol epigallocatechin-3-gallate, an abundant polyphenol found in green tea, reduces FASN activity to approximately 22% when given in 150 μM for 48 h and further, while suppressing proliferation and inducing apoptosis (Brusselmans et al. 2003).

1.1.4 Cell Cycle Targeted Cancer Therapies

Cell division cycle governs the events of both the transition to proliferative stages from quiescence as well as consecutive cell divisions. Preservation of genetic information in progeny of a parental cell is the ultimate goal of these cell cycle transitions (Hunt et al. 2011). Traditionally, the cell cycle is considered in four stages; S phase associated with DNA synthesis, M phase associated with cell division, and G1 and G2 gap phases, that intervene the S and M phases. G0 is the period where cells are in quiescence meaning they are not dividing, like in the case of neural stem cells that can maintain most of their life span in this resting state unless they are stimulated. A tightly regulated control system called Checkpoints oversees that transition from one phase of the cell cycle to the next proceeds properly so that genetic fidelity is guarded (Morgan 1992). The first checkpoint is called "the Start" or the G1/S checkpoint. When conditions are ideal for proliferation, cells enter the division cycle whereby DNA replication, centrosome duplication, and other early cell-cycle events take place. Eventually, progression through the second major checkpoint right before the entry into mitosis (G2/M checkpoint) surveys the fidelity of spindle assembly, allowing cells to proceed to metaphase. The third major checkpoint takes place at the metaphase-to-anaphase transition, which precedes sister chromatid segregation and completion of mitosis (Morgan 1992). Mutations in several checkpoint regulatory molecules have been reported across a wide variety of human cancers. Therefore, tremendous effort has been dedicated to the development of small molecules that target deregulated checkpoints to reverse their function in protecting cancer genome (Peyressatre et al. 2015).

1.1.5 Targeting G1 Phase: Effect of Algal Sulfated Polysaccharide Extract from Laurencia papillosa

Early in the cell cycle G1 progression is controlled by mitogen-regulated D-type cyclins (cyclin D1, D2, and D3). Cell cycle arrest is known as the stalling of the cells at the G0/G1 border when withdrawal of mitogens results in accumulation of cells in quiescence, an event triggered by rapid turnover of cyclin D1 (Sherr 1995). In the absence of their regulatory subunit of cyclin D, the catalytic subunits, Cyclin-dependent kinases 4 and 6 (CDK4 and CDK6) cannot be activated to phosphorylate their substrate panel, an event that conducts the molecular events of G0-G1 transition. CDK4 and CDK6-dependent catalytic activities are crucial particularly because they phosphorylate and inactivate the inhibitory members of the retinoblastoma family which function as transcriptional co-repressors and adaptors for chromatin modifiers for the activities of those genes that are required for the entry and progression into S phase (Bockstaele et al. 2009; Malumbres 2014). For example, a transcription factor called E2F upregulate the expression of those genes associated with cell cycle progression including genomic DNA synthesis. However, the transcriptional activity of E2F is negatively regulated by the members of the retinoblastoma family (also called as Pocket Protein family) such as pRB, p130, and p107 that bind and hinder E2F from initiating transcription (Khleif et al. 1996; Weinberg 1995). Therefore, preventing E2F-dependent transcription program from driving cells through further phases of the cell cycle is considered as a promising therapeutic approach (Knudsen and Witkiewicz 2017). In that regard, numerous attempts were made to block the CDK 4 and CDK 6-associated activity. For example, in a 2016 study published by Hossam Murad and his collogues demonstrated

that algal sulfated polysaccharide extract (ASPE) from *Laurencia papillosa* could inhibit proliferation and arrest the proliferating MDA-MB-231 cells in G1 phase in association with the downregulation of D1, D2 and E1 cyclin transcripts as well as their CDK-binding partners; CDK4, CDK6, and CDK2, respectively. This effect was observed at concentrations as low as (10 μg/ mL). In the case of high concentration treatments of ASPE (50 μg/mL), an upregulation of BAX family members and Caspase 3 cleavage was observed, whereas expression of Bcl-2 was downdregulated promoting the apoptosis of the breast cancer cells via the intrinsic pathway. *Laurencia papillosa* itself belongs to red algae family and its routine consumption in daily diet is found in association with low prevalence of breast cancer in East- Asia. The sulfated polysaccharides from red seaweeds are known for their anti-oxidant, anti-proliferative, anti-tumor, anti-viral, and anti-coagulant effects. Hence, there has been a major research interest to understand their therapeutic activity in numerous studies for many years (Murad et al. 2016; Parmar and Uludag 2016).

1.2 Targeting S Phase: Xanthohumol; Major Prenylated Chalcone of *Humulus lupulus*

S phase is where the DNA synthesis takes place. The synthesis of DNA is accomplished by the coordinated action of several enzymes such as topoisomerases, helicases, and DNA polymerases. It is surely beyond any doubt that targeting of the enzymes that are responsible for the DNA synthesis is one of the most important approaches taken in cancer therapy. For example, anti-metabolites that block the activity of essential enzymes for DNA synthesis as well as intercalating agents that have the ability to interfere with the replication of their resident strands have displayed therapeutic effects (Mills et al. 2018).

Xanthohumol is a prenylated flavonoid the anti-cancer effect of which is also exerted through blockade of S phase. Prior to the discovery of its anti-tumoral activity, this agent was recognized for its various therapeutic effects on conditions such as neurodegenerative and cardiovascular diseases (Ooi et al. 2018; Yao et al. 2015). However, growing amount of evidence points to the promising therapeutic effects of Xanthohumol via impairing mitochondrial function, inhibiting proliferation and the angiogenesis, and modulating immune-therapeutic responses (Magalhaes et al. 2009). Major Xanthohumol up-take in human diet occurs through the consumption of fermented alcoholic beverages, particularly beer. It is only found in *Humulus lupulus*, a hop plant and core prenylflavonoid of female blooms. In the plant itself, prenyyflavonoids are responsible for defending the plant against pathogenic microorganisms (Yang et al. 2015). As being bioactive compounds, they are known as antioxidants, immune modulators, antibacterial, antifungal, and anticancer agents. In cancer, this agent is used in a chemopreventive and/or therapeutic manner. Due to its inhibitory role in metabolic activation of procarcinogens, xanthohumol is a member of a 'broad- spectrum' of cancer chemopreventive agents. It has been shown that xanthohumol promotes activation of enzymes that are responsible for carcinogen detoxification and hence has a potential to inhibit tumor growth in early stages (Yang et al. 2015). In a study of Jessica G. Drenzek et al., they demonstrated that xanthohumol is a potent inhibitor of ovarian cancer cell growth and the underlying mechanism-of-action could involve the downregulation of Notch1 pathway. A therapeutic effect of xanthohumol is reported in SKOV3 ovarian cancer cell line in a dose-dependent manner whereby cells underwent cell cycle arrest. In this study a 30 μM xanthohumol treatment resulted in accumulation of cells in S and G2/M phases compared to the vehicle control (Drenzek et al. 2011; Stevens and Page 2004).

1.3 Targeting G2 Phase: A Contribution of Ginkgetin from Ginko Biloba Leaves

G2 phase is the premitotic phase which starts after the DNA replication. During G2 there is an increase in cellular biomass so that there is enough cytoplasmic material (like organelles) to partition between the two daughter cells at the end of mitosis. Therefore, not too surprisingly, inhibition of protein synthesis results in abrogation of progression through G2 and impairment of the cells to undergo mitosis (Kousholt et al. 2012).

B-myb is a transcription factor and a major regulator of differentiation, survival, and cell cycle progression. The expression of b-myb is controlled by its dimerization partner, RB-like proteins, E2Fs and MuvB core (DREAM complex). DREAM complex is crucial in gene expression related to the coordination of cell cycle progression (Fischer and Muller 2017; Sadasivam and DeCaprio 2013). Frequently, b-myb is often found deregulated in cancer cells and, therefore, associated with initiation and progression of cancer (Nath et al. 2015; Sala 2005). Ginkgetin is a biflavonoid enriched in *Ginkgo biloba* plant and its anti-tumoral activity has been studied in various cancer studies. In terms of its mechanism-of-action for its anti-cancer effects its ability to inhibit proliferation has been demonstrated. Biflavonoids contain two flavones groups which constitute a distinct subclass of flavonoids. They are characterized as an example of unsaturated carbon compounds that has three carbons harboring a double bond between the carbon 2 and carbon (Musa et al. 2017).

Ginkgetin, a type of bioflavonoids that is extracted from the leaves of ginkgo plant, induces a G2 arrest in HCT116 colon carcinoma cell line by downregulating b-myb by 80% (Lee et al. 2017). In another study, following the treatment with ginkgo fruit extract of the breast cancer cell line (MDA-MB-231) an enhanced expression of cytochrome P450 (CYP) 1B1 (an enzyme involved in biochemical processes such as decomposition of drugs and production of certain lipids) was detected compared to the treatment with leave extract of *Ginkgo biloba* (Zhao et al. 2013). Despite these promising results there has been reports showing the activation of stress-responsive MAPK pathway in liver metastasis of colon cancer suggesting a pro-metastatic effect of the compound (Wang et al. 2017). Therefore, future studies should address the controversial effects Gingko Bloba extracts in exacerbating metastatic growth as opposed to its preventive or anti-tumorigenic effects.

1.4 Targeting Mitotic Phase: Ethanolic Extract of *Origanum majorana*

At the end of the mitotic phase two daughter cells are formed by the division of cytoplasm and chromosomes, a phenomena termed as cytokinesis. Mitosis proceeds in 5 stages; prophase, prometaphase, metaphase, anaphase, and telophase (McIntosh 2016). Prophase is the stage at beginning of the mitosis when the chromosome condensation begins. Each centrosome in the duplicated pair moves to opposite poles and the spindle formation begins via synthesis of microtubules. Prometaphase is the stage where the nuclear envelope breaks down. In metaphase, chromosomes reach their most condensed stage lining up at the equator of the spindle. During anaphase a protease named separase degrades the cohesion molecules that functions for joint of the sister chromatids. Therefore, at this stage sister chromatids separate from each other. Telophase is the final stage of the mitosis marked by the reaching of the chromosomes to the poles, re-formation of the nuclear membrane, and the de-condensation of the chromosomes within the newly formed nuclei. The final stage is followed up by cytokinesis which is the division of the cytoplasma into two daughter cells. The daughter cells which are the products of mitosis have identical genetic material. Deregulation of cell cycle

in the cancer cells is a hallmark, hence the preventive or therapeutic approaches targeting the mitosis is an effective point of intervention to block the abnormal proliferation of the cancer cells (McIntosh 2016). A study on breast cancer shows that mitotic arrest and apoptosis are induced by treatment of *Origanum majorana* extract. This plant is rich in phenolic compounds. This content is associated with antioxidant properties due to ability of them to capture the free radicals. *Origanum majorana* extract is ethanolic so the general type of the extract is considered as alkaloid. Alkaloids are naturally occurring compounds in many organism such as fungi, bacteria, and plant. Besides having basic nitrogen atoms, they have neutral even weak acid properties. The alkaloids can be isolated from crude extract by acid-base extraction method. They serve to a broad range when it comes to pharmaceutical field. Examples can be anti-asthma, anti-bacterial, and anti-cancer drugs. The first alkaloid purified from *Papaver somniferum* was morphine (Chan et al. 2012). Ethanolic extract of *Origanum majorana* induces mitotic arrest as well as apoptosis in a dose- and time-dependent manner and the mechanism involves the upregulation of Tumor Necrosis Factor Alpha (TNF-α) as well as the downregulation of Survivin, and mutant p53 (Al Dhaheri et al. 2013).

2 Conclusion

Plants have been in use for disease treatment for centuries. Moreover, several herbal treatments are incorporated in the contemporary applications of traditional medicines over the world. The scientific research will definitely offer the answers why medicinal plants reveal such a remarkable therapeutic potential. By developing a precise understanding on the biology of the plant as well as that of the cancer combined with a thorough understanding of the drug chemistry, the plant-based treatments will continue to be part of either chemopreventive or chemotherapeutic approaches. When the hallmarks of cancer are considered there are many mechanisms that an agent can potentially target. However, seeing them as independent processes could fail to be rational. In this review, possible targets for cancer prevention and therapy are examined and the mechanism of action for some of the plant based agents such as flavonoids and alkaloids are exemplified based on the findings from the recent studies. We can conclude that plant based approaches can target different steps of carcinogenesis, including disruptions in cell cycle check points, proliferation, and metastatic progression.

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Differentiation Potential of Mesenchymal Stem Cells into Pancreatic β-Cells

Safa Aydin, Derya Sağraç, and Fikrettin Şahin

Abstract

Stem cells having the capability to differentiate into other type of cells and renewing themselves, gained so much importance in recent years. Investigations in stem cells revealed that mesenchymal stem cells can successfully differentiate into other type of cells like adipocytes, hepatocytes, osteocytes, neurocytes and chondrocytes. In addition, these cells can also differentiate into insulin-producing beta cells. Insulin is a crucial hormone for glucose balance of the body. Insufficiency or unavailability of insulin is called diabetes. External insulin intake, as well as pancreas or islet transplantation, is the most basic treatment of diabetes. *In vivo* and *in vitro* studies demonstrate that stem cell therapy is also used in the cure of diabetes. Differentiation process of stem cells into beta cells releasing insulin is quite complicated. There are many different reports for the differentiation of stem cells in the literature. The success of differentiation of stem cells into beta cells depends on several factors like the source of stem cells, chemicals added into the differentiation medium and the duration of differentiation protocol. Distinct studies for the differentiation of stem cells into insulin-secreting cells are available in the literature. Moreover, thanks to the superior differentiation capacity of stem cells, they are being preferred in clinical studies. Stem cells were clinically used to heal diabetic ulcer, to increase c-peptide level and insulin secretion in both type 1 and type 2 diabetes. Mesenchymal stem cells having high differentiation potential to insulin-secreting cells are encouraging vehicles for both *in vivo* and *in vitro* studies together with clinical trials for diabetes mellitus.

Keywords

Mesenchymal · Stem cells · Diabetes · Differentiation · Pancreatic β cells · Stem cells

Abbreviations

ASCs	Adult stem cells
bHLH	Basic helix-loop-helix
BME	β-mercaptoethanol
DAPT	N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester
DE	Definitive endoderm
DEX	Dexamethasone
Dhh	Desert hedgehog
Dmso	Dimethyl sulfoxide
Dvl	Dishevelled
ESCs	Embryonic stem cells
Fox	Forkhead box
FoxA2	Forkhead box A2

S. Aydin (✉), D. Sağraç, and F. Şahin
Department of Genetics and Bioengineering, Faculty of Engineering, Yeditepe University, İstanbul, Turkey
e-mail: safa.aydin@yeditepe.edu.tr

Fzd	Frizzled
Gck	Glucokinase
GLP	Glucagon-like peptide
Glut-2	Glucose transporter 2
HDACs	Histone deacetylases
Hes1	Hairy and enhancer of split-1
HGF	Hepatocyte Growth Factor
HSCs	Hematopoietic stem cells
IAPP	Islet amyloid poly peptide
Ihh	Indian
iPSCs	Induced pluripotent stem cells
Isl1	Islet-1
LEF/TCF	T-cell 13 factor/lymphoid enhancer factor
MSCs	Mesenchymal stem cells
Ngn3	Neurogenin 3
Pdx-1	Pancreatic and duodenal homeobox 1
Ptc1	Patched1
Ptc2	Patched2
PTF1a	Pancreatic Transcription Factor 1 a
RA	Retinoic acid
RALDH	Retinaldehyde dehydrogenase
Smo	Smoothened
Sonic	Shh
TFs	Transcription factors
Wnt	Wingless-related integration site

1 Introduction

Cells having self-renewal ability and multilineage differentiation capacity are described as stem cells (Bacakova et al. 2018). Responsibilities of stem cells are to provide homeostasis, to repair of damage to the body and differentiation into new cells if needed (Suchánek et al. 2007). According to their sources, stem cells are basically categorized into three groups; adult stem cells (ASCs), embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) (Lakshmipathy and Verfaillie 2005; Takahashi et al. 2007; Takahashi et al. 2007). As known, inner cell mass of the blostocyte is the source of ESCs. These cells have the capacity of pluripotency and can create all embryonic cell types (Ilic and Ogilvie 2017). Reprogramming of somatic cells into embryonic-like cells having pluripotency is called induced pluripotent stem cells (iPSCs). iPSCs obtained by transferring transcription factors like Sox2, Klf4, Oct3/4 and c-Myc into somatic cells were firstly achieved in 2006 by Yamanaka (Takahashi and Yamanaka 2006). The main cell type to get iPSCs was Mouse fibroblast. However, studies indicated that cells obtained from dental tissues are also used in the generation of iPSCs (Demirci et al. 2016). Mesenchymal stem cells (MSCs), neural stem cells and hematopoietic stem cells (HSCs) can be given in the group of adult stem cells (Yalvac et al. 2010). The primary role of ASCs is to provide renewal of cells in adult tissues. MSCs are obtained from distinct sources like bone marrow, skin, synovial membrane, liver, adipose, nerve and dental tissues (Doğan et al. 2015). MSCs are firstly isolated by Friedenstein in 1970 (Friedenstein et al. 1970). It is very easy to maintain MSCs in cell culture conditions. MSCs express surface antigens like CD 29, CD105, CD90 and CD73 which are mesenchymal stem cell markers as they express no hematopoietic stem cell markers such as CD14, CD45 and CD133 (Aydin et al. 2016). MSCs have high differentiation capacity. It was demonstrated that MSCs can be turned into adipocytes, chondrocytes and osteoblasts (Al-Nbaheen et al. 2013). Moreover, neurocytes and hepatocytes are successfully obtained by differentiation of MSCs *in vitro* (Ullah et al. 2015). It was indicated that human bone marrow stem cells included in MSCs are able to successfully be differentiated into islet-like cell (Chen et al. 2004; Zanini et al. 2011; Milanesi et al. 2012).

There are other crucial features of MSCs except their differentiation ability. It was shown that MSCs have the capacity of secrete various growth factors, chemokines and cytokines (Squillaro et al. 2016). At present, it was indicated that these released agents from MSCs may protect peripheral cells from apoptosis and induce their proliferation which accelerates regeneration of injured tissues (Wang et al. 2011). Apart from their differentiation potential, MSCs possess immunomodulatory features both *in vitro* and *in vivo* (Gebler et al. 2012). MSCs can regulate

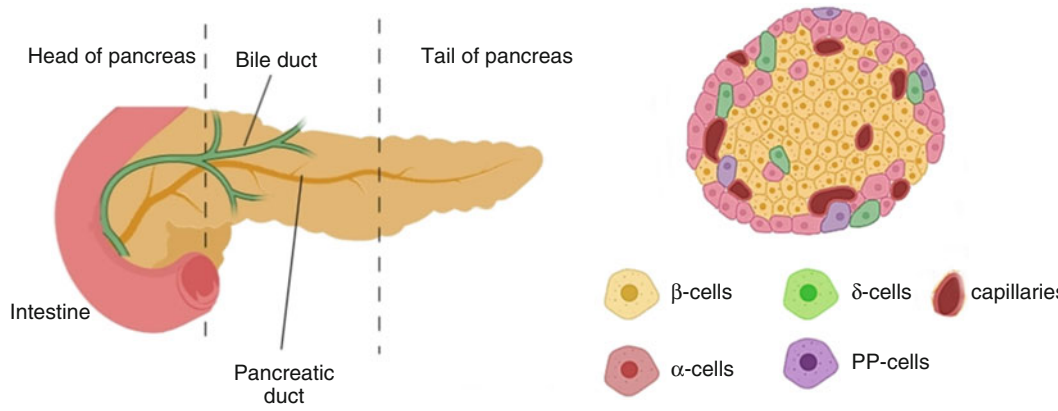

Fig. 1 Composition of distinct cell types in Pancreas

various factors such as NK cells (Spaggiari et al. 2008), macrophages (Singer and Caplan 2011), B cells (Corcione et al. 2006) and regulatory T cells (Maccario et al. 2005) involved in the immune systems. MSCs are preferred in cell therapy applications due to their useful features.

2 Pancreatic Development

The pancreas originates from foregut derived from definitive endoderm in the fifth month of pregnancy. Definitive endoderm folds into a primitive gut tube with the help of growth factors secreted from adjacent tissues. The epithelial tissue extending toward the mesoderm forms the ventral and the dorsal pancreatic buds. (Can 2014). Pancreas consists of two parts; ventral and dorsal buds. Along the developmental process, these buds grow, branch and the ventral bud switches to the other side. Therefore, two buds combine to create the head of pancreas. A whole pancreas is consisted of the head, body and tail sections.

Definitive endoderm differentiates into several cell types like ductal, acinar, and pancreatic multipotent cells. Since it composes of many cell types, the pancreas has different characteristics. The pancreas contains 90% exocrine cells called ductal and acinar cells and 10% endocrine cells which are pancreatic multipotent cells. While exocrine cells are responsible for providing the secretion of enzymes for digestion to the duodenum, endocrine cells secrete hormones into the blood (Fig. 1).

When looking at the development of pancreas closely, the multipotent progenitor cells can transform into many types of pancreatic cells like stated before. Each cell group has a different role on body homeostasis. As Alpha (α) cells produce glucagon, while Delta (δ) cells are important for the secretion of somatostatin. Beta (β) and Gamma (γ) cells produce insulin and pancreas related polypeptides, respectively. There is another group of cells found in the pancreas. Epsilon (ε) cells are responsible for secreting *ghrelin* hormone of the pancreas with the stomach and small intestine.

Dorsal pancreatic buds release insulin more than ventral buds because of the difference between beta cell composition (Kim et al. 2001). These differences based on the development during gestation. The destiny of a cell is determined by silencing the genes necessary for differentiation or absence of signaling pathways important for the maturation of pancreatic cells. In the presence of Neurogenin3 and Isl1 and absence of Notch signaling pathway, the progenitor cells transform to the endocrine precursor cells. Although cells are different from each other, they co-express the same transcriptional factors like Pdx-1 and Nkx6.1. The origin of Alpha and

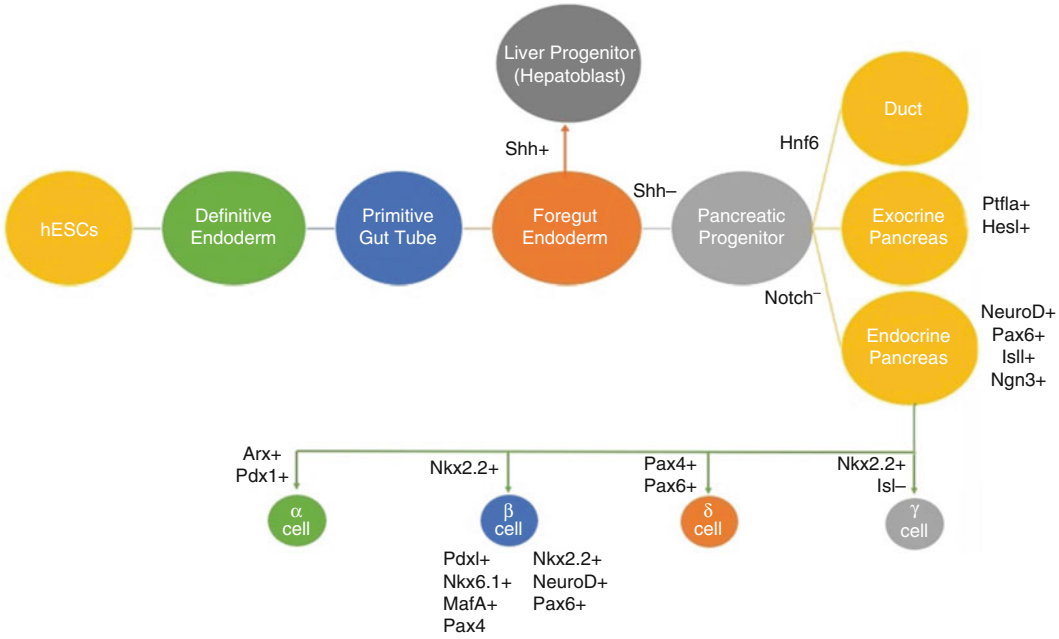

Fig. 2 Pancreatic Linage. (hESCs, human embryonic stem cells)

gamma cells is slightly different as well as delta and beta cells. In other words, the cells expressing Neurogenin-3 gene are divided into two groups Pax4 expressing and Arx expressing groups. The Pax4 precursors form the Beta and Delta cells. The differences are based on the expression of different genes. For example, to produce beta cells, the Nkx2.2, Nkx6.1 and MafA genes must be expressed, while Isl1 and Pax6 are required to produce Delta cells. The same situation is also present in Arx precursors. To achieve alpha cells, Arx precursors express Brn4, MafB, and Pax6 and they express Nkx2.2 for delta cells (Fig. 2). Scientists working with mice recommend that the transformation of endocrine cells continue after birth (Brennand et al. 2007; Dor et al. 2004).

3 Transcription Factors

Transcription factors (TFs) are important signal transduction molecules in whole cellular actions by binding promoter and enhancer regions. They help to transfer signals to the cytoplasm or nucleus or vice versa. Transcription factors regulate gene levels by repressing or promoting them.

3.1 Pancreatic and Duodenal Homeobox 1 (Pdx-1)

Pancreatic and duodenal homeobox 1 (Pdx; well-known as Insulin Promoting Factor 1 (Ipf1)) is a transcription factor that contains homeobox sequences. Pdx gene is required for the differentiation of progenitor cells into the β-cell phenotype and the whole pancreatic development. The expression of Pdx-1 gene is seen the pancreas, intestine and stomach. (Fukuda et al. 2006; Jørgensen et al. 2007). Pdx-1 is regulated as a response to the amount of glucose by phosphorylation and translocation of the Pdx1 protein into the nucleus (MacFarlane et al. 1994; Rafiq et al. 1998). Level of Pdx-1 expression changes between stages of pancreatic development. It decreases during early stages but then the

expression increases in murine pancreatic β-cells (Bernardo et al. 2008). On e8.5, Pdx-1 expression is firstly observed in murine foregut endoderm. The expression of Pdx-1 is observed in both dorsal and ventral pancreatic buds on e9.5 (Offield et al. 1996). Between e11.5 and e13.5, the expression of Pdx-1 is seen along the developing ductal formation. While the exocrine pancreas comes out and the islets start to create cells producing hormone (e14–e15), the expression of Pdx-1 shifts to the endocrine compartment. When the exocrine portion of pancreas shows up and islets start to create cells which produce hormone on e14 and e15, Pdx-1 is expressed in the endocrine portion. On e16.5, Pdx-1 expression markedly diminishes and it is almost unseen in the exocrine pancreas of adult mice. On e18.5 (in later stages), Pdx-1 expression is limited to endocrine pancreas (Habener et al. 2005). Pdx-1 is initially indicated as confined to β and δ cells of islets in the mature pancreas (Stoffers et al. 1997), but in some cases such as pancreatic injury it is re-expressed in acinar cells. Since Pdx-1 is determined as gamma and beta cell-specific regulator for the expression of somatostatin and insulin genes like stated before. But glucagon producing alpha cells rarely express the Pdx-1 gene (Habener et al. 2005). Pdx-1 gene is also found to control the gene expression of other islet-specific factors, including islet amyloid poly peptide (IAPP), glucokinase (Gck) and glucose transporter 2 (Glut-2). (Ahlgren et al. 1998; Al-Khawaga et al. 2017). Pdx-1 expression begins at week 4 and becomes confined to the beta cells in human pancreas (Lyttle et al. 2008; Jennings et al. 2013). Interestingly, Pdx-1 expression is detected in islet cells, exocrine cells of adult pancreas in both murine and humans (Pan and Wright 2011; Rooman et al. 2000; Castaing et al. 2005). It is reported that the development of the pancreas from the gut by budding can be seen without branching and expansion in Pdx null mice. Hence, it can be said that the activity of the Pdx-1 gene is vital for differentiation of the pancreas. (Offield et al. 1996; Jonsson et al. 1994).

Heterozygous Pdx-1 (Pdx-1+/−) mice develop normally, but the insulin deficiency increases in time because of the genetic mutation. This suggests that Pdx-1 should be expressed at a high rate for insulin production. Pdx-1 gene mutations are connected to the development of Diabetes mellitus type 2 in humans (Hani et al. 1999). MODY4, which is portrayed by agenesis of pancreas, is known to occur as a consequence of homozygous mutations in the Pdx-1 gene (Stoffers et al. 1997).

3.2 Pancreatic Transcription Factor 1A (PTF1a)

Pancreatic Transcription Factor 1a (PTF1a; also known as p48) is a crucial gene for the development of the acinar composition. In the pancreas, the knockout of the Ptf1a gene pioneers to an absence of acinar cells. In mice, deletion of Ptf1a causes the death of the animal after birth. In the literature, it was shown that Ptf1a is found in the ductal, exocrine, and endocrine progenitors, whereas inactivation of the gene indicated that these progenitors changed to intestinal epithelial progenitors (Kawaguchi et al. 2002).

3.3 Islet-1(Isl1)

Islet 1 (Isl1) included in LIM/homeodomain transcription factors family found in the developing pancreas and some neural cells during embryogenesis (Karlsson et al. 1990). Isl-1 expression is firstly detected in the mesenchyme, and then in all hormone secreting pancreatic islet cells. Interestingly, it is rarely expressed in insulin-producing beta cells due to the regulation of insulin secretion. Knocking out of Isl-1 gene causes the blocking the development of pancreas at e9.5 (Thor et al. 1991). Especially, the development of dorsal pancreas is not observed and endocrine cells are not present. However, the progression of

ventral pancreatic epithelium and mesenchyme continues to develop normally in the Isl null mice (Habener et al. 2005). These outcomes indicate that Isl1 is necessary for the progression of the dorsal pancreatic bud and it also necessary for the differentiation of the dorsal pancreatic epithelium to the endocrine cells.

3.4 Neurogenin 3 (Ngn3)

Neurogenin-3 is a TF encoded by the NEUROG3 gene in humans and classified to the basic helix-loop-helix transcription factors (bHLH) family. The fate of endocrine cells starts with Ngn3 protein. Induction of Ngn3 causes the expression of other transcription factors such as Pax4, Isl, Arx and Nkx family proteins (Gouzi et al. 2011). Ngn3 is one of the leading markers that initiates the differentiation of cell in the primary endocrine direction. In humans, Ngn3 positive cells appear from 9th week of pregnancy and the appearance continues until the week 17. Then this amount decreases rapidly (Jeon et al. 2009). The expression of Ngn3 begins at e9.5, reaches the highest point during endocrine cell production at e15.5, and is reduced with birth, in mice. Ngn3 is almost non-existent in the adult pancreas (Rukstalis and Habener 2009). Timing of the expression of Ngn3 is very important during embryonic development. The expression at later phases causes formation of β, γ, and δ cells; while it causes formation of glucagon-expressing cells at the early phase of expression (Al-Khawaga et al. 2017).

Studies with mice showed that neither endocrine cells nor endocrine progenitor cells are formed during development in the Ngn3 null group, whereas the exocrine section remains intact (Gradwohl et al. 2000). Thus, it was confirmed that Ngn3 own a vital role in the pancreatic improvement. Hes1 (hairy and enhancer of split-1) is another TF that involves in Notch pathway. It works antagonistically with Ngn3 for the protection of pancreatic precursor cell pool. (Jensen et al. 2000). In another study with mice indicated that Ngn3-expressing cells were able to secrete insulin and other hormones after injection into the pancreas of Ngn3 defective mice. However, mice in the control group did not secrete any hormones (Xu et al. 2008).

3.5 SRY (Sex Determining Region Y)-Box (Sox)

The SRY-box gene family is largely protected during development. Two of them, Sox9 and Sox17 own a role especially in the progression of the pancreas. Sox17 is associated with the early stages of development. According to studies on xenopus and zebrafish, the expression of Sox17 is vital for endodermal growth.

Sox9 is related to proliferation and maintenance of progenitor cells and expressed with Pdx-1 between e9-e12.5. However, the accumulation of Sox9 is limited with Pdx-1 expressing cells in epithelial cord. Sox9 knockout studies show that Pdx-Cre-mediated deletion increased glucose level of the blood and caused death on postnatal day 4. This is the evidence of the importance of Sox9 expression for progenitor cells (Seymour et al. 2007).

In adults, Sox9 is accumulated in the acinar cells and duct and targeted deletion of the gene is caused cysts. This situation also demonstrates the role of Sox9 in ductal cell maintenance by reducing the expression of ductal markers (Delous et al. 2012; Magenheim et al. 2011). Conversely, Sox9 initiates the expression of Neurogenin3 gene (Lynn et al. 2007). Scientists discovered that haplo-insufficiency of Sox9 invoke aberrant acinar phenotype and the formation of endocrine pancreas in humans (Piper et al. 2002). Current evidences demonstrate the considerable role of Sox9 in controlling and maintaining progenitor missions of the pancreas.

3.6 Homeobox Protein Nkx Family (Nkx2.2, Nkx 6.1)

Homeobox protein Nk-gene family is expressed during pancreatic progression. Henseleit and colleagues proved that mutation in Nkx6.1 gene causes defect in only β cells, while mutation in Nkx6.2 gene provokes no certain pancreatic

deformities in mice. Remarkably, when mutation occurred in both genes, pancreatic development continued with diminished amounts of α and β cells (Henseleit et al. 2005). Thus, it is suggested that both genes are vital for the development of islet cells; however, the solo mutation of Nkx6.2 is compensated by Nkx6.1. The Nkx6.1 expression starts at embryonic day 9.5 and continues until at e13 in beta cells. The Nkx6.1 transcription factor is involved in crucial events such as beta cell development or insulin production. According to studies in adult mice, the presence of a mutation in Nkx6.1 did not show any effect in other cell groups, but the mutation reduced the number and the growth of beta cells (Sander et al. 2000).

Nkx2.2 is another homeobox TF basic for pancreatic progenitor cell pattern (Doyle and Sussel 2007). As in Nkx6.1, expression of Nkx2.2 begins early in multipotent pancreatic progenitor cells at e9.5 and keeps on in α, β, and γ cells (Al-Khawaga et al. 2018). Nkx2.2 is expressed with Pdx-1 during the pancreatic progenitor cells; however, its expression becomes controlled to the Ngn3 endocrine progenitors (Doyle and Sussel 2007). When the transcription factor is silenced, the formation of various cell types is not observed (Prado et al. 2004). Papizan et al. revealed that Nkx2.2 is vital for the maintenance of beta cell in adults (Papizan et al. 2011).

3.7 Forkhead Box Factors (Fox)

Fox-O is another key transcriptional factor essential for the maintenance and beta cell differentiation during the early improving of the pancreas (Wang et al. 2010). Insulin negatively regulates working mechanism of Fox proteins. The absence of growth factors or insulin results in nuclear translocation of Fox-O proteins and increased expression of Fox-O activated genes (Brunet et al. 1999). Fox-O1 is the most abundant form of Fox-O genes in the adult pancreas. In mice, Fox-O1 rivaled with Forkhead box A2 (FoxA2) in the case of binding on the Pdx1 promoter. Thus, it acts as a negatory regulator for the expression of the gene. Through murine pancreatic development, Fox-O1 and Pdx-1 have identical generation design. Between e9.5-e14.5 they are thoroughly produced in the pancreatic epithelium. These discoveries recommend that Fox-O1 is involved in different steps of the pancreatic development in mice (Kitamura and Ido Kitamura 2007). FoxA1-KO mice own phenotypically normal appearance but with abnormal function of beta cell (Vatamaniuk et al. 2006).

4 Signaling Pathways

Signaling pathways conduct and regulate all cellular communications. It has been documented that the related-cells use different pathways throughout the embryonic development of the pancreas. The main signaling pathways underlying the development process are in Table 1.

4.1 Hedgehog Signaling Pathway

The hedgehog pathway is a negative regulator of pancreatic development and owns a vital role for tissue homeostasis in adult, embryonic development, and taking part in the division and differentiation of cells. Three hedgehog genes have been discovered in mammals, Indian (Ihh), Desert hedgehog (Dhh) and Sonic (Shh) which are well expressed throughout embryogenesis and also important for the growth of several organs (Chuang and Kornberg 2000). Shh is the most expressed form of genes in embryonic and adult mammalian tissues. However, Shh is kept out from pancreatic epithelium throughout development (Apelqvist et al. 1997; Hebrok et al. 1998).

Hedgehog proteins are ligand of Patched2 (Ptc2) and Patched1 (Ptc1) transmembrane receptors found in cells contiguous to hedgehog

Table 1 Signal pathways

1	Hedgehog
2	Notch
3	Wnt/β-catenin
4	Retinoic acid
5	TGF-β superfamily

producing cells (Motoyama et al. 1998). When Shh binds to the receptors, it activates the pathway. The absence of ligands, Ptch blocks another receptor-like protein named as Smoothened (Smo) (Motoyama et al. 1998; Stone et al. 1996). Patched receptors inhibit the binding of Smo agonist, an endogenous intracellular small molecule, to Smo and carries this molecule out of the cell. In the presence of hedgehod ligands, Patched receptors become inactivated by binding to ligands and the suppressive effect on smo is eliminated. Thus, Smo is activated and the hh signal is transferred to the cytoplasm. In cytoplasm, active-Smo separates and activates the Gli molecule from SuFu-Gli complex. The active Gli transcriptional factors bind to the nucleus and stimulate target genes which will respond hedgehod ligands (Fig. 3).

It was indicated that inhibition of sonic hedgehog gene is crucial for initialization of early pancreatic development (Hebrok et al. 1998; Martí et al. 1995). Furthermore, in the early pancreatic development, abnormal activation of the hedgehog pathway leads to loss of pancreatic marker genes and pancreatic tissue. On the other hand, the abnormal activation increases duodenal properties (Fendrich et al. 2008). Activation of the pathway is limited to beta cells of endocrine pancreas in the regulation of insulin production in the adult pancreas. However, it is necessary for regeneration of exocrine pancreas in case of injury or disease (Algül et al. 2002). Also, the abnormal activation of the Hh pathway in human pancreatic tissue has been documented that it causes the pancreatic cancer (He et al. 2016).

4.2 Notch Signaling Pathway

Notch signaling is tightly conserved pathway that determines cellular fate through embryonic development. It is crucial for the regulation of survival/apoptosis, cell differentiation and the cell cycle in adults and is also good example of the direct cell to cell contact. On the other hand, ongoing investigations in mice state that Notch pathway controls endocrine and exocrine fate of pancreatic progenitors (Habener et al. 2005).

Notch is a large protein which has a transmembrane region. It co-acts as a receptor in signal transduction with other transmembrane proteins (ligands) located on the surface adjacent cells.

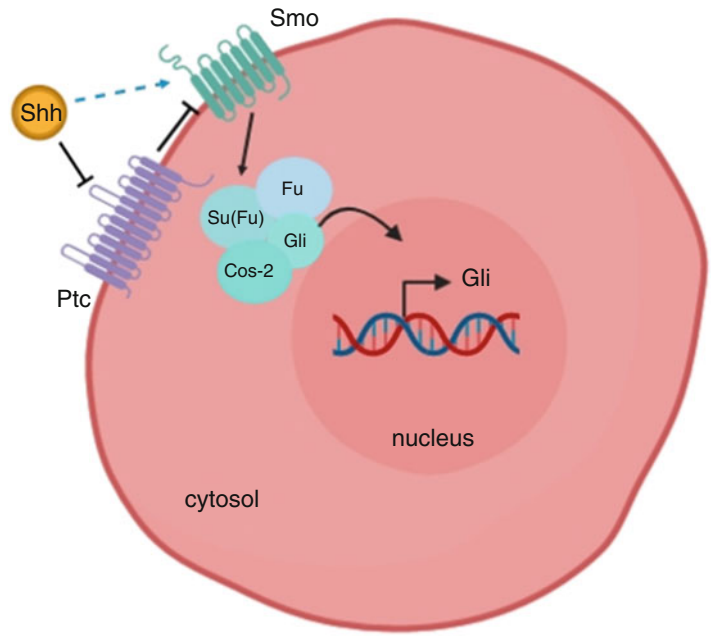

Fig. 3 Hedgehog signaling pathway

Notch signaling pathway has two main classes of ligands in mammals; Delta ligands (Delta-4, Delta-3 and Delta-1) and Jagged ligands (Jagged-2 and Jagged-1). When the ligand attached to the receptor, it causes proteolytic cleavage of Notch protein. Thus, intracellular part of Notch (ICDN) passes to the nucleus and it connects with the transcriptional factor (Suppressor of Hairless, Su (H), in Drosophila and CBF1 in mammals,). So, the genes that determine cell fate are expressed. Various targets of Notch signaling pathway are well-known. One of them in mammals and Drosophila is the Hes (hairy/enhancer of split) repressors belonging to bHLH (Iso et al. 2003; Kim et al. 2010). Hes repressor negatively regulates the gene expression by hiding the transcriptional activators. In addition, ICDN directly promotes the expression of transcription factors, growth factor receptors and cell cycle regulators. This complicated situation shows that Notch signaling is included in various cellular events. The necessary activation of Notch signaling in pancreatic progenitors inhibits their differentiation into the exocrine or endocrine cell lineage (Li et al. 2016; Ahnfelt-Ronne et al. 2007). Interestingly, the activation of the pathway induces untimely differentiation of the progenitor cells to endocrine cells (Nakhai et al. 2008). In pancreatic development, pro-endocrine factor neurogenin3 (Ngn3)-expressing cells have to become endocrine cells, and these cells have higher expression of Notch ligands Serrated, Delta (Dll), and Jagged. Likewise, some researchers have suggested that the Notch pathway refers to pancreatic progenitors that differentiate into the endocrine lineage or the inactivation of Notch pathway supports the differentiation of acinar cells (Afelik et al. 2012; Hosokawa et al. 2015) (Fig. 4).

As mentioned before, Notch signaling leads to the transformation of progenitor cells into endocrine cell types with two possible mechanisms (Field et al. 2003). The first one is "lateral inhibition" blocking contiguous cells from having the identical phenotype. Lateral inhibition is necessary for many types of cell fate determination. The second one is "suppressive maintenance" which clarifies the function of the Notch signals in pancreatic differentiation (Jensen 2004).

Studies on animal pancreatic regeneration models and diseases have demonstrated that Notch signals play a role in controlling the plasticity of fully differentiated adult pancreatic cells (Li et al. 2016).

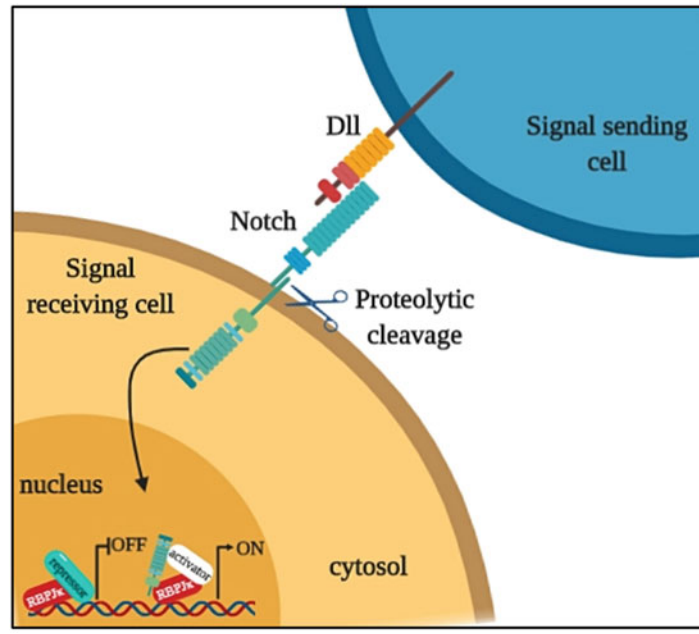

Fig. 4 Notch signaling pathway

4.3 Wnt/β-Catenin Pathway

As Hedgehog and Notch signaling pathways, Wnt (Wingless-related integration site)/β-catenin pathway is also evolutionary conserved for cell-fate and cell proliferation during embryonic development (McMahon and Bradley 1990; Pin and Fenech 2017). Wnt signaling contains various signaling pathways; β-catenin-mediated canonical pathway and two non-canonical pathways which are planar cell polarity and Wnt/calcium. In pancreatic development, the canonical form of the pathway controls the expression of key transcriptional factors (114, 163), whereas the non-canonical forms of the pathway only regulate the cellular shape and calcium uptake (Wells et al. 2007; Pin and Fenech 2017). Some Wnt ligands such as WNT2b, WNT4, WNT5a, and WNT7b were announced for the process of pancreatic development (Heller et al. 2002).

In the presence of Wnt protein, the protein binds to the Frizzled (Fzd) receptors and they induce Dishevelled (Dvl) in the canonical form. Through the cascade, Wnt pathway is activated. Then, APC/Axin/GSK3β complex become inactivated. Thus β-catenin remains intact and accumulates in the cytoplasm. The accumulated proteins relocate to the core and bind to the LEF/TCF (T-cell 13 factor/lymphoid enhancer factor) proteins which bind to the DNA, fundamentally. However, in the absence of Wnt protein, the Fzd-Dvl complex does not occur and APC/Axin/GSK3β/CK1 cleavage complex phosphorylates and digests the β-catenin by ubiquination. Therefore, β-catenin cannot pass to the nucleus and the signal is blocked (Behrens et al. 1996).

Before taking the final version of the pancreas, enforced expression of the WNT signal prevents the development of the liver and pancreas (Heller et al. 2002). Direct erasure of β-catenin exhibits the loss of exocrine pancreatic tissue and increases the tubular forms. Recent investigations demonstrate that β-catenin is primarily necessary for the development of pancreatic epithelium prior to differentiation. Besides loss of β -catenin in pancreatic progenitors which are Pdx-1-positive prevent creation of acinar cells (Wells et al. 2007) (Fig. 5).

4.4 Retinoic Acid Pathway

Retinoic acid (RA) is a metabolite of vitamin A and assumes a role in assigning the polarity of the body during embryonic development with the help of retinoic acid signaling molecules. This pathway owns a vital role in the creation of gut endoderm. In order to produce a continuous RA signal in pancreatic progenitors, Pdx-1 expression must be initiated (Serup 2012). In the pathway, the enzyme called Retinaldehyde dehydrogenase type II is responsible for generation of various retinoic acid derivatives (Niederreither and Dollé 2008). The enzyme is present until

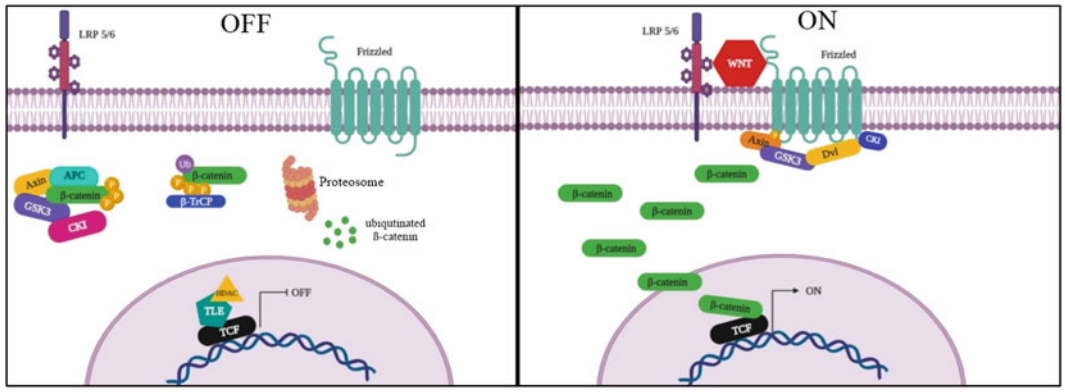

Fig. 5 Schematic representation of Wnt/β-Catenin Pathway

embryonic day 12.5 in the primitive pancreas of mice. Thus, the RA is necessary for the development of pancreas at an early stage (Martín et al. 2005). Tulachan and Shen separately showed that the retinoids support the differentiation of beta cells (Tulachan et al. 2003; Shen et al. 2007).

4.5 TGF-β Superfamily Signaling Pathway

TGF- β superfamily is a class of various transcriptional factors including TGF-β factors, Activins, Nodal and Bone morphogenetic proteins (BMPs). The pathway plays vital role in most of cellular process during the whole life (Kashima and Hata et al. 2018).

4.5.1 TGF- β

The expression of distinct TGF-β proteins is seen in the pancreatic epithelium till E12.5 and they are limited to acinar cells over time (Crisera et al. 2000). A pilot research demonstrated that the mutation on TGF-β1, TGF-β2, TGF-β3 and TGF receptor type 2 are resulted in death in the embryonic period (Serup 2012).

4.5.2 Activins & NODAL

Activins are included in various biological processes during the life of the individual (Namwanje and Brown 2016). The expression of activins is seen in gut endoderm and the early pancreas (Serup 2012). Two different types of Activin (Activin A and B) are actually closely related peptides which show 63% identity and are almost similar in their domains (Namwanje and Brown 2016). Activin A and B are limited to progressing glucagon-positive cells. Hebrok et al. drew attention to that the Activin B can block the sonic hedgehog pathway in the pancreatic endoderm at the early stage (Hebrok et al. 1998). Furthermore, presence of Activin A inhibits ductal and acinar differentiation (Ritvos et al. 1995). Studies on transgenic mice exhibited that the activin receptors type II A and B knocking mice did not show any pancreatic developmental abnormalities in type IIA mutated group whereas type IIB mutated group shows phenotypically defective pancreas (Kim et al. 2000). Serup et al. reported that this might be related to the failure of Hh signaling (Serup 2012). It seems that Activin assumes a role by repressing the expression of a vital transcriptional factor for alpha cell differentiation and improves the expression of Neurogenin3 (Zhang et al. 2001). These points determine the importance of activins in early pancreatic development by provoking endocrine differentiation and blocking ductal and acinar differentiation (Al-Khawaga et al. 2017).

Nodal protein is included in TGF- β superfamily and effector for downstream signaling. It plays a role in cellular transformation in early embryogenesis. The absence of Nodal can not initiate the primitive streak, the line in the blastula.

5 Methods for β Cell Differentiation

As mentioned before, beta cells are very important group of cells for body homeostasis and digestion processes. Defective or dysfunctional beta cells cause many diseases, especially diabetes. There are several approaches to cure this situation. Pancreatic or beta-cell transplantation is important in type 1 diabetes. However, transplanted pancreas or beta cells might not be accepted by the immune system. In such cases, the differentiation of stem cells form the patient into beta cells *in vitro* and subsequent injection to the dysfunctional area are considered as an important issue for today's physiologists and researchers. The first study related to pancreatic beta cell differentiation was done by using human pluripotent stem cells in 2001 (Assady et al. 2001) and the number of studies about differentiation of stem cells into beta cells significantly increased in recent years.

The most important factor in the differentiation period is that proliferation does not prevent differentiation. During differentiation, cells need to use the source for the differentiation instead of the proliferation. For this situation, scientists have used proliferation reducing agents. Therefore, toxic substances such as dimethyl sulfoxide (DMSO) or β-mercaptoethanol (BME) are added

to the differentiating medium. Reducing the oxygen level or nutrients can be given as examples to stop proliferation of stem cells.

In the literature, various differentiation methods have been published. (Table 2). But the main idea is based on direct differentiation of stem cells from any source, into early pancreatic development stages *in vitro*. Some studies have successfully established insulin-secreting β cells, but these studies have established that hormone expressing cells are not only β cells, they determined that differentiation only reaches until the primitive-fetal pancreatic period.

Protocols for producing insulin-secreting cells *in vitro* modify depending on the cell line, but there are plenty of mutual subjects among them. Table 2 summarizes stem cell differentiation from several sources into into β cells capable of secreting insulin using diverse chemicals at different time points.

5.1 Extrinsic Factors

The beta cell differentiation process is completed in 5 stages, induction to definitive endoderm, primitive gut tube formation, development of foregut, pancreatic progenitor cell development and maturation of β cell, respectively.

5.1.1 Induction to Definitive Endoderm

Definitive endoderm (DE) is the first station of differentiation. Definitive endoderm is one of the three germ layers of an embryo. It forms gastrointestinal organs and gut tube which is origin of primitive pancreas. Several components have been used to back definitive endoderm, experimentally.

Activin A is necessary for stem cell transformation for the creation of definitive endoderm (Kumar et al., Bose et al). Activin A is used with another substance, Wnt3a and they have found that Activin a and wnt3a triggered the meso-endoderm transition (D'Amour et al.; Jiang et al). Activin A blocks the ductal and acinar differentiation of DE cells. For this purpose, Pagliuca et al. used Activin A through the next stage of differentiation (Pagliuca et al. 2014). Kumar et al., have demonstrated that activin A, with sodium butyrate, initiates differentiation of hESCs into pancreatic cells during the first a few stages of endoderm development (Kumar et al. 2014).

Wnt3a is the co-worker of Activin A to form definitive endoderm through the differentiation. According to Table 2, D'Amour et al. added Wnt3a in induction medium for 2 days. Then it was withdrawn after 2 days. This alteration augments the effect of mesoendoderm formation and transition to DE.

Wortmannin is a chemical substance that inhibits PI3K pathway directly, so it promotes pancreatic development by initiating Nodal and TGFb pathways throughout DE formation (Kumar et al. 2014).

Histone deacetylases (HDACs) are inhibited by sodium butyrate (SB) which is a short chain fatty acid. SB also blocks the dedifferentiation of DE cells. It was demonstrated that sodium butyrate aids to trigger the early stage of β cell differentiation with Activin A in hESCs. Moreover, it also enhances the secretion of insulin and glucagon at high level (Kumar et al. 2014). As known, sodium butyrate stimulates the transition of stem cells into DE, too. Studies have shown that removal of sodium butyrate from inducing media reduces PDX1 gene expression (Goicoa, et al. 2006).

5.1.2 Primitive Gut Tube Formation and Development of Foregut

The second station of beta cell differentiation is the creation of the foregut endoderm. Foregut endoderm is the period in which the cell fate is determined in the direction of the liver or pancreas. Various activities of some transcriptional factors lead the differentiation into pancreatic direction *in vivo*. However, different stimulators have been used to mimic the same way *in vitro* studies.

FGF Family (FGF2, FGF7, FGF10): Fibroblast growth factor family members are widely used in cellular events, especially differentiation. These members participate in the developmental connection between the pancreas and other gastroenteric organs. FGF2 controls the transition

Table 2 Different approaches in order to generate pancreatic β cells

Author	Source	Differentiation Approach					
Moshtagh et al. (2013)	Human Adipose stem cells (2×10^5 per well of 6 well plate)	Three stages	**Stage1**	**Stage2**	**Stage3**	**Stage 4**	**Stage 5**
			To induce differentiation (definitive endoderm)	**Primitive gut tube**	**Foregut endoderm**	**Pancreatic progenitor**	**Mature insulin producing cells**
			DMEM-low glucose +5% FBS + nicotinamide **For 2 days**	DMEM-high glucose +2.5% FBS + BME + NA **For 10 days**			DMEM-high glucose +1.5% FBS + BME + nicotinamide + Exendin-4 **For 14 days**
Govindasamy et al. (2011)	Human dental pulp stem cells (1×10^6 cells/cm^2)	Three stages	DMEM-KO + BSA + ITS + Activin A, sodium butyrate + BME **For 2 days**	SFM B: BSA + DMEM-KO + taurine + ITS **For 2 days**			SFM C: Taurine + DMEM-KO + ITS + BSA + GLP-1 + nicotinamide, + NEAA **For 2 days**
				SFM B and SFM C change for 5 days, alternating every 2 days			
Chandra et al. (2009)	Mouse Adipose stem cells (1×10^6 cells/cm^2)	Three stages	DMEM/F12 (1:1) + BSA + additive glucose + ITS + sodium butyrate + BME + Activin	SFM B: DMEM/F12(1:1) + additive glucose + BSA + ITS + taurine			SFM C: DMEM/F12(1:1) + additive glucose + taurine + BSA + ITS + nicotinamide + GLP-1 + NEAA
			For 2 days	**For 2 days**			**For 2 days**
				SFM B and SFM C change for 5 days, alternating every 2 days			
Zhang et al. (2009)	Human induced pluripotent stem cells and	Four stages	DMEM/F12 (1:1) + N2 + B27 + BSA + Wortmannin + Activin A	DMEM/F12 / IMDM + RA + BSA + B27 + ITS + KGF + noggin	DMEM-high + BSA + ITS + N2 + EGF		DMEM/F12(1:1) + ITS + BMP4 + bFGF + Exendin-4 + nicotinamide

(continued)

Table 2 (continued)

Author	Source	Differentiation Approach							
	human embryonic stem cells		**For 4 days**	**For 4 days**	**For 5 days**		**For 7–9 days**		
Jiang et al. (2007)	Human embryonic stem cells	Four stages	RPMI 1640 + glucose + B27 (RPMI 1640/ B27) + Sodium Butyrate + Activin A	RPMI1640/ B27 + EGF + bFGF + noggin	RPMI1640/B27 + EGF + noggin		RPMI 1640 + BSA + Nicotinamide + IGF-II		
			For a day After; RPMI 1640/B27 + Sodium Butyrate + Activin A	**For 14 days**	**For 7 days**		**For 5 days** w/o IGF-II another 2 days		
			For 6 days						
D'Amour et al. (2006)	Human embryonic stem cells	Five stages	RPMI + Activin A + Wnt3a **For 2 days**	RPMI + FGF10 + Cyclopamine +2% FBS	DMEM + B27 + RA + Cyclopamine + FGF10	DMEM + B27 + DAPT + Exendin-4	CMRL + B27 + Exendin-4 + IGF1 + HGF		
Pagliuca et al. (2014)	Human embryonic stem cells and human induced pluripotent stem cells (6×10^5 cells/ml)	Five stages	w/o Wnt another 2 days S1 + Activin A + Chir99021	**For 2–4 days** S1 + Activin A	**For 2–4 days** S2 + KGF	**For 2–3 days** S3 + KGF + RA + SANT1 + LDN193189 (for a day) + PdBU	**For 3+ days** S5 + Alk5i + RA + T3 + xxi + SANT1 + Betacellulin	S6+ Alk5i II+ T3	
			For a day	**For 3 days**	**For 3 days**	**For 2 days** w/o LDN193189 + PdbU another 5 days	**For 7 days** w/o Sant1 for 4 days	**For 7–14 days**	
Rezania et al. (2014)	Human embryonic stem cells (1.3–1.5 ×	Seven stages	MCDB 131 + BSA + GDF8 + MCX-928	MCDB 131 + BSA + FGF7 + ascorbic acid	BLAR medium* + BSA + ascorbic acid + FGF7 + SANT-1 + RA +	BLAR + BSA + ascorbic acid + FGF7 + RA + SANT-1 +	BLAR medium + BSA + SANT-1 + RA + LDN193189 + ITS + T3 + ALK5	BLAR + BSA + LDN193189 + ITS-X + T3 + ALK5 inhibitor	BLAR + BSA + ITS + T3 + ALK5i II + zinc Sulphate +

	10^5 cells/ cm^2)				LDN193189 + ITS + TPB	LDN193189 + ITS + TPB	Inh II + zinc Sulphate + heparin	II + zinc sulfate + xxi	N-Cys + Trolox + R428 + heparin
			For 2 days On day 3 MCX-928 replaced by CHIR-99021	**For 2 days**	**For 2 days**	**For 3 days**	**For 3 days**	**For 7 days** With heparin another 7 days	**For 7–15 days**
Chao et al. (2008)	Human Umblical cord stem cells	Four stages	10% FBS + DMEM	10% FBS + DMEM + AraC	DMEM/F12 + 2% FBS + B27 + NA	DMEM/F12 + 2% FBS + glutamine+ NA			
			For 3–6 days	**For 7 days**	**For 7 days**	**For 14 days**			

NA Nicotinamide, *BME* Beta Mercaptoethanol, *BSA* bovine serum albumin, *ITS* insulin-transferrin-selenium, *DMEM-KO* DMEM Knock out, *GLP-1* glucagon-like peptide 1, *NEAA* Non-essential amino acid, *KGF* keratinocyte growth factor, *RA* retinoic acid, *EGF* epithelial growth factor, *bFGF2* basic fibroblast growth factor 2, *BMP-4* bone morphogenic protein 4, *IGF* insulin growth factor, *HGF* hepatocyte growth factor, *DAPT* γ secretase inhibitor, *Chir99021* glycogen synthase kinase 3b inhibitor, *SANT1* sonic hedgehog pathway inhibitor, *LDN193189* inhibitor of the BMP pathway, *PdBU* protein kinase C activator, *Alk5i* inhibitor of TGF-β, *Xxi* γ-secretase inhibitor, *MCX-928* ALK inhibitor

of DE cells into various foregut lineages in time dependent manner. Furthermore, Jiang et al. indicated that addition of FGF2 into induction medium with noggin resulted in the termination of liver formation (Mfopou et al. 2010). According to Cai et al., PDX1 gene expression has enhanced in hESCs more than 70%, when FGF7 is used in differentiation procedure (Kumar et al. 2014; Cai et al. 2010). Both FGF2 and FGF7 help the formation of islet-like clusters. FGF10 owns a major impact on the creation of pancreatic epithelium from MSCs. FGF10 is necessary during pancreatic development together with KAAD-cyclopamine which is hedgehod-signaling inhibitor (Kumar et al. 2014; D'Amour et al. 2006). In another study, it was displayed that addition of FGF10 with KAAD-Cylopamine caused 160-fold increase of Insulin mRNA compared with activin removal alone (D'Amour et al. 2006).

RA is a precursor of Vitamin A and formed in the mesoderm by retinaldehyde dehydrogenase (RALDH) (Oström et al. 2008). It also regulates pancreatic differentiation of hESCs at early phase by stimulating the PDX1 gene expression. However, in this situation, RA requires distinct compounds such as FGFs or Noggin. Studies have shown that the level of PDX1 gene decreases when RA is used alone (Kumar et al. 2014).

Cyclopamine (or KAAD-Cyclopamine) is a plant derivative that inhibits Hedgehog signaling pathway protein Smoothened, thus also inhibiting that pathway (Chen et al. 2006). As mentioned earlier, enhanced expression of HH pathway proteins allows the formation of liver by cell fate. Therefore, the inhibition of HH pathway triggers pancreatic development. The addition of FGF10, RA and indolactam V in the differentiation medium together with cyclopamine promotes gut formation. This leads to high expression of PDX1, NeuroD1 and Neurogenin3.

Noggin is an antagonist that binds and inactivates the member of TGF-β signaling proteins such as BMP-2, BMP-4, and BMP-7 (Rifas 2007). The inactivation of TGF-β signaling protein encourages pancreatic development at the later stage. Noggin also induces PDX1 gene expression from DE cells (Takeuchi et al. 2014). Noggin works with RA to decrease the formation of liver precursor cells in the foregut development (Kumar et al. 2014).

5.1.3 Pancreatic Progenitor Cell Development

The third station is differentiation into the pancreatic progenitors which are origin of pancreatic cells, duct and acinar cells. *In vivo*, silencing of Notch and expression of Hnf6 transcription factors cause different type and roles of cells. To consist of duct cells, Hnf6 must be expressed or Hes1 and Ptf1a must be shown for exocrine pancreas. To form of endocrine cells, various TFs are expressed such as NeuroD, Pax6, Isl1 or Ngn3. As mentioned before, different chemicals are used to catch same effects *in vitro*.

Exendin-4 is a peptide acting as an agonist of the glucagon-like peptide (GLP) receptor that encourages the secretion of insulin (Ding et al. 2006). In the pancreatic differentiation process, Exendin-4 accelerates the maturation of pancreatic cells and enhances β cell proliferation alone or in combine with Hepatocyte Growth Factor (HGF) and betacellulin (Bose and Shenoy 2015).

EGF is the acronym of epithelial growth factor family and it is vital for the progression of pancreatic progenitors and islet growth by rising the number of cells expressing PDX1 (Zhang et al. 2009).

ALK5 Inhibitor II is another inhibitor of TGF-β type I receptor. Expression of NKX6.1 necessary for β-cell development increased four-fold when Alk5i II and Noggin were added into the medium at the pancreatic endoderm stage Kumar et al. 2014).

5.1.4 Maturation of β Cell

β cell maturation is the terminal stage of endocrine cell differentiation. At this stage, it is important to separate and mature the cell secreting the desired hormone.

Nicotinamide is an inhibitor of poly ADP-ribose synthetase that promotes

proliferation, regeneration and β cell differentiation (Hosoya 2012; Kumar et al. 2014).

Betacellulin belongs to Epithelial Growth Factor (EGF) superfamily. In literature, it is combined with another TGFβ family protein, Activin A and this combination promote the secretion of insulin from islet-like cells, also encourages the growth of differentiated β cells (Bose and Shenoy 2015).

BME is a chemical compound that act as reducing agent *in vitro* systems and used for various cellular processes such as mimicking cell environment, protecting from oxidative stress or differentiating, on the other hand, inducing cells. Pruett and his colleagues discovered that BME promotes survival and cell growth of murine lymphocytes by increasing protein synthesis as two-fold (Inui et al. 1997; Pruett et al. 1989).

Sant1 is a kind of inhibitor of sonic hedgehog pathway proteins which decides the cell fate to the formation of the liver. Therefore, SANT promotes pancreatic endoderm from foregut precursor cells.

DAPT is abbreviation of N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester which is γ secretase inhibitor and blocks the Notch signaling pathway inhibitor (Hosoya 2012; Akinci et al. 2013).

CHIR99021 is a selective glycogen synthase kinase 3b inhibitor that activates the canonical Wnt pathway. It works interactively with Wortmannin to induce through definitive endoderm of hPSCs (Hosoya 2012; Takeuchi et al. 2014).

Ascorbic Acid is a portion of Vitamin C usually used in differentiation process and treatment of various cancers (Chen et al. 2005; Du et al. 2012; Doğan et al. 2015). It is known that Ascorbic Acid plays a role in cell fate and several epigenetic demethylations. In differentiation processes, it was also determined as a supporter for the transformation and maturation of the mouse pancreatic β-cell. (Zhu et al. 2016).

Dexamethasone (DEX) is a synthetic glucocorticoid that is comparable to natural glucocorticoid hydrocortisone. Dexamethasone is an agonist of glucocorticoid receptors (Zhu et al. 2016). It induces reprogramming of hepatocytes from pancreatic cells of murine (Al-Adsani et al. 2010).

Lastly, forskolin is an activator of Adenylyl Cyclase. It synergistically works with DEX and they have been shown to promote maturation of cells that differentiated (Hosoya 2012; Zhu et al. 2016).

6 Clinic Use of Mesenchymal Stem Cells

Based on the official website of US National Institute of Health, it was reported that 759 clinical studies conducted with MSCs. Most of MSCs-based clinical trials were performed in diabetes, immune system-based disease, cardiovascular disease, bone and cartilage-based disease and neurological diseases (Squillaro et al. 2016).

The application of MSCs together with tissue engineering is one of the most commonly used procedures in clinical studies. As known, MSCs may boost cell proliferation and angiogenesis as well as immune regulation. Thanks to these features of MSCs, they can be used in wound healing studies. Autologous MSCs, bone marrow-derived, have efficiently been used to treat a diabetic ulcer in a combination with fibroblasts and Coladerm, which is a biodegradable collagen membrane (Vojtaššák et al. 2006). In a pilot study conducted in 2011, 10 type 2 diabetic patients were given injections of MSCs derived from placenta three times with one-month interval. Results showed that daily insulin intake of patients with type 2 diabetes undergoing placenta-derived MSCs injection decreased while C-peptide level of the patients increased (Jiang et al. 2011). In a study performed in 2015, it was demonstrated that autologous MSCs treatment leads to increased C-peptide response with no side effect in type 1 diabetic patients (Carlsson et al. 2015). It was reported that isolated MSCs from adipose tissue was differentiated into cells which can secrete insulin and these cells were given into the patients having type 1 diabetes. Results indicated that the requirement of exogenous insulin in the patients with type 1 diabetes was

decreased while the c-peptide level was increased (Thakkar et al. 2015).

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Zooming in across the Skin: A Macro-to-Molecular Panorama

Ayşen Aslı Hızlı Deniz, Ezgi Avşar Abdik, Hüseyin Abdik, Safa Aydın, Fikrettin Şahin, and Pakize Neslihan Taşlı

Abstract

Maintaining integrity of the skin and its appendages still preserves its top-ranking in priorities of survival for the modern human as it probably once did for the ancient individual, −not only- because it is the primary barrier to external assaults, but also because of social and psychological impact of healthy skin during their life-span. Healing wounds in order to shield off the internal organs from infections and damage, restoring its ability to adapt to various environmental stimuli, and slowing-down and reversing aging of the skin in the quest for an everlasting youth can be named as a few of the main drivers behind the multi-million investments dedicated to the advancement of our understanding of skin's physiology. Over the years, these tremendous efforts culminated in the breakthrough discovery of skin stem cells the regenerative capacity of which accounted for the resilience of the skin through their unique capacity as a special cell type that can both self-renew and differentiate into various lineages. In this review, first we summarize the current knowledge on this amazing organ both at a structural and functional level. Next, we provide a comprehensive -in depth- discussion on epidermal as well as dermal stem cells in terms of the key regulatory pathways as well as the main genetic factors that have been implicated in the orchestration of the skin stem cell biology in regards to the shifts between quiescence and entry into distinct differentiation programs.

Keywords

Dermis · Differentiation · Epidermis · Skin tissue · Stem cells

Abbreviations

AEG	Apoeccrine Sweat Glands
APM	Arrector Pili Muscle
BCCs	Basal Cell Carcinomas
BMP	Bone Morphogenetic Protein
BMPs	Bone Morphogenetic Proteins
Brdurd	Bromodeoxyuridine
Bu-SC	Bulge Lack Bulge Stem Cell
CGRP	Calcitonin Gene-Related Peptide
CSCs	Cancer Stem Cells
DETCs	Dendritic Epidermal Γδ T Cells
DP	Dermal Papilla

A. A. H. Deniz
Health Institutes of Turkey, Turkish Cancer Institute, Istanbul, Turkey
e-mail: asli.deniz@tuseb.gov.tr; asli.hizli@gmail.com

E. A. Abdik, H. Abdik, S. Aydın, F. Şahin, and P. N. Taşlı (✉)
Faculty of Engineering and Architecture, Department of Genetics and Bioengineering, Yeditepe University, Istanbul, Turkey
e-mail: ezgi.avsar@yeditepe.edu.tr; huseyin.abdik@yeditepe.edu.tr; safa.aydin@yeditepe.edu.tr; fsahin@yeditepe.edu.tr; pneslihan.tasli@yeditepe.edu.tr

DP	Dermal Papilla
DS	Dermal Sheath
DSCs	Dermal Stem Cells
E6	Embryonic Week 6
EGFR	Epidermal Growth Factor Receptor
FGF-7	Fibroblast Growth Factor 7
GSI	Gamma Secretase İnhibitor
GVHD	Graft Versus Host Disease
HF	Hair Follicle
HFSC	Hair Follicular Stem Cell
Hh/Ptc	Hedgehog/Patched
H3K27	Histone H3 Lys
Hox	Homeobox
Hescs	Human Embryonic Stem Cells
EPI-Ncscs	Human Epidermal Neural Crest Stem Cells
IRS	Inner Root Sheath
IGF-1	Insulin-Like Growth Factor 1
IFE	Interfollicular Epidermis
IFs	Intermediate Filaments
K14	Keratin 14
K5	Keratin 5
LRCs	Label Retaining Cells
LLP	Lower Permanent Portion
M-SCs	Melanocyte Stem Cells
MSCs	Mesenchymal Stem Cells
NKC	Natural Killer Cell
NID	Notch İntracellular Domain
ORS	Outer Root Sheath
PSU	Pilosebaceous Unit
PDGF-Alpha	Platelet-Derived-Growth-Factor Alpha
SOA	Solid Organ Allograft
SOT	Solid Organ Transplantation
SCCs	Squamous Cell Carcinomas
TGF Alpha	Transforming Growth Factor Alpha
TACS	Transiently Amplifying Cells
Wg/Wnt/Catenin	Wingless/Armadillo

1 Introduction

With roughly 1.85 m^2 surface area and accounting for about 15% of the total body weight skin is -not only- the largest organ for most mammals, but also an essential barrier that protects the organisms from external insults such as pathogens, toxic chemicals, UV from the sun, and mechanical injury (Park 2015). In addition, skin also executes vital functions such as regulation of body temperature, prevention of excessive water loss, removal of waste metabolites through sweat, and production of pigments against the sunlight (Kolarsick et al. 2011). Furthermore, skin serves as a major site for the metabolic and secretory processes that yield an array of biomolecules, including lipids, proteins, glycans, and hormones. For example, it is one of the major endocrine sites where peripheral Vitamin D synthesis takes place (Gaur et al. 2017).

Anatomically human skin is composed of three main layers, including the outer layer of epidermis, the inner layer of dermis underlying the epidermis, and the inner-most layer of subcutaneous fat (also known as hypodermis or panniculus) (Arron 2016). Together with its appendages such as hair, nail, and mucous membranes of sudoriferous (sweat) and sebaceous (oil-secreting) glands, it forms the continuous integumentary system (McGrath et al. 2004). Moreover, several accessory structures such as specialized nerve receptors for regulation of responses to external as well as internal stimuli (such as touch, heat, pain, and pressure) aid the skin in execution of its vital functions (Garland 2012). The types of the cells in each layer and their thickness at a given anatomical location varies to a great extent. For example, while epidermis is the thinnest in the eyelids (0.1 mm) it reaches its thickest value in the palms and soles of the feet (1.5 mm). On the other hand, dermis is the thickest in the back (approximately 30–40 times thicker) than the overlaying epidermis in the same location (Kolarsick et al. 2011).

In this chapter, we aim first to introduce our reader to this organ that is recognized for its remarkable ability in tissue regeneration both in normal and repair homeostasis. Next, we continue our discussion by dissecting out the biology of skin stem cells which sets the basis of skin's resilience. In a thorough summary, we report the findings of several elegant studies which unveiled distinct types of skin stem cells, their cell intrinsic

as well as extrinsic signalling pathways, their complex interaction with local immune cells all of which play essential roles in proper operation of this "fountain of youth" in times of need. Finally, the contribution of cases to the oncogenic transformation in the skin when these signalling pathways lose their harmony and go astray is iterated.

1.1 The Epidermis

The layer of epidermis is renewed in a natural process known as cell (skin) turnover continually throughout life albeit with slowing kinetics with aging (Fuchs 2009; Li and Clevers 2010). In this natural cycle of turnover cells generated at the basal layer of the epidermis continually change form, which is known as differentiation in stem cell biology, until they reach the skin surface where they become shed having undergone apoptosis (Arron 2016).

Epidermis takes its embryonic roots from the ectoderm where in the course of the embryonic development mesenchymal cells populate the skin as they transmit instructive signals for the stratification of the epidermis and the positioning of down-growths that mark the initiation of formation of hair follicle (HF).

Epidermis is composed of several layers so-called *strata*, each with a unique composition of a number of main cell types, including keratinocytes at various stages of differentiation, dendritic cells, melanocytes, Merkel's cells, and Langerhans' cells. Keratinocytes constitute 95% of the cells in the epidermis (Arda et al. 2014). Numerous catabolic enzymes including lipases, phosphatases, esterases, nucleotides, and proteases remodel the extracellular space (Gaur et al. 2017).

In a way the tissue architecture of the epidermal layer can be envisioned as a continuous array of a binary unit that is compiled in countless numbers in an ordered and repeated fashion (Rasouli et al. 2018). The interfollicular epidermis (IFE), which is a stratified epithelium and hence forms the protective barrier against the outside environment and pilosebaceous unit (PSU) (Forni et al. 2012), which harbours some of the skin appendages such as the secretory glands and the hair follicle, constitute the two main components of binary unit that are associated with one another in this continuous array (Schepeler et al. 2014; Ceafalan et al. 2012).

Being the most abundant cell type of the epidermis, the main function of keratinocytes is to produce keratin, a protein that accounts for maintaining flexibility the skin and its ability to resist mechanical as well as hydraulic stress (Kolarsick et al. 2011). They can be readily distinguished from the "clear" dendritic cells by their relatively larger stainable amount of cytoplasm and their intercellular bridges (Gaur et al. 2017). In fact, the concerted morphological as well as spatial changes in keratinocyte population of each layer determines the distinct transitions from one stratum to the next (Bikle 2012). For example, the three bottom layers, including the basal cell layer of Stratum Germinativum bordering the Basal Membrane, the squamous cell layer of Stratum Spinosum, and the granular cell layer of Stratum Granulosum constitute the zone of the epidermis with living and nucleated cells all three of which are also collectively termed as Stratum Malpighii. While Stratum Lucidum and Stratum Corneum constitute the zone of the epidermis with dead and cornified or horny enucleated cells (corneocytes) (Kolarsick et al. 2011).

Hence, going outward from the Basement Membrane the dividing populations of the basal cells undergo proliferation cycles followed by the commitment to a *terminal differentiation* program that allows generation of the auxiliary structures of the integumentary systems such as the nails and the sweat glands (Leung et al. 2014). Considering the loss of thousands of cells upon each touch, these tightly controlled sprouts of self-renewal provided by the epidermal stem cells -not only- replenish the protective shell of the outer epidermal layer, but also ensure maintenance of a constant cell number constructed with relevant cell-to-cell and cell-to-basement membrane adhesions (Yang et al. 2019). The farther away from the Basement Membrane the more differentiated and the less viable the cells become, because in the absence of a capillary

network carrying nutrients, the cells of the upper epidermal layers undergo a morphologically and biochemically distinct apoptotic program that eliminates cells without causing injury (Sigismund et al. 2012). In a sense the epidermal differentiation program is proposed as a type of apoptotic program overseeing proper conversion of keratinocytes into corneocytes (Gaur et al. 2017). In other words, during their migration the terminally differentiating cells alter their morphology on their way to death transcending multiple stages epidermal differentiation under the control of tightly regulated transcriptional programs (Lippens et al. 2005). Although these transcriptional read-out remains mostly the same at the early stages throughout the spinous and granular layers, ultimately they change to result in the dead flattened cells of the squamous layer and become removed from the skin surface very much like the process found in the gut (Deo and Deshmukh 2018; Fuchs and Nowak 2008). For example, advancing towards a more differentiated phenotype expression of Keratin 5 and 14 (*KRT5/K5* and *KRT14*) is turned off, and expression of *KRT1* and *KRT10* become transcriptionally upregulated (Torma 2011; Alam et al. 2011). This change in transcription profile sets a pivotal switch in the keratin production because it establishes an intermediate filament protein network interlinked with desmosomes that serves as resilient structural scaffold fortifying cell-to-cell junctions and resisting against mechanical stresses (Gaur et al. 2017).

Almost all the nutrients feeding the epidermal layer is provided by the capillaries of the deeper dermis and are taken up by single layer of dividing cells of the Stratum Germinativum (Losquadro 2017). This strata hosts column-shaped keratinocytes attached to basement membrane as well as one another through desmosomal junctions at their short and long axes, respectively (Suzuki et al. 2000). Contrary to the predictions for this mitotically active compartment not all epidermal stem cells divide under normal conditions, they rather progress slowly through their long cell cycle (Alcolea and Jones 2014). However, stimuli such as wounding can increase the number dividing stem cells by inducing withdrawal of these non-dividing clones from quiescence. The migration of a basal cell from the basal layer reaching the surface of the skin is estimated as 28 days (Kolarsick et al. 2011).

A variety of cell types found in Stratum Spinosum present with highly different morphology, cellular structure, and properties (Freeman and Sonthalia 2019). In an interwoven architecture they provide support to the overall skin through the desmosomal plaques where -in fact- the keratin filaments are anchored. The spinose forms of these desmosomal plaques that mark the periphery of these cells gives this layer its name (Delva et al. 2009).

In the next upper layer of Stratum Granulosum further keratin production and the concomitant cell death takes place in its flattened cells with cytoplasmic keratohyaline granules (Bragulla and Homberger 2009). Owing to its elevated levels of lysosomal enzymes compared to those found in the lower to layers of Germinativum and Spinosum, Stratum Granulosum is known as the keratogenous layer where abrupt terminal differentiation converts these granular cells to the horny cells of the cornified layer (Kolarsick et al. 2011).

In the next layer up towards the surface of the skin the corneocytes of the thick layer of Stratum Lucindum serves as the barrier against UV damage from the sun or water loss (Yousef et al. 2019). Corneocytes are dense in protein, but not in lipid content while their extracellular millieu is a continuous lipid matrix (Haftek 2015). Desquamation, a term derived from the Latin verb "desquamare" (meaning scraping the scales of a fish) is namely peeling of the skin and describes the process for the shedding of the outermost layer of the skin tissue which involves physical and biochemical properties of the corneocytes at the cellular level (Murphrey and Zito 2019). For example, compact and tightly attached arrangement of the cells to one another in the lower levels becomes more scattered as they proceed to the surface of the skin through increased degradation of desmosomes at the intercellular attachments (Kolarsick et al. 2011). During this process keratinocytes move outward toward the surface of the skin, while millions of dead cells full with

keratin are disposed daily from the outermost layer of Stratum Corneum, depositing the soft keratin that gives the skin its elasticity and protection to the underlying dermal and hypodermal tissues (Agarwal and Krishnamurthy 2019b). Overall, in every 35–45 days a new epidermal layer becomes created through the stunningly concordant orchestration of mitotic activity coupled to differentiation and followed by cell death, allowing the epidermis to sustain a dynamic tissue homeostasis in terms of the cell number and to be a selective barrier that keeps damaging microorganisms out and important body fluids in (Kolarsick et al. 2011).

Similar to the case of keratinocytes, melanocytes produce a complex polymer derived from the amino acid tyrosine called melanin -a dark pigment that -not only- protects core epidermal cells from UV damage, but also determines the color of both skin and hair- in membrane-bound organelles of melanosomes in a series of hormone-induced, receptor-mediated biochemical reaction cascades (Agarwal and Krishnamurthy 2019b). In healthy skin melanocytes become shed via skin turnover. However, interruptions to the normal skin turnover results in long term retention of melanin causing hyperpigmentation and formation of freckles and dark spots. Together with carotene (yellow to orange pigment) melanin gives skin it color (Chadwick et al. 2012).

Melanocytes are derived from the neural crest and are located at the basal layer (Cichorek et al. 2013). As they progress toward the surface of the skin they come in contact with the keratinocytes to which they transfer their melanin content without forming cellular junctions (Feller et al. 2014). Abundance of this skin-and-hair-coloring pigment is directly proportional to the abundance of melanosomes and their release into the keratinocytes that -overall- is associated with how much a human population is exposed to sun historically in a given geographical region (Schalka et al. 2014). Thereby, increased sun exposure is counteracted through increased melanogenesis in conjunction with increased surfacing of the melanin through its transfer to the keratinocytes (i.e., tanning of the skin), resulting in improved UV-absorbance for the ultimate goal of preserving genetic information from the radiation damage. Increase in sun exposure results in increases in melanin production changing the skin color temporarily (suntan). Similarly, on a permanent basis darker skin produces more melanin. The differences in skin colors amongst individuals is reflected rather through the differences in kind and amount of melanin, not the number of melanocytes (Del Bino et al. 2018). For example, while oriental skin color is a result of carotene in the stratum corneum, albinism is a skin color defect, in which skin does not produce melanin (Fajuyigbe and Young 2016). For example, individuals of African descent have larger melanosomes than Caucasians, who has membrane-bound melanosomes in smaller size and with distinct morphological differences compared to the spherical melanosomes of red-haired individuals (Del Bino et al. 2018). Conversely, loss of melanosomes in conjunction with loss of melanocytes results in graying of hair (Del Bino et al. 2018).

Merkel's cells are type I mechanoreceptors that are found densely in high tactile sensitivity regions such as the finger tips, oral cavity, lips, and outer root sheath of hair follicles. They account for high touch sensitivity in these anatomical locations (Haeberle and Lumpkin 2008). Upon changes in their interaction within their assemblies of so-called "touch domes" they secrete neurotransmitters creating an action potential in the neighboring Sensory Neurons to relay the touch reception to the brain.

Langerhans cells that mediate various T Cell responses are present in all layers of the epidermis are densely present in the Stratum Spinosum and mainly function in the protection of the body by preventing pathogens from entering the body (Upadhyay et al. 2013). They are a type of dendritic cells (antigen-presenting immune cells) of the skin originating from bone marrow. Constituting 2–8% of the total epidermal cell population Langerhans are distributed in constant numbers in various squamous epithelia of the body, including epidermis, oral cavity, esophagus, vagina, lymphoid organs, and in normal dermis (Westerterp et al. 2005). Due to their key

roles mediating T Cell responses, the hydrolytic enzymes of their phagolyosomes process the antigens found in the contents of their specialized organelles called Birbeck granules and ultimately contribute to T cell activation (Suhail et al. 2019).

The basement membrane residents, basal keratinocytes and the dermal fibroblasts, form the backbone of the Dermal-Epidermal junction at the interface between the epidermis and dermis and produce key extracellular matrix (ECM) components such as Collagen type IV, anchoring fibrils, and dermal microfibrils (Breitkreutz et al. 2013). Together with this dense meshwork of extracellular molecules the zone of Dermal-Epidermal junction provide support for the epidermis. In addition to housing the mitotically active basal cells it is also in charge of guiding cell polarity, direction of cell growth, organization of cytoskeleton in basal cells, providing growth stimulatory signals, and serving as a semipermeable barrier that controls trafficking of fluids and exchange of cells (Agarwal and Krishnamurthy 2019a; Yousef and Sharma 2018). For example, Laminin 5, which is an abundantly found factor in the architecture of ECM, utilizes α3β1-integrin for its assembly. As the cells of the basal layer leave the mitotically active compartment they exit the cell division cycle, commit to a terminal differentiation program, they switch off integrin and laminin expression (Kolarsick et al. 2011).

1.1.1 Skin Appendages

As mentioned above a group of auxiliary structures that grow down in the direction from the epidermis toward dermis become embedded in this zone, assist skin's function in touch, temperature sensation, removal of toxins, perspiration, and thermoregulation (Brohem et al. 2011). These auxiliary structures that are derived from the ectoderm are collectively known as skin appendages (or adnexa), including eccrine and apocrine glands, ducts, pilosebaceous unit (PSU) that is comprised of hair, hair follicle, arrector pili muscle, and sebaceous gland. During wound healing these adnexal structures can be regenerated via migration of the keratinocytes from adnexal epithelium to the surface of the epidermis in a process called reepithelialization that takes place rather more rapidly in areas with higher number of pilosebaceous units (like the face and scalp) than those that have less (like the back) following an injury (Yousef and Badri 2019). Sudoriferous (from the latin word sudor for sweat) glands, Sebaceous glands, Ceruminous glands (present in the external auditory canal), and Mammary glands (present in the breast epithelium) are the four main types of glands of the integumentary system (Murphrey and Vaidya 2019).

Eccrine Sweat Glands

Eccrine sweat glands are formed by the downward growth of a group of epithelial cells from the epidermis towards the dermis in three compartments (Diao et al. 2019). Out of ~ 3,000,000 present in total overall the skin, highest number of eccrine sweat glands are found in anatomical areas such as palms, soles of the feet, forehead, and armpits while their number is the fewest on the back (Rittie et al. 2013). Each eccrine unit, transcends from a pore on the surface of the skin all the way to the depth of dermis. The overall function of the gland in heat control and electrolyte homeostasis is executed through the concerted operation of various cell types present in each compartment of the gland (Hodge and Brodell 2019; Lu and Fuchs 2014). In response to thermal stimuli a hypotonic solution is generated by the sodium-absorbing action of the cells in the straight dermal portion and the lowest coiled secretory duct where primarily glycogen-rich inner epithelial cells produce the sweat (Lu et al. 2012). Dark mucoidal cells and myoepithelial cells of the lowest coiled secretory duct compartment contribute to the formation of the sweat which then is transferred to the skin surface through the upper part of the straight dermal duct and the spiral intra-epidermal duct to be excreted from the skin pores (Flament et al. 2015).

Apocrine Sweat Glands

Apocrine glands are secretory glands of thick fluidic mixtures with characteristic odors distinct from those produced by bacteria that decompose

skin secretions (Patel et al. 2019). Secretory fluids of the apocrine gland are more viscous than those secreted by the eccrine glands that are smaller in size are located closer to skin surface than the apocrine glands (Lu and Fuchs 2014). These highly viscous secretions by the apocrine glands contain pheromones, substances that mediate communication with other members of the species olfactory stimuli (sensing the environment through smell-detection of airborne substances) (Doty 2014). They regulate scent release as opposed to eccrine glands that control thermal adaptation. With distinct anatomical and physiological differences in comparison to the eccrine and apoeccrine sweat glands, intraepithelial ducts of the apocrine glands do not open directly to the skin surface, but open into the pilosebaceous duct (Briggman et al. 1981). Their secretory coiled base is entirely based in the subcutaneous fat and comprises of soly secretory cells without ductal cells as it is the case for the eccrinal coiled secretory base (George et al. 2004). Although exact composition of their secretion remains largely unknown due to difficulties in obtaining pure samples, it is a viscous fluid that is rich protein content (Witwer et al. 2013). Since apocrine gland activity is induced right before onset of puberty it is thought to be under a hormonal regulation. Specialized apocrine glands such as ceruminous and mammary glands have specific secreted cargo (Ohki and Kikuchi 2019). Ceruminous glands located in the external ear canal lining secrete the sticky cerumen (earwax) that repels foreign material. In the case of mammary glands the secreted fluid is the milk (Shokry and Filho 2017).

Apoeccrine Sweat Glands

Being derived from eccrine-like precursors during puberty Apoeccrine Sweat Glands (AEGs) open directly onto surface of the skin like the eccrine glands. However, the secretory rate of AEGs is 10 times higher than that of eccrine glands while their total number may vary in each individual (Cui and Schlessinger 2015).

Sebaceous Glands

Sebaceous glands are present in groups of two or more per hair follicle (Martel and Badri 2019). Major role of this gland is to keep hair soft and pliable by flushing the hair shaft constantly with its secretion called *sebum*, which is the term given to the lipid droplets available in ample amounts within the cytoplasm of the sebaceous cells, but in reality it is a cocktail of fats, waxes, and hydrocarbons (Martel and Badri 2019). Morphologically the gland has a lobular shape which is surrounded by the sebaceous gland connective tissue sheath on the periphery where a collagenous layer often contains blood vessels, nerve cells, and those from an immunogenic origin such as the Mast cells (Hoover and Krishnamurthy 2019). These cells are located in the upper segment of the hair follicle in a lobular arrangement. The lipid-packed cells are derived from an underlying germinative basal layer. Resting on this connective tissue zone there is a pool of proliferative sebocytes that forms the innermost cell layer of the gland (Blanpain and Fuchs 2006). Very much like the case seen in epidermal self-renewal, the basal sebocytes enter a maturation process whereby they become committed to a differentiation program as they migrate to the necrotic mid-zone of the gland that is aligned with the sebaceous duct concordant with their increased accumulation of lipid droplets (Niemann 2009; Zouboulis 2009). Hence, sebocytes at various points in their differentiation program can be visualized with distinct staining patterns (i.e., nuclear, membranous, and cytoplasmic) in immunohistochemical analyses (Xu et al. 2002). At the central zone of the gland lobule fully differentiated sebocytes undergo holocrine secretion releasing their sebum contents. Sebum then becomes transferred to the infundibular segment of the hair follicle inside the Follicular Canal via the Sebaceous Duct to be emitted on the hair shaft and skin surface together with the necrotic cellular debris (Schneider and Zouboulis 2018). Sebaceous glands are most densely populated in the face and scalp and are present

in other anatomical parts of human body to lesser extent, however, they are absent in the palms, soles and dorsal sides of the feet (Taylor and Machado-Moreira 2013). Since lipids are poor conductors of heat, sebaceous glands help prevent water and heat loss (Pappas 2009). Throughout lifetime with the elevation in the plasma levels of sex hormones sebaceous glands become activated during puberty. The over-secretory activity of the glands may result in excessive sebum production, clogging of the gland and hair follicle, leading to lesions of "*acne*", a common disorder seen in teenagers. From an evolutionary perspective sebaceous glands are proposed to be important for extra lubrication to facilitate birth during the passage through the birth canal (Kolarsick et al. 2011).

Nails

Fingernails are another important appendage of the integumentary system providing protection, improved sensation, and ability to grasp small sized objects (Shirato et al. 2017). The nail bed underneath the nail plate is part of the nail-matrix that has blood vessels, nerves, melanocytes, and keratinocytes (Brahs and Bolla 2019). The nail plate itself is formed from the keratin-producing keratinocytes in distinct matrices of the nail bed (Rice et al. 2010). The ventral (where the nail emerges), dorsal, and the intermediate/deep nail layers are produced by the nail bed, proximal matrix, and the intermediate matrix, respectively (Baswan et al. 2017). Fingernails (0.1 mm per day) grow 2–3 times faster than the toenails (Yaemsiri et al. 2010). Due to their slow growth rate toenails can provide information about toxic exposure of an individual such as the Mees lines -a form of horizontal hypopigmentation across the nail plate- are characteristic of arsenic poisoning (Kolarsick et al. 2011).

Hair Follicles

Although biological function of hair such as protection from external agents, providing insulation, and spreading glandular secretion products evenly were of higher degree of significance for the caveman, social and psychological role of hair has taken precedence over its former biological roles for the individuals of the modern society. To battle either hair loss (alopecia) or presence of excessive hair in undesired areas (hirsutism and hypertrichosis) or decolouring of hair sets the drive for a giant pharma-cosmetics-academic ecosystem that works with the goal of developing improved hair products annually (Sachdeva 2010). Hence, today, much of what we have learned about hair growth is the fruition of this momentum that propels a multibillion industry willing to prevent the emotional distress associated with having these conditions. Moreover, several investigators studying different biological processes picked the hair follicle as a model system resulting in an explosion of our knowledge on this particular skin appendage that regenerates in cycles (Paus and Cotsarelis 1999; Chuong et al. 2012).

The decisions towards establishing the number and distribution of hair follicles is made during the fetal stages and these decisions are not amended after birth (Lothian 2000). For example, density of the hair follicles in a given area of the skin are determined by early-expression gene products that are involved in the morphogenesis of the follicles. Although the size and shape of a hair follicle may vary depending on the location, its structure will be the same as others (Balana et al. 2015; Nowak et al. 2008). During embryonic development mesenchymal cells in the fetal dermis become congregated below the basal layer of the epidermis, an event that stimulates the basophilic cells of the epidermis resident to the basal cell layer to grow at a downward angle into the dermis (Schlessinger and Sonthalia 2019). In the second-half of the 8-staged-morphological development program the hair follicle continues to grow up until a *bulb* forms around those mesenchymal stem cells from which dermal papilla is derived (Rompolas and Greco 2014). Differentiation at the lower portion of the follicle gives rise to structures such as hair cone, the hair shaft, the cuticle, and the two inner-root sheaths, while the differentiation ongoing in the upper segments of the follicle gives rise to hair canal that spans from upper dermis throughout to the surface of the epidermis (Kobielak et al. 2003). Further structures such as the sebaceous gland and a

groups of smooth muscle cells, called the *Arrector Pili Muscle* (APM), that attach the follicle to the external root sheath, and hair bulge forms from two distinct buds (Martel and Badri 2019). The hair bulge becomes located where the APMs are attached to the hair follicle while the opposite end of the APM is embedded in the papillary dermis. As it is discussed in detail in the next section, the bulge is thought to be a reservoir of stem cells that are in charge of regenerating follicles. Opposite to the side of the sebaceous gland a third bud emerges to give rise to the apocrine gland.

In a sense, HF is the compartment of the PSU where the sebaceous gland, the apocrine gland, and the AMP are housed (Mistriotis and Andreadis 2013). HF is further conceptualized as a two-compartment system where it has an upper part that includes the *infundibulum* and *isthmus*, whereas the lower part includes the hair bulb, hair bulge, the matrix, and the dermal papilla (DP) (Mistriotis and Andreadis 2013; Hsu et al. 2014). In collaboration with IFE, the main compartment of the epidermis, where progressive differentiation of keratinocytes after leaving the basal compartment form the barrier against the outside environment, PSU enables waterproofing of the skin through the secretion of sebum by the sebaceous gland (Schepeler et al. 2014; Fuchs et al. 2003).

The infundibulum compartment of the HF in a given PSU neighbours IFE and -hence- opens to the skin surface on one end and isthmus on the other (Schepeler et al. 2014). Isthmus is the mid-segment of the HF spanning from the infundibulum above and the top of the hair bulge below (Schepeler et al. 2014). The upper portion of the isthmus is defined as the *junctional zone* (JZ) which covers the region between the duct of the sebaceous gland and APM attachment (Schepeler et al. 2014).

Depending on the source, the lower compartment of the HF of a given PSU can be conceptualized differently. For example, while the hair bulge can be considered to reside in the isthmus according to one source (Mistriotis and Andreadis 2013), it is considered as a distinct segment beneath the isthmus as the upper segment of the permanent PSU which is proposed to consist of hair bulge and hair germ (Schepeler et al. 2014). Irrespective of the lack of consensus on its location, hair bulge is recognized as the reservoir for the hair follicular stem cell (HFSC) that is capable of regenerating the HF in cases of normalcy and damage. Committed HFSCs migrate from the bulge region toward the hair bulb where they proliferate and differentiate to generate the hair shaft and the rest of the epithelial cells of the HF such as the inner and outer sheaths composed of keratinocytes (Myung and Ito 2012; Woo and Oro 2011).

While the, inferior segment experiences cycles of involution and regeneration throughout life, same is not true for the infundibular and isthmus layers. Both the hair shaft and the inner & outer sheaths are derived from the proliferating cells of the hair bulb and these cells are called matrix cells (Martel and Badri 2019). Similar to the case with epidermal and sebaceous gland epithelial homeostasis, matrix cells of the follicle move upward as the hair grows, becoming more compressed as they enter the rigid inner root sheath which sheds when the growing hair (also in an upward direction) reaches the isthmus. Hence, it is not surprising that the number of cells entering the sheath determine the size of the hair while the dimensions and the curvature of the inner root sheath determine the shape of the hair (Alibardi 2004; Thibaut et al. 2005). Meanwhile, the color of the hair is determined by the number and shape of a melanosomes stretch lined up in the hair shaft after being synthesized by the melanocytes which transfer them to keratinocytes inside the bulb matrix (Slominski et al. 2005).

In contrast to the case of continually regenerating epidermis, hair grows in cycles stemming from each hair follicle that operates independent of others in humans. Each hair cycle comprises of three distinct phases called Anagen, Catagen, and Telogen phases. While these cycles could be out of synchrony for the human HF units, in mice the first two cycles take place in synchrony (Hsu et al. 2014). *Anagen* phase is known as the active growth phase during which hair growth is approximately at 0.33 mm and generally lasts about three to 5 years on the

scalp. With age anagen phase lasts progressively shorter and it is profoundly shortened in individuals who suffer from alopecia (Qi and Garza 2014). During *Catagen* phase involution takes place whereby apoptosis prevails in many cells of the outer root sheath and this phase lasts about 2 weeks (Botchkareva et al. 2006). During the resting phase of Telogen, hairs of the scalp become pushed out by the growing hair shaft that are in anagen phase for about 3–5 months, while hairs in the other parts of the body present with shorter anagen, but longer telogen phases resulting in their shorter length, but longer retention on the skin (Pierard-Franchimont and Pierard 2013).

Interactions between the epithelial and mesenchymal cells determine the development of hair follicle (Sennett and Rendl 2012). As it will be discussed in detail in the "Stem Cells of the Skin" section, genes that play key roles in hair development are also important for the cycling of the hair follicle (Paus and Cotsarelis 1999). Insulin-like growth factor 1 (IGF-1) and fibroblast growth factor 7 (FGF-7) are the two key molecules that regulate the development as well as cycling of hair follicles. In mice both are secreted by the dermal papilla and stimulate their receptors embedded in the membranes of overlying matrix cells (Seo et al. 2016). Estrogens, thyroid hormones, glucocorticoids, retinoids, prolactin, and growth factor are a few examples of hormonal factors that impact hair growth. Androgens such as testosterone and its active metabolite dihydrotestosterone have potent effects on hair growth through their receptor-mediated action exerted on dermal papilla cells by increasing hair follicle size, like seen in the case of beard area during puberty. Intriguingly, this promoting effect can become suppressive for the follicles in the scalp resulting in androgen alopecia later on in life (Zhang et al. 2018; Chen and Zouboulis 2009).

With the exception of congenital hair disorders that may be consequences of genetic mutations in keratins or other structural proteins, pathologies such as alopecia, hair loss, and undesired hair growth result from deviations from hair follicle cycling and, therefore, can be reversed (Zernov et al. 2016). A number of factors impact hair cycle, for example, telogen phase can be prolonged during pregnancy, while number of scalp hairs in anagen phase can be increased (Chueh et al. 2013). Upon equilibration of estrogen levels following childbirth telogen hairs become lost and anagen hairs simultaneously are converted to telogen hair which eventually becomes lost in 3–5 months. Another striking example of hair cycle disorder is seen in cases of telogen effluvium whereby synchronous termination of anagen or telogen results in massive hair loss in scalp, face, and other body parts (Ting and Barankin 2006). Severe trauma, childbirth, surgery, weight loss, severe stress, drug side effect, endocrine disorders, anemia, and malnutrition are found in association with telogen effluvium (Guo and Katta 2017).

Strikingly, hair follicle is the only organ that epitomizes its pre-natal development in each hair follicle cycle as it regenerates during postnatal stages of life. Several gene products including growth factors and their receptors, growth factor antagonists, transcription factors, adhesion molecules, and intracellular signal transduction components regulate both hair follicle development and hair follicle cycling (Lee and Tumbar 2012). Among these gene products many were historically discovered in Drosophila Melanogaster and hence are named after the phenotypes stemming from their specific mutant versions. For example, Decapentaplegic (Dpp/bone morphogenetic protein (BMP)), Engrailed (en), Homeobox (hox), hedgehog/patched (hh/ptc), notch, wingless/armadillo (wg/wnt/catenin) genes are known for their critical roles both for hair follicle and vertebrate development (O'Connor et al. 2006; Mizutani and Bier 2008).

1.2 Dermis

The dermis, also known as the "true skin", is the layer of skin that lies between the epidermis and subcutaneous tissues of hypodermis as a thick layer of fibrous, filamentous, amorphous, and elastic tissue containing predominantly collagen

(protein that gives skin its strength), reticular fibers (protein fibers that provide support) and elastin (protein that accounts for the skin's elasticity) (Brown and Krishnamurthy 2018). In the dermal layer, those bio compounds that help maintain skin hydration and firmness (such as hyaluronic acid, collagen, and elastin) are manufactured by fibroblasts of various lineages (Ganceviciene et al. 2012).

While the epidermis serves as a protective barrier and hosts cell turnover, the main function of the dermis is to maintain skin's firmness and elasticity. Dermis is about 2 mm accounting for 90% of skin's thickness (Yousef et al. 2019). Collagen that maintain skin firmness, elastin that provides elasticity, and hyaluronic acid that maintains hydration make up approximately 70% of the dermis. The dermis plays a greater role on skin firmness and elasticity than the epidermis in that upon its damage skin becomes more prone to wrinkles and sagging that are harder to reverse (Yousef et al. 2019; Zhang and Duan 2018). Factors such as aging, inflammation, and UV exposure cause skin deterioration which is aggravated with slowed down metabolism, cell turnover, and fibroblastic cell division, and hence the amount of collagen (Phillip et al. 2015).

The high abundance of these elaborate filamentous dermal protein networks in the dermal layer accounts for the tensile strength, pliability, and elasticity of the skin. Moreover, due to housing receptors of sensory stimuli such as heat and touch, it regulates body temperature, protects the body from injury, and binds water (Phillip et al. 2015; Wang et al. 2015). Upon various stimuli dermal tissue allows an array of cell types, including cells of the nervous system and vascular epidermally-derived appendages, fibroblasts, macrophages, mast cells, lymphocytes, plasma cells, and leukocytes enter the dermis (Wang et al. 2015). The sustained interaction of the dermis with the epidermis promotes maintenance for the properties of both tissues. For example, collaboration of both is seen both during the morphogenesis of the dermal-epidermal junction and epidermal appendages during the development and during wound healing to accomplish proper repairing and remodelling (Pastar et al. 2014). Although dermis is not known to undergo waves of differentiation conspicuously like in the case of epidermis, distinct connective tissue compartments can be predicted depending on depth across the dermal cross-section. Likewise, depending -not only- on depth, but also on turnover and remodelling processes that may be governed by external stimuli in normal as well as diseased states of the skin, abundance of ECM components such as collagen and elastic connective tissue also vary (Bonnans et al. 2014).

In terms of embryonic origin, dermal layer is heterogeneous in nature in that various types of residential cells are derived from different embryonic fate. For example, while the constituents of the dermis originate from mesoderm, others such as nerve cells, melanocytes are descendants of the neural crest. Up to E6 (embryonic week 6) dermis is full with precursors of the fibroblasts which are dendritic shaped cells containing acid-mucopolysaccharides. By E12 fibroblasts commence synthesizing reticulum fibers as well as collagen and elastic fibers. Later on (by E24) both fats cells of an adipose layer and those of the vasculature emerge underneath the dermal layer (Domowicz et al. 2008; Agarwal and Krishnamurthy 2019b). Strikingly, only a few of the many fibroblasts present in infant dermis persist throughout adulthood where small collagen bundles are typical. However, it is noteworthy that infant dermis that comprises of small collagen bundles converts to an architecture that contains thicker collagen bundles (Lakos et al. 2004).

As the principal component of the dermis collagen is highly enriched in amino acids such as glycine, hydroxyproline, and hydroxylysine, and encoded by 15 distinct genetic variants that become translated into the members of a fibrous family of proteins in human skin (Shoulders and Raines 2009). For example, Type I collagen is the sub-type intrinsic to the dermis and while Types IV is found in the basement membrane zone, Type VII -produced by the keratinocytes- is important for the infrastructure of the anchoring fibrils. As a stress-resistant protein, collagen is a key structural component that is widespread throughout the body being present in tendons,

ligaments, bones, and the dermis, while the elastic fibers of the dermal layer contribute marginally to the stress-resistance property of the skin in the face of mechanical injury (Shoulders and Raines 2009). The members of the fibrillar collagens found in the skin is predominating group of proteins in terms of abundance throughout the body. In line with the tissue texture pertained to the layers of the dermis, while loosely positioned collagen fibrils are typical of the papillary and adventitial dermis, heftier collagen bundles are more of a characteristic of the reticular layer of the dermis (Prost-Squarcioni et al. 2008).

In contrast to the structural and biochemical properties of the collagen fibers, elastin fibers have a binary structure where there is a protein filament and the amorphous protein component of elastin (van Eldijk et al. 2012). Elastin fibers are anchored into the glycosaminoglycan-rich ECM of the dermis via the fibroblasts. Parallel to the case of collagen fiber network, finer elastin fibers are found in the papillary dermis, while more coarse versions are found in the reticular layer. Although hyaluronic acid is a minor component of the normal dermis, becomes the highly accumulating mucopolysaccharide of the pathological states (Ushiki 2002; Tracy et al. 2016).

The layer of dermis can be envisioned in two such intermingled sub-layers that they are often hard to tell apart. One that consists of the loose connective tissue is called the Papillary Layer while the one that has the denser connective tissue is called the Reticular Layer (Brown and Krishnamurthy 2019a).

Papillary layer owes its name to the finger-like projections of papillae and in certain regions it entails a network of fine capillaries that nourish the epidermis, while other regions contain the so-called Meissner's corpuscles (Tactile Corpuscles), which are a type of nerve ending mediating sensitivity to light touch (Piccinin and Schwartz 2019). The intricate network of capillaries serve the crucial functions of carrying nutrients to and removing waste metabolites from the local cells as well as maintain optimal body temperature by increasing or decreasing blood flow through pertinent contraction and relaxation cycles. Interestingly, the papillary layer of the dermis in the fingertips determines the pattern of the fingertips (Joyner and Casey 2015).

On the other hand, as the deepest layer of the dermis the reticular layer is composed of an elaborate meshwork of elastin and collagen fibers (which makes up about 70% of the extracellular matrix) (Frantz et al. 2010). The reticular layer collagen is produced by the resident fibroblasts. The strength and elasticity of the skin is attributed to its reversible property of viscoelasticity which provides resuming back to the resting state following a stretching up to a physiological limit upon elevation of the mechanical stress (Tepole et al. 2012). In other words, viscoelasticity of the skin gives its resilience to insults by external forces due to the tightly-woven elastin and collagen meshwork. While sliding and re-arrangement of these collagen fibers underlies the ability to persist a physical load by guarding tissue-integrity through allowing skin deformation whilst preventing damage, elastic fibers provide the ability to bounce/relapse back to the resting state once that physical load is removed (Ehrlich and Hunt 2012). However, when this property falters the architecture of the skin in terms of structural properties of collagen and elastin networks are subject to change like seen in cases of cancer, aging, toxic UV, and sunlight exposure (Marionnet et al. 2014).

In that regard, the collagen network exist in a rather dynamic than static state where its degradation due to catalytic activities such as spare collagenases is counteracted by its constant assembly following its synthesis and processing by the fibroblasts where a pro-collagen polypeptide chain becomes integrated and then secreted to be used in the construction of collagen fibrils (Abou Neel et al. 2016).

A set of sensory receptors called Pacinian corpuscles that are involved in reception of deep pressure are also found within the reticular layer which cushions the deep projections of skin appendages such as sweat glands, lymph vessels, smooth muscle, and hair follicles (Slominski et al. 2012).

1.3 Other Cell Types of the Dermal Architecture

Nerve cells, an intricate network of blood vessels, hair follicles, sebaceous, and sweat glands constitute the skin appendages that are embedded in the dermal layer. In addition, dermal adipose cells, mast cells, and infiltrating leukocytes also reside in the dermis. In this section we will brief our reader about these minority cell types of the dermal layer (Randall et al. 2018).

1.3.1 Vasculature

Most of the skin vasculature is embedded in the reticular dermis, however, according to the recent reports a branching and intricate network of capillaries is placed right above the bulge where they modulate hair growth by Hair Follicle Stem cells (HFSCs) via secretion of angiogenesis-derived factors. Major function of the skin vasculature is to carry nutrients, hormones, and immune cells (Hsu et al. 2014). Two types of intercommunicating plexuses encompass the dermal vasculature architecture. The first is known as the *subpapillary* (or *superficial) plexus*, that comprises of postcapillary venules, and it is located at the papillary-reticular junction of the dermis (Imanishi et al. 2008). The second is the *lower plexus* which is found at the dermal-subcutaneous interface. The capillaries, end arterioles, and venules of the subpapillary plexus supply to the papillae of the dermis. Meanwhile, the deeper plexus, −which is supplied by the larger blood vessels and more complex in structure than the subpapillary plexus-, supply to the adnexal structures. Being regulated by the preoptic-anterior hypothalamus blood flow, the skin is modulated in response to thermal stress in humans. (Ye and De 2017). Being regulated by the preoptic-anterior hypothalamus blood flow, the skin is modulated in response to thermal stress in humans. In order to cope with increased heat, vasodilation, increased skin blood flow, and sweating are important responses to disseminate heat (Greaney et al. 2016). Conversely, in response to cold, vasoconstriction in the skin helps preventing heat loss and hypothermia. Disturbance to the skin blood flow can significantly debilitate maintenance of normal body temperature as seen in the case of patients with type II Diabetes who may experience heat stroke and heat exhaustion upon elevation in external temperature and menopausal women who experience hot flashes induced by hormonal imbalance (Hifumi et al. 2018).

1.3.2 Muscles

As mentioned earlier the APM that are attached to the hair follicles below the sebaceous glands make up one of the muscle groups of the dermis (Fujiwara et al. 2011). Being situated in the connective tissue of the upper dermis APM fibers exist at such an angle to the hair follicle that upon contraction hair follicle becomes pulled into a vertical position resulting in a type of skin deformation known as "gooseflesh" or "goosebumps" (Brown and Krishnamurthy 2019b). Another group of smooth muscle bundle is found surrounding the veins and arteries of the skin. The specialized smooth muscles of glomus is in between the arterioles and venules. Striated (voluntary muscle groups) resident to the skin of the neck and face are known as muscle of expression (Haddad et al. 2001). Likewise, subpapillary muscles of the *aponeurotic system* (a network of aponeuroses connecting muscles and fascia) mediate movement of body parts.

1.3.3 Nerves

Together with the arterioles and venules the highly abundant nerve bundles make up the neurovascular bundles of the dermis. Among the sensory organs skin is the largest due to its dense innervation by innumerable primary sensory neuron fibers (Andreone et al. 2015). The cell bodies of this heterogeneous population of neurons including nociceptors, mechanoreceptors, and proprioceptors, are located in trigeminal and dorsal root ganglia (Hsu et al. 2014). For example, Meissner corpuscles -densely found in the ventral sides of the hands and feet and fingertips- are resident to the dermal papillae and convey the signals induced by touch to the central nervous system. Hence, sensory nerves are in close

contact with the cells of the epidermis and the hair follicle with the nerve endings anchoring at the different layers of the epidermis (Hsu et al. 2014; Andreone et al. 2015). A region just above the hair bulge is surrounded by the mechanoreceptive nerve endings. Being located in the deeper portion of the dermis in the weight-bearing surfaces of the body Vater-Pacini corpuscles are large nerve-endings and mediate sense of pressure (Bell et al. 1994). The unmyelinated nerve fibers found around the hair follicles and papillary dermis transmit sensations such as pain, temperature, and itching (Park and Kim 2013). The postganglionic adrenergic fibers of the autonomic nervous system regulate the vasoconstriction. The latter also controls the secretions of the apocrine gland and the contractions of the AP muscles of the hair follicles. Secretions of the eccrine sweat glands are regulated by the cholinergic fibers. Signals emanating from the skin can affect the sensory innervation and dendritic arborisation (McCorry 2007; Gordan et al. 2015). Conversely, signals from peripheral nerves may influence hair follicles, in return. For example, neuropeptides such as substance P and calcitonin gene-related peptide (CGRP; a pro-inflammatory neurogenic bio compound), can induce hair follicle regression (Hsu et al. 2014).

1.3.4 Mast Cells

Mast cells are a sub-type of immune cells that originate in the bone marrow in a progenitor form of myeloid lineage and they localize widespread in the peripheral tissues bordering external environment, including the mucous-producing tissues of the gut and lungs as well as the skin and blood vessels (Krystel-Whittemore et al. 2016). Given that the skin is one such interface, it houses a large mast cell population (more densely found in papillary dermis than in hypodermis) as the first responders to the presence of parasitic invaders as well as allergens (da Silva et al. 2014). While Type I (connective tissue) mast cells are inherent to the dermis and submucosa, Type II (or mucosal Mast cells) are found in the mucosa of the respiratory tract and the bowel. Mast cell maturation takes place in response to the c-kit ligand, stem cell factor, and other stimuli released by their microenvironment (Krystel-Whittemore et al. 2016). Numerous peripheral large and long vili and round, oval or angular membrane-bound cytoplasmic granules encapsulating chemokines such as histamine and heparin, certain cytokines, serine proteinases, leukotrienes, and prostanoids are hallmarks of their morphology (Kunder et al. 2011). Anchoring of a variety of stimuli such as superoxides, complement proteins, neuropeptides, and lipoproteins to the immunoglobulin E (IgE) and consequential binding of IgE to its receptors embedded on mast cell surface triggers a process called "degranulation of mast cells" whereby -within seconds- inflammatory content of cytoplasmic granules are delivered to the microenvironment. In other words, crosslinking of hundreds of thousands of FcεRI glycoprotein membrane receptors to their ligand IgE as a consequence of engaging stimulatory signals is the initiating event of Mast Cell activation (Shakoory et al. 2004). While these cells participate in regulation of vascular homeostasis, angiogenesis, venom detoxification as well as innate and adaptive immune responses in normal physiology, they have emerged as players with either promoting or suppressive roles in pathologies of allergy, asthma, atherosclerosis, several types of cancers, and gastrointestinal disorders when deregulation of their accumulation, proliferation, clearance or migration prevails (Chen et al. 2018).

1.3.5 The Hypodermis (Subcutaneous Fat)

Hypodermis is a cushioning layer present underneath the dermis. It insulates the body against physical trauma, heat, and cold, while serving as an energy storage area. The fat is exclusively deposited in adipocytes, held together by fibrous tissue (Driskell et al. 2014; Labusca and Zugun-Eloae 2018). This layer of the skin begins to develop toward the end of the fifth month with the appearance of the fat cells in the subcutaneous tissue. The fat cells are the most predominant cell type found in this layer while mesenchymal stem cells are also present in the hypodermis layer. Adipocytes are separated by large blood and lymph vessels (Labusca and Zugun-Eloae 2018).

In fact, recent findings rapidly established the notion that subcutaneous fat is a major endocrine organ that secretes an array of stimulatory factors, also termed as *adipokines*, that exert influential roles on lipid metabolism, energy balance, insulin sensitivity all of which are important in angiogenesis, immunomodulation, and inflammatory response. In other words, adipokines collectively constitute the secretory repertoire of the adipose tissue and they participate in maintenance of organ homeostasis at the autocrine, paracrine, and/or endocrine level by mediating communication between multiple cell types (Gaur et al. 2017; Al-Suhaimi and Shehzad 2013; Stern et al. 2016). For example, conversion of androstenedione into estrone by aromatase, production of leptin by lipocytes to control sateity takes place in this layer. Another example is seen in tissue repair processes of the skin. During epidermal and dermal repair adipokine repertoire participates in coordinating both proliferation and migration of keratinocytes and fibroblasts (Schmidt and Horsley 2013).

The fact that the mesenchymal stem cell content per gram of tissue is 500 times higher in subcutaneous adipose tissue (hypodermis) than bone marrow was probably one of the most exciting findings for the history of regenerative medicine, an exploding field which was summarized comprehensively in the previous issue of this journal by Cankirili et al. (2019). For the most part it is thought that the therapeutic effects of the dermal layer (discussed further in the following section) and subcutaneous fat compartment are executed by the mesenchymal stem cell residents of these layers through their healing capacity on sites of injury and inflammation through actions of their endogenous secretory repertoire including pro-regenerative, anti-fibrotic, anti-apoptotic, and growth factors required for the tissue repair processes (Gaur et al. 2017). Mesenchymal stem cells derived from the adipose tissue is proposed to be the "endogenous factories" that supply trophic factors capable of supporting all layers of the skin "in sickness (repair) and in health" (Gaur et al. 2017). For that matter we believe there will be benefit to introduce the common properties of mesenchymal stem cells before plunging into the amazing depths of skin stem cell biology.

2 Mesenchymal Stem Cells of the Skin

Mesenchymal Stem cells have the capacity to renovate the mesodermal tissues such as the connective, cartilage, and bone tissues by replenishing their cellular context. In addition to subcutaneous fat (hypodermis) they can easily be isolated from blood, fat, bone marrow, and foreskin in adequate quantities and this property is only one of their attractive features for the purposes of regenerative medicine (Augustine 2018). Second feature involves their ability to retain their stemness in tissue culture conditions over relatively high number of passages (Shim et al. 2013). Thirdly, under appropriately provided *ex vivo* conditions they can take the fate of various cell types, including myocytes, adipocytes, osteoblasts, neuronal sub-types, and chondrocytes (Jumabay and Bostrom 2015). Finally, the immunomodulatory properties of mesenchymal stem cells (especially those that are immunosuppressive in nature) allow them to execute an ideal management of tissue repair processes. Hence, in recent years numerous methodologies have emerged in the clinic, whereby mesenchymal stem cells were manipulated in order to accelerate the wound healing processes induced in response to a wide variety of injuries such as severe burns, myocardiac infarction, neurodegenerative damage disorders, hepatic injury, muscle degenerative disorders, bone injury, and chondrocyte erosion (Zachar et al. 2016).

2.1 Stem Cells and Their Niches in the Skin

Skin is home to a diversified community of stem cells and other cell types where each community is located in a friendly neighbourhood of various niches each which are resided by other cell types assisting stem cells at different stages of their lifetime from self-renewal to terminal differentiation

(Lutolf and Blau 2009). In this section, we brief our reader with the current knowledge on different types of stem cells and their niches at both cellular and molecular scope. Although initial thought was that cells forming the niche come from a lineage distinct from the stem cells they regulate, recent reports underscore the co-presence of stem cells with their differentiated progeny, suggesting that cues from the descendant cells are as much important as those coming from the rest of cells forming the niche in governing the biology of their stem cell parents (Hsu et al. 2014).

Considering the constant regeneration capacity that has come forth repeatedly for several of the skin compartments described in the previous sections, it is not too surprising that replenishment of each compartment is provided by a unique set of stem cells that concertedly exit quiescence and execute a program of differentiation until the desired tissues are formed, a phenomenon underlying the maintenance of skin in normal homeostasis and wound healing (Gaur et al. 2017). Since the first exploitation of skin stem cells (keratinocytes) in treatment of burn patients (two children whose body surface was burnt by more than 90%), there has been prodigious amount of information accumulating about skin stem cell biology (Wabik and Jones 2015). The first hints about the presence of skin stem cells was coined by their ability to retain diploidy even after hundreds of clonal passages without any requirement for immortalization procedures when grown in the presence of feeder fibroblast layer to make tissue based on the findings obtained in Howard Green's laboratory at MIT (Adam et al. 2018). Following this landmark work introducing us to the immense clinical potential of these skin stem cells, which are contemporarily known as epidermal (Fuchs et al. 2003) stem cells (also known as the epithelial stem cells of the skin), numerous studies continued the discovery of different types of stem cells resident to differential niches found in the skin. For example, over the years increasing evidence have pointed to the presence of a diversified presence of adult stem cells, including mesenchymal, hematopoietic, and neural stem cells residing in the skin (Shi et al. 2006).

In line with the current definition of stem cells, skin stem cells and/or progenitor cells can -not only- renew themselves (*self-renewal*) and commit to specific differentiation programs to generate various lineages of the skin (*multipotency*), but also produce cell types of other tissue types when provided proper *ex vivo* conditions (*plasticity*) (Shi et al. 2006). Furthermore, *cellular quiescence*, which is relatively unrelated to the these three traits, is also recognized as a stem cell property and methods involving uptake and retention of labelled nucleotide analogues are used for the identification of long-lived quiescent stem cell populations (Lang et al. 2013).

According to the results of the engraftment studies where labelled epidermal cultures are allowed to reconstitute epidermal tissue *in vivo*, about 10–12% of the basal cells were capable of generating a single column of differentiating cells (Potten and Booth 2002). Another method for labeling stem cells involves pulse-labeling of newly synthesized DNA in all dividing cells of a tissue with radiolabeled nucleotide analogs (such as bromodeoxyuridine (BrdUrd) or tritiated (Hrckulak et al. 2016) thymidine) and follows up the rarely dividing cells that retain the label in the tissue (Podgorny et al. 2018). Shedding light to the slow cycling nature of tissue stem cells, this pulse-chase method lead to the development of a model for the skin epithelial maintenance where periodic division of slow cycling *so-called* Label Retaining Cells LRCs (putative stem cells) in the basal layer generates a pool of cells termed as *transiently amplifying cells* (TACs). TACs that populate most of the basal layer typically divide two or three times before their commitment to a differentiation into mature skin cells as they migrate upward (Fuchs 2009; Li et al. 2017).

Later on studies done in mice, rats, and human pointed out that bulge region of the HF is one of the important niches where majority of LRS find -in fact- sanctuary. Historically, bulge region of the hair was characterized as a thickening area in the upper portion of the follicle where slow-growing LRCs are found (Lang et al. 2013). It is proposed that the reason for the hair bulge to be a niche of preference for most clonogenic cells and

LRCs of high label retaining capacity is -most likely- because in mammals the hair bulge is fortressed amongst the upper column of cells and heavily keratinized hair shaft above and the supportive, innervated, and nourishing vasculature of the dermal pocket below (Fuchs et al. 2003).

In normal skin homeostasis, stem cells from various niches of the skin such as hair follicle (HF), interfollicular epidermis (IFE), and sebaceous glands play key roles in maintaining healthy epidermal and dermal layers (Gonzales and Fuchs 2017). Both intrinsic signalling pathways at the genetic and epigenetic levels and extrinsic crosstalk between the stem cells and resident cells of their niche are mediated via the secreted cytokines, chemokines, and growth factors, accounting for the overall regenerative capacity of the skin (Psarras et al. 2019; Kizil et al. 2015). In this section we summarize the most prominent long-term stem cells and progenitors found in the skin epithelium as well as current understanding of how stem cells and progenitor cells interact with each other to mobilize the tightly regulated sequence of events that result in adequate amount of tissues and stem cell pool.

The unipotent populations of epidermal stem cells that occupy the niche of the basal layer differentiate into keratinocytes to regenerate the epidermis in normal and injured adult skin. More specifically, these unipotent stem cells are proposed to originate from the multipotent progenitors of the bulge region of the hair follicle (Shi et al. 2006). In case of injury a subset of these follicle-derived multipotent stem cells can migrate out of the hair follicles to the wound site and participate in the repair of the damaged epithelium, while their contribution to the maintenance of the epidermis in normal homeostasis is limited. As it is discussed in detail below, the follicle-derived stem cells can give rise to the tissues of outer root sheath, inner root sheath, hair shaft, and sebaceous gland. Notably, being positive for the neural stem cell marker of Nestin, follicular stem cells have a capacity to differentiate into neurons, glia, keratinocytes, smooth muscle cells, melanocytes, and even blood vessels under appropriate conditions (Shi et al. 2006; Chen et al. 2009).

As mentioned earlier, IFE units comprise of differentiated layers of the stratified epithelium constituting the main component of the epidermis and the foundation of the protective barrier. The layers of epithelia are fueled by the epidermal stem cells found in the basal layer (Schepeler et al. 2014). As the basal cells depart from the Basement Membrane and commit to the terminal differentiation program an extensive transcriptional and post-translational remodeling take place involving modification of intracellular proteins, intercellular junctions, and nuclear fragmentation whereby dense cytoskeletal architecture of keratinocytes forms the highly crosslinked 10-nm intermediate filaments (IFs) that are key to confront external insults (Schepeler et al. 2014; Hsu et al. 2014). The undifferentiated proliferative progenitors expressing Keratin 5 (K5) and 14 (K14) participate in self-renewal as well as give rise to epidermal epithelium. Hierarchically they are proceeded with an increasing degree of differentiation by the nonproliferative, but transcriptionally active spinous and granular layers, that express K1, K10, and involucrin, and eventually by the to-be-shed cells of the dead stratum corneum (Hsu et al. 2014; Alam et al. 2011; Srivastava et al. 2018).

Results from mouse studies utilising various Cre-lineage tracer methodologies engineered under the control of Keratin promoters specific to basal layer support two models: According to the first, "Hierarchical model" a slow-cycling stem cell nested in the conceptual proliferative basal unit of each IFE gives rise to the short-lived transiently amplifying cells (TACs) which then exits the proliferative layer after a certain number of cell divisions to replenish the differentiating cells of the upper layers in a columnar fashion (Hsu et al. 2014; Zhang and Hsu 2017; Rangel-Huerta and Maldonado 2017). According to the second, "stochastic model" basal IFE layer comprises of a single type of proliferative progenitor, descendants of which randomly decide either retain their progenitor identity or to differentiate in which case both daughter cells have equal chances of remaining as

stem cells or committing to a differentiation program (Hsu et al. 2014). Hence, it is tempting to speculate that the former model prevails under homeostatic conditions, whereby the stem cell pool is maintained through asymmetric division, where the parent stem cell divides into two cells one retaining stemness (particularly self-renewal) and the other assuming a more differentiated phenotype (differentiation). This way hierarchical model assumes that the body reserves a powerful reservoir of cells that can be readily engaged when tissue repair becomes needed while supplying a differentiated progeny for normal tissue maintenance (Bryder et al. 2006). On the other hand, in the case of engaging in a repair activity stochastic model could predominate. For the two stem cells generated in the symmetric division from each parent stem cell, it could completely depend on the signaling conjecture of the microenvironment whether these two daughter cells will continue to be stem cells or commit to a differentiation program to replenish the damaged tissue (Mistriotis and Andreadis 2013). Therefore, like other adult SCs, skin stem cells typically remain quiescent until they are coaxed to proliferate and/or differentiate *in vivo*, while *in vitro* they display a noteworthy proliferative as well as differentiation potential (Mistriotis and Andreadis 2013; Horsley et al. 2008).

Mitogens such as insulin-like growth factor (IGFs), fibroblast growth factor 7 (FGF-7), FGF-10, and epidermal growth factor receptor (EGFR) ligands are produced by dermal fibroblasts that facilitate potent pathways for epidermal proliferation (Seeger and Paller 2015). Upregulation of transforming growth factor alpha (TGF a), which is a positive regulator of EGFR signalling, or abrogation of Mig6 or LRIG1 (in humans), which is an inhibitory to EGFR-dependent signalling, stimulates epidermal proliferation (Hsu et al. 2014). Basal epidermal cells are anchored to the major basement membrane component laminin-5 through their receptors such as integrin alpha3beta1 and alpha6beta4 that signals through GTPase RAC1. For example, in humans higher beta1 integrin expression is indicative of greater stem cell potential (Hsu et al. 2014; Hamill et al. 2009; DiPersio et al. 2000).

Furthermore, certain factors of epigenetic modifications are also implicated in epidermal proliferation and differentiation homeostasis. For example, histone H3 Lys (H3K27) methyltransferases EZH1 and EZH2, histone H3K27 demethylase JMJD3 associated activities are essential for epidermal differentiation, respectively through modulating transcription of alpha 6 and beta1 integrin genes. Intriguingly, epidermal proliferation is proposed also to be under a temporal regulation exerted by the core clock factors of the circadian rhythm machine (Mistriotis and Andreadis 2013; Chen et al. 2012).

Epidermal stratification requires delamination of basal cells, whereby they lose their attachment to the basement membrane. Their journey moving upward is presumed to begin with an asymmetrical cell division which generates a committed suprabasal cell and a proliferative basal cell as a result of cytokinesis perpendicular to the basement membrane. The first commitment step in differentiating to spinous cells is dependent on Notch signaling which execute important roles in developmental processes (Hsu et al. 2014; Berika et al. 2014). Binding to its ligands triggers cleavage of Notch receptor proteins by gamma-secretase and the cytosolic domain then translocates to the nucleus to alleviate the transcriptional repression exerted by RBP-J -thereby-enabling the induction of Hes/Hey-dependent transcriptome. For example, expression of Notch ligand DELTA by the basal cells and consequent induction of Notch signaling as a result of DELTA binding to the receptors allows commitment to spinous cell differentiation by promoting detachment from the basement membrane and mediating downstream events of asymmetric cell division to balance epidermal cell proliferation and differentiation (Hsu et al. 2014; Bazzoni and Bentivegna 2019).

2.1.1 Hair Follicle Stem Cells

The dynamism of the HFs is unanimously attributed to a diversified and rich pool of stem cells known as Hair Follicle Stem Cells (HFSCs) that are continuously self-renewing, differentiating, and regulating hair growth. HFSCs emanate from distinct

developmental origins and localizing to distinct anatomical locations within the hair follicle (Soteriou et al. 2016). Because HFSCs are easily accessible they have been extensively studied *in vitro* demonstrating a highly proliferative and multipotent characteristic which -*in vivo*- is believed to be the major contributory factor to skin homeostasis (Mistriotis and Andreadis 2013). Observations made in the *in vitro* studies of HFSCs, have advanced to enabling engineering of various tissues for organ replacement. Furthermore, combined with the tools of genetic engineering HFSCs offer encouraging venues for the treatment of genetic diseases of skin or hair disorders (Ormandy et al. 2011).

Several anatomic locations within the HF -by itself- are home to distinct type of stem cell populations, including HFSCs and MSCs (Mistriotis and Andreadis 2013; Lang et al. 2013). Both HFSCs in charge of regenerating hair and MSCs in charge of regenerating UV-absorbing melanocytes co-reside in the hair bulge and hair germ (Hsu et al. 2014).

In regards to the exact identity of the stem cell population harboured in the bulge region, current model accepts that the bulge niche includes both proliferative (CD34+ and LGR5+) and quiescent (label retaining; CD34+ but LGR5-) stem cells (Mistriotis and Andreadis 2013). Interestingly, LGR5+ cells that are fully capable of regenerating the HF, do not coincide with the label retaining cell populations of the bulge.

Intriguingly, cells from the Isthmus/Infundibulum region display multipotent properties due to the observation that they can -not only- differentiate into the epithelial cells of the HF, but also those of sebaceous gland and the epidermis (Mistriotis and Andreadis 2013). These cells isolated from the region between the sebaceous gland and the bulge lack bulge stem cell (Bu-SC) markers (KRT15- and CD34-), but they are highly proliferative *in vivo*, remain clonogenic *in vitro*, and give rise to new HFs upon transplantation or epidermis upon injury (Mistriotis and Andreadis 2013).

With a quiescent subpopulation residing in the bulge Bu-Scs and another with higher propensity to proliferate residing in the hair germ just neighboring the bulge, HFSCs are generally considered in two subpopulations (Hsu et al. 2014). While Bu-SCs ultimately proceed to generate the outer root sheath (ORS) cells, those derived from the hair germ differentiate to form the TACs of the matrix that give rise to the inner root sheath (IRS) (a channel that surrounds the hair shaft during anagen) cells. In catagen, the next generation of HFSCs become deposited in the newly formed bulge and hair germ that are derived from the upper ORS and middle ORS cells, respectively, for the consecutive hair cycle. In a series of elegant experiments Hsu and co-workers demonstrated that during anagen although some of the Bu-SCs that exit the bulge, they retain their stemness including remaining quiescent. This fraction of BuSCs constitute the reservoir of stem cells for the next cycle, some differentiate, yet still return to the bulge having lost their stemness despite expressing stem cell markers (Mistriotis and Andreadis 2013). In case of injury, but not in normal skin homeostasis, Bu-SCs can migrate to the wound site and differentiate into keratinocytes (Mistriotis and Andreadis 2013). Further studies demonstrated the robust multipotent capacity of Bu-SCs both *in vivo* where they took part in angiogenesis and *in vitro* where they were able to differentiate into keratinocytes as well as cells of neuronal (such as neurons, glial cells, and melanocytes) and mesenchymal origin (Mistriotis and Andreadis 2013).

Although pool of HFSCs remain mostly in quiescence throughout the hair cycle, their proliferation and differentiation become triggered due to the action of several factors secreted by the stem cell progeny and dermal cells in anagen (Hsu et al. 2014). One such factor is the bone morphogenetic proteins (BMPs) that play important roles in bone and cartilage formation. Strikingly, recent studies uncovered that BMPs contribute to the maintenance of quiescence of HFSCs while they execute additional roles that are essential, in embryogenesis, organ homeostasis and other developmental processes. For example, BMP4 secreted by the dermal fibroblasts, BMP2 expressed by the subcutaneous fat, BMP6 secreted by the K6+ cells of the inner bulge layer, as well as the quiescence factor

FGF-18, all function in maintenance of quiescence of both Bu-SCs and hair germ SCs during telogen (Hsu et al. 2014). In support of these findings, conditional ablation of the bone morphogenetic protein receptor 1α (Bmpr1α) gene promotes beta-catenin stabilization, expansion of HFSCs that fail to enter terminal differentiation, and reduction in slow-cycling cell population (Kobielak et al. 2007). Conversely, overexpression of Bmpr1α promotes precocious commitment of HF-SCs to differentiation (Mistriotis and Andreadis 2013; Sotiropoulou and Blanpain 2012). In agreement with the inhibitory role of BMP in hair cycling, expression of BMP antagonists -such as NOGGIN, FGF-7, FGF-10, TGF-β2 (hair germ activating factors) in dermal papillae concordant with fall in BMP4 and BMP2 levels in dermal fibroblasts and adipocytes, respectively, allows the HFSC pool to promote hair growth (Sotiropoulou and Blanpain 2012).

In addition to its widely renowned pro-oncogenic effects and those in development, accumulation of the WNT effector, nuclear beta-catenin and activation of its target genes following complex formation with TCF/LEF transcription factor, is a major propeller in hair germ and dermal papillae stimulation (Hrckulak et al. 2016; Li et al. 2018). Indeed, phenotype of mouse models where WNT inhibitor DKK1 is overexpressed under the control of Krt14 is hairless revealing the potent inhibitory role of DKK1 in HF development confirming the essential role of WNT-dependent signalling in hair growth (Dela Cruz et al. 2012). Conversely, overexpression of a constitutively active truncated beta-catenin under the control of an epidermal promoter permanently promotes formation of new HFs as well as tumors in adult mice, while transient activation of beta-catenin results in increased hair formation (Hsu et al. 2014; Enshell-Seijffers et al. 2010).

An activated state of WNT signalling within hair germ as well as dermal papillae is crucial for the normal transition of hair follicles from telogen to the next anagen. This fact is underscored by the findings that abrogation of beta-catenin nuclear accumulation either in hair germ or dermal papillae results in a telogen arrest of the hair cycle or delayed regeneration of the hair follicles, respectively (Hsu et al. 2014). Although precise compartments of the HF supplying WNT stimulatory signals are to be determined, hair germ itself and dermal fibroblasts are strong candidate sources (Hsu et al. 2014). Reduction of matrix cell proliferation and subsequent precocious entry into catagen in response to loss of beta-catenin nuclear accumulation in DP points to the importance of WNT signalling in maintenance of the mesenchymal and epithelial interactions between the DP and bulge cells (Sotiropoulou and Blanpain 2012; Lowry et al. 2005). Moreover, appearance of nuclear beta-catenin and other WNT effectors in hair germ during the initial stages of anagen preceding that by the dermal papillae is required for normal entry into anagen, because targeted ablation of WNT ligand secretion in the HF epithelium in telogen results in suppression of WNT signalling in DP during anagen and a subsequent potent arrest of hair cycle (Sotiropoulou and Blanpain 2012; Myung et al. 2013).

Parallel to the HF-activating output of WNT signalling, activated TGFβ family member TGF-β2 also contributes to regenerative cues in the HF (Vaidya and Kale 2015). For example, prevention of TGF-β2-dependent signalling within HFSCs through SMAD2/3 resulted in delayed coat recovery in conditional TGF-β reporter II knock-out mouse model (Oshimori and Fuchs 2012b). Furthermore, elevating TGF-β2 levels exogenously was stimulatory to HFSC proliferation both in *in vivo* and *in vitro* conditions by antagonizing BMP-mediated maintenance of quiescence through transcriptional upregulation of TGF-β2-target gene *Tmeff1* (tomoregulin) (Vaidya and Kale 2015) (Sakaki-Yumoto et al. 2013). Hence, together with other BMP-inhibitory factors secreted by DP during late telogen, TGF-β2 overrides quiescence signals to drive follicular regeneration into the next anagen.

Dermal papilla underlying the hair germ produces several factors such as FGF-7, FGF-10, TGF-β2, BMP2 inhibitor NOGGIN during early-to-late telogen to stimulate hair follicle activation for the consecutive hair cycle (Plikus

2012). Meanwhile, expression levels of pro-quiescence genes BMP4 in dermal fibroblasts and BMP2 in mature adipocytes become decreased to perpetuate HFSC activation. Finally, platelet-derived-growth-factor alpha (PDGF-alpha) produced by the adipocyte precursor cells turn PDGF signalling in dermal papillae on (Andrae et al. 2008; Gonzales and Fuchs 2017).

Most likely, this fine balance within the bulge niche and the interaction between the bulge and DP rests on an elaborate stimulatory and inhibitory signalling network in a manner that couples HFSC quiescence/activation to the hair cycling. Therefore, the signalling events discussed above will –most likely- be followed by the discovery of additional ones (Oshimori and Fuchs 2012a).

Although murine models have been instrumental in advancing our knowledge on molecular details of HF biology, findings from these invaluable studies should be evaluated with caution in the sense that there are several differences between the murine HF biology and that of human. For example, in contrast to humans who have only two types of hair (vellus and heavily pigmented hairs), mice have several distinct types of hair, including pelage, vibrissae, cilia, hairs on the tail, ear, genital, nipples, perianal area, and around the feet) (Mistriotis and Andreadis 2013). Apart from type, human HFs cycle independently after birth, while those of mouse (e.g., pelage hair) cycle in synchrony. Finally, there are profound differences between biological markers used in characterization of hair follicular stem cell populations of human and mouse all of which are described in detail in a comprehensive review (Mistriotis and Andreadis 2013).

Several challenges exist in path of stem cell marker discovery. First of all, these markers are rare and often not linked to stem cell function (Lang et al. 2013). Second, these putative markers can be transiently expressed by a small stem cell population or can be co-expressed by another non-dividing cell resident to the niche raising reliability issues (Carulli et al. 2014). Hence, a true stem cell marker must be expressed by such candidate stem cell populations in the niche that that population must meet all of the criteria for stemness including self-renewal, multipotency, and plasticity. Moreover, these hallmarks of stem cells must be cross-confirmed by the use of other stem cell characterization/identification methods, including measurement of quiescence and behavior of the cells in culture (the ability to behave as stem cells in in vivo lineage tracing experiments) upon transplantation (Lang et al. 2013; Cai et al. 2004). All of these methods have pros and cons in terms of their accuracy in diagnosing stemness (Zhu et al. 2017). For a detailed description, we recommend two comprehensive reviews written by Mistriotis and Andreadis (2013) and Lang et al. (2013). Nonetheless, novel stem cell markers are in need in the isolation of these populations to aid therapeutic means of regenerative medicine. In this direction, engineering reliable *in vitro* models that can mimic the *in vivo* HF microenvironment sets the foci of several research groups (Mistriotis and Andreadis 2013).

In contrast to the case of hematopoietic stem cells, where both parent stem cell and every member of its progeny across the differentiation hierarchy can be identified, such specific markers for the precise identification of epidermal stem cells are still lacking impeding the development of reliable isolation protocols (Firth and Yuan 2012). Members of the integrin transmembrane receptor family that mediate the attachment of the basal layer to the substratum of the epidermis are proposed as candidate stem cell markers (Barczyk et al. 2010; Alvares et al. 2008). If epidermal stem cells residing either in the hair follicle bulge or inter-follicular epidermis require strong adherence to the basement membrane to maintain both their stemness and their location in the niche, molecules of anchorage to the cell-substratum attachment can be putative markers for stem cells (Chermnykh et al. 2018). Transferrin receptor is another surface marker, the expression of which differs between stem cells and their proliferating progeny, the former presenting with reduced expression of the transferrin receptor (Brekelmans et al. 1994).

So far several stem cell markers were suggested for the murine HFs, however, human HFs remain relatively underexplored. One reason is the difficulty in isolation of human cells due to

the challenges in identification of human bulge region anatomically in contrast to the case of murine bulge (Joulai Veijouye et al. 2017). According to the results of a recent screen carried out by Klöpper and co-workers CD200, KRT15, and KRT19 were proposed as putative bulge stem cells markers, although their location is not restricted to the bulge, but extends to a wider area of isthmus as well (Mistriotis and Andreadis 2013; Tiede et al. 2007). In contrast to mouse, human bulge cells are CD34, NES, or LHX2 negative (Kloepper et al. 2008). In this study although CD200 positive cells were isolated from a population of LRCs using laser capture microdissection and they showed an increased clonogenic potential *in vitro*, their multipotency was not examined (Mistriotis and Andreadis 2013; Ghadially 2012). On the other hand, KRT15high/CD200+/CD34-/CD271- bulge-derived cells had an increased clonogenic potential as compared to KRT15low/CD200+/CD34-/CD271- cells (Mistriotis and Andreadis 2013; Sari et al. 2010). More recently, it was reported by the Andreadis group that human DP/DS cells displayed a cell surface profile characteristic of MSCs being positive for CD90, CD44, CD49b, CD105, and CD73. These cells were clonally multipotent as individual clones could be induced to differentiate into fat, bone, cartilage, and smooth muscle with high efficiency (Mistriotis and Andreadis 2013).

Given the differential slower activation kinetics of Bu-SC compared to those of the hair germ which gives rise to the matrix TACs upon anagen initiation, it is conceivable that the HFSC subpopulations are stimulated by distinct set of signals (Panteleyev 2018). For example, production of the potent mutagenic signalling factor Sonic hedgehog (SHH) by the newly formed matrix TACs is shown to trigger and maintain the Bu-SC activation (Guo et al. 2018). In addition to the bulge activation, SHH-dependent signalling enhances the expression of anti-quiescence genes such as noggin and FGF-7 in dermal papillae to sustain a highly proliferative state in the matrix and lower ORS (Che et al. 2012). Therefore, once proliferating TACs start sending mutagenic factors -not only- the Bu-SCs are stimulated, but also they mediate a cross-stimulatory signalling between the hair germ and dermal papillae parallel to the progression throughout the anagen (Ren et al. 2017). Impact of core circadian clock genes on HFSC biology will need to be addressed in further studies.

HFSCs also appear in control of the formation and attachment of the APM. Bu-SCs express nephronectin, an ECM protein from the same family of proteins including EGFL6 (Hsu et al. 2014; Linton et al. 2007). Both nephronectin, which is enriched in the basement membrane of the bulge, and EGFL6 are ligands for α8β1 integrin and nephronectin recruits α8β1+ dermal cells. Upon nephronectin-integrin engagement dermal cells up-regulate the expression of smooth muscle actin, an APM marker (Linton et al. 2007). Remarkably, in nephronectin null mice, fewer APMs are formed and their anchorage becomes shifted to the EGFL6-expressing cells above the bulge, suggesting that EGFL6 may compensate for the loss of nephronectin-dependent anchorage (Hsu et al. 2014; Tsepkolenko et al. 2019). Overall these findings suggest that different hair follicle compartments recruit and assemble different hair follicle–associated structures, including peripheral nerves, blood vessels, and APMs via involvement of different ECM proteins (Tsepkolenko et al. 2019; Shimoda et al. 2014).

Sebaceous gland is also thought to be derived from the Bu-SCs that have migrated out of the bulge. Another view advocates differentiation of a unipotent stem cell population located above the bulge and that express BLIMP1 to replenish the sebocyte pool (Mistriotis and Andreadis 2013; Blanpain and Fuchs 2009).

Another niche that is proposed to contain HFSCs is the Dermal Papilla (DP) and Dermal Sheath (DS) compartments which are derived from the mesoderm, unlike the hair bulge that is derived from the ectoderm (Mistriotis and Andreadis 2013; Morgan 2014). Both DP and DS cells crosstalk to the bulge in regulation of the hair cycling. Both DP and DS stem cells display a profound differentiation potential that is demonstrated in ability to form hematopoietic cell subtypes upon engraftment in lethally irradiated mice (Morgan 2014). Moreover, there

is evidence to DP and DS stem cells being the precursor's dermal stem cells that function in dermal maintenance in normalcy and tissue repair. Strikingly, both rat and human-derived DP and DS stem cells reveal broad plasticity to generate myogenic, osteogenic, chondrogenic, and adipogenic lineages which is comparable to that seen with mesenchymal stem cells of bone marrow under appropriately provided culture conditions (Mistriotis and Andreadis 2013; Fitzsimmons et al. 2018). Taken together with the ability of single human DP and DS cell to differentiate into mesenchymal lineages these HFSC subpopulations are recognized for their multipotent trait.

2.1.2 Melanocyte Stem Cells (MSCs)

Proliferation of the Melanocyte stem cells (MSC), which co-reside together with HFSCs in the bulge and hair germ, seems to be closely coupled to that of the latter in the sense that MSCs also initiate generating differentiating progenitor melanocytes at the beginning of the anagen (Mull et al. 2015). Therefore, it is not too surprising that the synchronization of MSC activation and differentiation with that of HFSCs is achieved by the signals originating from HFSCs and dermal papilla. Factors such as the KIT ligand (produced by the dermal papilla) and endothelins (produced by the matrix) conduct differentiation of melanocytes in the hair bulb during anagen. Endothelin-1 is a downstream target of WNT signalling in HFs of early anagen, therefore, elevation of WNT signaling or conditional deletions of Nftb results in expansion of melanocytes which can be rescued by the injection of endohelin receptor B antagonists (Hsu et al. 2014; Chang et al. 2013). As the new hair cycle initiates, MSCs are co-activated along with the HFSCs to produce proliferative committed progenitor melanocytes (Hsu et al. 2014). Later in mature HFs these melanocytes are found in the inner core of the matrix where they transfer the melanin synthesized to the differentiating HFSCs (Hsu et al. 2014; Gola et al. 2012). During the following phase of catagen melanocytes also degenerate together with the rest of the matrix cells. Several findings support the notion that activation of the WNT signaling and beta-catenin-associated activity in MSCs promotes proliferation and differentiation of MSCs (Lang et al. 2013). Strikingly, stabilization of the beta-catenin in the HFSCs induced an extrinsic effect on the MSCs in the sense that the melanocyte pool was expanded, the bulge region was enlarged in a endothelin-dependent manner (Lang et al. 2013). Several Wnt inhibitors are present in the MSC niche, including DKK3, Sfrp1, and Dab2 and in MSCs themselves, including DKK4, Sfrp1, and Wif1 (Lang et al. 2013; Svensson et al. 2008). Hence, these findings suggest presence of a seesaw-like mechanism whereby suppression of WNT signalling promotes stemness of MSCs while its activation induces differentiation of MSCs into melanocytes (Lang et al. 2013; Gola et al. 2012).

In addition to WNT ligands TGF-betas are also presumed to be produced by HFSCs which -in return- might be responding and coordinating signals released by MSCs (Hsu et al. 2014; Svensson et al. 2008; Bogaerts et al. 2014). TGF-beta-dependent signalling is implicated in maintaining MSC quiescence and their undifferentiated state (Lang et al. 2013). TGF-beta signalling is normally involved in cellular growth and survival processes, however, in the HF biology TGF-beta proteins become up-regulated (indicated by the presence of the nuclear phospho-Smad2) as the HF regresses during catagen and promote degeneration of the epithelial-derived components of the HF (Lang et al. 2013; Bogaerts et al. 2014). Both during normal hair growth and following UV exposure, changes in TGF-beta expression are reported. Transcription factors such as MITF and PAX3, melanin-associated enzymes such as tyrosinase decrease upon activation of TGF-beta-dependent signalling (Lang et al. 2013).

Disruption of Notch signaling also hampers MSCs from self-renewal (Lang et al. 2013). The four known Notch receptors can be activated by ligands (Jagged 1 and 2, Delta-like 1,3, 4) which stimulates the cleavage of the receptor by gamma secretase to release Notch intracellular domain (NID) (Rutz et al. 2005). The release of NID from the membrane and its subsequent translocation to the nucleus results in activation of CBF1-

dependent transcription (RBP-J kappa in mice). Blockade of Notch signalling (through use of gamma secretase inhibitor (GSI)) results in permanent hair greying due to complete depletion of both melanocytes and MSC pool, in contrast to the reversible de-coloration seen with the blockade of KIT receptors (Lang et al. 2013; Rutz et al. 2005).

HFSCs regulate themselves and the MSCs by their increased expression of ECM proteins such as the hemi-desmosomal transmembrane collagen (Collagen XVII also known as COL171A, BP180 or BPAG2) which mediates anchorage to the basement membrane (Lang et al. 2013). Therefore, loss of this protein results in graying and loss of hair as well as loss of HF integrity, suggesting this anchorage protein provides docking both to the HFSC and MSC population (Walko et al. 2015).

Coupling of hair cycle events to those of the HFSC life cycle from self-renewal throughout expansion and differentiation demands intricate crosstalk amongst the HFSC subpopulations (Mulloy et al. 2003). In this respect it is quite remarkable that during the generation of a pigmented hair shaft Bu-SC subpopulations that come from diverse developmental origins such as follicular epithelium ectoderm, melanocyte stem cell-neural crest, DP/DS-mesoderm all crosstalk to one another in a concerted fashion (Mulloy et al. 2003; Sakaki-Yumoto et al. 2013).

The hair bulge is also home to the MSCs that are TACs activated during anagen to produce melanocytes to maintain hair pigmentation during each hair cycle (Mistriotis and Andreadis 2013). Due to the relative ease to harvest MSCs from skin and their close synchrony with the expansion and differentiation of HFSCs during the hair cycle have promoted them as a popular model to studies of stem cell biology (Hsu et al. 2014; Tobin 2009). Furthermore, quiescence or growth of melanocytes can be controlled through depilation while any dysfunction in this population can be readily traced through loss in pigmentation, for example, BcL2 loss-associated apoptosis of MSCs results in greying of the hair (Lang et al. 2013; Jo et al. 2018). Having their embryonic roots in the highly plastic tissue of neural crest, being very multipotent and prolific, and capable of migrating to new locations are inherent qualities of MSCs that allow them to be an excellent stem cell source for the application of regenerative medicine (Achilleos and Trainor 2012). Moreover, because of their relatively longer lifespan compared to that of keratinocytes and their ability to work as a single-cell unit the growth and differentiation capacities of which can be controlled by surrounding cells enables manipulation of MSCs in *in vivo* and *ex vivo* in stem cell based therapies (Lang et al. 2013; Zakrzewski et al. 2019). As it will be discussed in detail in "Stem Cells and Cancer" section the very same properties could account for the aggressiveness and highly malignant nature of the tumors derived from melanocytes. Melanomas the incidence of which is rising steadily in contrast to the case of other types of cancers tend to metastasize very early raising fatality (Lang et al. 2013; Schatton and Frank 2008).

Due to the pairing of proliferation and apoptosis of the melanocytes to the hair growth cycle in mice, most of the studies on MSCs tend to be based on this model system (Preston et al. 2018). Using a transgenic mouse model where beta-galactosidase as a reporter under the control of the dopachrome tautomerase promoter (gene encoding an enzyme involved in melanin synthesis, it was understood that bulge region (also defined as the lower permanent portion (LLP)) is the niche for the MSCs (Hsu et al. 2014). The bulge microenvironment provides direct contact with the adjacent cells, scaffold proteins of the ECM, and secreted signaling factors to regulate MCSs (Hsu et al. 2014; Gentile and Garcovich 2019).

Nevertheless, our understanding of MSC biology is beginning to take in terms of how melanocyte stem cells function within their niche, how their quiescence and proliferation is controlled by external signals, and how plastic this population is (Lang et al. 2013). MSCs, that locate to the bulge of the hair follicle are impacted by other cell types such as HFSCs, extracellular matrix proteins, and a number of secreted factors that either promote or suppress multipotency or self

renewal (Hsu et al. 2014). Deregulation of these regulatory signals lead to inability of the stem cell to maintain their stem cell pools or provide pigmented progeny and could potentially contribute to transformation processes in the context of acquiring oncogenic mutations (Lang et al. 2013; Aponte and Caicedo 2017).

2.1.3 Dermal Stem Cells

Dermal Stem Cells (DSCs) are another stem cell population the presence of which has gained evidence parallel to those obtained for epidermal stem cells (Blanpain and Fuchs 2006; Martin et al. 2016). In recent years, DSCs has entered the clinic as an accessible and abundant stem cell source for cell-based therapies (Vapniarsky et al. 2015). For that matter their isolation, purity, safety, viability, characterization, and *in vitro* propagation have been main focus of research in several laboratories around the world. DSCs display plasticity in the sense that they can be coaxed to generate mesenchymal, ectodermal as well as endodermal cell lineages under properly provided *ex vivo* conditions (Vapniarsky et al. 2015; Ojeh et al. 2015). Various lineages of dermal fibroblasts as the most predominant cell type of the dermis has been studied extensively (Driskell and Watt 2015). Particularly they have been considered as the source that is mobilized to wound site to accomplish dermal regeneration. However, recent evidence obtained in the past decade-and-a-half points out that dermal fibroblast/myoblast pool might be derived from other sources (Vapniarsky et al. 2015). Among candidate sources there are bone marrow-derived, tissue-derived mesenchymal stem cells as well as a source that ensues "epithelial-mesenchymal-transition" a process that involves de-differentiation of epithelial cells (Vapniarsky et al. 2015; Stone et al. 2016).

In this respect, hair follicle DP and DS were the first niches proposed for the DSCs that can give rise to cells of the hair-supporting papilla in the lower region of the follicle and migrate out into the dermis of the adjoining skin (Agabalyan et al. 2017). For example, hair follicle dermal sheath cells of the (Martin et al. 2016; Balañá et al. 2015). Conversely, transitions of dermal sheath cells into dermal papillae cells have been reported (Lachgar et al. 1996; Darby et al. 2014). For example, dermal stem cells derived from the follicle are transplanted onto wounds (Cha and Falanga 2007), they contribute to the new dermal tissues in a manner reminiscent of the wound-healing fibroblasts. In this regard, DP and DS-associated cells can differentiate into several mesenchymal, neuronal, and glial lineages (Vapniarsky et al. 2015).

Although, hair follicle is taken as an epidermal stem cell source, −especially the bulge region- emerge as a key source for the regeneration of both epidermal and dermal cell populations (Cheng et al. 2018). It was demonstrated that the hair bulge contains stem cells of neural origin such as the human epidermal neural crest stem cells (EPI-NCSCs), most likely by the epidermal ORS being in strong association with DS of the hair bulge (Vapniarsky et al. 2015; Hu et al. 2006). These cells display multipotency covering major neural derivatives such as the bone, cartilage, neurons, Schwann cells, myofibroblasts, and melanocytes (Vapniarsky et al. 2015).

As it will be discussed in detail in the next section cells isolated from foreskin, reveal that hair follicle is not the only source for the DSCs. The perivascular niche consists of stem cells in the adipose tissue, placenta, skeletal muscle, pancreas, and others (Corselli et al. 2010; da Silva Meirelles et al. 2008). Like the DSCs of the perivascular niche, those recently identified in the stroma of the sweat glands can also differentiate into adipogenic, chondrogenic, and osteogenic lineages (Vapniarsky et al. 2015).

2.1.4 Stem Cells of the Foreskin

Circumcision is described as the removal of foreskin tissue from the tip of the penis. It is done due to religious, cultural or medical reasons. Removal of foreskin tissue is surgically performed by professionals under sterile conditions. Circumcision should be carried out during the neonatal stage due to the higher risk of complications that may arise during later stages of development like puberty (Schoen et al. 2000). Basically, towards the end of the first trimester, a twist of skin evolves at the tip of the penis. Skin distally

expands folding to turn into the foreskin. In the presence of certain androgens, the cells keratinize, spread, and migrate to the prepuce. This process is not completed at birth, but rather continues progressing throughout the childhood (Taylor et al. 1996).

As mentioned in the previous sections mesenchymal stem cells are important for gene therapy and regenerative medicine. Recent studies show that human adult stem cells offer to be a therapeutic alternative to embryonic stem cells. Adult stem cells can be isolated from different sources such as bone marrow (Tasli et al. 2014), adipose (Kalinina et al. 2011), placenta (Li et al. 2012), and dental tissues (Tasli and Sahin 2014). It was shown that skin is also a source of MSCs (Blanpain and Fuchs 2006). Like in the case of fibroblasts derived from dermal skin which are multipotent, express mesenchymal stem cell markers, and display immunosuppressive properties in common with those derived from the bone marrow. Fibroblastic tissue derived from the human foreskin tissue is one of the most important sources of mesenchymal stem cells. Moreover, it was reported that fibroblast-like cells derived from tissue support pluripotency and self-renewal of human embryonic stem cells (hESCs) (Unger et al. 2009; Mamidi et al. 2011). Human foreskin tissue is preferable because it is accessible, cheap, and can be acquired through ways that do not entail ethical issues (Ullah et al. 2015).

There are different types of studies performed using foreskin cells. In 2015, Somuncu et al. showed that foreskin cells have the capability to turn into adipogenic, chondrogenic, osteogenic cells as well as neurogenic and epithelial cells *in vitro* (Ullah et al. 2015). Normally, mesenchymal stem cells do not express hematopoietic stem cell markers. However, it was also reported that foreskin cells express mesenchymal and hematopoietic stem cell markers (Somuncu et al. 2015).

In another study, foreskin cells have been used as feeder cell lines for the human Embryonic Stem (ES) cells. It was shown that ES cells preserved their stem cell properties such as proliferation capacity, immortality, and pluripotency up to 70 passages. The main role of foreskin feeder cells is their potency to be cultured for 42 passages that allows suitable diagnosis for unknown agents and some genetic modifications (Hovatta et al. 2003; Mamidi et al. 2011).

There are studies investigating the migration potential of human foreskin cells. As known, fibronectin is described as a glycoprotein and found in an insoluble form on the surfaces of fibroblasts. It was shown that the presence of fibronectin averted the migration capacity of human foreskin cells through the matrix. These studies are given advises about cell-matrix and cell-cell interactions of human foreskin cells (Schor et al. 1981; Hovatta et al. 2003).

As emphasized, human foreskin cells is a promising tool for cell therapy and tissue engineering applications. They have the ability to differentiate into neurogenic, osteogenic, chondrogenic, and adipogenic cells. Due to their neurogenic differentiation capacity, human new-born foreskin cells can be used in neurodegenerative diseases such as Parkinson's or Alzheimer's disease (Bredesen et al. 2006). Moreover, they express hematopoietic stem cell markers making them appropriate candidates for cancer types such as multiple myeloma and leukaemia. Moreover, human foreskin tissue is abundant and can be easily obtained from circumcision treatments. Mostly, circumcised tissues are thrown away but these tissues can be used for stem cell isolation and further experiments (Somuncu et al. 2015).

3 Immunomodulatory Properties of Skin Stem Cells

Hair follicle displays unique immunological profile that accounts for its immune privilege described by the virtual absence of MHC Class I expression and relatively low numbers of immune cells in the hair follicle. Due to this immune privilege property, transplantation of follicle dermal-sheath cells from an individual to another does not involve tissue rejection processes (which are typically encountered in allograft procedures) while permitting new hair growth. Thus, being

universal donors skin-derived stem cells became an attractive therapeutic source in cell-based therapies.

Highly proliferative Mesenchymal Stem Cells (MSCs) can be harvested from almost every tissue type in human body such as adipose (Zuk et al. 2001), cartilage (Conrad 1979), liver (Campagnoli et al. 2001), amniotic fluid (Scherjon et al. 2003), tooth germ (Taşlı and Şahin 2014), hair follicle (Rahmani et al. 2014), foreskin (Somuncu et al. 2015), and so on (Benvenuto et al. 2007; Gucciardo et al. 2009; Phinney and Prockop 2007). Also according to the data obtained from immunology-related studies, these MSCs can be used in treatment of immune disorders. For example, systematic infusion of MSCs proved them as useful tools in management of Graft Versus Host Disease (GVHD) due to their immunosuppressive properties (Le Blanc et al. 2003; Prasad et al. 2011). Modulation, activation, and proliferation ability of MSCs may help tissue renewal that would also be applicable for curing of variety immune diseases including GVHD.

It is not surprising that skin is home to a diversified pool of immune cells due to its essential function of forming the barrier to external assaults. Therefore, there is a dynamic crosstalk between the epithelial skin stem cells and resident immune cells whereby function and action of both cell types are coupled to each other to sustain or resume tissue homeostasis during normalcy or tissue repair. While dendritic epidermal γδ T cells (DETCs) and Langerhans cells reside in the epidermal layer, dendritic cells, mast cells, macrophages, γδ T cells, and αβ T cells are typically found in the dermal layer. Upon injury or infection, repair processes are initiated by the inflammatory cues, which are collectively known as "damage associated molecular patterns (DAMPS) or "pathogen associated molecular patterns (PAMPS)" disseminating from either dying cells or pathogens, respectively, to alert the immune system. In case of muscle repair, muscle stem cells (also known as satellite cells) rely on signals emanating from macrophages and T_{regs} to initiate the tissue repair processes such as proliferation of myoblasts (Naik et al. 2018). Release of distinct factors by the inflammation-activated macrophages (M1) provides suppression of NOTCH function to allow muscle stem cells to exit quiescence. During later stages of the repair, differentiation of the myoblast pool into myocytes ensues when regenerative M2 macrophages take over. For proper completion of the repair of the injured muscle tissue damage-induced inflammatory response must be subdued by the T_{regs}, expansion of which takes place at the time when macrophages phenotype switches from M1 and M2 and secrete stimulatory factors for regeneration. In contrast, during recovery from hair depilation release of CCL2 by the HFSCs guide the congregation of macrophages in the follicular niche possibly through augmented WNT signalling the loss of which delays hair cycling (Naik et al. 2018).

DETCs in injured skin produce FGF-7, FGF-10 and IGF-1, which are significant for endurance, proliferation, and relocation of epidermal cells. It has been shown that in mice secretion of fibroblast growth factor (FGF-9) by the dermal γδ T cells stimulates WNT expression and signalling in the dermal fibroblasts of the wound site promoting hair follicle neogenesis (Gay et al. 2013). In a positive feedback mechanism FGF9 produced by the wound fibroblasts fuels further WNT stimulation to augment regenerative processes of hair follicle neogenesis in the injured epidermis. However, human dermal layer lacks such an abundant dermal γδ T cell population which accounts for the failure to restore hair growth following damage in humans (Gay et al. 2013). Similar or varying mechanisms of immune cell participation in wound healing are reported in skin and other tissue types. For example, in an organ culture model activated human T cells likewise upregulate IGF-1 contributing to wound healing. Strikingly, same study reports that while both αβ T cells and γδ T cells demonstrate secretion of IGF1 when they are isolated from acute wounds, same is not true for these epidermal immune cell pools, which remain unresponsive to stimulation, when they are isolated from chronic wounds (Toulon et al. 2009).

Throughout the hair cycle the distribution and utility of immune cells change proposing that morphogenetic changes in the hair follicles may

orchestrate concordant immune cell alignment in the skin. One such example is seen in the association of the pre-Langerhans Cells (pre-LCs, precursors of epidermal dendritic cells, see above) with the HFs before they appear in the IFE both in humans and rodents (Nagao et al. 2012). In this elegant study authors report that upon external stress, entry of the pre-LCs was permitted to the desired regions of HF via CCR2 and CCR6 chemokine receptor-dependent *recruitment-permissive*, but CCR8 chemokine receptor-dependent *recruitment-nonpermissive* mechanisms. Further evidence unveils the stunningly well-orchestrated biology of LC-recruitment to the HFs by demonstrating that ligands for the recruiter receptors of CCR2 and CCR6 are expressed by the distinct regions of the HF. While chemokine ligand CCL20 (activates CCR6 receptor) is secreted by the keratinocytes in the infundibulum region and chemokine ligand CCL2 (activates its CCR2 receptor) by the keratinocytes in the isthmus region, secretion of CCL8 is executed by the keratinocytes in the bulge region that probably accounts for evasion of pre-LC and LC repopulation in the bulge region. On the contrary, absence of immunogenic rather predominance of immunosuppressive signalling in the bulge region, which is recognized as the "stem cell sanctuary" and the matrix, reconciles with the "immune privilege' property of the HFs. Nonetheless, restrictions to the "immune privilege" properties of the HF conceivably exist as suggested by the fact that besiege of both hair follicular and epidermal tissues by the immune cells can be encountered in allotransplantation procedures (Paus et al. 2003).

Understanding impact of wounding on both skin stem cells and resident immune cells can aid treatment of autoimmune diseases like alopecia areata, pathology of which involves sustained activity of inflammatory immune cells aiming the hair bulb and the matrix, avoiding only Bu-SCs, and results in hair loss (Ito et al. 2005). In the cases of disorders such as discoid lupus erythematosus and lichen planopilaris, Bu-SCs are demolished and, therefore, ensuing hair loss is permanent (Harries et al. 2013; Al-Refu and Goodfield 2009). Therefore, precise identification of the molecules in charge of the interaction between the epithelial stem cells and immune cells will undoubtedly result in fruition of novel treatments that tap into the capacity of the skin in fine-tuning the adequate stimulation of immune response upon damages and pathogens without allowing over-stimulus of inflammatory responses to prevent autoimmune disorders (Patzelt et al. 2008).

Finally, lessons learned from the p120-catenin conditional knock-out mice underscore the intimate relation between the epidermal cells and resident immune cells (Perez-Moreno et al. 2006). As a crucial component of the adherens junctions (AJs), that serve as the intercellular glue-molecules in all epithelial tissues, p120 is a sister molecule of β-Catenin in the family of Armedillo repeat catenins. Both p120 and β-Catenin docks and stabilizes to the core E-Cadherin transmembrane protein at distinct sites of the cytoplasmic regions to bridge the dynamics of the intra-cellular actin skeleton to the extracellular domain of E-Cadherin that functions as the intercellular physical connector. While β-Catenin turns on Lef1/Tcf-dependent gene expression in response to WNT stimuli, p120 turns on Kaiso-dependent transcription. At the neonatal stage, despite of having decreased levels of intercellular AJ components, the epidermis of the p120-Catenin null mice displays no overt failure of the epidermal barrier function. However, as these animals age, they develop epidermal hyperplasia, chronic inflammation, hair degeneration, and profoundly decreased abdominal fat. All of these pathologies are linked to aberrant activation of proinflammatory signals downstream to NFκB-dependent transcriptional cascade. These findings propose that in the context of combating infectious assaults pathogen-induced proinflammatory signalling cascades that coordinate immune responses in the skin, possess a potential to participate in neoplastic progression in the absence of pathogens (Perez-Moreno et al. 2006).

3.1 T Lymphocyte-Mediated Suppression

T cells play the most important role in the memory creation of the adaptive immunity reaction which is generated as a specialized immune response to specific pathogens (Zhang et al. 2012). T cell activation can be hampered from direct cell to cell interaction with MSCs. These interactions between MSCs and cytotoxic ($CD8^+$) or regulatory ($CD4^+$) T cells, which control the secretion of related cytokines for signaling and inhibition of T cell activation, are necessary for effective immune suppression (Nauta and Fibbe 2007; Uccelli et al. 2008). Also, it has been shown that MSCs can express the Fas ligand (FasL), also known as a binding partner for the death receptor, that prevents T cell migration (Zhang et al. 2012), by prompting initiated T cell death via direct cell-to-cell interaction (Akiyama et al. 2012). Researchers demonstrated the inhibitory effect of MSCs on T cell proliferation, however, these suppressive effects of MSCs haven't been fully understood either in cases of autologous or allogeneic cell transfer (Krampera et al. 2003; Le Blanc et al. 2003). Cytokine secretion from MSCs and immune cells increase the levels of interferon γ (IFNγ) and interleukin (IL)-17, which trigger T helper cells (TH^0) to produce IL-4 that is in charge of inducing differentiation of regulatory T cells (TH^2) cells (Aggarwal and Pittenger 2005; Sun et al. 2009). The (observation) conclusion that MSCs can suppress immunity might, at first, appear as an unwanted effect, however, it becomes a desired consequence in cases of organ transplantation of this type of stem cells, especially in cases of Solid Organ Transplantation (SOT). While there isn't any research showing the effect of MSCs against Solid Organ Allograft (SOA) refusal, a plentiful and increasing amount of facts obtained from *in vitro* and *in vivo* studies claim that this approach might be a promising in management of SOA (Bartholomew et al. 2002; Zhou et al. 2006; Crop et al. 2009; Renner et al. 2009). While results from the human clinical studies are not satisfactory yet, preclinical studies demonstrated that MSCs could become usable therapeutic agents for OR applications. Furthermore, not just that they can modulate the host immune reaction in a way that may enhance the tolerance of the transplanted organ, but also their ability to trigger tissue regeneration and induce unique gene expression profile may assist the processes involved in reduction of the inflammatory reaction to the allograft. Further studies must be designed and carefully carried out to explain the MSC-dependent immune suppression. Nonetheless, the less toxicity and possible long term suppression of immunity by MSCs make them a potentially outstanding therapeutic tool compared to the traditional T cell suppressive agents (Corry et al. 1999; Kawai et al. 2014).

3.2 B Lymphocyte-Mediated Suppression

While T cells are the main player of an immune reaction, B cells have important roles in antibody secretion to modulate immune response and also they closely work together with T cells. While many studies have shown that MSCs can inhibit B cell proliferation, differentiation, and cytokine secretion (Augello et al. 2005; Gerdoni et al. 2007; Rasmusson et al. 2007; Asari et al. 2009), it has also been shown that MSCs can increase B cell proliferation and cytokine secretion from B cells in *in vitro* and *in vivo* studies (Rasmusson et al. 2007; Traggiai et al. 2008). Even though, the exact mechanism-of-action how MSC-mediated B cell suppression operates remains unknown, researchers claim that this suppression can take place as a result of differentiated B cell or the direct effect of the local stimulating signals. Co-culture studies displayed that triggering MSC-mediated immune response suppression needs some inducing cues from B cells, suggesting that in order for MSCs to suppress an immune reaction they need to be activated by relevant stimuli derived from B cells in the first place. This back-and-forth crosstalk between MSCs and B cells ultimately result in the inhibition of B cell proliferation, pointing to a feedback

loop inhibition type mechanism in control of B Cell proliferation. An alternate mechanism for the inhibition of B cell proliferation involves the effect of MSCs on the activity of T cells through which they might inhibit B cells indirectly (Gerdoni et al. 2007). One other important point about these experiments is that majority them were done in *in vitro* conditions not *in vivo* or *ex vivo*.

3.3 Dendritic Cell-Mediated Suppression

Additionally, MSCs are effective against monocytes, monocyte-derived dendritic cells, macrophages, natural killer cells, and neutrophils. Few studies demonstrated that MSCs are able to suppress the generation dendritic cells from monocytes by blocking the antigen presentation, *in vitro* (Gerdoni et al. 2007; Ramasamy et al. 2007). Also, it has been claimed that dendritic cells are key factors of immune response and tolerance, depending on the activation and maturation stage and the cytokine milieu at sites of inflammation (Rutella et al. 2006). Suggesting that, inhibition of differentiation, maturation, and action of dendritic cells might occur by suppressing the $CD14^+$ monocyte differentiation into mature dendritic cells and through promoting cytokine secretion (Beyth et al. 2005; Jiang et al. 2005).

3.4 Natural Killer Cell (NKs)-Mediated Suppression

NK cells play important roles in the intrinsic pathway, mainly during anti-tumor and anti-viral infections. As a result of their highly cytotoxic function, and they have the ability to secrete large amount of pro-inflammatory cytokines, including TNFα and IFNγ (Malhotra and Shanker 2011). Moreover, MSCs inhibit IL-2 and IL-15 mediated NK cell proliferation, IFNγ secretion, and cytotoxicity of both latent and stimulated NK cells (Rasmusson et al. 2003; Meisel et al. 2004; Aggarwal and Pittenger 2005).

Immunosuppression ability of MSCs are making them outstanding candidates for immunosuppressive agents for the prevention and treatment of various inflammatory and autoimmune diseases (Uccelli et al. 2008; Rasmusson et al. 2007). Starting from the 2000, unique MSC sources have been obtained from several dental tissues, which exhibit remarkable tissue regenerative and immunosuppressive properties (Yamaza et al. 2010). Other than having spectacular self-renewal property and multipotency, hFSSCs have powerful immunosuppressive functions comparable to other stem cell types, making them promising cell sources for MSC mediated transplantation treatment.

Foreskin is a waste tissue that's why they might be more beneficial than other cell sources as obtainability of MSCs. Additionally FSSCs have a high growth and clonogenic capacity (Najar et al. 2016). Several immunomodulatory cytokines and factors including IL-6, IL-12 and TNF-α secreted from FSSCs, potentially involved in FSK-MSC immunomodulation were identified. Hence, this immunomodulation function of FSSCs might be make these cells hopeful tolerogenic agent for developing stem cell-based immunotherapy.

4 Stem Cells in Skin Cancer

Skin cancer is a common form of cancer diagnosed in men and women. During the last decade, there has been a rise in the prevalence of skin cancer globally (Bergers et al. 2016). Skin phototype, hair color, multiple nevi, family history, and the degree of exposure to ultraviolet (UV) radiation are cited amongst the etiologic factors that can cause skin cancer (Hernando et al. 2016; Pfeifer 2015; Raimondi et al. 2008). There are three major types of skin cancer: (i) malignant melanoma (ii) basal cell carcinomas (BCCs) and (iii) squamous cell carcinomas (SCCs) (Simões et al. 2015). Melanoma is one of the most aggressive, complex, and heterogeneous cancer type with 132,000 new cases diagnosed worldwide each year (Lohcharoenkal et al. 2018; WHO 2017).

Human malignant melanoma is an extremely aggressive type of skin cancer which is characterized by its astonishing heterogeneity, tendency for spreading throughout the body, and developing resistance to cytotoxic mediators.

Even though standard care such as chemotherapy and immunotherapy have been assessed in clinical studies, most of these therapeutic agents fall short in providing an effective treatment for those with progressive disease. Lack of effective treatment in melanoma patients is ascribed primarily to a high degree of tumor heterogeneity that results in countless number of genetically distinct subpopulations. Therefore, in a given tumor mass, some of these subpopulations belong to cancer stem cells (CSCs), while the rest are composed of non-CSCs which constitute majority of the tumor (Alamodi et al. 2016). The consensus on the commonly found features of a CSCs include: (i) their potential to initiate tumor growth, ability for (ii) self-renewal and (iii) differentiate into tumor cells from different epithelial origin that make up the bulk of the tumor (Aponte and Caicedo 2017).

The CSC model includes various subpopulations of malignant cells and for long time it has been suggested that the carcinogenic component of primary melanoma is not homogeneous, as one would expect in a stochastic model of clonal carcinogenesis (Zabala et al. 2016). Fairly, melanoma display 'polyclonism' (Laga and Murphy 2010), in the sense that structurally, cytologically, and immune-histochemically distinct populations often co-exist inside of a single tumor nodule. In consistence with this model of melanoma, a hierarchical order of cell types, that is a consequence of tumor differentiation, is documented in vivo. Each of these cell types has distinct capacity for self-renewal that can propel tumorigenesis through the engagement of signature signalling pathways (Maniotis et al. 1999).

Several malignant features of melanomas such as intratumoral heterogeneity, tumor progression, and drug resistance are determined by their stem cells, also known as melanoma stem cells (MSCs) (Nguyen et al. 2015). MCSs are a subpopulation of melanoma tumors and their molecular characteristics may either account for increased progression, drug resistance, and recurrence of melanomas or are a consequence of these malignant events (Alamodi et al. 2016). Distinct populations of MSCs are described by their signature proteomic as well as genomic context that includes several driver-mutations promoting tumor growth. There are certain features of melanoma stem cells that offer an excellent disease model to study (El-Khattouti et al. 2014, 2015; Leikam et al. 2015; Li et al. 2015). First, MSCs may be isolated from the skin through moderately non-invasive methods as they are mainly present in the interior of a particular anatomic niche inside of the skin, thus allowing their easy isolation. For example, MSCs are located within the bulge area of the hair follicle in the murine skin (El-Khattouti et al. 2015). Due to the developmental origins of MSCs, they are multipotent and they have the innate property to migrate to different sites (Stecca and Santini 2015).

Both BCCs and SCCs are thought to originate from a stem cell ancestry although this idea needs further proof. The rationale for this claim bases on the fact that the continuously renewing tissue of epidermis is a niche for all keratinocytes that undergo a well-defined differentiation program upon leaving the stem cell compartment and terminate this process by the generation of dead horn squames (Leikam et al. 2015). In relevance with this concept, should a damage take place in the cells of a tissue-type other than the stem cell compartment, these cells will most likely be cleared due to the differentiation process. On the other hand, in case of an insult to the pool of epidermal stem cells, several genetic changes that can potentially prevent cell cycle control and stimulate neoplastic growth, may accumulate due to the long-lived nature of these cells that are much less frequently divided (Li et al. 2015). Alternatively, non-stem cells may prevail and -thereby- contribute to transformation through acquiring genetic changes, all of which may hinder their clearance. Support for this hypothesis comes from the observation for the heterogeneity found in human BCCs. It was demonstrated previously that inactivation of p53 through several mutations -mostly those caused by UV exposure- is an early event in the development of skin

carcinoma (Brash et al. 2010; Shannan et al. 2016). For example, nuclear accumulation of mutant p53 protein was detected in clones of keratinocytes with normal morphology as well as potential stem cell populations isolated from skin samples of individuals who were chronically exposed to sun. Since mutant p53 is devoid of function, normally executed by the wild type protein, UV-induced apoptosis would be abrogated (Pacifico et al. 2017). Moreover, there is a striking heterogeneity in the mutations characterized for p53 in human BCCs based on several reports (Benjamin and Ananthaswamy 2016). Studies, where clonality of tumors was delineated by microdissection-based approaches, indicated that cellular composition of these tumors are shaped by a dominating clone with a high degree of genomic instability. Nonetheless, a second or -even- a third genomic variation in p53 was detected in subclones found within the same tumor area (Pontén et al. 1997).

Overall, subpopulations found in melanoma lesions display a CSC-like phenotype including tumorigenesis, self-renewal, and differentiation characteristics in that these putative melanoma CSCs have enhanced ability of forming and maintaining melanoma tumors with a propensity to resist treatment.

5 Conclusion

Due to their exceptional regenerative properties stem cells of the skin will continue being an attractive research area-of-interest for numerous research studies in future. There is no doubt understanding the precise nature of the communication of the distinct stem cell populations with the niche components, their progeny, and with one another will hold promise for the improvement of clinical methods of regenerative medicine. Improvement of the current clinical practice will be -further- enabled by the accurate identification of skin stem cell subtypes that is dependent on the characterization of subtype-specific markers. With increased sensitivity of isolation methods, advanced understanding of the biology for a specific sub-type of skin stem cells, their therapeutic manipulation could offer more effective treatment modalities for a wide variety of degenerative disorders of the skin.

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