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Kursad Turksen *Editor*

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Perspectives in Diverse Human Diseases and Their Therapeutic Options

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Kursad Turksen
Editor

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Preface

In this next volume in the Cell Biology and Translational Medicine series, we continue to explore how insights from state-of-the-art cell biological studies can impact translational medicine. Amongst topics explored here are recent developments that provide important new understanding of a number of human diseases such as infection with monkeypox and others that underscore where we stand with respect to translational medicine and novel cell- and/or drug-based therapeutic options for a variety of other diseases including leukemia and skin cancer. As with each of the volumes in this series, we continue to highlight timely, often emerging, topics and novel approaches with potential to accelerate our understanding of various diseases with the ultimate goal of improving therapeutic options.

I remain very grateful to Gonzalo Cordova, the Publishing Editor of the series, and wish to acknowledge his continued support.

A special thank you goes to Shanthi Ramamoorthy and Anju Baskar 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 willingness to share their insights and all their efforts to capture both the advances and the 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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Monkeypox and Sexually Transmitted Diseases

Rujittika Mungmunpuntipantip and Viroj Wiwanitkit

Abstract

Monkeypox is a unique variety of pox infection. Despite being around for a long time, there hasn't been a large outbreak of this disease. WHO has already acknowledged the seriousness of the large monkeypox outbreak in 2022. Africa's unusual outbreak outside of endemic regions is a problem. Numerous observations have been made about the new clinical epidemiological pattern. Human monkeypox is currently spreading outside of endemic African countries, and the majority of those affected are gay and bisexual men in linked sexual networks. As was already mentioned, the current public health concern is the spread of monkeypox through sexual contact. The link between monkeypox and other sexually transmitted diseases is a growing concern because it is believed that the disease can be transferred through sexual contact. This page provides a summary of the interactions between other significant STDs such as HIV infection, syphilis, and gonorrhea. A fascinating research topic at the moment is the connection between monkeypox and other sexually transmitted diseases.

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Keywords

Infection · Monkeypox · Sexually transmitted disease

Abbreviation

CD	cluster of differentiation
HIV	human immunodeficiency virus
WHO	World Health Organization

1 Introduction

A specific type of pox infection is monkeypox. There hasn't been a significant outbreak of this disease despite its long existence. The large monkeypox outbreak in 2022 already has WHO recognizing its significance (Srikanth Bhagavathula and Khubchandani 2022). There are many more things that should be said about monkeypox. Monkeypox is now widely considered to be a serious hazard to public health worldwide. Skin lesions and severe febrile sickness frequently coexist. There is a growing possibility that the virus will transmit from one person to another. Some of today's most urgent issues have been clarified by recent research on human settings, particularly sexual interaction. Since effective illness therapies depend on early detection and treatment, understanding the issue is essential. Given the increased likelihood that pregnant women would contract monkeypox during the

outbreak, early detection is essential. On the other hand, a fever or a skin lesion is unusual (Sookaromdee and Wiwanitkit 2022).

Without the unusual appearance, the doctor might have overlooked the issue and given the wrong diagnosis. This may be an unidentified underlying reason causing the current epidemic. A patient may need to stay in the hospital if they have a critical clinical condition and the right therapy is available. Clinical samples isolated from crust or vesicles are often investigated in-depth. This suggests that clinical problems connected to skin lesions are widespread. It's crucial to keep in mind that certain people, such those who have neurological or digestive problems, only display particular symptoms (Mungmunpuntipantip and Wiwanitkit 2022a).

It is essential to check for contamination during the investigation. According to a recent study on laboratory quality assurance, the monkeypox virus test has a significant probability of being inaccurate; thus proper laboratory procedures, beginning with precise specimen collection, are necessary. Atypical sickness symptoms need to be taken into account. The last major concern is prevention. The necessity of comprehensive prevention is a crucial impediment to dealing with this deteriorating public health crisis. A universal preventive standard is required. More research is required to entirely eradicate monkeypox. But conventional sickness prevention is worthwhile and efficient, given that the situation is erratic and might proceed in the manner of SARS-CoV-2 infection outbreak. It should go without saying that the problem needs to be located and dealt with as quickly as feasible. Given that the SARS-CoV-2 outbreak issue is still a serious one, it is feasible that many lessons have been learnt regarding how to treat an outbreak of a new infectious disease. While errors might still occur, they should be less common than those we saw in the early phases of the SARS-CoV-2 crisis.

2 Sexual Transmission of Monkeypox

There has always been monkeypox. The name comes from the fact that it was initially identified in a monkey in 1958. The first human infection, however, was found in a little kid in the Democratic Republic of the Congo in 1970 (Joob and Wiwanitkit 2022). Since then, more and more cases have been identified and especially over the previous 5–10 years (Wiwanitkit and Wiwanitkit 2018). What has changed is that we are now observing instances in nations that don't typically have monkeypox and it's quite unusual. The occasional epidemic or isolated case involving a traveler from West Africa has been reported. However, this outbreak is unlike any we have ever seen before.

As of July 2022, there has been more than 10,000 instances documented internationally, prompting the declaration of a public health emergency of international concern. The monkeypox virus spreads from animal to human by infected sores or fluids and is spread from human to human through fomites, droplets, or direct contact. Most illnesses are self-limiting, but severe sickness can develop in particular populations, including children, pregnant women, and those with weakened immune systems (Huang et al. 2022). Human-to-human transmission has been the main mode of infection in the current pandemic (Lai et al. 2022). Although the virus can be transmitted through sexual contact, specifically through contaminated body fluids, it is yet unknown if monkeypox can be transmitted through sexual contact (Lai et al. 2022).

The atypical outbreak outside endemic areas in Africa is a challenge. There are many observations on new clinical epidemiology pattern. The majority of persons afflicted with human monkeypox, which is currently expanding outside of endemic African nations, are gay and bisexual men in connected sexual networks (Ogoina and Yinka-Ogunleye 2022). Anogenital lesions are the most common manifestation, and sexual contact is the main method of transmission, especially among males who have sex with men (MSM) (Srivastava and Srivastava 2022). According to a recent UK investigation, gay and bisexual men have been transmitted in international sexual networks since April 2022 (Vusirikala et al. 2022). Interventions aimed against geographic dating services, sex-on-premises locations, and sexual health services are likely to be essential for outbreak containment

Table 1 Monkeypox and other important sexually transmitted diseases

Sexually transmitted diseases	Details
HIV infection	Since homosexual cases are the main group affected by monkeypox in the current 2022 monkeypox outbreak, there are many reports on coinfection. Nevertheless, there is no proof on the relationship with CD4+ count
Syphilis	The coinfection between syphilis and monkeypox is reported, and it usually forms the triad with HIV infection
Gonorrhea	There is a report on high rate of monkeypox among clinical sample tested for gonorrhea
Chlamydia infection	There is a report on high rate of monkeypox among clinical sample tested for chlamydia. There is also a report on coinfection between monkeypox and several pathogens including chlamydia

(Vusirikala et al. 2022). Despite the fact that the condition is self-limiting, some patients need to be admitted due to severe anorectal discomfort, pharyngitis, eye lesions, kidney damage, myocarditis, or soft tissue superinfections. Antiviral treatment has been recommended (Srivastava and Srivastava 2022). As already noted, the sexual contact and transmission of monkeypox becomes the present public health concern. Since it is likely that the disease can be transmitted due to sexual contact, the emerging issue is the interrelationship between monkeypox and other sexual transmitted disease (Table 1).

3 Monkeypox and HIV Infection

HIV is an important sexually transmitted disease. This disease can be detected in any sexual active groups including heterosexual and homosexual cases. The concurrence between monkeypox and HIV infection is possible (Mungmunpuntipantip and Wiwanitkit 2022b). Up to 25% of cases of monkeypox had HIV testing results that were positive. Additionally, it was confirmed that a secondary syphilis infection existed (Bížová et al. 2022). It is a fundamental question to ask whether HIV patients with a low CD4+ count are vulnerable to monkeypox. It's crucial to be aware that the monkeypox virus can infect people with healthy immune systems and is very contagious. According to a recent research (Hammerschlag et al. 2022), monkeypox appeared in HIV patients with a suppressed viral load and a normal CD4 count. These data suggest that the immunodeficiency of HIV cases may not be the cause of the co-occurrence of HIV and monkeypox in the current epidemic in Europe and America. Anal sexual contact, which is frequent among homosexual male HIV patients and raises the risk of developing an infected skin lesion in a concealed area like the genital or perianal areas, may also be a contributing factor. Last but not least, it is unknown if HIV-positive people can receive cross protection against monkeypox from the smallpox vaccine that is now available. If the CD4 count is lower than 300, an animal model's attempt to produce cross IgG antibodies against the monkeypox virus is unsuccessful (Smith et al. 2005). However, it should be noted that severe monkeypox virus infection is possible in undiagnosed advanced HIV infection (Boesecke et al. 2022).

For MSM patients with monkeypox whose HIV status is known, available summary surveillance data from the European Union, England, and the United States show that 28–51% have HIV infection (O'Shea et al. 2022). According to Mungmunpuntipantip and Wiwanitkit (2022b), HIV-positive people can contract monkeypox regardless of CD4+ count, and the clinical presentation is often a large skin lesion at the vaginal region. Coinfection with syphilis is possible. Death can happen if the patient's CD4+ count is very low. If the CD4+ count is normal, the patients' clinical characteristics resemble those of non-HIV infected cases. Some clinical aspects of monkeypox in HIV patients may differ from those in non-HIV people. Patients who are not HIV-positive typically arrive with a feverish illness and a widespread skin

rash (Wiwanitkit and Wiwanitkit 2018). Only genital or perinatal areas may have a concealed skin lesion in HIV-infected people. Skin vesicles and other minor clinical manifestations could be the initial clinical problem (Mungmunpuntipantip and Wiwanitkit 2022b). Only genital or perinatal areas may have a concealed skin lesion in HIV-infected people, and skin vesicles alone could be the only clinical manifestation at first, with no sign of fever (Mungmunpuntipantip and Wiwanitkit 2022b). When taking into account the clinical history, the death rate among non-HIV infected people in the endemic area, the western half of Africa, is roughly 3.6% (Bunge et al. 2022). This study's HIV-infected individuals have a higher observed mortality rate, and those with low CD4+ counts are more likely to progress to severe illness (Mungmunpuntipantip and Wiwanitkit 2022b). The treatment of the patients must be cautious in light of the aforementioned characteristics of monkeypox in HIV-infected patients. Monkeypox should be present on the body of any HIV-positive person presenting with a genital or anorectal lesion (Mungmunpuntipantip and Wiwanitkit 2022b).

The CDC has an experimental medicine protocol available for the compassionate use of tecovirimat as a first-line treatment for monkeypox. The coadministration of tecovirimat with antiretroviral therapy (ART) for HIV infection is not prohibited by any known medication interactions (O'Shea et al. 2022). If necessary, pre- and postexposure prophylaxis with the JYNNEOS vaccination can be considered. Despite the paucity of data about monkeypox in HIV patients, early detection, treatment, and prevention may lower the probability of unfavorable outcomes and restrict the spread of monkeypox (O'Shea et al. 2022). Considerations for prevention and therapy will be updated when there are more available clinical data.

4 Monkeypox and Syphilis

Syphilis is a dangerous illness that can coexist with monkeypox in a patient in addition to HIV. As far as we are aware, there are at least three cases of syphilis with monkeypox. In one patient, HIV infection was also present (Bížová et al. 2022; Hammerschlag et al. 2022; Ogoina et al. 2019). The three examples presented are all male and exhibit gay conduct. All were negative for overt syphilitic symptoms; however syphilis was initially diagnosed via a reactive VDRL test. Every patient exhibits the standard monkeypox signs and symptoms, such as fever and vesicular skin rash. All of them had skin sores at the genitalia. All subjects had mild monkeypox symptoms and recovered completely without any issues. As earlier mentioned in the topic of HIV, syphilis and monkeypox can also coinfect individuals. It will be beneficial to carry out for possible coinfection. It will be beneficial to perform a serological examination on a patient with monkeypox to check for syphilis and HIV. On the other hand, if a syphilis case has a peculiar skin lesion, monkeypox should be investigated. The fact that monkeypox and syphilis, a condition that can only be contracted through sexual contact, co-occur, suggests that the current outbreak of monkeypox may have been spread through sexual contact.

5 Monkeypox and Gonorrhea

Gonorrhea is a well-known sexually transmitted disease. According to our best knowledge, there is still no case report on concurrent monkeypox and gonorrhea infection. However, there is a report that the rate of monkeypox PCR positive is very high among the collected samples for gonorrhea test (De Baetselier et al. 2022).

6 Monkeypox and Chlamydia Infection

Another well-known sexually transmitted disease is chlamydia infection. According to a survey, a significant percentage of the samples acquired for the chlamydia test had positive monkeypox PCR results (De Baetselier et al. 2022). There is an interesting case report on coinfection between monkeypox and chlamydia trachomatis

(Raccagni et al. 2022). There are numerous pathogens present in that situation. A man who had sex with males who also had monkeypox, pan-resistant *Campylobacter* spp. infection, and *Entamoeba histolytica* infection developed Chlamydia trachomatis re-infection (Raccagni et al. 2022).

7 Monkeypox and Herpes Simplex Type 2 Infection

Herpes simplex type 2 infection might be transmitted via sexual contact. There is a report on coinfection between monkeypox and herpes simplex type 2. A case of monkeypox and herpes simplex type 2 coinfection in an HIV-positive MSM patient was described by Zlámal et al. in 2022. An MSM patient with a history of STIs is described in this case report as having a diagnostic approach for a papular rash in the anal area (Zlámal et al. 2022).

8 Monkeypox and Genital Wart

There is no report on the coinfection between monkeypox and condyloma or molluscum contagiosum. However, when the disease widely expands, it is possible that there will be a co-occurrence.

9 Conclusion

Monkeypox can coinfect with several kinds of sexual transmitted diseases. The important present concern is on the coinfection between HIV infection and monkeypox. Since there is a possibility of sexual transmission of monkeypox, it is recommended to seek for possible existence of monkeypox in any patients visiting to the physician with problem of sexually transmitted disease. Sexual health professionals and the MSM communities they serve became especially aware of monkeypox and its clinical appearance because many of the earliest cases were diagnosed among MSM. Compared to other communities where clinician and patient awareness is not as strong, the MSM community may have had a higher probability of having cases recognized early on because of this initial vigilance. It is possible to avoid missed opportunities to diagnose monkeypox and stop transmission in all groups by raising knowledge of monkeypox among all clinical practitioners and assuring the availability of diagnostic testing. Finally, stigmatization is a negative phenomenon in real life. There shouldn't be any bias in the treatment of patients or the investigation of suspicious cases, and communication must be effective. To enable people to make choices that will better safeguard their own health and the health of their community, it is crucial to convey health risks in a way that is understandable and culturally appropriate for this demographic. This includes using trusted messengers and platforms.

Conflict of Interest None.

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Clinical Manifestations of Monkeypox

Haneen Abaza, Kuchalambal Agadi, Ayush Anand, and Mohamed Elsaid

Abstract

Monkeypox is a global health issue caused by the monkeypox virus. It can spread from person to person through respiratory secretions, direct exposure to dermatological lesions of infected patients, or exposure to contaminated objects. It is more common in homosexual men, and most patients are asymptomatic. The gold standard for diagnosis is a real-time polymerase chain reaction. In the absence of testing facilities, clinicians rely upon detailed history to exclude other causes of fever with rashes. Initially, there is a prodrome phase of a few days, which is followed by the appearance of rashes. The dermatological manifestations are in the form of an exanthematous rash, which transforms through a macular, papular, and vesicular phase and disappears after crusting in approximately 3 weeks. There can be associated lymphadenopathy in these patients. Respiratory manifestations include nasal congestion and shortness of breath that may result in secondary bacterial infections. Additionally, patients can have neurological involvement in the form of encephalitis. Furthermore, ocular involvement can occur in the form of conjunctivitis, keratitis, and corneal ulceration. Other symptoms can include diarrhea, vomiting, myalgia, and backache. Since most patients do not require hospitalization, the approach to treatment is mainly vigilant monitoring, antiviral therapy, and management of associated complications.

Keywords

Dermatological · Monkeypox · Neurological · Respiratory · Signs · Symptoms

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Abbreviations

CDC	Centers for Disease Control and Prevention
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay

MPXV Monkeypox virus
MVA Modified vaccinia Ankara
PCR Polymerase chain reaction
STI Sexually transmitted infection

1 Introduction

Monkeypox, the first human case reported in Congo in 1970, has evolved into a global public threat (WHO Director-General declares the ongoing monkeypox outbreak a Public Health Emergency of International Concern 2022; Monkeypox 2022). As of September 2, 2022, more than 50 thousand cases with 15 mortalities were reported globally in the year 2022 (Monkeypox 2022). It is caused by the monkeypox virus (MPVX), a double-stranded virus belonging to the genus orthopoxvirus of the poxviridae family (Berthet et al. 2021; Shchelkunov et al. 2002). Person-to-person transmission is achieved by close contact with respiratory secretions, direct contact with dermatological lesions of an infected patient, or touching contaminated objects (Monkeypox 2022; How It Spreads 2022). To our knowledge, most cases have occurred in homosexual men (Tarín-Vicente et al. 2022; Girometti et al. 2022; Thornhill et al. 2022; Adler et al. 2022). Most frequently, patients present with rash, fever, pruritus, and lymphadenopathy within days of infection (Thornhill et al. 2022; Benites-Zapata et al. 2022; Patrocinio-Jesus and Peruzzu 2022). Other generalized symptoms can include sore throat, cough, malaise, headache, photophobia, joint pain, myalgia, rectal pain, abdominal pain, and conjunctivitis (2022 Monkeypox 2022; Thornhill et al. 2022; Benites-Zapata et al. 2022). A polymerase chain reaction (PCR) is the gold standard for laboratory diagnosis of monkeypox; however, the testing facilities for monkeypox are not widely available, especially in low-to-middle income countries (Monkeypox 2022). Hence, a detailed history of rash and lymphadenopathy is crucial to distinguish monkeypox from other causes of rash-like illness, like smallpox (Monkeypox 2022). Approximately one-third of patients with monkeypox require hospitalization, with fatality in 1 to 9% of positive cases (Benites-Zapata et al. 2022). The mainstay of pharmacological management includes using antivirals such as tecovirimat, cidofovir, and brincidofovir (Webb et al. 2022). In addition, monitoring hydration, nutrition, and management of associated complications are recommended (Monkeypox 2022; Webb et al. 2022). In this review, we aim to discuss the clinical manifestations and management of monkeypox.

2 Dermatological Manifestations

Almost 80% of patients with monkeypox were asymptomatic, while 90% of the symptomatic patients had a rash, and 65% presented with arthralgia (Bothra et al. 2021). The classical symptoms and signs of monkeypox resemble that of smallpox by having prodromal symptoms and signs that are followed by exanthematous rash within 1–4 days. The initial skin manifestations in patients were found to be influenced by the route of transmission (Reynolds et al. 2006). Initially, the cutaneous lesions appear either in the anogenital region or in the facial region and then spread centrifugally to involve the trunk, hands, legs, and feet (Huhn et al. 2005; Ogoina et al. 2020; Bothra et al. 2021). In a recent study by Thornhill et al. (2022), they discovered that 95% of the patients with monkeypox presented with a rash, of which 73% had the rash in the anogenital region, and 41% had mucosal lesions while a small percentage presented with a single genital lesion. Moreover, the lesions were often painful (Thornhill et al. 2022). On the other hand, the exanthematous rash starts as macular, which then progresses to a papular, vesicular (umbilicated), and finally the pustular phase. After scabbing, the crusting disappears in about 3 weeks. Also, 80% of the patients had a range of 5–100 lesions dispersed in different body parts, and all the rashes in each body part were in the same stage of development (Huhn et al. 2005). In the current outbreak of 2022, skin manifestations

can be present simultaneously with prodromal or subtler symptoms. When dermatological signs are present, the lesions can be in various stages of development, and rashes on the palm and soles might be absent. However, lymphadenopathy might be absent (Koenig et al. 2022). Monkeypox can be differentiated from smallpox, in that lymphadenopathy in the prodromal stage is classic in the former (Reynolds et al. 2006). Other sexually transmitted infections (STIs) or other common diseases can be difficult. This is further complicated by the fact that monkeypox can coexist with other STIs.

3 Other Clinical Manifestations

In addition to dermatological manifestations of monkeypox, other symptoms have been reported. As of August 2022; the latest update from CDC reported several nondermatological symptoms of monkeypox including the following:

3.1 Respiratory Symptoms

Respiratory symptoms like nasal congestion, dyspnea, and coughing among other respiratory side effects have been reported in monkeypox-infected patients (Orviz et al. 2022). However, pharyngeal inflammation (pharyngitis) and bronchopneumonia were reported as a result of secondary bacterial infections of the respiratory system including *Streptococcus* pneumonia and *Mycoplasma* pneumonia and can result in respiratory distress and difficulty breathing (Damon 2011; Petersen et al. 2019). Therefore, monkeypox can be transmitted via airborne droplets, which come in line with other new findings that the DNA particles of the human monkeypox virus are tested positive in infected patients for more than 30 days post-diagnosis. Hence, it is recommended to wear personal protective equipment close to infected patients to protect them from airborne transmission routes, i.e., large respiratory droplets and fomite spread (Adler et al. 2022; Orviz et al. 2022).

3.2 Triggering the Second-Line Immune System

Fever is a frequently documented symptom of monkeypox. The fever onset ranged from 10 to 14 days post-exposure, which precedes the dermal rash onset by 1 to 5 days (12 to 16 days following exposure), and the degree ranged between 38.5 and 40.5 °C (Damon 2011; Ježek et al. 1987). In a study conducted in 1987, it was noted that the density of dermal lesions was related to the degree of fever and severity of symptoms (Ježek et al. 1987). Hence comes the importance of monitoring the temperature of suspected patients. Unfortunately, as with smallpox, patients are asymptomatic before the fever onset and can transmit the disease. In developed countries, where hospitals have more capacity and better isolation standards, a suspected case of monkeypox with fever and dermal lesions is immediately isolated in negative air pressure rooms and monitored closely for other symptoms (Petersen et al. 2019).

Lymphadenopathy (swelling of lymph nodes) is also a common sign that is observed in 90% of patients and is associated with secondary bacterial infections (Petersen et al. 2019). Lymphadenopathy can be used as a feature to distinguish patients who are infected with monkeypox from those infected with smallpox and usually occurs in the early stage of the infection, in most cases 1 to 2 days after the fever onset (Damon 2011; Ježek et al. 1987). The swollen lymph nodes, especially cervical and submaxillary lymph nodes, are usually tender, firm, and painful. Their diameter can range between 1 and 4 cm (the size of a pigeon's egg) (Ježek et al. 1987).

3.3 Myalgia and Backache

Other common generalized physical symptoms including headache, myalgia (muscle pain), back pain, and fatigue have been reported by patients and usually start with the fever onset (Petersen et al. 2019; Kumar et al. 2022). These symptoms are not disease-specific, and infected patients can

be misdiagnosed with flu-like symptoms until the dermal rash starts to appear.

3.4 Encephalitis

Encephalitis is a rare complication in severely ill patients and was reported in the US outbreak in 2003 (Damon 2011). It can also occur in vaccinated patients as an adverse event of the vaccine (Petersen et al. 2019).

3.5 Gastrointestinal Symptoms

Involvement of the gastrointestinal system is another rare complication of the virus that usually occurs in the second week of the infection. It includes vomiting and diarrhea, resulting in dehydration and deterioration of overall health (Damon 2011; Petersen et al. 2019). The symptoms are curable, and patients with these signs were only hospitalized for infection control purposes (Adler et al. 2022).

3.6 Ocular Symptoms

Conjunctivitis and edema of the eyelids are complications that are caused by secondary bacterial infection rather than the monkeypox virus. They are curable with the proper antibacterial within the normal response rate (Adler et al. 2022). Corneal ulceration and keratitis with ensuing vision loss and opacity were also seen in some patients. Luckily, the symptoms rapidly resolve with the use of antimicrobials (Adler et al. 2022; Damon 2011).

4 Management of Monkeypox

Classical monkeypox can be diagnosed by its clinical presentation. However, a definite diagnosis is made by PCR or ELISA techniques of a sample collected by either an oropharyngeal swab or from the skin rash, exudate, or scab (Petersen et al. 2019; Nasir et al. 2018). The early identification and isolation of the patients are necessary because the monkeypox virus is infectious in the first week of rash and can transmit rapidly by either skin-to-skin contact, droplet, or aerosol. Being declared a Public Health Emergency of International concern (PHEIC), suspected and confirmed cases are to be reported by the healthcare professional to be managed properly (World Health Organization 2022). The recommended therapy for monkeypox is cidofovir and smallpox vaccination within 14 days of exposure (Learned et al. 2005). A vaccinia-based vaccine, modified vaccinia Ankara (MVA), was approved by WHO for monkeypox in 2019, which is a two-dose vaccine with restricted accessibility (World Health Organization 2022).

Although hospitalization of monkeypox patients is rare, it is either done to treat secondary infections or to manage the painful lesions (Koenig et al. 2022). The reported case fatality rate ranges between 1% and 10%, with scarring being one of the complications of these skin lesions that may lead to keratitis or blindness if it affects the cornea (Ježek et al. 1987; Beer and Rao 2019).

5 Conclusion

Monkeypox is a global public threat manifesting as a rash-like illness. The clinicians should take a detailed history to differentiate it from other rash-like illnesses. The mainstay of diagnosis is PCR testing. Most cases are of mild severity, which can be managed by antiviral therapy, vigilant monitoring of hydration and nutrition, and treatment of complications. Stakeholders should be proactive in detecting the cases with early intervention to curtail the spread of disease.

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Laboratory Diagnosis for Monkeypox: Clinical Practice and Important Points to Be Recognized

Rujittika Mungmunpuntipantip and Viroj Wiwanitkit

Abstract

Monkeypox is a unique variety of pox infection. WHO has already acknowledged the seriousness of the large monkeypox outbreak in 2022. The disease commonly presents as acute febrile illness with skin lesion. However, a fever or a skin lesion, however, is sometimes not detectable. Without the peculiar appearance, the physician might have missed the problem and made a false diagnosis. Basically, the clinical diagnosis is the simplest procedure and must be used by practitioners. Laboratory methods can help confirm diagnosis. The gold standard for diagnosis is the molecular-based diagnosis. There are also other available approaches such as point of care testing. In this article, we also summarize and discuss important practical points in laboratory diagnosis for monkeypox.

Keywords

Diagnosis · Laboratory · Monkeypox

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Abbreviations

DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
POCT	Point-of-care testing
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
WHO	World Health Organization

1 Introduction to Monkeypox

Monkeypox is a unique variety of pox infection. Despite being around for a long time, there hasn't been a large outbreak of this disease. WHO has already acknowledged the seriousness of the large monkeypox outbreak in 2022 (Srikanth Bhagavathula and Khubchandani 2022). Regarding monkeypox, there is a lot more that needs to be mentioned. Nowadays, monkeypox is commonly regarded as posing a severe risk to public health on a global scale. Severe febrile illness and skin lesions frequently coexist. The likelihood that the virus will spread from one person to another is increasing. Recent research on human settings, notably sexual contact, has shed light on some of today's most pressing concerns. Understanding the problem is crucial because effective sickness remedies depend on early detection and treatment.

A fever or a skin lesion, however, is sometimes not detectable. Without the peculiar appearance,

the physician might have missed the problem and made a false diagnosis (Joob and Wiwanitkit 2022). This could be the current epidemic's undiscovered underlying cause. If a patient has a serious clinical condition and the appropriate treatment is available, they might need to stay in the hospital. Clinical samples that are isolated from crust or vesicles are frequently thoroughly studied. This may indicate that clinical issues related to skin lesions are common. It's important to remember that some people only exhibit certain symptoms, such as those who have neurological or intestinal issues (Mungmunpuntipantip and Wiwanitkit 2022b).

During the investigation, it is critical to look for contamination. The monkeypox virus test has a substantial likelihood of being erroneous, according to a recent study on laboratory quality assurance; therefore good laboratory practices, starting with exact specimen collection, are required. The presence of unusual illness symptoms must be considered. The final main issue is prevention. The need for thorough prevention is a major barrier to addressing this escalating public health concern. There needs to be a global preventive standard. For monkeypox to be completely eliminated, more study is needed. However, traditional illness prevention is beneficial and effective. Because of the unpredictability of the scenario and the possibility of a SARS-CoV-2 infection breakout, it should be obvious that the issue has to be identified and solved.

It's possible that many lessons have been learned about how to handle an outbreak of a novel infectious illness, despite the fact that the SARS-CoV-2 outbreak problem is still a significant one. Even though mistakes could still happen, they ought to be less frequent than those we noticed in the early stages of the SARS-CoV-2 epidemic.

2 Diagnosing Monkeypox in Clinical Practice

Basically, the clinical diagnosis is the simplest procedure and must be used by practitioners. The first step is the history taking. Clinical history taking is very important for diagnosing any medical problems. Apart from the clinical history taking, the good physical examination is required to gather the information. The presumptive diagnosis of a medical disorder might be based on the data from clinical history and physical examination results. The laboratory investigation is the supplementation for confirming the presumptive clinical diagnosis.

2.1 Clinical History

As of August 2022, instances of the monkeypox virus had been confirmed in more than countries, including many countries where the virus is not endemic. Most recorded cases have identified gay and bisexual men between the ages of 20 and 50, albeit not always. When people come into contact with, consume, or are exposed directly to the blood or bodily fluids of an animal that has the disease, they can become infected (Hraib et al. 2022), in addition to contacting the lesions, bodily fluids, and contaminated personal objects of a patient (Hraib et al. 2022).

The clinical symptoms of monkeypox include mild headache, lymphadenopathy, body aches, extreme fatigue, and an acute onset of fever more than 38.5 °C (Meo and Klonoff 2022). Initially appearing as macules or papules, a skin rash develops into pustules and vesicles, ulcers, and finally crusted scabs (Meo and Klonoff 2022). Monkeypox infection is usually characterized by a majority of moderate symptoms, such as headache, body aches, extreme weakness, and an immediate onset of high fever (Meo and Klonoff 2022). Initially appearing as macules or papules, a skin rash develops into pustules and vesicles, ulcers, and finally crusted scabs (Meo and Klonoff 2022). Shivering, headaches, fainting, backaches, and myodynia are some of the symptoms that can be difficult to diagnose because they don't have any telltale signs (Hraib et al. 2022). Lymphatic hyperplasia, one of the most prevalent symptoms of monkeypox, might, nevertheless, aid in the diagnosis of the condition (Hraib et al. 2022). Illness is usually self-limiting, but severe disease can occur in specific groups – particularly children, and people who are immunocompromised

or pregnant (Huang et al. 2022). Complications can include secondary bacterial infection of skin lesions, vision loss from corneal involvement, pneumonia, sepsis, and encephalitis (Huang et al. 2022).

As early noted, the difficulty is existed in case of atypical clinical presentation. There are many possible atypical presentations of monkeypox that can result in difficulty in diagnosis. The examples of important atypical clinical presentation will be further discussed.

- Neurological presentation.
  Headache is a frequent clinical symptom that has a variety of medical causes, including neurological disorders. Despite the fact that none of these individuals reported experiencing a headache, an investigation of cases during an outbreak in the United States discovered that headache was one of the presenting symptoms in roughly one-third of the patients (Huhn et al. 2005). Concurrent clinical issues with a cutaneous lesion and lymphadenopathy are frequent (Huhn et al. 2005). In the traditional African tale (Yinka-Ogunleye et al. 2019), practically all patients with monkeypox manifested clinically as monkeys, in contrast to the US examples (Huhn et al. 2005; Yinka-Ogunleye et al. 2019). The headache may only be a clinical concern for up to 1 week before the pathognomonic skin rash appears (Eseigbe et al. 2021). To the best of our knowledge, no prior research has been done on the headache signs and symptoms of monkeypox patients. Despite the fact that these patients did not come with only a single headache, a review of cases during an outbreak in the United States in 2003 found that headache was one among the presenting symptoms in 9 of 34 people (Huhn et al. 2005). As a result, a clinician would be alerted by a variety of signs and indicators that the headache is likely secondary in nature in these cases. Lesions on the skin, febrile illness, and other clinical signs may occur more frequently than previously thought. One of the earliest possible clinical indications is headache.
  The prospect of a serious illness is rarely investigated, even though consciousness modification can manifest itself in a variety of ways. Although it can happen in any new infectious disease condition, confusion is a clinical sign that is rarely highlighted. To the best of our knowledge, no prior research has been done on the perplexing symptoms seen by patients with monkeypox.
  Monkeypox may also present with stiff neck as its initial clinical symptom. In a previous report, 3 of the 34 patients who were studied had a stiff neck as their primary presenting symptom. Not all of these persons developed fevers, and some also developed rashes (Huhn et al. 2005). Because an outbreak in a new area is conceivable, it is crucial in contemporary clinical practice to be ready for suspected monkeypox.
- Gastrointestinal presentation.
  There are many possible gastrointestinal clinical problems in monkeypox. Diarrhea is a common problem to be noted (Mungmunpuntipantip and Wiwanitkit 2022a). A recent study found that 5.9% of patients with monkey pox experience diarrhea [5]. Watery diarrhea is commonly present (Mungmunpuntipantip and Wiwanitkit 2022a). In this group, no one had a fever, and a few others had a rash (Huhn et al. 2005). Nausea and vomiting are the most frequent clinical manifestations that are associated with diarrhea (Huhn et al. 2005). The clinical issues typically appear on the same day as the monkeypox rash and symptoms (Wiwanitkit and Wiwanitkit 2018). Additionally, the pain might also present with an acute abdomen. According to a recent study, 10% of those with monkeypox experience stomach pain (Huhn et al. 2005), guarding or discomfort along with abdominal pain in all four quadrants (Huhn et al. 2005). The majority of the people in this group had rashes, and none of them had fevers (Huhn et al. 2005). Additionally, a common problem that might cause melena passing is low platelet levels, which can misdiagnose a simple gastrointestinal bleeding. Stomach ache may

initially be the sign of monkeypox. Even though some people may initially have diarrhea, many only experience severe abdominal pain (Mungmunpuntipantip and Wiwanitkit 2022a).

- Urological presentation.
  It's intriguing to talk about how monkeypox manifests urologically. No individuals with monkeypox had abnormal urine test findings, according to a prior investigation. There is no complaint or dysuria. The cutaneous lesion, however, might just affect the urogenital region. It's significant to remember that not everyone had a fever or the characteristic rash. Disaster planning is therefore essential in contemporary medicine. Due to the likelihood of a new monkeypox epidemic, it is crucial in current clinical practice to be prepared for suspected monkeypox and to provide the best care for all patients who present with unexplained atypical urological symptoms. The only available conventional vaccine at the moment is the smallpox shot. Cross immunity against monkeypox can be generated by this conventional vaccine. This antiquated immunization might be employed once more in the event of an outbreak. A significant adverse reaction to this vaccination is glomerulonephritis, and those who receive it may experience unexplained hematuria (von Vacano 1968). In order to be ready for a potential monkeypox outbreak, the practitioner has to be aware of this information.
- Psychiatric presentation.
  Confusion is a potential early indicator. Because a widespread epidemic of monkeypox is likely, it is crucial to be ready for it in contemporary clinical practice. Anxiety and despair are more prevalent in monkeypox patients. In fact, anxiety or depression can be brought on by any illness. According to reports, 25% of monkeypox patients experience depressive symptoms (Adler et al. 2022). Therefore, it is crucial to identify and take care of monkeypox sufferers who have mental health problems.

2.2 Physical Examination Findings

Enlarged lymph nodes, particularly those in the submental, submandibular, cervical, and inguinal regions, are one of the most accurate clinical indicators separating monkeypox from smallpox and chickenpox (Hraib et al. 2022). Upon physical examination, a distinctive rash and sometimes lymphadenopathy are frequently seen. It is necessary to examine the skin and mucosa thoroughly (including the anal, vaginal, oral, nasal, and ocular areas) for the recognizable vesiculo-pustular rash (Hraib et al. 2022).

Vessel-like rash is a common finding from physical examination. Immediately following the prodromal phase, a distinctive rash appears (Kabuga and El Zowalaty 2019). The rash often appears 1–3 days after the severe febrile fever starts, and it spreads to all body parts within 24 h. Palms and soles are frequently affected by the rash (Kabuga and El Zowalaty 2019). The lesions normally scab over and resolve over the course of 2–4 weeks as they simultaneously advance through the four stages of macular, papular, vesicular, and pustular. Until they begin to heal, lesions are frequently regarded as painful until they become itchy (Kabuga and El Zowalaty 2019). On any area of the body, lesions are all at the same stage of development and tend to be more concentrated on the face and extremities (centrifugal) than on the trunk. Lesions can have a diameter of 0.5–1 cm (although they might be larger), and there may be one or thousands of them. Lesions may be confluent or isolated, inflammatory, and resulting in mild erythema and/or skin discoloration (Kabuga and El Zowalaty 2019).

2.3 Laboratory Diagnosis

In essence, sampling is necessary for diagnostic tests. Monkeypox is identified via a PCR test on a viral swab obtained from one or more vesicles or ulcers, which is the current gold standard testing (McCollum and Damon 2014). Swabs in viral

Table 1 Specimen collection for monkeypox testing

Sample type and quantity	Collecting and delivering samples
Vesicular swabs (0.5–1 mL fluid required)	Clean the blister with 70% alcohol. Use a disposable syringe with a needle. Absorb water in the blister and store in sterile tubes. Close the lid and store in an ice container (4 °C) immediately
Wound swab	Clean the blister with 70% alcohol. Use scissors to cut the skin covering the blister. Then use a sterile lancet to scrape the wound until the skin is moist but no bleeding is applied to the wound with a sterile cotton swab and immediately soak the swab into the VTM tube. Delivery container should contain ice to maintain appropriate temperature to be 4 degree Celsius

delivery medium are necessary during delivery to the laboratory (McCollum and Damon 2014). The common molecular diagnostic test included monkeypox virus analysis by testing the genetic material for the genus virus *Orthopoxvirus* by real-time PCR technique and monkeypox virus identification by genome sequencing test. The specific specimen collection is various depending on type of specimen and described in Table 1. The standard clinical specimen is usually the sample collected from skin lesion. However, other samples, such as rectal swabs, nasopharyngeal swabs, semen, urine, and feces, are frequently positive (Peiró-Mestres et al. 2022). These findings advance our understanding of virus shedding and the potential involvement of bodily fluids in the spread of disease (Peiró-Mestres et al. 2022).

For detection of genetic material by real-time PCR technique, duration of examination is 24–48 h. For nucleotide sequencing by DNA sequencing technique, the required duration is 4–7 days. The advantages of the PCR method are high sensitivity and specificity. It can also be distinguished between monkeypox disease and smallpox disease (Maksyutov et al. 2016). At present, the real-time PCR assays are available and proven for good diagnostic property and can provide a fast diagnosis (Li et al. 2006, 2010).

One of the most updated PCR-based technique for diagnosis monkeypox is the multiplex assay. Using the GeneXpert platform, a portable quick diagnostic device that might be used as a point-of-care test to identify illnesses in endemic areas, Li et al. (2017) assessed the precision and usefulness of a multiplex assay. According to Li et al. (207) the GeneXpert assay performed well with both crust and vesicle samples, and the multiple assay had a sensitivity and specificity of 98.8% and 100%, respectively. The GeneXpert MPX/OPX test, according to Li et al. (2017) has a straightforward approach that works well in both lab and field settings, indicating its feasibility as a diagnostic platform that could improve and accelerate current MPX detection capabilities.

The use of serology is not recommended because it can result in a false-positive result, cross-reactive with other orthopoxviruses. In addition, people previously vaccinated against smallpox are immune and can cause false positives as well. Nevertheless, the serology test still has usefulness for serosurveillance, which is not the application for definitive diagnosis of the infection. An illustration of a serological test that can be used for monitoring is a radioimmunoassay adsorption test for the presence of antibodies specific for monkeypox or vaccinia (Jezek et al. 1987).

Finally, while the standard molecular diagnostic test is acceptable as gold standard for definitive diagnosis of monkeypox, the requirement for a rapid diagnostic test to urgently contain the outbreak is important. PCR tests that are timely and scalable are essential for stopping the spread of monkeypox (Nörz et al. 2022). High-throughput molecular testing is presently under developed and trialed (Nörz et al. 2022). The development of point-of-care testing (POCT) might be the solution. At present, there are some newly available POCT tests for diagnosis of monkeypox. Important repots on this specific issue are listed in Table 2.

Table 2 Some important reports on POCT for monkeypox rapid diagnosis

Authors	Details
Stern et al. (2016)	A point-of-care test for the detection of orthopoxviruses was presented by Stern et al. (2016). Given the high sensitivity and ease of handling, Stern et al. (2016) came to the conclusion that the novel assay could be very helpful for rapid on-site testing of suspected bioterrorism samples as well as on-site diagnosis of suspected monkeypox virus in the current outbreak situation
Townsend et al. (2013)	In locations without adequate laboratory infrastructure, Townsend et al. (2013) published an evaluation of the Tetracore Orthopox BioThreat® antigen detection assay utilizing laboratory-grown orthopoxviruses and clinical specimens of rash illnesses. The BioThreat® Alert assay may be used as a quick screening assay and point-of-care diagnosis for suspected human monkeypox cases, according to the results

3 Quality Management for Laboratory Diagnosis for Monkeypox Diagnosis

As already mentioned, the laboratory diagnosis plays important role for monkeypox diagnosis. Similar to any laboratory investigations, laboratory management is required. The laboratory quality cycle principle is applicable. The quality management must cover all phase according to laboratory cycles, pre-analytical, analytical, and post-analytical phases.

A. Pre-analytical phase.
   Pre-analytical phase starts from the proper laboratory investigation request. The patient preparation and specimen collection are the important process during the pre-analytical phase. It should note that the different samples required different collection techniques. Veintimilla et al. (2022) used real-time PCR to analyze 140 different clinical specimens and discovered that skin lesions on any area of the body had the highest positivity rates (97%) followed by plasma, pharyngeal, and anal swabs. Testing samples from various areas may increase sensitivity and decrease false-negative test findings, according to Veintimilla et al. (2022). This step is the first and earliest step. The problem of false result can easily occur due to any problem in this step. The error in this step is common and can affect the quality of the laboratory investigation.
B. Analytical phase.
   There are many analytical tools and techniques for diagnosing monkeypox. The error in this step is possible and might be systematical or sporadic error. As earlier noted, the only technique that can be used quickly enough to offer feedback on any public health interventions is PCR. The manner in which those PCR assays are being standardized between laboratories is currently not well understood. The main clinical requirement is detection, not quantity, of monkeypox virus DNA, and testing is currently restricted globally for this reason, among others (Huggett et al. 2022). However, we shouldn't let PCR performance make us complacent (Huggett et al. 2022). To support standardization and facilitate regulatory compliance, it would be prudent to guarantee PCR accuracy from the outset as testing requirements rise quickly and specimens grow increasingly varied (Huggett et al. 2022).
C. Post-analytical phase.
   The post-analytical phase includes the validation of the laboratory result and reporting system. It also deals with the proper laboratory result interpretation.

Quality management has to cover all three phases. Additionally, there must be the quality control internally and externally. Finally, the plan for continuous improvement of the quality is needed according to the concept of total laboratory management and continuous laboratory improvement. In a recent report, not all referencing laboratories passed the quality control criteria according to the external quality assessment program (Niedrig et al. 2006). The current problem on quality of monkeypox laboratory testing still exists, and it might be the problem for successful containment of the current outbreak.

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Regulatory Mechanisms of Muscle Mass: The Critical Role of Resistance Training in Children and Adolescent

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Abstract

Muscle mass and strength are subjected to several regulations. We found endocrine signals such as growth hormone, insulin-like growth factor 1, testosterone, thyroid hormones, and glucocorticoids among them. Neural inputs also influence muscle development, modulating mass and strength. Among the external stimuli that modulate these muscular features is physical training such as resistance and endurance training. Specifically, resistance training can mediate an increase in muscle mass by hypertrophy in adults, but the effects in children and adolescents are full of myths for most of the population. However, the evidence shows that the impact of resistance training on children and adolescents is clear and provides a wide range of benefits. However, qualified professionals must be available since exercise prescription and subsequent supervision must follow this population's abilities, needs, and interests.

Keywords

Adolescent · Children · Cortisol · Glucocorticoids · Growth hormone · Hypertrophy · Muscle mass · Resistance training · Strength

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Abbreviations

AR	Androgen receptors
D2	5´-deiodinase type 2
ERE	Elite referenced excellence
FMS	Fundamental movement skills
GH	Growth hormone
GHBP	GH-binding protein
GHRH	GH-releasing hormone
IGBP	IGF-binding proteins
IGF-1	Insulin-like growth factor
LTAD	Long-Term Athlete Development
MRF	Myogenic regulatory factors
mTOR	Mammalian target of rapamycin
PPW	Participation for personal well-being
PRE	Personal referenced excellence
TGFβ	Transforming growth factor β
T3	Triiodothyronine
T4	Thyroxine
WHO	World Health Organization
YPD	Youth Physical Development

1 Introduction

The development of muscle mass and strength is mainly mediated by cellular mechanisms, neural control, and endocrine pathways. The cellular mechanisms are primarily associated with embryogenesis, a process that gives way to the formation of skeletal muscle in the dorsal domain of the somites. The role of myogenic regulatory factors (MRF) is fundamental in this period, and postnatal events, such as muscle regeneration, due to the activation of satellite cells in the process called myogenesis. Moreover, neural control is necessary for force production, which is related to the electrical activity dependent on the motor neuron, which presents extensive anatomical and physiological properties. The generation of force will depend on the recruitment of the motor unit, the frequency of the action potential, and the modulation of the discharge, among others. Finally, hormones also play a role in developing muscle mass and strength. Some anabolic compounds participating in these processes are testosterone, insulin-like growth factor (IGF-1), and growth hormone (GH). The mechanisms above will be addressed in greater detail in the following sections. On the other hand, developing muscle strength in children and adolescents has gained support over the last decade due to the critical findings. The training focused on developing muscle strength is also part of combating obesity in this population. However, resistance training for children and adolescents is highly controversial and full of questions, especially among ordinary people. Questions such as: does it interfere negatively with the growth plate? Or does it generate hormonal misbalances in young children? There is abundant evidence that proper resistance training in children is beneficial at multiple levels, with minimal risks (Drenowatz and Greier 2018; Malina 2006; Rians et al. 1987). In this line, it is vital to know the fundamental mechanisms underlying the development of muscle mass and strength and the effect that resistance training exerts on them. Therefore, this review deals first with the cellular mechanisms, neural control of muscle development, and endocrine pathways involved in muscular mass and strength, and how these factors are related to the response to resistance training in children and adolescents. Then, we will present the specific benefits of resistance training. We will also discuss the most common myths and risks about resistance training and give some proposals for training programs focused on children and adolescents.

2 Cellular Mechanisms of Muscle Development and Growth

Skeletal muscle formation during embryogenesis occurs at the somites dorsal domain (dermomyotome). Myotome formation is a multistage process where muscle progenitors are added on sequential waves. A main myotomal structure is formed by a first wave that arises along the medial section of the epithelial somite. The second wave of cells migrates from the rostral and caudal lips of the epithelial dermomyotome, and the dorsomedial lip contributes to the cellular expansion (Kahane et al. 1998; Kalcheim et al. 1999; Perry and Rudnick 2000). As the embryo

develops, muscle progenitor cells in the myotome establish their myogenic identity trigged by the expression of transcription factors and myogenic regulatory factors (MRFs). The role of MRFs is to regulate the differentiation of muscle progenitor cells into myoblasts that will later bring about the different muscle groups of the adult body (Chang and Kioussi 2018; Murphy and Kardon 2011).

At birth, skeletal muscle comprises multinucleated myofibers derived from embryonic and fetal myogenic cells. Postnatal myogenesis occurs in skeletal muscles to compensate for physiological turnover and repair tissue damage. Muscle tissue maintenance and repair are fundamentally directed by activating satellite cells (SC) (Chargé and Rudnicki 2004; Schmalbruch 2006). SC are mononucleated myogenic cells located underneath the basal lamina and adjacent to the plasma membrane of the muscle fiber (Mauro 1961). SC remain in a quiescent and undifferentiated state in undamaged adult muscles. However, after injury, SC are activated to give rise to myogenic progenitors that proliferate, differentiate, and fuse to form new muscle fibers. At the same time, a proportion of these cells are capable of self-renewal, returning to inactivity to replenish the cell pool and thus maintain the regenerative capacity of skeletal muscle (Chargé and Rudnicki 2004; Wang et al. 2014).

3 Neural Control of Muscular Mass

The muscular ability to contract and develop gradual strength depends on the anatomic characteristics, fiber composition, and activation of neural connections. These connections occur through action potentials from motor neurons to muscle fibers across the neuromuscular synaptic junction or muscle endplate. At this level, the arrival electrical excitation is transmitted to the muscle by releasing acetylcholine into the synaptic cleft. On binding the receptor, acetylcholine can excite the endplate and generate muscle action potentials that propagate along the entire surface of the muscle fiber membrane, penetrating the T tubules. Further, calcium ions are released from the sarcoplasmic reticulum, causing interaction between thick and thin filaments of the sarcomere to finally lead to muscle fiber contraction (Monti et al. 2001).

The innervation has a critical role in forming muscle fibers since, in the earliest stages of myotube formation, there is a dependence on the number of secondary myotubes and neural connections (Harris 1981). Similarly, alterations in presynaptic formation can affect innervation, influencing muscle development (Schiaffino and Reggiani 2011). Besides, the neural influence is also observed in the gene expression of crucial proteins determining muscle contractile properties (slow- versus fast-twitch), such as acetylcholine receptors and myosin isoforms (Washabaugh et al. 1998). In addition, evidence indicates that normal muscle electrical activity can ensure that the endplates reach their appropriate size, efficiency, and spatial distribution in each muscle fiber (Lømo 2003). The impairment in endplate functions leads to muscle weakness or paralysis, leading to severe muscle wasting (Tintignac et al. 2015).

The motor neuron-dependent electrical activity is a critical factor in the generation of muscle force (Adrian and Bronk 1929), which increases by a rise in the number (motor unit recruitment) or frequency of motor neuron action potentials (discharge rate). The variability and modulation of motor unit discharge (trains of action potentials) and the mean discharge rate significantly impact muscle force (Duchateau et al. 2006).

4 Endocrine Regulators of Muscle Mass and Strength

Essential hormones such as insulin-like growth factor-1 (IGF-1), growth hormone (GH), and testosterone are anabolic compounds that play a critical role in the regulation of skeletal muscle mass and strength (Gharahdaghi et al. 2020). However, the underlying mechanisms controlling these processes are unclear. Below is described the contribution of the main hormonal axes in developing muscle strength.

4.1 Growth Hormone and IGF-1

The most critical pathway implicated in human growth is the GHRH–GH-IGF-1 axis. GH, also known as somatotropin, is a single chain peptide composed of 191 amino acids synthesized in the anterior pituitary gland. It is released into the blood by the hypothalamic GH-releasing hormone (GHRH). It circulates in the plasma bound to the GH binding protein (GHBP), which is the extracellular domain of the GH receptor (Leung et al. 1987). Several physiological stimuli increase GH production and release, including hypoglycemia, stress, sleep, exercise, nutrition, and thyroid/pubertal hormones (Gharahdaghi et al. 2020; Giustina and Veldhuis 1998).

GH induces the musculoskeletal system growth and almost organs in the human body. GH promotes primarily longitudinal growth in children and adolescents. GH acts through two direct mechanisms on the target cells to enhance response. An indirect mode of action occurs mainly via IGF-1, the main GH effector, whose synthesis and secretion are promoted by GH binding principally to hepatic surface receptors (GHR) (Bidlingmaier and Strasburger 2010). This binding leads to an increased IGF-1 and IGF binding proteins (IGBPs) synthesis and subsequent secretion (Laron 2001).

When IGF-1 reaches the target tissues promote biological effects such as inducing cell growth, proliferation, and differentiation, enhancing protein synthesis, and promoting metabolic changes related to cell survival.

4.2 Testosterone

Another hormone that influences muscle mass and strength is testosterone, an androgenic steroid hormone mainly produced from cholesterol in the testes Leydig cells and, to a lesser extent, in the ovaries. Testosterone is under the control of the hypothalamic-pituitary-gonadal axis, being produced and released by luteinizing hormone after binding to its receptors in the testis (Casarini et al. 2018).

Skeletal muscle cells are sensitive to testosterone by binding to membrane-bound or cytoplasmic androgen receptors (AR), which dimerize and translocate to the nucleus. Therein, the dimer binds to androgen-responsive elements of DNA and initiates a cascade of molecular signaling leading to alterations in muscle metabolism, expression of genes associated with the regulation of skeletal muscle structure and fiber type (Dubois et al. 2014; Kraemer et al. 2020; MacLean et al. 2008).

Testosterone can be converted to dihydrotestosterone, a form more biologically active mediated by AR, by the enzymatic action of 5-α reductase (Davey and Grossmann 2016) (Wilborn et al. 2010). Testosterone performs many anabolic, anti-catabolic, and ergogenic functions in the skeletal muscle and neural tissues resulting in increased muscle power, strength, endurance, and hypertrophy dose-dependent (Bhasin et al. 2003; Kraemer et al. 2017). Testosterone enhances the transition of muscle fibers towards isoform IIB (fast fibers with larger cross-sectional areas), making muscles powerful and less resistant to fatigue (Haizlip et al. 2015). The response of skeletal muscle to testosterone depends on the AR amount, which is differentially expressed according to the type of muscle fibers, sex, training condition, and androgen levels (Kraemer et al. 2020).

4.3 Thyroid Hormones

The thyroid is essential for metabolic regulation, thermogenesis, growth, and development of the human body (Mullur et al. 2014). The HPT axis regulates levels of thyroid hormones, triiodothyronine (T3), and thyroxine (T4) (Citterio et al. 2019). T3 and the more active form T4 are the hormones that will exert their effect at the systemic level. The secretion to the bloodstream is mainly T4, whereas a significant T3 amount originates from T4 by 5´-deiodinase type 2 (D2) enzyme activity at the peripheral level. D2 is expressed in the skeletal muscle, adipose tissue, and hypothalamus (Mullur et al. 2014).

Skeletal muscle is one of the most critical targets of thyroid hormones by regulating the contractile and metabolic properties and muscle development and regeneration. Such as it was previously discussed, the types of muscle fibers differ in contractile and metabolic features, primarily determined by the genetic background and kind of innervation (Simonides and van Hardeveld 2008). However, the thyroid hormones modulate the physiological characteristics of these fibers. The T3 regulation of myosin and the energy from the oxidation of mitochondrial substrates determine muscle contraction and relaxation. The balance of D2 and D3 expression within the muscle determines the content and half-life of thyroid hormones, influencing the proliferation and differentiation of SC. Therefore, thyroid hormones play a critical role in muscle repair and myogenesis (Bloise et al. 2018).

Musculoskeletal alterations are common in patients with thyroid dysfunction (Cakir et al. 2003). Thus, hyperthyroidism leads to weakness and muscle loss without elevated creatinine kinase, a marker of muscle fiber disruption. In contrast, hypothyroidism shows cramps and myalgia with high plasma levels of creatinine kinase, suggesting the presence of myopathies (Duyff et al. 2000; Olson et al. 1991). It is still debatable whether subclinical hypo- and hyperthyroidism have any clinically significant negative impact on muscle function and physical performance (Spira et al. 2019).

4.4 Glucocorticoids

The hypothalamic-pituitary-adrenal axis controls glucocorticoid secretion (Lee et al. 2015). Cortisol, the main glucocorticoid, is synthesized in the fascicular area of the adrenal gland cortex. Once released into circulation, it is distributed to many tissues, including the skeletal muscle, adipose tissue, and liver. The increase in cortisol levels enhances gluconeogenesis and glycogenolysis, thus raising glycemia. It also increases blood pressure and cardiovascular activity through catecholamines, water, and sodium retention (Miller 2018).

Glucocorticoids have a crucial role in determining body composition. Some adverse effects of glucocorticoids on body composition include the redistribution of body fat, leading to fatty tissue deposits in the abdomen/trunk. In addition, glucocorticoids lead to muscular atrophy. On the other hand, cortisol promotes protein muscle catabolism and alters the state of the growth cartilage (Dobson et al. 2001).

Hypercortisolemia causes a reduction of skeletal muscle mass due to augmented proteolysis and diminished protein synthesis (Stefanaki et al. 2018). Cortisol activates the ubiquitin-proteasome and autophagy lysosomal systems through the increased expression of genes involved in muscular atrophy, such as FOXO transcription factors, Atrogin-1, and MuRF-1 (muscle-specific E3 ubiquitin ligases). The glucocorticoid-induced reduction of the mammalian target of rapamycin (mTOR) signaling pathway is primarily responsible for the suppression of muscle protein synthesis (Kuo et al. 2013; Schakman et al. 2013). In addition, the blockade of IGF-1 signaling and the induction of myostatin production, a member of the transforming growth factor β (TGFβ) superfamily that has a regulatory effect on muscle size, glucocorticoids exacerbate the loss of protein and muscle mass (Schakman et al. 2008).

4.5 Adaptations in Muscle Mass and Strength in Children and Adolescents Following Resistance Training

Physical activity corresponds to the bodily movement produced by skeletal muscles that generate energy expenditure (Caspersen et al. 1985). It is well recognized that physical activity is essential both for physical and psychological development and the future health of children and adolescents (Janssen and Leblanc 2010; Smith et al. 2014). The positive influence of good health habits during childhood may carry over into adulthood (Faigenbaum and Myer 2010a, b, c; Stricker et al. 2020; Legerlotz 2020). Adherence to physical activity is necessary since surveys have shown

that levels of physical activity decrease as children approach adolescence, showing increasingly sedentary behaviors (Marques and de Matos 2014). In fact, physical inactivity has been determined as the fourth leading risk factor for global mortality (World Health Organization 2010).

Physical activity is a broad term that encompasses exercise, a more specific activity with a purpose. Exercise is characterized by being planned, structured, repetitive, and intentional (Caspersen et al. 1985). Commonly, exercise is divided into endurance exercise and resistance exercise. Endurance exercise is performed against a relatively low load for a long time, increasing mitochondrial density and, in turn, generating an increase in maximal oxygen uptake. On the other hand, resistance exercises are performed against a relatively high load for a short time, increasing muscle size and strength (Hughes et al. 2018; Watson and Baar 2014). Therefore, resistance training is mainly dominated by mechanical stress, while metabolic disturbances are more prominent in endurance training.

In particular, the effect of exercise in children is associated with improvements in physical and mental health, increased self-esteem and well-being, and promotion of behaviors to be physically active adults. The appropriate frequency, intensity, and duration of exercise training can induce several benefits on musculoskeletal tissue, the cardiorespiratory system, metabolism, and neuromuscular activity, among other effects. The evidence has shown that regular exercise results in physiological and psychological benefits and cognitive improvements (Gouveia et al. 2021; Myer et al. 2011). Although the beneficial effects are known, the levels of physical activity reported in young people are insufficient (Kohl III and Cook 2013; Townsend et al. 2015). Recent studies have shown that 81% of adolescents worldwide do not comply with the recommendations given by the World Health Organization (WHO) (Guthold et al. 2020). Interestingly, low muscular fitness has been suggested to contribute to insufficient physical activity levels in children (Avery D Faigenbaum and McFarland 2016). In addition, low levels of strength have been associated with less participation in recreational activities and sports, as well as an increased risk of cardiovascular diseases in children and adolescents (D'Hondt et al. 2013; Lopes et al. 2011; Steene-Johannessen et al. 2009). In 2010, the WHO developed global recommendations on physical activity for health. Curiously, no muscle-strengthening activities were suggested for children aged 5–17 (World Health Organization 2010). However, this type of activity has shown beneficial effects, so the suggestions above were updated, and muscle-strengthening activities are already being proposed for children and adolescents (Bull et al. 2020a, b).

The adaptive response to resistance training in healthy adults is related to neural factors and muscle fiber morphology changes, specifically muscle hypertrophy. It is well documented that these adaptations contribute to increased muscle strength in this population (Carroll et al. 2011; Duchateau et al. 2006; Sale 1988). However, these adaptations that increase muscle strength due to resistance training differ in children. Neural adaptations are the primary and responsible mechanism in contrast to the morphological changes of muscle fibers observed in adults (Behm et al. 2008; Granacher et al. 2011; Kraemer and Fleck 2005; Malina 2006; Waugh et al. 2014a). Several neural factors influencing strength improvement after resistance training, such as preactivation, reflex control, and co-contraction, have been reported. However, whether these factors could improve force production capacity in children has not yet been evaluated. It has been reported that increases in muscle strength in children appear to be related to greater synchronization and recruitment of type II motor units (Dotan et al. 2012; Dotan et al. 2013). Changes in the muscular system of adults are different compared to children and adolescents due to maturation. The low levels of circulating anabolic hormones in children could influence those adaptations to resistance training that are mainly neural and not at the level of skeletal muscle architecture since circulating androgens are necessary to stimulate muscle hypertrophy (Herbst and Bhasin 2004; Vingren et al. 2010).

For example, pubertal children have been reported to have higher GH levels in response to acute exercise than earlier children. In this sense, training variables, such as intensity, volume, workload, and rest, play an essential role in the magnitude of the response to GH after resistance exercise (Viru et al. 1998). After puberty, some increases in testosterone levels have been observed following resistance exercise. However, the effect of resistance training may be masked in this population due to increases in growth factors and circulating hormones at the onset of puberty that stimulate tissue growth and development (Armstrong and Van Mechelen 2017). Therefore, it is a challenge to distinguish between the processes associated with the effect of resistance training and growth. Different studies have shown that practically no changes in muscle morphology occur in children after resistance training (Fukunaga et al. 1992; Granacher et al. 2011; Ramsay et al. 1990; Waugh et al. 2014b). However, in adolescents, significant changes have been reported. Therefore, the adaptations in the increase of muscular strength in prepubertal children are mainly mediated by neuronal aspects (Behm et al. 2008; Falk and Tenenbaum 1996; Lillegard et al. 1997). On the other hand, the effect of resistance exercise as an acute or chronic stimulus in children and adolescents has not been extensively evaluated. For example, there is very little evidence to date on the impact of exercise on thyroid hormones, and most studies have shown practically no change (Jahreis et al. 1991; Tremblay et al. 1997). A similar situation occurs with the cortisol response since the results have been highly variable among the few studies, so much remains to be elucidated regarding these axes (del Corral et al. 1994; Filaire et al. 2013).

4.6 Benefits of Resistance Training

Several major organizations, such as The British Association of Exercise and Sport Sciences, The National Strength and Conditioning Association, and the UK Strength and Conditioning Association, have made statements supporting the beneficial effects of resistance training on youth (Faigenbaum et al. 2009a, b; Lloyd et al. 2014a, b; Stratton et al. 2004). Systematic reviews have shown that physical activity interventions effectively combat low levels in children and adolescents. However, very few studies consider interventions aimed at muscle strengthening (van Sluijs et al. 2008). Resistance training can offer ample benefits for children and adolescents as long as they are appropriately prescribed and supervised. The positive impact of resistance training on youth includes improvements in motor skill performance, increased muscle strength and power, gains in speed and power, reduction of injury risks, and development of physical performance. In addition, resistance training can also improve body composition, insulin sensitivity, and blood lipid profile (Faigenbaum and Myer 2010a, b, c; Stricker et al. 2020; Legerlotz 2020). Resistance training may positively affect weight status in healthy and overweight/obese youths, providing a potential benefit for treating and preventing obesity (Collins et al. 2018). Other research has reported that it improves endothelial function, hemodynamic and metabolic profiles, and physical fitness. In addition, it reduces cardiovascular and metabolic risk profiles in non-diabetic obese adolescents regardless of body mass changes (Dias et al. 2015). Nevertheless, fears and concerns associated with resistance training are common. These concerns are often misinformed and disadvantage children and adolescents by missing out on strength activities within their training regimens and routines. However, a large body of evidence has shown that when properly supervised, resistance training is effective and safe for this population (Behringer et al. 2010; Faigenbaum and Myer 2010a, b, c; Lloyd et al. 2014a, b).

4.7 Concerns and Risks of Resistance Training

Resistance training is an adequate stimulus to enhance muscular strength and endurance through the progressive use of a wide range of resistive loads, different movement velocities,

and a variety of exercise modalities (Faigenbaum and Myer 2010a, b, c; Stricker et al. 2020). Usually, people associate resistance training solely with weightlifting and immediately think of the associated risks of such training in children. However, a wide range of movements considers the use of own body weight to improve the physical fitness of children and adolescents (Stricker et al. 2020).

One of the concerns is resistance training is often considered unsafe (ten Hoor et al. 2015). However, the risks associated with this type of training are not more significant than in other sports activities when qualified instruction and progression are delivered with realistic and personalized objectives (Faigenbaum and Myer 2010a, b, c; Lloyd et al. 2014a, b). Resistance training is generally recommended only for athletes, which is wrong as it should be recommended for all young people due to its wide variety of benefits. For example, overweight youth are often unwilling or unable to perform resistance exercises for prolonged periods, whereas resistance training is a more tolerable strategy.

One of the great fears associated with resistance training is that it can stunt children's growth. The high forces produced by resistance training would potentially cause damage when imposed on the immature skeleton, leading to growth plate injuries and inhibition of the development of long bones. However, no evidence supports this concern (Lloyd et al. 2014a, b). Resistance training during childhood has been shown to promote bone growth and development in children (Behringer et al. 2014). Also, it was a general belief that resistance training could be ineffective in prepubertal boys because the low quantities of circulating androgenic hormones would prevent improvements in muscular strength, and conversely, this type of exercise might develop bulky muscles in girls (Faigenbaum and McFarland 2016; Lloyd et al. 2014a, b).

Contrary to the above beliefs, many studies have found that resistance training has a favorable impact on children's health while ignoring the occurrence of frequent and substantial side outcomes. A review dealing with experimental resistance training protocols on pre-and early-pubertal youth shows that supervised training with weights and resistance machines and low instructor/participant ratios do not influence the growth in height and weight and are relatively safe (Malina 2006). The supervised resistance training in prepubescent males (8.3 ± 1.2 years) results in a low injury rate and does not adversely affect bone, muscle, or epiphyses; nor does it negatively affect growth, development, flexibility, or motor performance in the short term (Rians et al. 1987). No evidence exists in the literature that resistance training is hazardous. Most injuries related to youth resistance training are caused by a lack of or insufficient education and supervision. These may be prevented with age-appropriate training programs supervised by qualified people (Drenowatz and Greier 2018; Hamill 1994) because safety depends on diverse external situations and individual child conditions (fitness level, muscle mass, disease, diet). Although a few case reports inform about injury to the growth cartilage in youth, most of these injuries arise from improper lifting techniques, poorly chosen training loads, or lack of qualified adult supervision (Faigenbaum and Myer 2010a, b. c). According to experts, most injuries occurring during resistance training are accidents generated by the inappropriate use of equipment (77.2% in 8–13 years old), excessive training load, defective execution technique, and lack of qualified supervision (Peña et al. 2016).

For example, understanding the limitations of each joint mobility range and avoiding postural positions that put the joint anatomical components at risk is essential to proper exercise technique. Some joints, such as the shoulder and spine, have a limited range of motion, so they can easily be injured when overcoming the critical angle of motion range. Even though the risk of injury with resistance training is low compared with other sports activities, the probability of soft tissue and joint injuries is higher without the guidance of expert professionals (Colado and García-Massó 2009). Another aspect to consider in injury prevention among children and adolescent athletes is overtraining. The overtraining

syndrome is a state of chronic fatigue with manifestations of chronic muscle or joint pain, personality changes, elevated resting heart rate, and decreased sports performance (Budgett 1998). Therefore, training programs can't be designed for all children, in the same way, even more so considering that there are certain specific health situations that, in many cases, require consultation with a medical professional before starting a resistance training program (Stricker et al. 2020). The initial evaluation of children is essential. The professionals who prescribe and supervise the training must observe that the children demonstrate competence in postural control and balance and accept and follow instructions. Subsequently, the principles for planning resistance training in children and adolescents (PROCESS) are relevant to achieving the objective. These principles suggest progression (gradual increase in the demands), regularity (continuous participation: 2–3 days per week), overload (stress the body beyond what it is accustomed to), creativity (variety and novelty in exercises), enjoyment (facilitation of prolonged participation), socialization (interact with others in a positive and supportive manner, meeting other people to work together towards a common goal), and supervision (safe exercise environment) (Avery D Faigenbaum and McFarland 2016).

Resistance training programs help increase daily levels of spontaneous activity in school-aged, implying that resistance training encourages children to become more active (Stricker et al. 2020). Training age, current strength level, motor skill competency, and psychological and maturational characteristics must be considered in resistance training programs (Drenowatz and Greier 2018). The adaptations of resistance training variables to the needs of children and adolescents will enhance motor skill performance, balance, core strength, and muscle power as part of an integrated training program. Resistance training programs integrating different elements of physical fitness are most likely to enhance sports performance and adherence to physical activity practice and active lifestyle (Faigenbaum and Myer 2010a, b, c). Fundamental movement skills (FMS) should be taught during preschool and primary school when children are at the proper age to learn locomotors, object control, and stability skills (Faigenbaum et al. 2011). According to several models, good training at the correct intervals throughout biological maturation can optimize healthy athletic development. One of the models used is the Long-Term Athlete Development (LTAD) (Balyi and Hamilton 2004), which proposes five general motor abilities to help enhance specific physical fitness components: suppleness (flexibility), speed, skills (coordination), endurance, and strength, and proposed sensitive periods based on biological and chronological age for boys and girls. The entire childhood can be considered a sensitive period for mastering fundamental movement skills (Balyi and Hamilton 2004). However, no evidence failing to take advantage of these “windows of opportunity” through proper training would impact physical performance (Ford et al. 2011). Despite the acceptance of the LTAD model, research has shown that chronological age is not a reliable indicator for training athletes between the ages of ten to sixteen. There are broad variations in the physical, cognitive, and emotional development within this age group. For this reason, individualization is the essential component of an effective training program (Ford et al. 2011). In addition, some research questions the validity of the sensitive periods in the LTAD model (Ford et al. 2012; Van Hooren and De Ste Croix 2020; De Ste Croix 2007; Van Hooren and De Ste Croix 2020) and propose that the training model should have a holistic orientation for training all physical attributes during the developmental stages rather than focusing on a single skill at a particular period (Ford et al. 2011). The LTAD model focuses on elite performance and winning athletes rather than sports participation per se to get the potential to improve the quality of life during childhood and adulthood. In contrast, the model presented by Bailey and coworkers termed the Three World Continuum proposes that the reason for participating in sports and physical activity should dynamically flow between different but interrelated involvement motives throughout life (Bailey et al. 2010). The model suggests that excellence can be considered in three different worlds. The

first two worlds are found in competitive sports, called elite referenced excellence (ERE), where achievement is measured against others, and personal referenced excellence (PRE), where achievement is related to improving personal bests. The third sports world corresponds to participation for personal well-being (PPW), which aims to perform physical activity to satisfy different individual needs (Collins et al. 2012). This model establishes the need for a continuum between the three worlds of sports participation to optimize physical activity throughout life. However, individual variation in maturation and athletic development makes it difficult to identify which child will achieve ERE compared to PRE or PPW and predict dropout during adolescence or throughout life (Ford et al. 2012).

Finally, The Youth Physical Development (YPD) model encompasses athletic development from early childhood (2 years of age) up to adulthood (21+ years of age) (Lloyd and Oliver 2012). The YPD model set down that during prepubescence, strength, fundamental movement skill (FMS), speed, and agility should be the principal physical qualities targeted. Once the child reaches adolescence, additional components (sport-specific skill, power, and hypertrophy) become important targets owing to the increased androgenic hormones associated with this stage of development. However, the individualization of the model should be considered when dealing with athletes of different sex, maturity status, and training history (Lloyd and Oliver 2012). The YPD model shows that the development of muscular strength should be prioritized at all stages of development for both males and females based on the observed association between muscular strength and all other fitness components, including FMS (Behringer et al. 2011). Muscle strength should be included in training programs to improve performance and reduce the risk of sports-related injuries (Faigenbaum et al. 2009a, b).

5 Conclusion

The effects of resistance training on children and adolescents are clear and provide a wide range of benefits. However, qualified professionals must be available since exercise prescription and subsequent supervision must follow the abilities, needs, and interests of this specific population. Beyond the goal of gains in muscular strength, muscular power, and muscular endurance, the physical demands of training must be balanced with the individual needs of children and adolescents to encourage continued interest in daily physical activity. Therefore, the pedagogical approach must respond effectively to individual developmental needs and learning styles.

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HSF1 and Its Role in Huntington's Disease Pathology

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Abstract

Purpose of review: Heat shock factor 1 (HSF1) is the master transcriptional regulator of the heat shock response (HSR) in mammalian cells and is a critical element in maintaining protein homeostasis. HSF1 functions at the center of many physiological processes like embryogenesis, metabolism, immune response, aging, cancer, and neurodegeneration. However, the mechanisms that allow HSF1 to control these different biological and pathophysiological processes are not fully understood. This review focuses on Huntington's disease (HD), a neurodegenerative disease characterized by severe protein aggregation of the huntingtin (HTT) protein. The aggregation of HTT, in turn, leads to a halt in the function of HSF1. Understanding the pathways that regulate HSF1 in different contexts like HD may hold the key to understanding the pathomechanisms underlying other proteinopathies. We provide the most current information on HSF1 structure, function, and regulation, emphasizing HD, and discussing its potential as a biological target for therapy.

Data sources: We performed PubMed search to find established and recent reports in HSF1, heat shock proteins (Hsp), HD, Hsp inhibitors, HSF1 activators, and HSF1 in aging, inflammation, cancer, brain development, mitochondria, synaptic plasticity, polyglutamine (polyQ) diseases, and HD.

Study selections: Research and review articles that described the mechanisms of action of HSF1 were selected based on terms used in PubMed search.

Results: HSF1 plays a crucial role in the progression of HD and other protein-misfolding related neurodegenerative diseases. Different animal models of HD, as well as postmortem brains of patients with HD, reveal a connection between the levels of HSF1 and HSF1 dysfunction to mutant HTT (mHTT)-induced toxicity and protein aggregation, dysregulation of the ubiquitin-proteasome system (UPS), oxidative stress, mitochondrial dysfunction, and disruption of the structural and functional integrity of synaptic connections, which eventually leads to neuronal loss. These features are shared with other neurodegenerative diseases (NDs). Currently, several inhibitors against negative regulators of HSF1, as well as HSF1 activators, are developed and hold promise to prevent neurodegeneration in HD and other NDs.

Conclusion: Understanding the role of HSF1 during protein aggregation and neurodegeneration in HD may help to develop therapeutic strategies that could be effective across different NDs.

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Keywords

Aggregation · Heat shock factor (HSF1) · Heat shock proteins (Hsp) · Huntington's diseases (HD) · Mitochondria

Abbreviations

17-AAG	17-allylamino-17-demethoxygeldanamycin
17-DMAG	17-dimethylaminoethylamino-17-demethoxygeldanamycin
8-OHDG	8-hydroxydeoxyguanosine
AD	Alzheimer's disease
AKT	Protein kinase B
ALFY	Autophagy-linked FYVE
ALS	Amyotrophic lateral sclerosis
AMPAR	α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptor
AMPK	5′-AMP-activated protein kinase
AOO	Age of onset
AR	Androgen receptor
ATF3	Activating transcription factor 3
Atro	ATROPHIN-1
BAG3	BCL-2–associated athanogene 3
BAX	BCL2-associated X
BDNF	Brain-derived neurotrophic factor
CACNA1A	α1A subunit of the voltage-dependent calcium channel Cav2.1
caHSF1	Constitutive active form of HSF1
CCT	Chaperonin containing TCP-1
CHIP	C-terminus of HSC70-interacting protein
ChIP-seq	Chromatin immunoprecipitation followed by sequencing
CK2	Casein kinase holoenzyme
CK2α'	CK2 α prime
CRE	cAMP-response elements
DBD	DNA binding domain
Dclk1	Doublecortin-like kinase 1
DDL-1	DAF16-dependent longevity-1
DRP-1	Dynamin-related protein 1
DRPLA	Dentatorubral-pallidoluysian atrophy
EF3	Elongation factor 3
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase
ETC	Electron transport chain
FBXW7	F-box and WD repeat domain containing 7
FILIP-1 L	Filamin A interacting protein 1-like
FRET	Fluorescence resonance energy transfer
FUS/TLS	Fused in sarcoma/translocated in liposarcoma
GCN5	General control non-repressed protein 5
GLT1	Glutamate transporter 1
GPX	Glutathione peroxidases
GSH	Glutathione
GSK3β	Glycogen synthase kinase 3β
HD	Huntington's disease
HDAC6	Histone deacetylase 6
HIF-1	Hypoxia-inducible factor 1
HMOX1	Heme oxygenase 1
HR-A/B	Heptad repeat-A/B
HSE	Heat shock element
HSFs	Heat shock transcription factors
Hsp	Heat shock protein(s)
HSR	Heat shock response
HTRA2/OMI	High-temperature requirement protein A2
HTT	Huntingtin
HuR	Hu-antigen R
IGF-1R	Insulin/insulin-like growth factor 1 receptor
IL-6	Interleukin-6
ILS	Insulin/insulin-like signaling
LZ	Leucine zipper domains
MEK	Mitogen-activated protein kinase kinase
MK2	MAPK-activated protein kinase 2
MMP	Mitochondrial membrane potential
MnSOD	Manganese-containing SOD
MPNST	Malignant peripheral nerve sheath tumor
mPTP	Mitochondrial permeability transition pore

MSNs	Medium-sized spiny neurons
N17	17-amino acid-long N-terminus
NDs	Neurodegenerative diseases
NEDD4	Neuronal precursor cell-expressed developmentally downregulated 4
NES	Nuclear export signal
NF1	Neurofibromatosis type 1
NF-IL6	Nuclear factor for interleukin-6
NLS	Nuclear localization sequence
NMDAR	N-methyl-D-aspartic acid receptor
NMP	Neuronal-specific 20S membrane proteasome complex
NOS	Nitric oxide synthase
NRF2	Nuclear factor E2-related factor 2
OD	Oligomerization domains
PD	Parkinson's disease
PGC-1α	Peroxisome proliferator-activated receptor-γ coactivator-1α
PIM2	Proviral integrations of Moloney virus 2
PLK1	Polo-like kinase 1
polyP	Polyproline
polyQ	Polyglutamine
PP2A	Protein phosphatase 2A
PR	Proteostasis regulators
PRDX	Peroxiredoxins
PSA-NCAM	Polysialylated-neural cell adhesion molecule
PTMs	Post-translational modifications
PUMA	p53 upregulated modulator of apoptosis
RD	Regulatory domain
RNS	ROS/reactive nitrogen species
ROS	Reactive oxygen species
SAE	SUMO-activating enzyme
SBMA	Spinal and bulbar muscular atrophy
SCAs	Spinocerebellar ataxias
SIRT1	Sirtuin 1
SOD	Superoxide dismutase
SQSTM1	Sequestosome 1
TAD	Transactivation domain
TARDBP	TAR DNA binding protein
TauT	Taurine transporter
TBP	TATA-box binding protein
TNF-α	Tumor necrosis factor-α
TOR1	Target of rapamycin 1
TPR	Translocated promoter region
TRiC	T-complex protein-1 ring complex
UPS	Ubiquitin proteasome system
USP19	Ubiquitin-specific protease 19
VCP	Valosin-containing protein
αB-crys	AlphaB-crystallin

1 Summary

Living organisms experience acute or chronic exposure to different endogenous and environmental insults during life, including elevated temperature, oxidative stress, and proteotoxic conditions. Cells have developed different self-defense mechanisms against such stressors to ensure cell survival. One of the most potent and successful mechanisms is the so-called heat shock response (HSR), characterized by the rapid induction of a group of molecular chaperones and heat shock proteins (Hsp) that fight the harmful effects of different stressors on proteins structure and function (Akerfelt et al. 2010; Anckar and Sistonen 2011). There is a regulation in the transcriptional activation of Hsp genes by a family of specialized heat shock transcription factors (HSFs) in eukaryotes. HSFs participate in many processes, including protein homeostasis, aging, innate immunity, and metabolism, and have a fundamental role in physiology and diseases like cancer and neurodegeneration. This chapter will focus on HSF1, the main HSF responsible for coordinating and executing the HSR in mammals. We will discuss fundamental features of HSF1, including structure, regulatory mechanisms, and physiological functions that go beyond the HSR, with particular emphasis on the neurodegenerative disease Huntington's disease (HD).

2 Introduction

HSF1 is a multifaceted factor, traditionally known for coordinating the cellular response to internal and external stimuli that disrupt cellular protein homeostasis (Akerfelt et al. 2010; Gomez-Pastor et al. 2018). This response is usually

mediated by the transcriptional regulation of several Hsp that have the task to prevent protein misfolding, refold misfolded proteins, and target damaged proteins for degradation (Ellis 2007). However, in the last few years, we have learned that HSF1 is much more complex, and it participates in numerous biological processes in both physiology and disease (Gomez-Pastor et al. 2018).

In unstressed cells, HSF1 exists as an inactive monomer in the cytoplasm due to the interaction by several regulatory proteins, including Hsp70, Hsp40, Hsp90, and the chaperonin complex TRiC (Gomez-Pastor et al. 2018). When cells encounter stress, HSF1 is released from its repressors and is activated. This activation process requires trimerization, several post-translational modifications (PTMs), and translocation to the nucleus where it binds to target genes. The transcriptional response elicited by HSF1 will then depend on the type of stress and the different protein-protein interactions in which HSF1 participates (Gomez-Pastor et al. 2018; Prince et al. 2020; Burchfiel et al. 2020). HSF1 recognizes a specific sequence in its target genes, composed of inverted repeats of a nGAAn sequence, called heat shock element (HSE) (Akerfelt et al. 2010; Vihervaara et al. 2013). Different studies conducting HSF1 chromatin immunoprecipitation followed by sequencing (ChIP-seq) revealed that numerous target genes contain HSE in their promoter and intergenic regions, but a subset of them do not (Korfanty et al. 2014; Riva et al. 2012; Vihervaara et al. 2017). However, it is important to note that the presence of an HSE within any given gene does not assure HSF1 binding. Another layer of complexity is that the expression of target genes of HSF1, with or without specific HSEs, can also be influenced by epigenetic modification of chromatin (Guertin and Lis 2010; Vihervaara et al. 2017). These studies imply that the regulatory events that control which set of genes are induced by HSF1 at any given time are much more complicated than we have previously anticipated. HSF1 is known for its role as a transcriptional activator. However, numerous studies confirmed its role in repressing transcription, i.e., interleukin-6 (IL-6) involved in inflammation and the microtubule-associated protein Tau involved in synaptic dysfunction in different tauopathies (Inouye et al. 2007; Kim et al. 2017).

HSF1 has been studied widely in the context of the HSR and proteotoxic stress, but its role is not limited to just regulating Hsp expression (Gomez-Pastor et al. 2018). Recent evidence now points to HSF1 influencing the expression of multiple genes that are essential for cell cycle regulation, glucose metabolism, inflammatory response, and development and maintenance of neuronal, reproductive, and sensory organs (Akerfelt et al. 2007; Nakai 2009; Page et al. 2006; Singh and Hasday 2013). Therefore, defects in the activity and levels of HSF1 result in devastating consequences. More studies now confirmed the role of HSF1 in age-related and neurodegenerative disorders like Alzheimer's (AD), Parkinson's (PD), and HD and its potential as a therapeutic target (Goetzl et al. 2015; Gomez-Pastor et al. 2017; Jiang et al. 2013; Khalsa 2015; Kim et al. 2016; Kozuki et al. 2011; Lee et al. 2014; Neef et al. 2010; Pierce et al. 2013; Soncin et al. 2003). Different molecules, i.e., Hsp90 inhibitors and proteotoxic stress inductors, have shown efficacy in activating HSF1 and ameliorating some neurodegeneration features in mouse models. Unfortunately, our incomplete understanding of the roles of HSF1 in the brain and the lack of direct activators of HSF1 that can penetrate the blood-brain barrier have negatively affected the translational potential of HSF1. Future studies need to address this need.

In this chapter, we have specifically focused on the pathological role of HSF1 in HD, a devastating neurodegenerative disease caused by a CAG repeat expansion in the HTT gene (Bates et al. 2015; MacDonald et al. 1993; Novak and Tabrizi 2010). A selective vulnerability characterizes HD with degeneration and death of medium spiny neurons (MSN) in the striatum (Gonitel et al. 2008; Goula et al. 2012; Kennedy et al. 2003; Lee et al. 2011; Mitchell and Griffiths 2003; Pickrell et al. 2011; Shelbourne et al. 2007) and deficits in behavioral, cognitive, and motor features (Group 1996; Kieburtz et al. 2001; Novak and Tabrizi 2010). Cumulative evidence

shows that HSF1 plays a crucial role in ameliorating disease progression in HD. Recent research has shown the inappropriate degradation of HSF1 in HD, exacerbates HTT aggregation and neuronal death (Gomez-Pastor et al. 2017). These studies also revealed a potential implication of HSF1 in regulating genes with synaptic functions and new avenues for controlling the levels of HSF1 in the brain.

Here, we will explore the fundamental features of HSF1, including the structure and regulatory mechanisms, and its implication in physiological and pathological conditions, mainly focusing on the role in the regulation of Hsp expression, proteasome-mediated degradation of abnormal proteins, oxidative stress, mitochondrial dysfunction, excitotoxicity, and synaptic function in HD.

3 Introduction to Heat Shock Factor 1

3.1 HSF1: Structure, Function, and Regulation

The human HSF family consists of six members: HSF1, HSF2, HSF4, HSF5, HSFX, and HSFY (Gomez-Pastor et al. 2018). Each of them possesses specialized tissue distribution, functions, and regulation. Among them, HSF1 is the most studied HSF due to its relevance during the stress response and cell survival (Akerfelt et al. 2010). The human HSF1 gene is located on chromosome 8q24 and is translated into 529 amino acids with a predicted molecular weight of 57 kDa. However, due to several PTMs (discussed below), the actual molecular size reaches approximately 75 kDa. HSF1 is composed of 4 functional domains distributed from N-terminus to C-terminus as follows: DNA binding domain (DBD), oligomerization domains (OD) composed of 4 leucine zipper domains (LZ1–3 and LZ4), regulatory domain (RD), and a transactivation domain (TAD) (Fig. 1).

The DBD, which is highly conserved within the human HSF family, binds to the major groove of the DNA by recognizing repeating units of a pentameric sequence motif (nGAAn, where n is any base) named HSE. Recent comprehensive ChIP-seq demonstrated that the architecture of HSEs is very diverse in the human genome, with deviations from the consensus sequence in the spacing, orientation, and extent of HSE repeats (Mahat et al. 2016; Pincus et al. 2018; Vihervaara et al. 2017; Vihervaara et al. 2013). These deviations can influence HSF1 DNA binding efficacy and the kinetics and magnitude of target gene expression. Several studies over the last decade have shown different types of HSE that can be classified as canonical and non-canonical HSE. Vihervaara et al. demonstrated that HSF1 prefers binding to triple inverted nGAAn pentamers (canonical HSE) in both mitotic and cycling human erythroleukemia K562 cells (Vihervaara et al. 2013). Studies in purified HSF1 using fluorescence polarization and thermal denaturation profiling showed that HSF1 prefers binding to extended HSE sequences. The HSE placement is on a head-to-head or tail-to-tail orientation (Jaeger et al. 2014). Studies from *S. cerevisiae* showed two types of non-canonical HSE, gap- and step-type HSE. In gap-type HSE, two nGAAn repeats are followed by a gap of 5 bp block and another repeat (nGAAnnTTCn(5 bp)nGAAn) (Morano et al. 2012), whereas, in step-type HSE, three of each nGAAn repeats are interrupted by 5-bp block (nGAAn(5 bp)nGAAn(5 bp)nGAAn) (Morano et al. 2012; Yamamoto et al. 2005). Recent studies using *C. albicans* found that GAAnnTTC and TTCn7TTC are another non-canonical binding site where HSF1 regulates virulence genes' expression (Leach et al. 2016). Different mechanisms can explain preferred binding to different HSEs, including sequence orientation and oligomerization with different HSFs (Jaeger et al. 2014, 2016; Korfanty et al. 2014). HSF1 and HSF2 can form heterotrimers in vitro and in vivo and cooperate under different stressful and physiological conditions to bind different HSEs (Jaeger et al. 2016; Korfanty et al. 2014; Sandqvist et al. 2009). However, the full implications of different HSF1-HSF2 oligomers' combination on tissue-specific genome regulation are still unknown.

The OD is composed of two amphiphilic helices, heptad repeat HR-A/B or leucine zipper

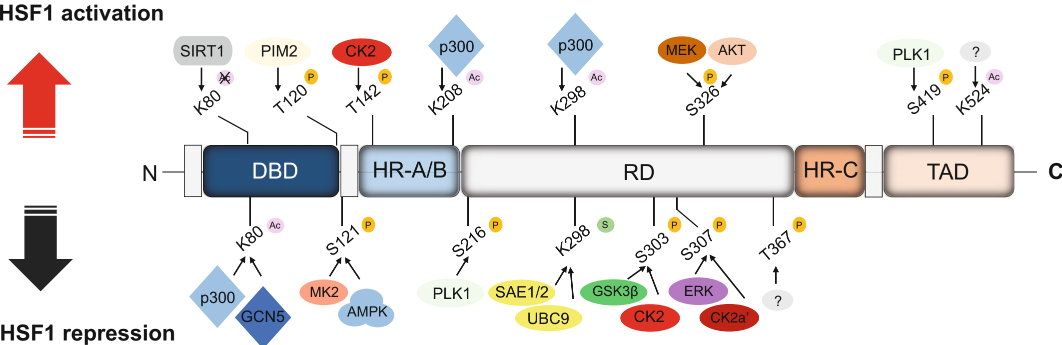

Fig. 1 Diagram of structural domains, regulatory enzymes, and PTMs of human HSF1. HSF1 can be divided into different structural domains: DBD (DNA binding domain), HR-A/B (heptad repeat-A/B), RD (regulatory domain), and TAD (transactivation domain). PTMs located at the top part of HSF1 are modifications with positive regulatory properties (red arrow), whereas PTMs located at the (bottom) represent modifications with repressive properties (black arrow). The different enzymes responsible for positive PTMs are SIRT1 (sirtuin 1), PIM2 (proviral integrations of Moloney virus 2, Pim-2 proto-oncogene, Ser/Thr kinase), CK2 (casein kinase holoenzyme), p300 (histone acetyltransferase p300), MEK (mitogen-activated protein kinase kinase), AKT (protein kinase B), and PLK1 (polo-like kinase 1). The enzymes responsive for the repressive PTMs are p300, GCN5 (general control non-repressed protein 5 histone acetyltransferase), MK2 (MAPK activated protein kinase 2), AMPK (5′-AMP-activated protein kinase), PLK1, SAE1/2 (SUMO-activating enzyme), UBC9 (RING-type E3 SUMO transferase), GSK3β (glycogen synthase kinase 3β), CK2, CK2α' (catalytic subunit CK2 holoenzyme), and ERK (extracellular signal-regulated kinase). *Ac* acetylation, *P* phosphorylation, and *S* sumoylation

LZ1–3, which consist of a repeating pattern of seven hydrophobic and charged amino acid residues and it is critical in the activation of HSF1. HSF1 exists in monomeric and oligomeric (mostly trimer) states, and transitions between these two states are critical for HSF1 activation. Interactions between HR-A/B and other heptad repeats HR-C (or LZ4), located between the RD and TAD, regulate oligomerization of HSF1. Under non-stressful conditions, HR-A/B and HR-C are permanently bound by intramolecular coiled-coil interactions forcing the protein to be in a monomeric state (Chen et al. 1993; Nakai et al. 1997; Rabindran et al. 1993). Different conditions can disrupt the interaction between HR-A/B and HR-C, allowing HSF1 trimerization. Recent research has shown that HSF1 possesses an intrinsic capacity to sense temperature and that HSF1 monomers exposed to different temperatures can promote HR-A/B and HR-C dissociation (Hentze et al. 2016). However, other mechanisms described below have shown the ability to regulate oligomerization, such as PTMs and interactions with different regulatory proteins (reviewed in (Gomez-Pastor et al. 2018)). Trimerization is thought to be a prerequisite for the transcriptional activity of HSF1 and induction of Hsp (Farkas et al. 1998; Lu et al. 2008, 2009; Orosz et al. 1996). However, recent studies using a mutant form of HSF1 lacking the OD (HSF1Δ156–226) showed that, although HSF1 is unable to trimerize, this form was capable of protecting cells exposed to proteotoxic conditions (Qu et al. 2018; Verma et al. 2014), suggesting the existence of alternative mechanisms to control HSF1 activation not described yet.

The RD is a highly unstructured domain targeted by many different PTMs (Green et al. 1995; Guo et al. 2001), which allow the RD to provide activating or repressing functions to HSF1 (Newton et al. 1996). Finally, the TAD located in the C-terminus is responsible for the transcriptional activation of target genes (Green et al. 1995; Newton et al. 1996). Recent HSF1 and HSF2 crystal structure studies have revolutionized the way we think about HSF binding to DNA and have opened the door for new

investigations regarding HSF DNA-binding regulation (Jaeger et al. 2016; Neudegger et al. 2016). Previous models suggested that the position of HSF1-DBD was so that the rest of the protein precluded access to the DBD once bound to the DNA, therefore limiting the interaction between the DBD and other potential regulatory proteins. Jaeger et al. and Neudegger et al. independently proposed a new HSF-DNA binding model in which the DBD wraps around the DNA, forcing the rest of the protein to be oriented in the opposite direction to the DBD and therefore leaving the DBD exposed for interaction with other potential regulators (Jaeger et al. 2016; Neudegger et al. 2016) (Fig. 2).

As stated above, the HSF1 activation/attenuation mechanism is highly influenced by its structural conformation and different protein-protein

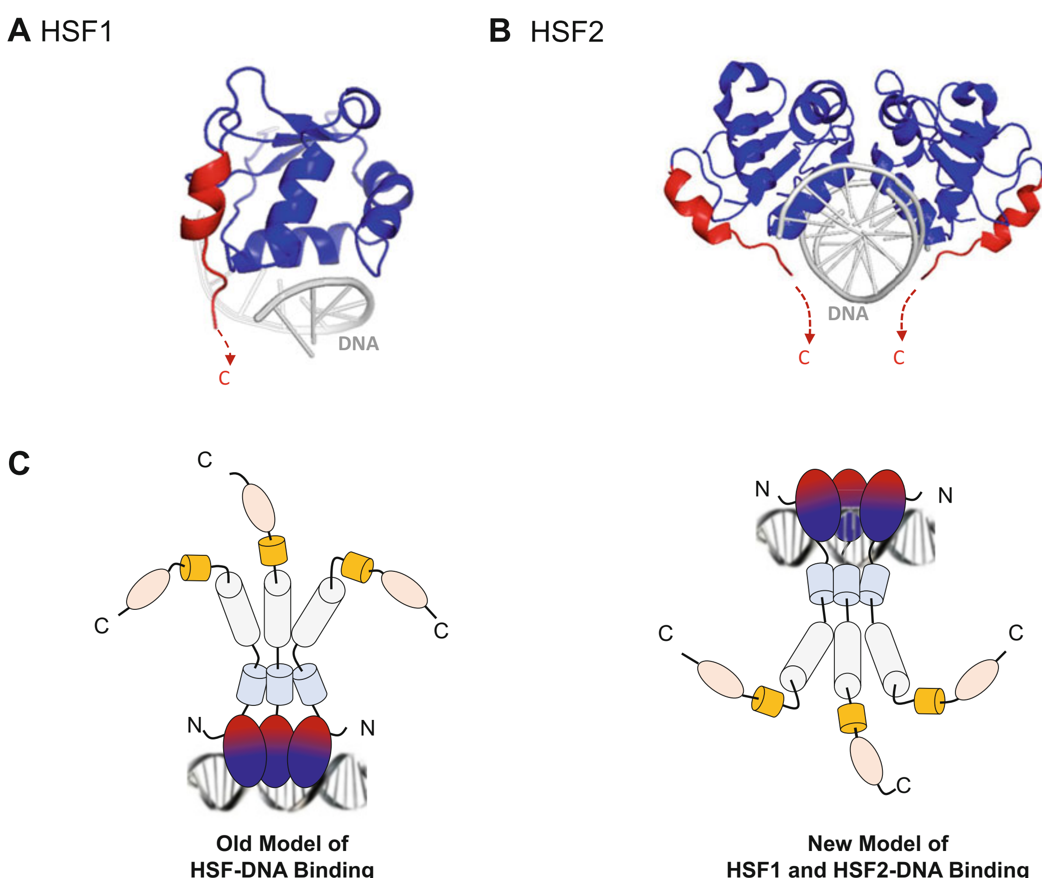

Fig. 2 Structural insights into HSF-DNA interaction topology. (**a**, **b**) Crystal structure of the DNA binding domain (DBD) of HSF1 and HSF2 bound to a two-site HSE as a dimer. These independently solved structures revealed that a previously unknown carboxy-terminal helix (red) that is conserved in both HSF1 and HSF2 directs these HSFs to wrap around the HSE DNA, resulting in a topology where the DBD and the remainder of the HSF1 protein (not present in the crystal structure) occupy opposite sides of the DNA. (**c**) A new model of the HSF-DNA interaction. Structural studies support a model in contrast with the previous model for the topology of DNA-bound HSF oligomers. In the old model (left), the oligomerization domains (light blue) were positioned on top of the DBD, such that the rest of the protein buried the free surface of the DBD (shown in red, in contrast to the DNA-bound portion of the DBD shown in blue). In the new model (right), this free surface of the DBD is solvent-exposed, making it available for interactions with regulatory proteins and accepting PTMs. (Figure adapted from (Gomez-Pastor et al. 2018) with authors' permission)

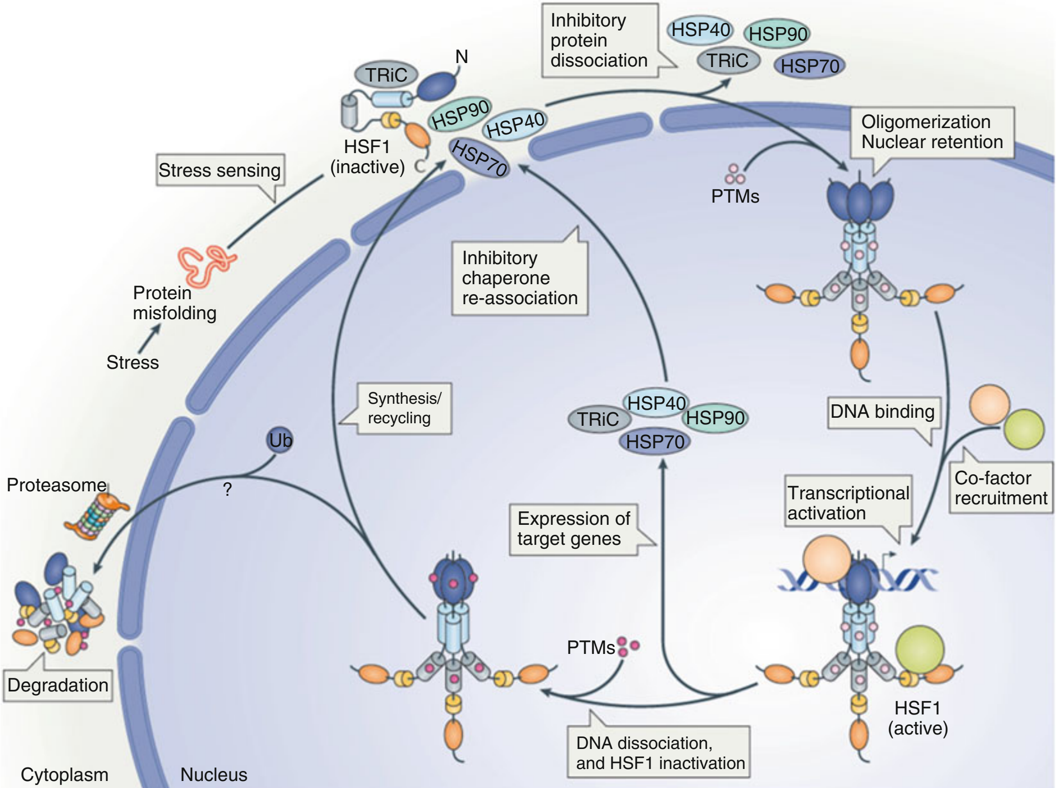

Fig. 3 HSF1 activation/attenuation cycle. In response to proteotoxic stress conditions, HSF1 is subject to a multi-step activation and attenuation cycle. Inactive HSF1 monomer is kept in the cytoplasm in a complex with regulatory proteins such as Hsp 40, 70, and 90, as well as the cytosolic chaperonin TCP1 ring complex (TRiC). Upon stress sensing, HSF1 is modified by several activating PTMs that promote DNA binding and transcriptional activation of target genes in concert with cofactor recruitment. HSF1 is then modified by different inhibitory PTMs and by p23 causing DNA dissociation, HSF1 inactivation, and HSF1 degradation (see Fig. 1) for PTMs details). It is currently unknown where HSF1 degradation occurs and the extent to which HSF1 is newly synthesized or recycled into the cytoplasm. Ultimately, after attenuation, HSF1 is maintained in the cytoplasm by an inhibitory protein complex in a negative feedback mechanism. Color code: DNA-binding domain (dark blue), leucine zipper oligomerization domain LZ1–3 (light blue), regulatory domain (gray-white), LZ4 (yellow), and activation domain (orange). (Figure adapted from (Gomez-Pastor et al. 2018) with authors' permission)

interactions (Fig. 3). Under non-stressful conditions, HSF1 exists in the cytoplasm as an inactive monomeric form due to intramolecular interactions between the HR-A/B and HR-C and direct protein-protein interactions with several inhibitory complexes. One of them is an auto-regulatory complex induced by HSF1-regulated proteins such as Hsp90, Hsp70, Hsp40, and T-complex protein-1 ring complex (TRiC)/chaperonin containing TCP-1 (CCT) (Akerfelt et al. 2010; Anckar and Sistonen 2011; Neef et al. 2014; Zheng et al. 2016). Previous research has shown that Hsp90 can inhibit HSF1 oligomerization and DNA binding (Zou et al. 1998), whereas Hsp70 and its co-chaperone Hsp40 regulate HSF1 transactivation by interacting with the TAD (Gomez et al. 2008; Shi et al. 1998). The cytosolic chaperonin TRiC/CCT also inhibits HSF1 activation by direct interaction with HSF1, although the exact mechanism by which TRiC/CCT inhibits HSF1 is not fully characterized (Akerfelt et al. 2010; Neef et al. 2014). Other repressive hetero-complexes include 14–3-3, histone deacetylase 6 (HDAC6), and the valosin-containing protein (VCP) that ultimately tune HSF1 activation (Pernet et al.

2014). Upon stress, there is a liberation of HSF1 from these repressive complexes allowing HSF1 trimerization and accumulation into the nucleus where HSF1 interacts with a different set of regulatory proteins that assist HSF1-DNA binding and transcriptional activation of its target genes (Amin et al. 1988; Pelham 1982; Sorger and Pelham 1988). HSF1 transcriptional activation results in increased levels of Hsp and other repressive proteins that hinder HSF1 activation by a negative feedback mechanism after the stress has subsided. Among these additional repressive proteins, filamin A interacting protein 1-like (FILIP-1 L) promotes HSF1 poly-ubiquitination and degradation by recruiting hHR23A, a ubiquitin receptor protein functioning as a transferrer of ubiquitinated proteins to the 19S proteasome (Hu and Mivechi 2011). In mammalian cells, FILIP-1 L forms complexes with HSF1 and Hsp72, and its ectopic overexpression reduces HSF1 protein levels leading to inhibition of HSF1-mediated transcription. However, the biological function of FILIP-1 L and the regulatory mechanisms responsible for HSF1-FILIP-1 L interaction are still unclear. Below we will discuss other regulatory events responsible for controlling HSF1 activity and stability in different contexts.

3.2 HSF1 PTMs: Pathophysiological Implications

The levels of HSF1 do not usually vary during stress-induced activation. In contrast, HSF1 undergoes numerous PTMs, including phosphorylation, sumoylation, and acetylation, that establish a complex code responsible for controlling every step of the HSF1 activation/attenuation cycle. Overall, HSF1-PTMs are classified into two main functions: positive and negative regulatory PTMs (Fig. 1). It is essential to mention that the enzymes responsible for HSF1 modifications and their effects are entirely dependent on the context. One example of the versatile-PTMs and the effects that they cause on HSF1 is led by polo-like kinase 1 (PLK1). During early mitosis, HSF1 is phosphorylated at Ser216 by PLK1, leading to HSF1 ubiquitin-dependent degradation by the SCFβTrCP E3 ligase (Lee et al. 2008). This event is critical to ensure mitosis progression. However, under heat shock conditions, PLK1 phosphorylates Ser419 and regulates HSF1 nuclear translocation. Similarly, protein kinase CK2 (casein kinase holoenzyme) regulates Thr142 phosphorylation and HSF1 DNA binding activation under heat shock conditions, but it controls phosphorylation of Ser303/307 and HSF1 degradation in the presence of pathogenic huntingtin (HTT) aggregates (Gomez-Pastor et al. 2017; Soncin et al. 2003).

Dozens of HSF1-PTM descriptions have been looked at under different experimental conditions, especially under heat shock. They have recently been reviewed in Gomez-Pastor et al. (2018), and their summaries can be seen in Fig. 1. This chapter will focus on some of those HSF1 PTMs that have relevance in pathophysiological stages. A fundamental set of PTMs that seem to control HSF1 activity and stability in different pathological conditions is Ser303 and Ser307 phosphorylation. In many cancer cells, there is a dramatic alteration in the levels of HSF1, contributing to cell proliferation and tumorigenesis (Jiang et al. 2015; Vydra et al. 2014). In melanoma cancer cells, the ubiquitin E3 ligase complex (Skp1-Cul1-F box) formed by the substrate-targeting subunit F-box and WD repeat domain containing 7 (FBXW7) interacts with and ubiquitylates HSF1. This interaction depends on Ser303/307 phosphorylation mediated by glycogen synthase kinase 3β (GSK3β) and extracellular signal-regulated kinase 1 (ERK1), respectively (Kourtis et al. 2015). Kourtis et al. suggested that reduced levels of FBXW7 or loss of function mutations present in many tumors may lead to increased levels of HSF1. However, a recent study in breast cancer cells showed that FBXW7 knockdown does not enhance HSF1 levels in those cells (Yang et al. 2019). Instead, elevated proviral integrations of Moloney virus 2 (PIM2) kinase phosphorylates HSF1 at Thr120, disrupting HSF1, and FBXW7 and promoting HSF1 accumulation (Yang et al. 2019). On the other hand, a very recent study by Gomez-Pastor et al. found that protein kinase CK2 α prime (CK2α') also phosphorylates Ser303/307 in HD (Gomez-Pastor et al. 2017).

CK2α’ is upregulated in HD, leading to increased FBXW7-dependent HSF1 degradation. Recently Jin et al. generated a knock-in mouse model where Ser303/307 were mutated to Ala and showed that HSF1 levels were stabilized and increased compared to wild-type mice (Jin et al. 2018). However, these mice presented age-dependent obesity, fatty liver disease, and insulin resistance, suggesting that phosphorylation of Ser303/307 may exert a positive effect in specific situations.

In general, phosphorylation of residues with inactivation functions is reduced in tumor cells, contributing to the hyperactivation of HSF1 previously reported in cancer. Mitogen-activated protein kinase kinase (MEK)-mediated Ser326 phosphorylation causes the stabilization of HSF1 by preventing it from poly-ubiquitination and subsequent proteasomal degradation (Tang et al. 2015). Repressive Thr367 phosphorylation is also reduced in cancer, although the specific kinase involved in this modification has not been identified yet (Asano et al. 2016). Other phosphorylation with repressive functions reduced in cancer is Ser121 involved in Hsp90 binding, mediated by MAPK-activated protein kinase 2 (MK2) and the 5′-AMP-activated protein kinase (AMPK). By contrast, during metabolic stress, AMPK-mediated phosphorylation of Ser121 increases and dictates HSF1 nuclear localization and stability (Asano et al. 2016; Dai et al. 2015; Guettouche et al. 2005). While several studies have revealed essential PTMs that contribute to HSF1 activity and stability in cancer, our knowledge about the different PTMs that regulate the role of HSF1 in other different diseases is very limited. These studies are necessary to fully understand the mechanisms that regulate HSF1 function under pathological conditions and may identify novel therapeutic targets.

Intriguingly, phosphorylation also serves as a platform for additional PTMs like sumoylation. GSK3β-induced phosphorylation at Ser303 is a prerequisite for Lys298 sumoylation, conjugation of SUMO-2/3, which results in inhibition of the transactivating capacity of HSF1 (Hietakangas et al. 2003). This sumoylation is mediated by the E1 SUMO-activating enzymes SAE1/2 and E2 SUMO-conjugating enzyme UBC9. The authors demonstrated the existence of a phosphorylation-dependent sumoylation motif within HSF1 (ΨKxExxSP, where Ψ is a branched hydrophobic amino acid and x is any amino acid), which resembles a consensus SUMO site (ΨKxE), that is utilized to prime proteins as a SUMO substrate. In general, sumoylation is facilitated by the aid of an E3 ligase, which increases sumoylation efficiency either by accelerating SUMO transfer from UBC9 to the substrate or merely by providing scaffolding support (Brunet Simioni et al. 2009). Site-specific mapping of the human SUMO proteome has revealed co-modifications with phosphorylation and the presence of several sumoylated-Lys on HSF1 (Hendriks et al. 2017). However, their roles in HSF1 regulation under physiological conditions are still unknown.

Another modification influencing HSF1’s function is Lys acetylation. Considering the studies reported, acetylation within the DBD has adverse effects on HSF1-DNA interaction. Acetylation at Lys80 in the DBD by p300 shortens the time of HSF1 on DNA, reducing HSF1 activation (Westerheide et al. 2009). In agreement with this, deacetylation at Lys80 by NAD + -dependent sirtuin 1 (SIRT1) accelerates HSF1 activity by increasing HSF1 DNA occupancy. Interestingly, the recognition of SIRT1 is as a nutrient sensor and longevity factor. The gradual loss of SIRT1 during aging correlates with the dissipation of HSF1 and reduced HSR and protein homeostasis in aging (Akerfelt et al. 2010). Zelin et al. demonstrated that general control non-repressed protein 5 (GCN5) histone acetyltransferases target HSF1 Lys80 in the presence of p23 chaperone, which disrupts HSF1 DNA binding (Zelin et al. 2012). An additional study identified that overexpression of histone deacetylases HDAC 7, HDAC9, and SIRT1 stimulated heat-triggered HSF1 DNA binding (Zelin and Freeman 2015). On the other hand, acetylation of Lys residues within the RD contributes to preventing HSF1 degradation. A study conducted in HeLa cells showed that acetylation at Lys208 and Lys298 by p300 prevents HSF1 from proteasomal degradation, maintaining HSF1 stability (Raychaudhuri et al. 2014). Also, HDAC6 has been implicated in the activation of HSF1 by

repressing the Hsp90-HSF1 complex and promoting HSP gene expression (Boyault et al. 2007; Pernet et al. 2014). Future studies are warranted to demonstrate the relevance of these modifications in pathological stages in which there is a dysregulation in HSF1. A summary of the most relevant regulatory proteins based on the HSF1-PTMs they are responsible for, and their role in the regulation of HSF1, is shown in Fig. 4.

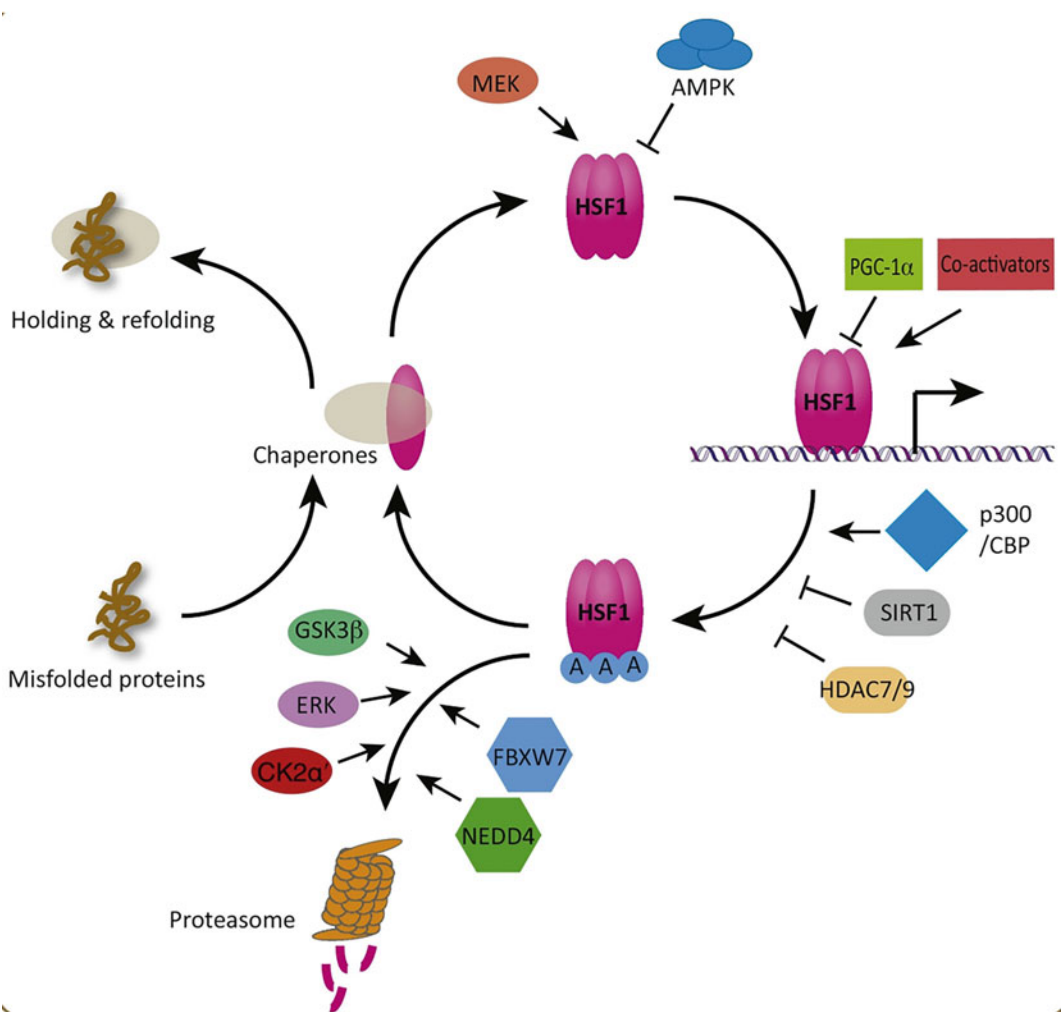

Fig. 4 HSF1-regulatory enzymes and their contribution in HSF1 activation cycle. Upon proteotoxic stress, misfolded proteins titrate away the Hsp repressive complex, allowing HSF1 trimerization and nuclear accumulation. The HSF1 trimer binds to HSE in the promoter region of HSF1 target genes (old model of HSF-DNA binding: see Fig. 2). HSF1 activation is modified by several PTMs (see Fig. 1). Enzymes responsible for controlling nuclear translocation and activation include MEK and AMPK (with opposite functions). PGC-1α and different co-activators modulate HSF1 transcriptional capacity. Enzymes like HDAC7, HDAC9, and SIRT1 prolong HSF1 binding to the DNA, while acetylation by p300/CBP has an opposite effect and mediate DNA dissociation. Ubiquitin proteasome-dependent HSF1 degradation occurs by E3 ligases FBXW7 and NEDD4. FBXW7 is recruited by phosphorylation of HSF1 mediated by GSK3β, ERK, and CK2α', whereas NEDD4 is accessed by p300/CBP-mediated acetylation (Figure reprinted from "Rethinking HSF1 in Stress, Development, and Organismal Health" by Li et al. 2017, with copyright permission from Elsevier. Figure legend has been modified accordingly for this publication)

4 Regulation of HSF1 and the HSR by Non-coding RNAs

Emerging evidence have shown that non-coding RNAs (ncRNAs) are actively involved in the regulation of HSF1 and the HSR (Place and Noonan 2014). A large class of small ncRNAs known as microRNAs (miRNAs) can regulate many biological processes by acting as post-transcriptional regulators of gene expression. Different miRNAs can bind in the 3'UTR of HSF1 altering its expression. miR-378 directly targets and represses the expression of HSF1 in cardiomyocytes (Jie Yuan et al. 2010) therefore affecting the induction of downstream HSPs in the heart while miR-608 operates as an HSF1 positive regulator in human breast cancer (Huang et al. 2012). Levels of HSF2 are also influenced by miRNAs (Björk et al. 2010; Cai et al. 2015). Other key determinant in the regulation of HSFs in human cells is the long non-coding RNAs (lncRNA). The lncRNA HSR1 (heat shock RNA-1), is upregulated during the HSR and plays an essential role in HSF1 trimerization and subsequent DNA binding activity forming a complex with the eukaryotic elongation factor 1A (eEF1A) (Shamovsky et al. 2006; Shamovsky and Nudler 2009). HSR1 is a foreign lncRNA derived from a bacterial genome, functioning as an exogenous auxiliary factor required for mammalian HSF1 activation upon stress conditions (Choi et al. 2015; Kim et al. 2010a, b).

HSF1 also regulates the expression of different non-coding RNAs (ncRNAs) involved in global suppression of transcription, translational processes, and protein aggregation (Lindquist 1986). Upon heat shock, HSF1 induces the expression of a class of lncRNAs known as Satellite III transcripts (Sat3) that accumulate at the site of transcription to form nuclear stress bodies (nSBs) (Jolly et al. 2004; Rizzi et al. 2004; Sengupta et al. 2009) and are known to co-localize with several RNA binding proteins and transcription factors such as HSF1 (Cotto et al. 1997; Jolly et al. 2004; Metz et al. 2004). Although knockdown of Sat3 transcripts does not affect HSF1 recruitment to the nSB-like structures, it has been recently demonstrated that Sat3 transcripts are essential for the recruitment of additional transcription regulators to the nSBs contributing to the heat-induced transcriptional silencing (Goenka et al. 2016). Transcription of the lncRNA satellite 2 (Sat2) is also strongly upregulated in the presence of heat shock in a HSF1-dependent manner (Tilman et al. 2012), and it has been involved in tumor progression (Tilman et al. 2012). Genome-wide studies revealed that HSF1 has also the ability to bind HSE present upstream of different miRNAs and regulate their expression under thermal stress (Srijit Das 2015). Interestingly, some of those HSF1 regulated miRNAs have shown inhibitory effects on HTT protein, and they are significantly depleted in HD, therefore contributing to increased mHTT expression and aggregation (Das and Bhattacharyya 2015). These studies demonstrate an integrated model of ncRNAs and HSF activity in the regulation of the HSR under physiological conditions and human diseases.

5 Role of HSF1 in Physiology and Disease

HSF1 is well known for its role in regulating the HSR, as discussed above. However, it has implications in many other processes, including aging, immune system maintenance, cancer, metabolic stress, neural development, and neurodegeneration. These functions can be accomplished by combining different protein-protein interactions and post-translational modifications that modulate HSF1 activity and stability and end up activating different transcriptional programs. Some of these functions are discussed below and summarized in Fig. 5.

5.1 HSF1 in Aging and Inflammation

For every single living cell, there is constant exposure to environmental and physiological stresses during its lifespan. Therefore, proper mechanisms are necessary to execute an adequate response to ensure cell survival. However, during

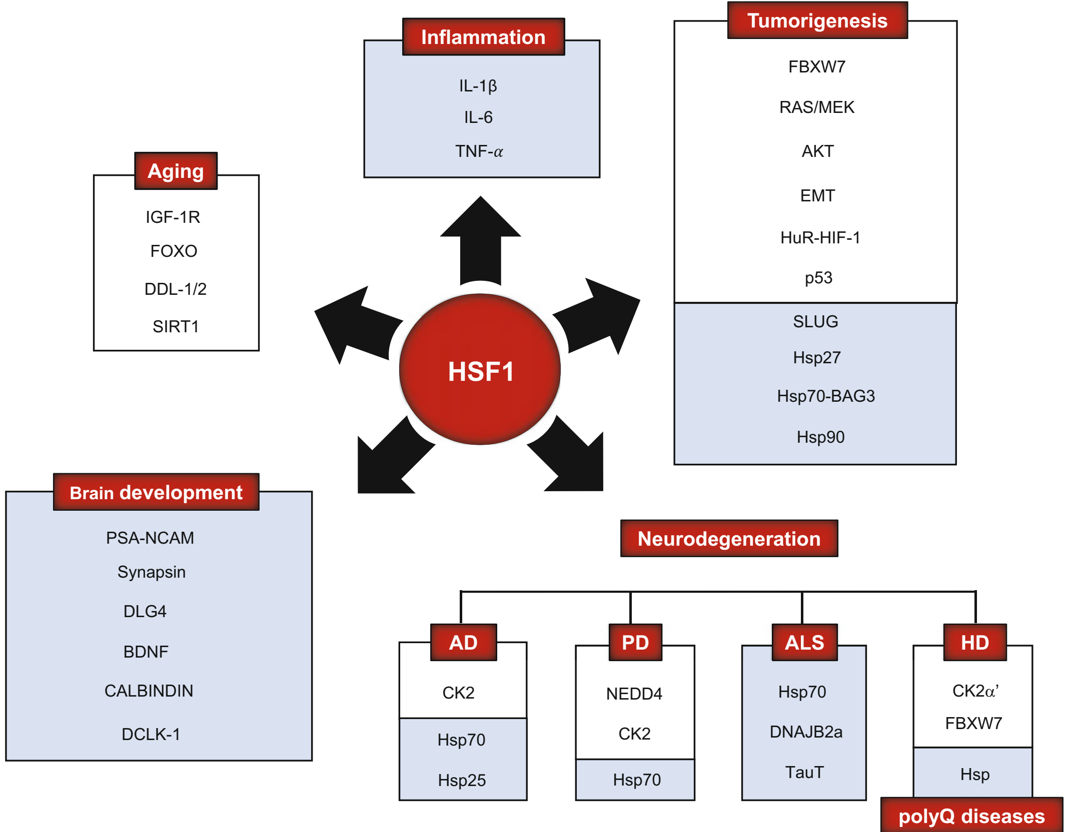

Fig. 5 Relationship between HSF1 and different physiological and pathological processes. Changes in activity and stability of HSF1 are responsible for regulating different cellular processes that are essential for life, including brain development, immune response, metabolism, and cell growth. Dysregulation of HSF1 contributes to different diseases like cancer and neurodegeneration. For each process, we included different proteins related to HSF1 and that contribute to the regulation of such a process. Proteins shown in a white box correspond to proteins that directly or indirectly regulate HSF1 activity or stability. In contrast, proteins shown in a blue box are proteins whose expression is influenced by HSF1

aging, there is a profound decline in the HSR that is accompanied by frail HSF1 activity and downregulation of its downstream target genes that compromise the survival of aged cells exposed to stressful conditions (Kregel 2002). Direct involvement of HSF1 in aging was reported by Garigan et al. showing that HSF1 knockdown speeded up the decline of tissue integrity and shortened lifespan in *C. elegans* (Garigan et al. 2002), while others showed that HSR significantly declines in this organism in early adulthood at reproductive maturity (reviewed in (Labbadia and Morimoto 2015)). These results have been supported by overexpression studies, in which HSF1 expression increased *C. elegans* lifespan by 20–40% (Hsu et al. 2003; Morley and Morimoto 2004). Examples in mammals include diminished HSF1-DNA binding capacity and Hsp accumulation in aged rats (21–26-month-old) upon heat shock and reduced HSF1-DNA binding in human lymphocytes and skin fibroblasts from old individuals (>70 years) when compared with those from a young age (20–40 years) (Fawcett et al. 1994; Gutsmann-Conrad et al. 1998; Jurivich et al. 1997; Locke and Tanguay 1996).

There is a coupling between longevity enhancing capacity of HSF1 with insulin/insulin-like

signaling (ILS) pathway (Barna et al. 2012; Chiang et al. 2012). In *C. elegans*, stimulation of the insulin/insulin-like growth factor 1 receptor DAF-2 (IGF-1R in mammals) inactivates anti-aging and HSP genes via activation of protein kinase B (AKT) and phosphorylation of the longevity-related transcription factor DAF-16 (FOXO in mammals). Increased DAF-2 signaling inhibits HSF1 activity by promoting HSF1 to compete with negative regulators DAF16-dependent longevity-1 (DDL-1) and DDL-2, which accelerates aging (Chiang et al. 2012). Although it is unclear the integration of DAF-2 signaling to HSF1, both HSF1 and DAF-16 are needed for DAF2-mediated extension of lifespan (Cohen et al. 2006; Hsu et al. 2003; Morley and Morimoto 2004). Moreover, both HSF1 and DAF-16 activate the same small hsp genes, hsp-12 and hsp-16, which are essential to promote longevity (Hsu et al. 2003). The ILS-dependent lifespan extension is observed in mammals as well. Heterozygous Igf-1r knockout mice have shown to live approximately 26% longer than their wild-type littermates (Holzenberger et al. 2003). Also, sequence analysis of IGF-1R genes displayed over-representation of heterozygous mutations of the IGF1-R gene in female centenarians (Suh et al. 2008). While these studies did not explore the involvement of HSF1 on long-lived life, given that HSF1 plays a critical role in IGF-1R-dependent lifespan regulation, it is reasonable to speculate that HSF1 might be a controlling factor in the longevity of mammals as it is in invertebrates.

Human aging is also characterized by chronic low-grade inflammation. Several studies indicate that HSF1 modulates normal immune response and inflammation by controlling the expression of different cytokines. For example, HSF1 suppresses tumor necrosis factor-α (TNF-α) by binding to the HSE-like sequences within the TNF-α promoter, while it represses IL-1β through direct interaction with a regulator of IL-1β transcription, a nuclear factor for interleukin-6 (NF-IL6) (Xiao et al. 1999; Xie et al. 2002a). In the case of IL-6, HSF1 represses IL-6 by recruiting an IL-6 repressor activating transcription factor 3 (ATF3) into the open chromatin structure of IL-6 (Inouye et al. 2007). Interestingly, reports have linked HSF1 with the repression of HIV-induced inflammation by impairing the HIV-dependent expression of IL-6 (Inouye et al. 2007; Takii et al. 2010; Xie et al. 2002b), TNF-α (Muralidharan et al. 2014), and IL-1β (Xie et al. 2002a). Additionally, active HSF1 increases the expression of anti-inflammatory cytokine IL-10 (Zhang et al. 2012). Therefore, the decline of HSF1 activity during aging may contribute to increased inflammatory cytokines expression exacerbating aging.

5.2 Tumorigenesis and HSF1

Cancer cells are in a hostile environment enriched with stress, including hypoxia, acidity, ATP depletion, and lack of nutrients (Hanahan and Weinberg 2011). Thus, it can be assumed that under these conditions HSF1 may remain constitutively activated. In 2007, Dai et al. demonstrated that Hsf1−/− mice were far more resistant to skin-induced tumor formation than Hsf1+/+ mice (Dai et al. 2007). The authors showed that HSF1 steers cancer growth by modulating proliferation, signal transduction, protein translation, and glucose metabolism. Since then, there has been growing evidence that shows the increase of HSF1 in many cancer types, including breast cancer, colorectal cancer, gastric cancer, myeloma, non-small-cell lung cancer, oral squamous cell carcinoma, prostate cancer, and many other cancer types (Cui et al. 2015; Fok et al. 2018; Ishiwata et al. 2012; Li et al. 2018; Santagata et al. 2011; Tong et al. 2018). However, it is essential to clarify that increased HSF1 expression is not causative for tumorigenesis. The different roles of HSF1 in cell proliferation and cancer were extensively reviewed recently (Carpenter and Gokmen-Polar 2019; Gomez-Pastor et al. 2018; Jiang et al. 2015). Therefore, we will focus on the most recent and groundbreaking studies that have set up HSF1 as an essential target in cancer biology.

The mechanisms underlying increased HSF1 expression and activity in cancer are complex and vary between different types of tumors and cell

types. Zhao et al. showed that the increased levels of HSF1 in ERBB2-overexpressing cancers are due to an elevation of HSF1 protein translation (Zhao et al. 2009). On the other hand, Kourtis et al. suggested that increased levels of HSF1 in melanoma are due to increased protein stability and decreased proteasomal degradation through decreased or mutated E3 ligase FBXW7 (see HSF1 PTMs section for further details) (Kourtis et al. 2015). As we previously mentioned in the above section, phosphorylation of Ser and Thr with repressive functions within HSF1 is significantly reduced in cancer cells, leading to increased HSF1 activity. In cells from human malignant peripheral nerve sheath tumor (MPNST) lacking neurofibromatosis type 1 (NF1), a tumor suppressor, there is an increase in RAS/MEK-mediated HSF1 Ser326 phosphorylation, which leads to HSF1 trimerization and nuclear translocation. Reports have indicated that this modification significantly contributes to cancer progression (Dai et al. 2012). Also, HSF1 acts in diverse signaling pathways driving cancer initiation, migration, invasion, and metastasis. For example, HSF1 enhances the initiation and progression of breast cancer by upregulating RNA-binding protein Hu-antigen R (HuR) and increasing hypoxia-inducible factor 1 (HIF-1) signaling (Gabai et al. 2012). Knockdown of HSF1 represses epithelial-mesenchymal transition (EMT), a facilitator of metastasis in cancer development, and reduces cell migration in ovarian cancer cells (Powell et al. 2016). Specifically, HSF1 is phosphorylated at Ser326 by AKT and binds to SLUG promoter, an EMT regulator, leading to the upregulation of SLUG in HER2-positive breast cancer cells (Carpenter et al. 2015). These findings suggest that the HSF1-SLUG axis is an essential pathway in cancer progression.

HSF1 exerts its effects on cancer progression, in part through the upregulation of Hsp. In MPNST, loss of NF1 increased HSF1 levels and Hsp90 expression, promoting carcinogenesis (Dai et al. 2012). HSF1 activation and Hsp90 expression are also essential in enhancing tumor growth in HER2-positive breast cancer (Schulz et al. 2014). Hsp70 also enhances tumorigenesis by acting as a survivor factor due to its anti-apoptotic effects. For example, Hsp70 and its co-chaperone BCL-2–associated athanogene 3 (BAG3) mediate apoptosis resistance in glioma cells by supporting cell survival through increasing the levels of pro-survival BCL-2 family members (Antonietti et al. 2017). In addition to the upregulation of Hsp90 and Hsp70, there is also an elevation in the expression of Hsp27 in a variety of different cancers, and it is involved in tumor progression and drug resistance (Fang et al. 2012; Vahid et al. 2016; Xu et al. 2006; Yu et al. 2014; Zhao et al. 2012). Interestingly, a study conducted in hepatocellular carcinoma showed that HSF1-mediated phosphorylation of Hsp27 rather than Hsp27 expression is necessary to promote migration and invasion of carcinoma cells (Fang et al. 2012).

Intriguingly, the upregulation of HSF1 in cancer is associated with the activity of tumor suppressor p53 (Toma-Jonik et al. 2019). Under stress conditions, HSF1 interacts with p53 in the nucleus and form a complex with DNA damage kinases to effect p53 phosphorylation in response to DNA damage (Logan et al. 2009). The presence of mutations in p53 protein is also connected to the activation of HSF1. Specifically, a gain-of-function p53 mutant variant (Arg280Lys) directly interacts with HSF1 phospho-Ser326, enhancing HSF1 transcriptional activity and Hsp90 expression in human breast cancer cells (Li et al. 2014a). Hence, the gain of function of p53 offers not only the drastic acceleration of oncogenic signaling but also the Hsp-induced survival capacity of cells through HSF1. On the other hand, DNA damage induces p53 activation and HSF1 downregulation, resulting in senescence (Kim et al. 2012). Moreover, HSF1 depletion causes growth inhibition of breast cancer cells by promoting p53-induced senescence (Meng et al. 2010), suggesting that lack or loss of p53 combined with HSF1 activation support tumorigenesis by regulating senescence and proliferation. These observations indicate that HSF1 may be a central mediator of the oncogenic function of different mutant variants of p53 observed in cancer cells.

Reports have shown that the transcriptional program triggered by HSF1 in malignant cells is different from those mediated by heat shock. The

genes activated in this program drive oncogenic transformation by accelerating protein folding, translation, mitosis, invasion, metabolism, and metastasis and obstructing immune functions and apoptotic response (Mendillo et al. 2012). Since there is an elevation in the expression levels of HSF1 in many solid tumors and there is an association between its levels with low survival rate and metastasis (Ciocca et al. 2013; Mendillo et al. 2012), HSF1 has been proposed as a prognostic factor in cancer. In addition, there is a link between increased HSF1 and reduced survival in many different tumors like estrogen receptor-positive breast cancer, osteosarcoma, pancreatic cancer, melanoma, and esophageal squamous cell carcinoma (Kourtis et al. 2015; Liang et al. 2017; Liao et al. 2015; Santagata et al. 2011; Santagata et al. 2013; Tsukao et al. 2017; Zhou et al. 2017). In patients with hepatocellular carcinoma (HCC), expression of HSF1 was also found to be correlated with poor overall survival (Fang et al. 2012) and, specifically, the high levels of HSF1 phospho-Ser326 has been reported in HCC progression and invasion (Li et al. 2014b). Notably, the levels of HSF1 phospho-Ser326 have clinical significance in shorter overall survival in ovarian cancer patients (Yasuda et al. 2017). Collectively, the expression and activity of HSF1 can serve as a potential clinical biomarker for patients with cancers. Due to the high levels of HSF1 and its relevance in tumor growth, HSF1 has become a desirable target to treat cancer (Carpenter and Gokmen-Polar 2019; Dong et al. 2019). However, although HSF1 has been validated as a potent target in cancers by genetic knockdown studies, HSF1 inhibitors reported to date have lacked specificity and potency for clinical evaluation (Dong et al. 2019). Hence, it is necessary to make a more systematic design to develop more potent and specific HSF1 inhibitors in the future.

5.3 HSF1 in the Central Nervous System (CNS)

Ubiquitous expression of HSF1 in the developing brain implies a need for HSF1 during neurodevelopment (El Fatimy et al. 2014). It is now known that not only HSF1 but also HSF2 plays essential roles in brain function through modulation of neuronal migration, formation, and maintenance of neuronal synapses. Lack of HSF1 dramatically alters brain structure, neuronal differentiation, and synaptic formation (Chen et al. 2014; Hooper et al. 2016; Uchida et al. 2011). In the hippocampus, a brain area involved in learning and memory, the absence of HSF1 causes a decrease in the dendrite length of the dentate gyrus granule neurons and pyramidal neurons. It reduces dendritic spine density, resulting in reduced synapse formation (Uchida et al. 2011). Additional observations demonstrated that lack of HSF1 in the hippocampus causes low expression of polysialylated-neural cell adhesion molecule (PSA-NCAM), which is fundamental in synapse formation. Other synaptic proteins, such as synapsin and discs large MAGUK scaffold protein 4 (DLG4) involved in synaptogenesis, also depend on HSF1 (Chen et al. 2014). Due to the specific role of HSF1 in regulating synaptic proteins, alteration in the levels of HSF1 during embryogenesis results in alterations in synaptic fidelity and memory consolidation. In this line, HSF1 also regulates the expression of brain-derived neurotrophic factor (BDNF), an essential regulator of synaptogenesis and synaptic plasticity mechanisms underlying learning and memory (Chen et al. 2014; Cunha et al. 2010). Other functions attributed to HSF1 are the regulation of lipid raft formation, the subdomains of plasma essential for postsynaptic consolidation of memory receptors and long-term memory retention (Nagy et al. 2007; Suzuki and Yao 2014), and regulation of Ca2+ homeostasis through the activation of CALBINDIN expression in cerebellar Purkinje cells (Ingenwerth et al. 2016). The deficiency of HSF1 also results in loss of oligodendrocytes and severe demyelination, astrogliosis, increased activated microglia, and neuronal apoptosis across different brain regions (Homma et al. 2007).

HSF1 has critical protective roles in response to stresses during brain development. Research has shown that exposure of Hsf1-/- mice to stressful conditions during prenatal stages increases

neuropsychiatric susceptibility like disorders in the newborns (Hashimoto-Torii et al. 2014). During exposure to stresses like alcohol or a maternal epileptic seizure, HSF1 is retained in the nucleus and activates Hsp70 expression. Intriguingly, under these conditions, HSF1 does not show a characteristic hyperphosphorylation pattern upon activation but rather presents reduced acetylation and sumoylation (El Fatimy et al. 2014). Interestingly, El Fatimy et al. demonstrated that prenatal alcohol exposure (fetal alcohol syndrome) causes brain structural abnormalities dependent on the formation of HSF1-HSF2 heterotrimers (El Fatimy et al. 2014). The group found that fetal alcohol exposure causes HSF1-HSF2 heterotrimers to bind to and downregulate doublecortin-like kinase 1 (Dclk1) expression, a gene that participates in neuronal migration and neurogenesis. The authors also demonstrated that lack of Hsf2 exerts a protective role during prenatal alcohol exposure facilitating HSF1 homotrimerization and regulating neuronal migration genes. These results indicate the complexity of the different HSF1 regulatory mechanisms during physiological conditions and the importance of these transcription factors in every step of an organismal life.

5.4 Neurodegenerative Diseases and HSF1

As we described in the previous section (HSF1 in the CNS), HSF1 participates in the development of the CNS by controlling diverse processes such as neuronal migration, neurogenesis, glycogenesis, synapse formation, and neuronal survival (El Fatimy et al. 2014; Homma et al. 2007; Hooper et al. 2016; Ingenwerth et al. 2016; Uchida et al. 2011). Therefore, maintaining proper levels of HSF1 is essential to ensure the appropriate development and maintenance of the CNS. However, in the adult brain, HSF1 activity significantly declines, contributing to inflammation and neuronal death (Calderwood et al. 2009; Murshid et al. 2013). In line with these observations, there is a significant reduction in HSF1 in many age-related neurodegenerative diseases (NDs), like AD, PD, and amyotrophic lateral sclerosis (ALS), which are characterized by protein aggregation and reduced expression of the protein quality-control machinery (Homma et al. 2007; Santos and Saraiva 2004). Lowered HSF1 has also been reported in other heritable NDs, such as those related to polyglutamine expansions (discussed below and in the section Implication of HSF1 in HD). The causes of HSF1 depletion during neurodegeneration are not well understood and may differ between different NDs. Nevertheless, numerous studies conducted in different animal models of NDs imply that increasing the levels or activity of HSF1 has therapeutic potential (Neef et al. 2011). In this book chapter, we will briefly discuss the role of HSF1 in different NDs with particular emphasis on the advances made in HD.

AD is a common age-associated neurodegenerative disease most often caused by extracellular depositions of amyloid-β, a neurotoxic peptide produced by the amyloid-β precursor protein proteolysis, leading to neuronal death (Mavroudis et al. 2010). Increasing reports show that intracellular levels of HSF1 are essential to maintain neuronal survival in AD. A study conducted in plasma neural-derived exosomes from patients with AD showed a remarkable reduction in the levels of HSF1 compared with unaffected individuals (Goetzl et al. 2015). There was also a reduction in the levels of HSF1 in the cerebellum of an AD rat model (Jiang et al. 2013). Notably, several studies have shown that overexpression of HSF1 results in an increased number of Purkinje cells in the cerebellum, the numbers of which are reduced in patients with AD and mouse models, lowering amyloid-β levels and improving cognitive deficits (Jiang et al. 2013; Khalsa 2015; Kozuki et al. 2011; Lee et al. 2014; Pierce et al. 2013). In line with the beneficial effects of increased HSF1 levels on neuronal survival, boosting HSF1 activity provides synaptic protection in AD. Pharmacological activation of HSF1 by using Hsp90 inhibitors led to the upregulation of Hsp70 and Hsp25 and improved synaptic integrity and memory consolidation in AD mouse models (Chen et al. 2014; Wang et al. 2017). Despite the lack of evidence as to whether the

decrease of HSF1 in AD is due to a pathological degradation, it is essential to note that increased CK2 has been reported in AD mouse models and patients with AD, contributing to the inflammatory phenotype characteristic of AD (Masliah et al. 1992; Perez et al. 2011; Rosenberger et al. 2016). However, the connection between CK2, HSF1, and inflammation in AD has yet to be explored.

PD is characterized by α-synuclein aggregation and an age-dependent loss of dopaminergic neurons in the midbrain (Jellinger 2014). In PD, reports have shown low levels of HSF1 in both mouse models and patients (Kim et al. 2016). Kim et al. showed that α-synuclein accumulation enhanced HSF1 poly-ubiquitination and degradation by elevated ubiquitin E3 ligase neuronal precursor cell-expressed developmentally downregulated 4 (NEDD4) expression. Notably, the authors found that acetylation at Lys80 within the DBD of HSF1 makes it more accessible for NEDD4-mediated ubiquitination (Kim et al. 2016). It is unknown whether phosphorylation contributes to NEDD4-mediated HSF1 degradation in PD. However, several kinases induce HSF1 phosphorylation, such as CK2 in PD (Lee et al. 2004a; Mavroudis et al. 2010). Therefore, it is tentative to speculate that phosphorylation events may control HSF1 degradation in PD, as it happens in HD (Gomez-Pastor et al. 2017; Soncin et al. 2003). While the degradation mechanism of HSF1 in PD is not fully characterized, its therapeutic potential has been demonstrated by studies in which a constitutive active form of HSF1 (caHSF1) is expressed in a human cellular model of PD (Liangliang et al. 2010). In this study, caHSF1 reduced α-synuclein aggregation and toxicity by inducing the expression of Hsp70. As proof of concept, direct overexpression of Hsp70 in a *Drosophila* model of PD inhibited α-synuclein induced neurodegeneration (Auluck et al. 2002). Recently, a new pharmacological activator of HSF1 (echinochrome derivative U-133) administered to a rat model of PD showed increased levels of Hsp70 and neuroprotective effects, including decreased microglia activation, a-synuclein aggregation, and improved motor behavior (Ekimova et al. 2018).

ALS is characterized by progressive dysfunction and death of motor neurons in the brain and spinal cord. Mutations in several genes can cause familial ALS and contribute to the development of sporadic ALS. Mutations in the chromosome 9 open reading frame 72 (C9orf72) gene account for approximately 40% of familial ALS; superoxide dismutase (SOD1) gene mutations cause approximately 20% of familial ALS. TAR DNA binding protein (TARDBP) (also known as TDP-43) and the RNA binding protein fused in sarcoma/translocated in liposarcoma (FUS/TLS) mutations each account for about 5% of cases. Increased microtubule-associated protein Tau has also been linked to ALS (Schreiber et al. 2018). The depletion of HSF1 in ALS significantly contributes to increased oxidative stress and aggregation of TDP-43 (Batulan et al. 2003; Chen et al. 2016). In the cell model of ALS, HSF1-induced TDP-43 clearance is partly mediated by Hsp70 and its co-chaperone DNAJB2a (Jung et al. 2013). HSF1 also regulates taurine transporter (TauT) levels under oxidative stress conditions, acting as an antioxidant to protect motor neurons (Jung et al. 2013). These studies suggest that HSF1 partially protects motor neurons by compensating for constitutive oxidative stress, which is thought to be a key mechanism contributing to ALS's pathogenesis.

Polyglutamine (polyQ) diseases are hereditary degenerative disorders characterized by the aberrant expansion of a CAG repeat in a specific gene, resulting in misfolding and aggregation of the disease-causing protein (La Spada and Taylor 2010). Multiple lines of evidence demonstrated that HSF1 and HSF1-dependent pathways strongly influence the pathogenesis of polyQ diseases. These studies are discussed in detail in the section Implication of HSF1 in HD. Understanding the specific roles of HSF1 in HD and other NDs may help develop new therapeutic strategies to increase the levels of HSF1 and prevent neurodegeneration across multiple NDs.

6 Protein Aggregation in HD

HD is a heritable neurodegenerative disease caused by a CAG trinucleotide (code for glutamine) repeat expansion within exon 1 of the HTT gene (MacDonald et al. 1993). The disease arises when the polyQ tract exceeds approximately 37 CAG repeats (Bates et al. 2015; Novak and Tabrizi 2010). The disease occurs in all populations but is most common in individuals of European ancestry, and it has an overall prevalence estimated to be 1 in every 10,000 individuals (Evans et al. 2013; Fisher and Hayden 2014; McColgan and Tabrizi 2018). Characteristic symptoms include progressive motor dysfunction (chorea, dystonia, bradykinesia, and incoordination), psychiatric disturbances (depression, obsessive-compulsive disorders, and anxiety) and cognitive decline (distractibility, impulsivity, and difficulty in multitasking) (Group 1996; Kieburtz et al. 2001; Novak and Tabrizi 2010). Other symptoms that accompany those mentioned above are weight loss and muscle wasting, metabolic dysfunction, and endocrine disturbance (Novak and Tabrizi 2010; Ross and Tabrizi 2011). Research has shown that such clinical aspects come from progressive and massive loss of neurons, predominantly GABAergic medium-sized spiny neurons (MSNs) located in the striatum. A brain region that controls movement and some forms of cognition (Albin et al. 1992; Deng et al. 2004; Ferrante et al. 1991; Ross and Tabrizi 2011; Rubinsztein 2003; Waldvogel et al. 2015). However, neurodegeneration in other brain regions, including cortex, thalamus, and hippocampus, is also observed as the disease progresses (Cepeda et al. 2007; Puigdellivol et al. 2016; Sieradzan and Mann 2001). The disease's symptoms usually begin in the adult-onset (around age 34–40 years with 15–20 years of progression). However, when the length of the CAG expansion exceeds about >60, it manifests before age 20 years (Juvenile HD) and accounts for 5–10% of all HD cases (Chen et al. 2001; Gusella and MacDonald 2000; Morley et al. 2002; Novak and Tabrizi 2010; Rubinsztein 2003). Despite numerous studies in the past two decades that have been addressing the importance of mutant HTT (mHTT) protein aggregation and MSN degeneration in HD pathology, very little is known about the exact molecular mechanisms by which mHTT induces neuronal death. There are several proposed pathophysiological pathways in this regard, many of which are associated with protein quality control impairment. In this chapter, we will discuss those pathways in which HSF1 may play a key role.

6.1 Structure and Function of HTT

The HTT gene encodes a large ~350 kDa widely expressed protein composed of multiple domains (Fig. 6) that control conformation, protein localization, and function (reviewed in (Jimenez-Sanchez et al. 2017)). PolyQ expansion induces conformational changes in HTT, affecting structural aspects of different regions or the protein proteolytic cleavage, unfolding, and aggregation (Almeida et al. 2013). While HTT is considered a cytoplasmic protein, it can partially accumulate in the nucleus, which is enhanced by the polyQ expansion and is believed to aggravate the disease. The highly conserved domain N17 (17-amino acid-long N-terminus) can modulate nuclear localization and mHTT toxicity (Desmond et al. 2012). N17 interacts with the nuclear pore protein called translocated promoter region (TPR) that is involved in nuclear export, facilitating the shuttling of HTT from the nucleus to the cytoplasm (Cornett et al. 2005). PolyQ expansions decrease this interaction and increase the nuclear accumulation of HTT (Cornett et al. 2005). The N17 is also subject to several PTMs, including sumoylation (Steffan et al. 2004), phosphorylation (Aiken et al. 2009; Thompson et al. 2009), acetylation (Thompson et al. 2009), and ubiquitination that control interactions between N17 and other proteins (Atwal et al. 2007; Graham et al. 2006; Kalchman et al. 1996; Maiuri et al. 2013; Steffan et al. 2004; Thompson et al. 2009; Wellington et al. 2002). The polyQ tract starts right after the N17, and it is composed of a series of CAG repeats interrupted by a CAA codon (also codes for glutamine) as follows (CAG)n-CAA-CAG (where $n \leq 36$ repeats in

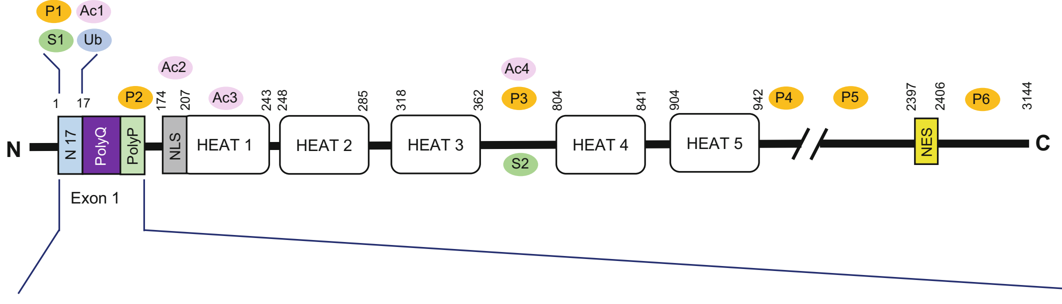

Fig. 6 Diagram of human wild-type HTT relative to its known domains and post-translational modifications. HTT is composed of different domains; N17 (containing the first 17 amino acids of the protein), polyQ tract (whose expansion is responsible for HD), polyP repeats, and five helical clusters of HEAT repeats. Several PTMs (e.g., acetylation [Ac, pink], phosphorylation [P, orange], sumoylation [S, green], and ubiquitination [Ub, blue]) alter HTT's cell biology and toxic properties. Within the N17 region, we find (P1) P-Ser13 and P-Ser16, (Ac1) Ac-Lys9, S-Lys6, 9 and 13, and (S1) S-Lys9. Other PTMs can also be detected along with the protein (P2) P-Ser116, (P3) Ser-419, 421, 434, 533, 535, and 536, (P4) P-Ser1181 and 1,201, (P5) P-Ser2076, P(6) P-Ser2653 and 2,657, (Ac2) Ac-Lys178 and 236, (Ac3) Ac-Lys 345, (Ac4) Ac-Lys444, and (S2) S-Lys444. For a detailed list of all the PTMs identified in vivo and in vitro, see (Arbez et al. 2017). The corresponding amino acid sequence from exon 1 is shown in blue for the N17, in purple for the polyQ repeat, and in green for the polyP-rich region. Sequences were obtained from the NCBI protein database (Human: NP_002102.4)

unaffected individuals). There is an inverse correlation between the polyQ tract length and the age of onset (AOO) (Langbehn et al. 2010). However, there is a lot of variability in the AOO that could not be explained by considering the number of polyQ. Two recent studies have shown that the number of the CAG uninterrupted track rather than the number of polyQ determines HD onset timing (Wright et al. 2019). Following the polyQ tract is a polyproline (polyP)-rich region that consists of 10–11 proline and functions in stabilizing polyQ conformation by forming proline-proline helixes (Bhattacharyya et al. 2006; Kim et al. 2009). These domains are followed by tandem arrays of a repeat called HEAT, which is named for the following four functionally characterized proteins: HTT, elongation factor 3 (EF3), the regulatory A subunit (65 kDa) of protein phosphatase 2A (PP2A), and target of rapamycin 1 (TORl). They form rod-like helical structures and serve as a scaffold for many protein interactions (Andrade and Bork 1995; Saudou and Humbert 2016). Recently, a nuclear localization sequence (NLS) has been described between amino acids 174 and 207, preceding the first HEAT domain, allowing the interaction with KARYOPHERIN β2. This protein mediates the nuclear import of proteins (Desmond et al. 2012). The authors showed that polyQ expansion decreased the interaction between the NLS containing region and karyopherin β2, increasing mHTT nuclear accumulation. A nuclear export signal (NES) can also be found in the C-terminus of HTT (Xia et al. 2003). Truant and colleagues have demonstrated that the C-terminus region's proteolytic cleavage occurs during HD and proposes that lack of the C-terminus containing NES facilitates the nuclear accumulation of mHTT and pathogenesis (Xia et al. 2003).

Another factor that contributes to HD pathogenesis is CAG repeat somatic instability. Meaning that the CAG track undergoes progressive length increases over time in somatic tissues, particularly in HD's most affected brain regions. Prior research demonstrated that CAG somatic instability could account for the increased susceptibility of MSNs to mHTT due to cell-type-specific transcriptional programs (Gonitel et al. 2008; Goula et al. 2012; Kennedy et al. 2003;

Lee et al. 2011; Shelbourne et al. 2007). It is known that CAG length variation is tissue-dependent, and it is enhanced in the striatum of HD mouse models and HD patients. A hypothesis for this phenomenon is the tissue-specific positioning of the RNA pol II at the HTT locus, which is enhanced in the striatum (Goula et al. 2012). Pharmacological suppression of CAG instability in the HdhQ150 mouse improved neuropathology and demonstrated the role of somatic instability is pathogenesis (Budworth et al. 2015; Kovtun et al. 2007; Massey and Jones 2018). Also, several mismatch repair genes, including MSH3, influence somatic instability of CAG repeats and are considered critical genetic modifiers for the disease (Flower et al. 2019). Recently, the Genetic Modifiers of Huntington's Disease (GeM-HD) Consortium demonstrated that the uninterrupted CAG repeat is a propensity for length instability leading to its somatic expansion (Lee et al. 2019). Other aspects contributing to the HD pathology include HTT transcriptional dysregulation and HTT mRNA abnormal splicing, two topics recently discussed in (Thomson and Leavitt 2018).

HTT is an essential protein required for normal development since the ablation of Htt in mice resulted in embryonic death (Zeitlin et al. 1995). However, the exact function of HTT is not known yet. There is an expression of HTT throughout the body, but it is highly active in the CNS, suggesting a potential role in brain physiology. Gain-of-function effects of mHTT have been proposed as the primary driver for neurodegeneration in HD. However, recent studies proposed an alternative hypothesis suggesting that loss-of-function may be at the forefront of the pathogenesis (reviewed in (Cattaneo 2003)). Studies using conditional deletion of HTT in mice's forebrain resulted in neurodegeneration, demonstrating that HTT is necessary for neuronal function and survival. A loss-of-function mechanism may contribute to HD pathogenesis (Dragatsis et al. 2000). In support of this study, McKinstry and colleagues demonstrated that silencing HTT in the developing mouse cortex was viable but resulted in alterations in cortical and striatal synaptic connectivity similarly to those deficits observed in mHTT expressing mice (McKinstry et al. 2014). Other studies have also shown that HTT plays a crucial role in neurogenesis by maintaining the lineage potential of primitive neural stem cells during neural induction and synapse formation (Nguyen et al. 2013; Sun et al. 2001). Additionally, HTT has been related to protein scaffolding, vesicular and organelle trafficking, and transcription regulation (Benn et al. 2008; Caviston et al. 2007; Gunawardena et al. 2003; Lee et al. 2004b; Li et al. 2001; Orr et al. 2008; Parker et al. 2001; Rong et al. 2006; Zuccato et al. 2001). While it is still unclear the direct role of HTT in all these processes, it seems evident that alteration in the structure and conformation of HTT can alter many different pathways that complicate the study of HD and difficult the development of effective therapeutic strategies.

Accumulation and aggregation of mHTT are positively associated with mitochondrial dysfunction, which is critical in promoting MSNs degeneration and death in HD. The mHTT disrupts mitochondria by interacting with several mitochondrial proteins and regulators. Consequently, the outcome of mitochondrial perturbation is ATP deficiency and increased reactive oxygen species (ROS) production. Both factors contribute to the exacerbation of mitochondrial damage and mHTT aggregation. Research has shown increased mitochondrial fragmentation in cellular and mouse models of HD and the brain of patients with HD (Cherubini and Gines 2017). Mitochondrial fragmentation is related to the abnormal interaction between mHTT with the central regulator of protein fission and dynamin-related protein 1 (DRP-1), resulting in DRP-1 dysfunction (Cherubini and Gines 2017). The mHTT also disrupts retrograde and anterograde mitochondrial trafficking along axons, resulting in a reduced transport of mitochondria to synapses with high energy demands and contributing to synaptic dysfunction (Orr et al. 2008). Also, mHTT interacts with TIM23, a component of the mitochondrial inner membrane translocase, altering mitochondrial protein import, and leading to respiratory dysfunction and neuronal cell death (Yano et al. 2014). Mitochondria dysfunction is also caused by decreased transcription of

nuclear-coded mitochondrial genes such as peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), a transcription coactivator that stimulates energy metabolism (Costa and Scorrano 2012; Cui et al. 2006; McConoughey et al. 2010; Weydt et al. 2006). One of the main pathways involved in the downregulation of PGC-1α in HD is the CREB/TAF4 signaling pathway, which is altered in the presence of mHTT (Cui et al. 2006). Very recently, Intihar et al. demonstrated that HSF1 also regulates the expression of PGC-1α in striatal like-cells, and deficits in HSF1 contribute to the downregulation of PGC-1α expression observed in HD (Intihar et al. 2019). The contribution of HSF1 to mitochondrial dysfunction in HD is discussed in the section HSF1 as a *Potential Regulator of PolyQ-Dependent Mitochondrial Dysfunction*.

6.2 HSR and Other Protein Quality Control Systems in HD

Full-length mHTT and proteolytic cleaved fragments have the propensity to misfold and aggregate, forming insoluble inclusion bodies, a hallmark of HD (Bates 2003; Davies et al. 1997; DiFiglia et al. 1997). Aggregates arise elsewhere in the cell, including cytoplasm, nucleus, dendrites, and axon terminals (DiFiglia et al. 2007; Vonsattel 2008). The insoluble aggregates have been observed in in vitro cell models of HD, mouse models of HD, and HD patients (Davies et al. 1997; DiFiglia et al. 1997; Gray et al. 2008; Sahl et al. 2012). However, it remains under debate whether the aggregates are a byproduct of a cellular attempt to protect neurons from misfolded mHTT protein or are instead the cause of pathology. Unfortunately, discriminating between these two hypotheses and correlating the presence of aggregates with the onset of a phenotype is technically tricky since in many cases quantifying small polyQ oligomers is challenging. When successful, it does not inform about all the different structural conformers present in the system. Studies conducted by Yang and colleagues demonstrated that preformed polyQ aggregates are highly toxic when directed to the nucleus, establishing proof that aggregates represent species with toxic properties (Yang et al. 2002). The pharmacological intervention aimed at inhibiting aggregate formation has shown beneficial effects in a mouse model of HD (Sánchez et al. 2003; Tanaka et al. 2004). Therefore, studying the regulatory mechanisms that lead to enhanced aggregation in HD may lead to more effective therapeutic strategies that can ameliorate neuronal death.

Neurons are post-mitotic cells in constant need of protein quality control to ensure protein homeostasis and keep cells functional. The HSR is a mechanism to cope with proteotoxic stress by inducing the expression of molecular chaperones and other Hsp, and it constitutes the first line of defense against aggregation. Due to the stress-inducible nature of Hsp, it would be expected to observe increased levels of Hsp in response to the accumulation and aggregation of mHTT. However, several studies demonstrated that Hsp shows a progressive decrease in cellular and mouse models of HD and that depletion of Hsp contributes to disease pathogenesis (Chafekar and Duennwald 2012; Hay et al. 2004). Research has shown decreased Hsp expression in postmortem brain tissue from patients with HD (Gomez-Pastor et al. 2017; Hodges et al. 2006). Multiple proposed hypotheses aim to explain the depletion of Hsp in HD. Hay et al. suggested that changes in the levels of Hsp are due to their sequestration into aggregates, a hypothesis supported by other studies (Park et al. 2013; Seidel et al. 2016; Yamanaka et al. 2008), while Labbadia et al. showed Hsp expression impairment in HD due to altered chromatin structure of the Hsp promoters (Labbadia et al. 2011). Finally, Gomez-Pastor et al. demonstrated that abnormal degradation of HSF1 during HD pathogenesis likely contributes to the downregulation of Hsp (Gomez-Pastor et al. 2017). Recently, a fascinating study conducted by Neueder and colleagues exposing wild-type and HD mice (HdhQ150 and R6/2) to a heat shock treatment demonstrated that HD mice are unable to induce Hsp in affected tissues, revealing a disruption in the HSR in HD mice (Neueder et al. 2017).

Interestingly, recent global chaperone gene expression analysis in the adult mouse brain identified that the striatum shows intrinsically lower levels of Hsp compared to the cortex (Tebbenkamp and Borchelt 2010), which may contribute to the explanation of enhanced abrogation in the HSR in the striatum and the increased susceptibility of MSNs in the presence of mHTT.

The ubiquitin-proteasome system (UPS), an intracellular pathway for degrading unfolded and unnecessary proteins, also constitutes an essential defense mechanism against protein aggregation. The prominent presence of ubiquitin characterizes inclusion bodies in HD. Recent work using the CAG140Q knock-in mice demonstrated that the UPS activity is lower in the striatum than in other brain regions, which correlates with decreased ubiquitin-activating enzyme (UBE1) levels (Wade et al. 2014). Decreased UPS activity, together with the observation that mutations in the UPS components give rise to some neurodegenerative diseases, suggests that UPS impairment may contribute to HD (reviewed in (Ortega and Lucas 2014; Schipper-Krom et al. 2012)). Another route for degradation of dysfunctional or aggregated proteins is via autophagy, a lysosome-mediated degradation pathway, whose role in HD has been widely studied (reviewed in (Croce and Yamamoto 2019)). One example that demonstrates impairment in the autophagy pathway is the decreased expression of high-temperature requirement protein A2 (HTRA2/OMI), a positive regulator of autophagy (Li et al. 2010), in cultured striatal neurons and the striatum of patients with HD (Inagaki et al. 2008). Inagaki et al. proposed that HTRA2/OMI is relevant to the selective vulnerability of striatal neurons in HD. Also, Yamamoto and colleagues identified a new protein, autophagy linked FYVE (ALFY), that mediates selective macroautophagy of aggregated proteins and whose expression is essential for the clearance of HTT aggregates (Yamamoto and Simonsen 2011). Recent studies indicate that the macroautophagic machinery, comprised of a core group of autophagy-related proteins, such as ATG5, ATG7, and LC3, is compromised in HD (Croce and Yamamoto 2019; Filimonenko et al. 2010; Martinez-Vicente et al. 2010). These findings demonstrate that degradative systems responsible for HTT clearance are affected and contribute to the age-dependent accumulation of neuronal aggregates. Very recently, a study led by Li and colleagues implemented the use of a small-molecule-microarray-based screening to identify specific linkers between the autophagy protein LC3 and mHTT that promote cargo recognition and uptake of mHTT into the macroautophagy pathway for degradation (Li et al. 2019). Administration of these linkers to HD mouse models decreased HTT aggregates' load and ameliorated motor deficits associated with HD (Li et al. 2019). This study establishes a start point for developing new therapies that exploit the degradative properties of different protein quality control systems as a strategy to remove mHTT aggregates and alleviate neurodegeneration.

7 Implication of HSF1 in HD

7.1 HSF1 and Hsp Function in HD

As discussed earlier, the HSR facilitates the folding of proteins and maintains protein homeostasis by inducing the expression of a set of molecular chaperones and Hsp (Bjork and Sistonen 2010; Lindquist 1986; Morimoto 1998). Hsp is a family of proteins named by their molecular weight whose functions require ATP (Fink 1999; Wang and Spector 2001). Some of the most critical Hsp players in the HSR include Hsp40, Hsp60, Hsp70, Hsp90, Hsp100, and the small Hsp (De Maio and Vazquez 2013; Kampinga et al. 2009). Among them, Hsp70 and its co-chaperone Hsp40 are central Hsp in the HSR. They are involved in folding proteins correctly and preserving polypeptides in a soluble conformation by binding to misfolded disease proteins, thereby preventing them from generating toxic protein structures (Hartl and Hayer-Hartl 2002). Early studies conducted in yeast expressing polyQ showed that Hsp70 and Hsp40 could alter the formation of detergent-insoluble mHTT

aggregates to detergent-soluble amorphous structures (Muchowski et al. 2000). Additionally, immunolabeling and fluorescence resonance energy transfer (FRET) experiments showed that Hsp70 and Hsp40 directly bind to the N-terminus region of HTT within the inclusion bodies and interfere with the intramolecular rearrangement of HTT by modulating the interaction between HTT and other proteins (Hay et al. 2004; Jana et al. 2000; Schaffar et al. 2004). Atomic force microscopy has also revealed that Hsp70 and Hsp40 reduce the formation of spherical and annular structures of mHTT fragments, resulting in the partition of monomeric conformations of mHTT and acceleration of fibrillization (Wacker et al. 2004). Consequently, deletion of Hsp70 in the R6/2 HD mouse model increased mHTT inclusion bodies' size and exacerbated HD-related physiological and neuropathological features (Wacker et al. 2009). Furthermore, single overexpression of different DNAJ proteins (Hsp40 family members) has shown protective properties in different HD animal models (Bason et al. 2019; Gillis et al. 2013; Kakkar et al. 2016). These studies demonstrated the fundamental role of Hsp70/Hsp40 in preventing polyQ aggregation.

In yeast, Hsp70 also assists Hsp104, a member of the Hsp100 family with disaggregase activity, by binding to protein aggregates and recruiting Hsp104 (Acebron et al. 2009; Okuda et al. 2015; Winkler et al. 2012). After Hsp104 is bound to protein aggregates, Hsp70 stimulates ATP-driven substrate-threading activity of Hsp104, leading to the extraction of single unfolded polypeptides from aggregates (Lum et al. 2004; Schlieker et al. 2004; Tessarz et al. 2008; Weibezahn et al. 2003; Zietkiewicz et al. 2004). Hsp110, the mammalian homolog of Hsp104, also interacts with and disassembles protein aggregates through its ATP-dependent activity (Gao et al. 2015; Garcia et al. 2017; Rampelt et al. 2012; Shorter 2011). In vitro and in vivo assays have recently shown that a trimeric chaperone complex formed by HSC70 (a member of the Hsp70 family), Hsp110, and DNAJB1 (Hsp40) completely suppresses fibrillization of HTT exon1 Q48, demonstrating the effective co-operation between the different Hsp (Scior et al. 2018). Hsp90 also plays a vital role in the triage of HTT. However, contrary to what would be expected, Hsp90 functions in promoting polyQ aggregation. Reports have shown that N-terminal fragments of HTT associate with Hsp90, and inhibition of Hsp90 increases ubiquitination and proteasomal degradation of mHTT by disrupting the Hsp90-mHTT complex (Baldo et al. 2012; He et al. 2017). Hsp90 diminishes the removal of mHTT aggregates by promoting the recruitment of a deubiquitinating enzyme (ubiquitin-specific protease 19 (USP19)) (He et al. 2017).

Several transcriptomic and proteomic studies conducted in different mouse models of HD and patients with HD have demonstrated that several Hsp are in low abundance in HD affected tissues (Ament et al. 2017; Hodges et al. 2006; Kumar et al. 2016; Langfelder et al. 2016). The thought is that this defect contributes to polyQ aggregation and neurodegeneration. The importance of restoring the levels of Hsp has been widely demonstrated in cell cultures, yeast, fly, and mouse models of HD in which overexpression of different Hsp including Hsp70, different members of the Hsp40 family as well as different small Hsp decreased mHTT aggregation and neuropathological features of HD (Bason et al. 2019; Carmichael et al. 2000; Fuchs et al. 2009; Gillis et al. 2013; Gunawardena et al. 2003; Jana et al. 2000; Kuo et al. 2013; Wacker et al. 2004, 1999a). There is an association between the decreased expression of Hsp and impairment in the HSR (Chafekar and Duennwald 2012; Cowan et al. 2003; Duennwald and Lindquist 2008; Maheshwari et al. 2014; Neueder et al. 2017; Riva et al. 2012), and different hypothesis have been proposed in this regard (discussed in HSR and Other Protein Quality Control Systems in HD section). Different studies suggested that Hsp depletion is caused by their sequestration into aggregates (Park et al. 2013; Seidel et al. 2016; Yamanaka et al. 2008), while Labbadia et al. proposed that impaired Hsp expression in HD occurs at the level of transcription due to hypoacetylation of histone H4 at Hsp promoters altering nucleosome landscape and chromatin architecture (Labbadia et al. 2011). However, these studies did not fully explain how Hsp

promoters are preferentially hypoacetylated over other HD gene promoters.

The transcriptional regulation of the HSR is primarily controlled by HSF1 (Parsell and Lindquist 1993; Wu 1995). The importance of HSF1 is demonstrated by the high susceptibility to heat-induced apoptosis in Hsf1-null fibroblasts (McMillan et al. 1998) and the failure to respond to thermal insults in Hsf1-null mice (Neueder et al. 2017; Xiao et al. 1999), whose defects are attributed to the improper induction of HSF1-induced Hsp. The fundamental role of HSF1 in HD was demonstrated by Hayashida and colleagues when they showed increased polyQ aggregation and shortened lifespan in a transgenic mouse model of HD (R6/2) lacking Hsf1 (Hayashida et al. 2010). Additionally, overexpression of a caHSF1 in the same mouse model enhanced Hsp expression, inhibited polyQ aggregation, and ameliorated HD-like symptoms (Fujimoto et al. 2005; Rimoldi et al. 2001). Other authors obtained similar results in *C. elegans*, *Drosophila*, and mammalian cells expressing polyQ (Homma et al. 2007; Hsu et al. 2003; Morley and Morimoto 2004), supporting the importance of HSF1 in HD. acknoHSF2 also plays an important role in polyQ aggregation since lack of HSF2 enhanced mHTT aggregation and shortened the lifespan of R6/2 mice (Shinkawa et al. 2011). Although the exact role of HSF2 in HD has not been fully characterized, it is possible that the defects observed in Hsf2 null mice are associated with an alteration in the ratio of HSF1-HSF2 heterotrimers and the impact on HSF1 activation.

Early studies conducted by Chafekar and Duennwald in immortalized striatal cells derived from wild-type mice expressing full-length HTT-Q7 (STHdhQ7) and knock-in HD mice expressing HTT-Q111 (STHdhQ111; HD cells) demonstrated a significant reduction in HSF1 in HD cells. HSF1 depletion leads to severe impairments when cells are exposed to heat shock conditions (Chafekar and Duennwald 2012). In the study, the authors demonstrated that both nuclear translocation and trimerization of HSF1 were significantly lower in STHdhQ111 cells than STHdhQ7, particularly after heat shock, presumably due to the lowered levels of HSF1 observed in the HD cells. Such deficits correlated with reduced expression of several Hsp upon HS in HD, a phenomenon widely observed in other studies (Chafekar and Duennwald 2012; Duennwald and Lindquist 2008; Maheshwari et al. 2014; Neueder et al. 2017). The authors also demonstrated a significant reduction of HSF1 levels, both mRNA and protein, in the striatum of STHdhQ111 knock-in mice compared to wild-type mice. As with the study above, studies conducted in R6/2 and zQ175 HD mice showed an age-dependent decrease in HSF1 levels in differentially affected brain regions (Gomez-Pastor et al. 2017; Maheshwari et al. 2014). HSF1 ChIP-seq in STHdhQ111 and STHdhQ7 cells under control (33 °C) or HS (42 °C) also showed a marked reduction in HSF1 DNA binding to its target genes in STHdhQ111 cells in both 33 and 42 °C compared to STHdhQ7 cells (Riva et al. 2012). Recently, studies conducted by Neueder et al. in which both R6/2 and CAG-140 HD mice were exposed to HS in vivo showed a significant impairment in the HSR, in which many affected genes were HSF1-dependent (Neueder et al. 2017). Further comparisons between HD and Hsf1 null mice transcriptomes in the absence of HS will be necessary to establish the extent to which HSF1 degradation contributes to large transcriptional changes in HD.

Very recently, Gomez-Pastor et al. demonstrated the existence of a pathological HSF1 degradation pathway in the presence of polyQ and suggested this process as a potential driving force for the HSR impairment in HD (Gomez-Pastor et al. 2017). The authors demonstrated that the presence of polyQ enhanced the expression of two proteins, protein kinase CK2α' and the E3 ligase FBXW7, which sequentially phosphorylated and ubiquitylated HSF1 and signaled the protein for proteasomal degradation. The authors showed that CK2α' mediated phosphorylation of Ser303/ 307 was a prerequisite for FBXW7-dependent ubiquitylation and degradation. Abrogation of HSF1 phosphorylation in vitro by either genetic mutation of the Ser303 into Ala and genetic and pharmacological

inhibition of CK2α’ resulted in increased levels of HSF1 and Hsp70 and Hsp25 expression and decreased polyQ aggregation. As proof of concept, the authors generated a zQ175 HD mouse lacking one allele of the CK2α’ kinase and demonstrated increased levels of HSF1 and Hsp, decreased polyQ aggregation in the striatum, and improved HD-like phenotypes such as amelioration of synaptic deficits and decreased weight loss (Gomez-Pastor et al. 2017). These studies proposed CK2α’ as a potential therapeutic strategy to prevent HSF1 degradation in HD, enhance Hsp expression, and ameliorate protein aggregation. These and other studies have developed different therapeutic strategies aimed at activating HSF1 to enhance the levels of multiple Hsp simultaneously and ameliorate protein aggregation and neurodegeneration (Neef et al. 2011) and discussed in HSF1 as a Therapeutic Target in HD and Other PolyQ Diseases section.

7.2 Crosstalk Between HSF1 and the UPS: Implications in HD

When there is a failure in the correct folding of abnormal proteins, they can be targeted by the UPS for degradation or the lysosome-mediated autophagy pathway. In conjunction with the HSR, these systems ensure protein homeostasis and prevent the accumulation of toxic peptides (Hipp et al. 2012, 2019). However, eukaryotic proteasomes cannot digest polyQ sequences longer than 25Q residues (Venkatraman et al. 2004). Typically, polyQ diseases like HD possess polyQ sequences from 37-300Qs, exceeding standard proteasome products’ length and interfering with proteasome function. Inclusion bodies in HD contain ubiquitin and components of the proteasome (20S core proteasome, 19S regulatory complex, and 11S proteasome-activating complex) whose levels increase in an age-dependent manner (Davies et al. 1997; DiFiglia et al. 1997; Waelter et al. 2001). These observations suggest an age-dependent failure of the UPS to degrade polyQ (Sherman and Goldberg 2001). Also, the continued accumulation of these polyQ inclusions may impact the folding of other proteins exceeding the proteasome’s capacity and eventually reduce the cell’s ability to degrade other proteins (Bence et al. 2001).

Many investigators have connected dysregulation of the UPS and mHTT-induced toxicity in cellular models of HD (Bence et al. 2001; Bennett et al. 2005; Duennwald and Lindquist 2008; Hunter et al. 2007; Maynard et al. 2009), mouse models of HD (Bennett et al. 2007; Wang et al. 2008), and tissues from patients with HD (Bennett et al. 2007; Seo et al. 2004). The HSR and UPS pathways are closely interconnected. When there is a block in proteasome function by inhibitors such as MG132, abnormal proteins accumulate, and the expression of Hsp is enhanced (Bush et al. 1997; Lecomte et al. 2010; Mathew et al. 1998; Pirkkala et al. 2000). Also, damaged UPS is a potent stressor that stimulates HSR (Bush et al. 1997). Various studies indicated that the UPS network’s impairment activates HSF1 and HSF2, which in turn elevates Hsp expression (Lecomte et al. 2010; Mathew et al. 1998; Pirkkala et al. 2000). UPS-mediated induction of Hsp70 facilitates the E3 ubiquitin ligase C-terminus of HSC70-interacting protein (CHIP)-dependent ubiquitylation and degradation of protein aggregates (Lackie et al. 2017). The role of CHIP in promoting mHTT ubiquitylation and suppression of mHTT aggregation and toxicity has been extensively studied in cells, flies, zebrafish, and mouse models of HD (Al-Ramahi et al. 2006; Jana et al. 2005; Miller et al. 2005). CHIP is confined to the cytoplasm in unstressed conditions, but it translocates to the nucleus in response to cellular stress (Dai et al. 2003). CHIP promotes HSF1 trimerization and transcriptional activation of HSF1 and is a requirement for protection against stress-induced apoptosis in murine fibroblasts. Lastly, CHIP leads to stable interactions between Hsp70 and activated forms of HSF1. The authors detected CHIP in DNA-bound HSF1-containing complexes that were transcriptionally activated by heat shock (Dai et al. 2003). CHIP exerts a central and unique role in tuning the response to stress at multiple levels by regulating protein quality control and transcriptional activation of stress response signaling via HSF1.

There is a broad acceptance that the UPS function is abrogated in HD, although the causes of such a problem are unclear. It is still under debate whether deficits in the UPS activity cause mHTT accumulation or rather are the consequence of polyQ aggregation. Considering the intricate relationship between HSF1, Hsp70, and the UPS and the fact that HSF1 degradation and Hsp70 expression are impaired during HD, we speculate that the HSR impairment may cause UPS dysfunction in HD. However, further studies are necessary to unravel the role of the UPS in HD since alternative studies have shown no impairment in this system (Bett et al. 2006; Bowman et al. 2005; Diaz-Hernandez et al. 2003; Maynard et al. 2009; Seo et al. 2008).

Recent studies have demonstrated the existence of a mammalian membrane-associated proteasome complex, specifically expressed in the nervous system, that modulates neuronal function by degrading intracellular proteins into extracellular peptides, which can stimulate neuronal signaling (Ramachandran and Margolis 2017). This study challenged the notion that proteasomes function primarily to maintain protein homeostasis. In a follow-up study, the authors indicated that this neuronal-specific 20S membrane proteasome complex (NMP) exclusively degrades a large fraction of ribosome-associated nascent polypeptides that are newly synthesized during neuronal stimulation (Ramachandran et al. 2018). Interestingly, this NMP-mediated degradation is independent of canonical ubiquitylation pathways. However, several aspects of these NMPs remain unknown, including their potential role in the UPS in neurons, whether there is an alteration in their function during HD, and the extent HSF1 influences the regulation of NMPs.

7.3 PolyQ-Induced Oxidative and Metabolic Stress: Convergence into HSF1

Cells possess antioxidant mechanisms responsible for the elimination of harmful byproducts generated during biological processes. However, the failure of these systems often results in oxidative stress and cellular cytotoxicity. Oxidative stress is the imbalance between ROS/reactive nitrogen species (RNS) generation and the biological antioxidant defense system. Accumulation of ROS/RNS leads to damage of proteins, DNA, and lipids and further damages tissues and organs. Many studies in both HD patients and experimental mouse models of HD have documented that oxidative stress is a critical player in HD neuropathology (Johri and Beal 2012; Paul and Snyder 2019). Studies conducted in the striatum of different mouse models of HD (R6/1 and R6/2) revealed the accumulation of ROS, alteration in nitric oxide synthase (NOS) and superoxide dismutase (SOD) activities (Deckel et al. 2002; Perez-Severiano et al. 2002; Santamaria et al. 2001; Tabrizi et al. 2000), and a decline of antioxidant molecules such as ascorbate and antioxidant vitamins (Rebec et al. 2002). Moreover, increased levels of 8-hydroxydeoxyguanosine (8-OHDG), reports have shown that a product of oxidative stress-derived DNA damage is present in the urine, plasma, samples from striatal microdialysis, and brain of R6/2 and BACHD mice (Browne and Beal 2006; Gray et al. 2008; Stack et al. 2008). Studies conducted in plasma and postmortem tissues from patients with HD have also shown increased global oxidative stress manifested by increased lipid peroxidation products (malondialdehyde and lipofuscin), reduction in the antioxidant molecules, and increased DNA damage and fragmentation (Browne et al. 1999; Butterworth et al. 1998; Chen et al. 2007; Hersch et al. 2006; Tunez et al. 2011). The latter correlated with CAG repeat length and instability (Massey and Jones 2018) (see Structure and function of HTT section for details). Biochemical and proteomic studies conducted in the cerebral cortex and the striatum of HD patients also revealed decreased glutathione (GSH), a potent intracellular redox buffer, and a substantial elevation of glutathione peroxidases (GPX1 and 6), peroxiredoxins (PRDX1,2, and 6), and manganese-containing SOD (MnSOD/SOD2) (Beal et al. 1992; Sorolla et al. 2008). Endogenous ROS mainly originates from mitochondria during the synthesis of ATP. There is a large amount of evidence showing that

mitochondrial dysfunction indeed plays a crucial role in polyQ-mediated oxidative stress and the pathogenesis of HD (Carmo et al. 2018b; Costa and Scorrano 2012; Damiano et al. 2010; Guedes-Dias et al. 2016; Lin and Beal 2006; Quintanilla and Johnson 2009). This topic is discussed in detail in the section HSF1 as a Potential Regulator of PolyQ-Dependent Mitochondrial Dysfunction.

HSF1 can sense and respond to oxidative stress by inducing the expression of several Hsp and other anti-apoptotic genes (Ahn and Thiele 2003; Kim et al. 2013; Raitt et al. 2000). Studies conducted by Ahn and Thiele using purified recombinant mammalian HSF1 and H2O2 demonstrated that HSF1 directly senses oxidative stress by forming a disulfide bond between two cysteine residues (C35 and C105) localized within the HSF1 DNA-binding domain (Ahn and Thiele 2003). C35-C105 disulfide bond formation is critical for HSF1 trimerization, nuclear accumulation, DNA binding, and target gene activation in the presence of H2O2 (Ahn and Thiele 2003). This process seems to be reversible since the addition of reducing agents such as DTT to an H2O2 activated-HSF1 trimer decreased binding to an Hsp70 HSE sequence. At the same time, removal of DTT by dialysis and re-exposure to H2O2 recovered HSF1 binding. Additional studies conducted in yeast showed that Hsf1 coordinates the expression of Hsp under oxidative stress conditions by interacting with the yeast oxidative stress response regulator Skn7 (Raitt et al. 2000). Interestingly, Skn7 contains a small polyQ at its C-terminus (Gutiérrez et al. 2017), which implies that polyQ dependent aggregation mechanisms may play a role in regulating the Hsf1-Skn7 mediated response to oxidative stress. While there are no human homologs described for Skn7, it seems to share sequence homology and function with the RHOA effector protein ROCK-I (Alberts et al. 1998), a modulator of dendritic spine morphogenesis upon Ca2+ signaling (Murakoshi et al. 2011). RHOA and ROCK-I mRNA and the induction of protein levels in HD human blood leukocytes and postmortem brain and R6/2 HD mouse brain tissue respond to oxidative stress conditions (Narayanan et al. 2016). Interestingly, studies conducted in cardiomyocytes showed that activation of RHOA negatively regulates the HSR via attenuation of the HSF1-HSE binding and significantly suppressed the proteotoxic stress-induced HSR (Meijering et al. 2015).

Upregulation of Hsp during oxidative stress is key to ensure proper protein folding and prevents further oxidative damage (Janowska et al. 2019). Rubinsztein and colleagues showed that Hsp27, an ATP-independent chaperone, has antioxidant effects on cells expressing mHTT and its overexpression decreased ROS and polyQ-mediated cell death. Surprisingly, the authors also showed that there was not an attribution of benefits exerted by Hsp27 to the suppression of polyQ aggregation but rather the amelioration of polyQ-mediated oxidative stress (Wyttenbach et al. 2002). Reports have shown that Hsp27 protects against oxidative stress through its ability to increase GSH levels and glucose-6-phosphate dehydrogenase activity, preventing the mitochondrial cytochrome-c release and maintaining optimal cellular detoxifying machinery (Paul et al. 2002; Preville et al. 1999). HSF1 plays a crucial role in regulating Hsp27 expression in HD (Gomez-Pastor et al. 2017). Both HSF1 depletion and subsequent downregulation of Hsp27 in HD can contribute to the damages caused by polyQ-induced oxidative stress (Gomez-Pastor et al. 2017). Interestingly, Hsp27 also modulates HSF1 transactivation activity by promoting conjugation of SUMO-2/3 at Lys298 on HSF1 (Brunet Simioni et al. 2009). These studies demonstrate how Hsp27 exerts a feedback inhibition of HSF1 transactivation and enlighten the strictly regulated interplay between HSF1, Hsp, and oxidative stress.

A critical transcription factor that regulates antioxidant proteins' expression and protects against oxidative damage is the nuclear factor E2-related factor 2 (NRF2). NRF2 activation mitigates multiple pathogenic processes involved in HD and other neurodegenerative disorders through upregulation of antioxidant defenses, inhibition of inflammation, improvement of mitochondrial function, and maintenance of protein homeostasis (Dinkova-Kostova et al. 2018). NRF2 has become an attractive therapeutic target

in neurodegeneration, and several small molecules activating NRF2 have shown protective effects in numerous models of neurodegenerative diseases. NRF2 have several common target genes with HSF1, including heme oxygenase 1 (HMOX1, also known as HSP32) (Maines and Ewing 1996; Prestera et al. 1995), HSP70 gene (Almeida et al. 2010), autophagy cargo protein sequestosome 1 (p62/SQSTM1) (Komatsu et al. 2010; Samarasinghe et al. 2014), and ATF3 (Dziunycz et al. 2014; Hoetzenecker et al. 2011). Recent studies conducted in MCF7 breast cancer cells have shown that NRF2 transcriptionally activates HSF1 under oxidative stress conditions, connecting these two critical regulators of cytoprotective mechanisms (Paul et al. 2018). While no studies have yet explored these two transcription factors' dual activities in HD, there is evidence that alteration of their expression levels and activities may affect HD pathogenesis (Dinkova-Kostova et al. 2018; Gomez-Pastor et al. 2017; Quinti et al. 2017).

Oxidative and metabolic stresses and mitochondrial dysfunction are interconnected HD processes (Lou et al. 2016). Proteomic analysis conducted in yeast revealed that cells challenged to menadione, a ROS generator, upregulated antioxidant enzymes, several Hsp, and the expression of metabolic enzymes in a Hsf1-dependent fashion (Kim et al. 2013). Additionally, overexpression of alphaB-crystallin (αB-crys) in astrocytes reversed HD related phenotypes and neuronal cell loss in BACHD mice by restoring antioxidant protection and glucose uptake in astrocytes (Oliveira et al. 2016). On the other hand, AMPK is another important sensor for cell survival by modulating energy homeostasis. Ju and colleagues showed that AMPK is activated in the brains of mice and patients with HD, suggesting that this abnormal activation may contribute to neuronal degeneration in HD (Ju et al. 2014). The authors showed that mHTT-induced ROS contributes to the activation of AMPK-α1 and subsequently facilitates neurotoxicity in STHdhQ109 cells and the striatum of R6/2 mice while AMPK inhibition reduced the level of oxidative stress (Ju et al. 2014). However, conflicting reports have shown the opposite indicating that activation of AMPK in HD protects from neuronal dysfunction and vulnerability (Vazquez-Manrique et al. 2016). Therefore, it is unclear to what extent AMPK activation can be considered a detrimental response. However, the fact that AMPK negatively regulates HSF1 activation via phosphorylation at Ser121 (Dai et al. 2015) points out the possibility that activation of AMPK-α1 could contribute to the impairment of the HSR in HD and therefore negatively contribute to pathology.

7.4 HSF1 as a Potential Regulator of PolyQ-Dependent Mitochondrial Dysfunction

7.4.1 Mitochondrial Dysfunction and Excitotoxicity

Mitochondria are distinct cellular organelles that control vital physiological processes such as energy production, lipid metabolism, and Ca2+ signaling. There is a strong association between mitochondrial function defects with different NDs, including HD (Wang et al. 2019). The overall manifestation of mitochondrial impairment in HD appears in the abnormal mitochondrial morphology (Jin et al. 2013a; Kim et al. 2010a, b; Panov et al. 2002; Squitieri et al. 2006), alteration in the components of the electron transport chain (Browne et al. 1997; Gu et al. 1996; Guidetti et al. 2001; Sawa et al. 1999), reduction in cellular glucose uptake and enzymes in the oxidative metabolism (Antonini et al. 1996; Butterworth et al. 1985; Dubinsky 2017; Feigin et al. 2001; Sorolla et al. 2008; Tabrizi et al. 1999), and increased mitochondrial DNA damage (Acevedo-Torres et al. 2009; Horton et al. 1995; Liu et al. 2008). Reports have shown that all these defects play an important role in promoting MSN degeneration (Carmo et al. 2018a; Costa and Scorrano 2012; Lin and Beal 2006; Quintanilla and Johnson 2009). Indeed, it has been proposed that mHTT-mediated mitochondrial abnormalities underlie the specific vulnerability of MSNs in HD since this type of neurons require a higher-energy demand compared to other cell types in the brain (Ferrante et al. 1991; Mitchell and Griffiths 2003;

Pickrell et al. 2011). This hypothesis is supported by additional studies in which administration of mitochondrial inhibitors in rodents (Beal et al. 1993; Borlongan et al. 1995; Brouillet et al. 1993) and non-human primates (Brouillet et al. 1995) produced selective striatal degeneration and behavioral defects evocative of HD (Brouillet et al. 1999; Brouillet et al. 2005).

The primary function of mitochondria is the generation of energy in the form of ATP. ATP is generated via oxidative phosphorylation through the action of the electron transport chain (ETC) in conjunction with the ATP synthase (Lenaz and Genova 2010). Several components of the ETC present altered levels in postmortem striatum and cortex of patients with HD and cell and mouse models of HD (Browne et al. 1997; Gu et al. 1996; Majumder et al. 2007; Quintanilla and Johnson 2009; Tabrizi et al. 1999; Tabrizi et al. 2000). Interestingly, lymphoblasts from patients with HD, but not from patients with ataxia type-1 (another polyQ disease), treated with toxins targeting different complexes of the ETC, showed increased mitochondrial depolarization and caspase 3-dependent apoptosis (Sawa et al. 1999). This study demonstrated the increased susceptibility of HD cells to mitochondrial dysfunction and mitochondrial-induced cell death. However, analyses conducted in pre-symptomatic patients with HD have not shown ETC abnormalities (Guidetti et al. 2001), suggesting that mitochondrial defects may be secondary to neurodegeneration.

The exact mechanism by which mHTT induces mitochondrial dysfunction is unclear, but several studies have linked these defects to alterations in Ca2+ buffering. The maintenance of mitochondrial Ca2+ levels is by the mitochondrial permeability transition pore (mPTP). When mitochondrial Ca2+ buffering capacity is overloaded, the mPTP opens and causes a decrease in mitochondrial membrane potential (MMP), uncoupling the oxidative phosphorylation and leading to cell death (Choo et al. 2004; Halestrap 2006; Krieger and Duchen 2002; Rasola et al. 2010). Alterations in Ca2+ buffering capacity and decreased threshold for mPTP opening are defects widely reported in HD (Brennan Jr. et al. 1985; Choo et al. 2004; Ciammola et al. 2006; Milakovic and Johnson 2005; Milakovic et al. 2006; Oliveira et al. 2006; Panov et al. 2002; Rockabrand et al. 2007; Squitieri et al. 2006). These defects are more significant in the striatum than in the rest of the brain (Gellerich et al. 2008). They are delayed in cyclosporin A and mPTP inhibitor (Milakovic and Johnson 2005). Additional studies conducted in mitochondria isolated from skeletal muscle from R6/2 mice have shown that reduced Ca2+ accumulation capacity and mPTP opening threshold are also responsible for energetic depression and muscle atrophy (Gizatullina et al. 2006). Further, immunocytochemistry and electron microscopy studies conducted in brain mitochondria obtained from YAC72 HD mice revealed a direct interaction between the N-terminal fragment of mHTT and the outer mitochondrial membrane (Choo et al. 2004; Jin et al. 2013b; Kim et al. 2010a, b; Panov et al. 2002; Rockabrand et al. 2007; Squitieri et al. 2006), suggesting a direct role of mHTT in the alteration of mPTP function.

Yan et al. initially reported that HSF1 plays a significant role in regulating mitochondrial activity (Yan et al. 2002). The authors showed that the heart of Hsf1-/- mice presented increased oxidative damage on a structural component of the mPTP (ANT1 protein), which resulted in decreased ANT1 catalytic activity and increased mPTP opening. Additional studies in oocytes and hepatocytes of Hsf1−/−mice showed mitochondrial functional deficits, ultrastructural abnormalities, and increased caspase-3 activation (Bierkamp et al. 2010; Canto 2017). Recent studies have connected HSF1 with increased mitochondrial membrane depolarization and decreased MMP observed in HD (Intihar et al. 2019). Intihar et al. showed that silencing Hsf1 in the wild-type striatal cell model STHdhQ7 resulted in decreased MMP compared to scramble conditions (Chafekar and Duennwald 2012; Cui et al. 2006; Gomez-Pastor et al. 2017; Intihar et al. 2019). More importantly, these HSF1-induced defects in STHhdQ7 cells recapitulated the MMP decrease observed in untreated STHhdQ111 HD cells (Chafekar and Duennwald 2012; Cui et al. 2006; Gomez-Pastor et al. 2017; Intihar et al. 2019). Due to the fact that HSF1 seems to regulate MMP and dramatically reduce its levels in HD

(Gomez-Pastor et al. 2017), it is reasonable to hypothesize that HSF1 dysfunction in MSNs could be at the forefront of the increased MSN mitochondrial susceptibility to polyQ.

Disrupted intracellular Ca2+ levels in neurons can be provoked by excessive glutamatergic signaling. Glutamate is the primary excitatory neurotransmitter in the CNS and has critical functions in controlling perception, reward circuitry, and cognition. In HD, glutamate release changes, glutamate uptake, and postsynaptic signaling converge into Ca2+ buffering dysregulation and promote mitochondrial energy failure and cell death. This phenomenon is known as excitotoxicity (Dong et al. 2009; Zhou et al. 2013). Different neuronal receptors respond to the extracellular levels of glutamate. However, ionotropic receptors such as N-methyl-D-aspartic acid receptor (NMDAR) and α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptor (AMPAR) are the major players (Danysz and Parsons 2003). Reports have shown that mHTT disrupts glutamatergic transmission by reducing the levels of glial glutamate transporter 1 (GLT1) and therefore reducing astrocytic glutamate reuptake (Behrens et al. 2002; Estrada-Sanchez et al. 2009; Li et al. 2000; Lievens et al. 2001; Shin et al. 2005). Decreased GLT1 expression in HD leads to increased extracellular glutamate concentration, and excessive Ca2+ influx through the NMDARs, and increased NMDAR-mediated currents in the striatum from HD mice (Fan et al. 2007; Laforet et al. 2001; Milnerwood et al. 2010; Okamoto et al. 2009; Qiu et al. 2013; Song et al. 2003; Sun et al. 2001; Zeron et al. 2002). Interestingly, in silico analyses have revealed several putative HSE in the promoter region of both human and mouse GLT1 (Liu et al. 2011). Liu et al. showed an increase in GLT1 expression in NG108–15 cells (a hybrid mouse neuroblastoma-rat glioma cell line) exposed to heat shock. The authors also showed that administration of the neuroprotective drug riluzole increased the amount of HSF1 in NG108–15 cells by slowing HSF1 turnover and increasing the levels of GLT1 (Liu et al. 2011). Also, cells exposed to glutamate excitotoxic stress survived better in the presence of riluzole, indicating that increased HSF1 and GLT1 levels protect cells from excitotoxicity. Despite all the evidence discussed, it is still unknown whether depletion of HSF1 in the striatum of HD mice is responsible for GLT1 downregulation and glutamate excitotoxicity in HD (Chafekar and Duennwald 2012; Maheshwari et al. 2014).

7.5 Crosstalk Between HSF1 and Mitochondrial-Mediated Apoptotic Pathways

Mitochondria are a reservoir for pro-apoptotic factors and play a fundamental role in regulating cell death (Dumollard et al. 2009; Suzuki et al. 1999). Dysregulation of two major transcription factors, tumor suppressor p53 and PGC-1α, has been connected to mitochondrial-mediated apoptosis in HD. p53 is responsible for regulating pro-apoptotic genes such as BCL2-associated X (BAX) and p53 upregulated modulator of apoptosis (PUMA), and its activation triggers mitochondrial-dependent intrinsic apoptosis. There is an increase in p53 levels in cell and mouse models of HD and the striatum and cortex of patients with HD (Bae et al. 2005; Reynolds et al. 2018). Also, transcriptomic analysis in the striatum of an allelic series of HD knock-in mice demonstrated CAG length-dependent activation of p53 signaling pathways (Langfelder et al. 2016). Bae et al. also showed that p53 interacts with HTT, and there is an enhancement in such interaction in the presence of polyQ. It is still unknown how mHTT increases p53, although the authors hypothesized a dysregulation in p53 turnover. They also showed that genetic and pharmacological inhibition of p53 provided neuroprotection by altering transcription and improving mitochondrial function (Bae et al. 2005). However, the mechanism by which p53 inhibition mediated such beneficial effects in HD is unknown. Additional studies showed that mHTT increased phosphorylation of p53 on Ser46, a key PTMs involved in decoupling p53 from the apoptosis inhibitor iASPP, thereby inducing the expression of apoptotic target genes (Bae et al. 2005; Grison et al. 2011; Yu

et al. 2001). Alternative studies suggested p53 induces mitochondrial damage and necrosis by directly binding to DRP-1, a primary mitochondrial fission protein (Guo et al. 2013, 2014).

Reciprocal regulation between p53 and HSF1 has been reported in different contexts such as DNA damage and cancer (Jin et al. 2009; Logan et al. 2009; Oda et al. 2018), suggesting that the abnormal degradation of HSF1 may accelerate the harmful effects of p53 on mitochondrial dysfunction in HD. The primary mechanism responsible for keeping p53 levels under control depends on an E3 ligase MDM2, a gene regulated by p53 that promotes p53 ubiquitylation and degradation (Barak et al. 1993; Haupt et al. 1997; Momand et al. 1992). When MDM2 is inefficient or fails, an alternative degradation mechanism involving the small chaperone αB-crys and the E3 ligase FBX4 is activated (Jin et al. 2009). The αB-crys interacts with p53 and facilitates the recruitment of FBX4 (Watanabe et al. 2009). The accumulation of p53 in αB-crys−/− cells is due to the inability of p53 to interact with FBX4 (Jin et al. 2009). The transcriptional regulation of αB-crys expression is made by HSF1 (Gomez-Pastor et al. 2017), and its expression is downregulated in HD (Gomez-Pastor et al. 2017; Hodges et al. 2006). Consistent with this observation, Hsf1 deficient cells express reduced levels of αB-crys and accumulate p53 (Jin et al. 2009). Interestingly, p53 positively regulates the expression of FBXW7 (Kimura et al. 2003; Mao et al. 2004), an E3 ligase involved in the degradation of HSF1 and whose levels are increased in HD (Gomez-Pastor et al. 2017; Kourtis et al. 2015). Therefore, p53 and HSF1 may operate on a unified pathological pathway that controls mitochondrial function and neuronal integrity in HD (Fig. 7).

PGC-1α governs an additional mechanism essential for the regulation of mitochondrial function. PGC-1α is a transcription factor that regulates the expression of nuclear-encoded mitochondrial genes and participates in the regulation of mitochondrial biogenesis, ROS detoxification, and oxidative phosphorylation (Johri et al. 2013; Puigserver and Spiegelman 2003; Wu et al. 1999). Downregulation of PGC-1α has been reported in cell and mouse models of HD as well as in postmortem brain, muscle biopsies, and myoblast cultures from HD patients (Chafekar and Duennwald 2012; Cui et al. 2006; Gomez-Pastor et al. 2017; Intihar et al. 2019; Turner and Schapira 2010; Weydt et al. 2006). Along with a decrease in the expression of PGC-1α, there is also a decrease in the expression of several PGC-1α-dependent targets and MSN markers (Lucas et al. 2012; Weydt et al. 2006). The role of PGC-1α in HD is supported by several studies conducted in different mouse models. Ppargc1a (a gene encoding PGC-1α) null mice displayed similar defects to those observed in HD such as mitochondrial dysfunction, myelination deficits, degeneration of striatal neurons, white matter atrophy, and motor alterations (Cui et al. 2006; Leone et al. 2005; Lin et al. 2004; Lucas et al. 2012; Weydt et al. 2006; Xiang et al. 2011). Recent studies where PGC-1α was knocked out in MSNs showed that PGC-1α is necessary for MSN transcriptional homeostasis and function (McMeekin et al. 2018). Although the loss of PGC-1α in MSNs does not replicate an HD-like phenocopy, its target genes are altered in a CAG-length and age-dependent fashion, suggesting a potential role of PGC-1α in the selective vulnerability of MSNs in HD (McMeekin et al. 2018). In contrast, overexpression of Ppargc1a rescued HD neurological phenotypes and neurodegeneration and decreased mHTT aggregation (La Spada 2012; St-Pierre et al. 2006). Similarly, pharmacological activation of PGC-1α ameliorated both neuropathological features and HD phenotype in different mouse models of HD (Chandra et al. 2016; Chiang et al. 2010; Jin et al. 2013a; Johri et al. 2013).

Despite all the studies showing the importance of PGC-1α in HD, the mechanism responsible for PGC-1α downregulation is still unclear. Different studies have suggested that an impairment in the CREB/TAF4 signaling pathway in HD (Cui et al. 2006; Gines et al. 2003; Weydt et al. 2006) controls the expression of PGC-1α by binding to cAMP-response elements (CRE) present in the PGC-1α promoter (Fig. 7). Other studies indicated that increased extrasynaptic NMDAR

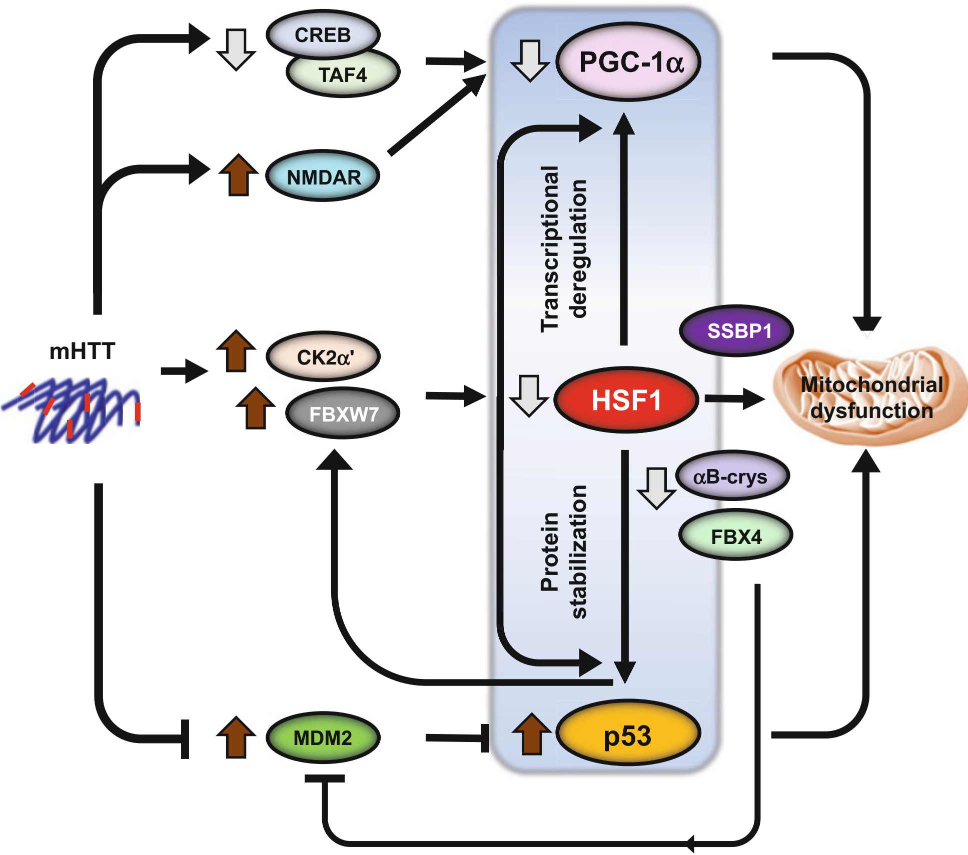

Fig. 7 Model for p53-HSF1-PGC-1α integrated responses in HD. Crosstalk between the transcription factors p53, HSF1, and PGC-1α in regulating transcription, protein homeostasis, mitochondrial function, and apoptosis. There are alterations in different pathways (CREB/TAF4, CK2α'/FBXW7, and MDM2) in the presence of mHTT, which independently leads to the deregulation of the levels and functions of all three transcription factors. However, HSF1 becomes a key player in the subsequent regulation of the levels of p53 and PGC-1α by directly regulating the transcription of PGC-1α and controlling p53 protein stability in HD. The potential role of p53 in regulating the HSF1 degradation pathway in HD would add a positive feedback loop into the p53-HSF1-PGC-1α axis, which triggers mitochondrial dysfunction and neuronal death. Reprinted and modified from "Mitochondrial Dysfunction in Huntington's Disease; Interplay Between HSF1, p53 and PGC-1α Transcription Factors" by Intihar et al. 2019, with permission from the original authors

activity causes PGC-1α depletion (Dickey et al. 2016). More recently, different studies showed that HSF1 directly binds to a non-canonical HSE present in the PGC-1α promoter and regulates its expression (Chafekar and Duennwald 2012; Cui et al. 2006; Gomez-Pastor et al. 2017; Intihar et al. 2019; Ma et al. 2015). Intihar et al. documented a reduction in HSF1 binding to PGC-1α promoter in STHdhQ111 cells compared to STHdhQ7 cells and that overexpression of HSF1 in STHdhQ111 cells restored PGC-1α expression and improved cell viability (Chafekar and Duennwald 2012; Cui et al. 2006; Gomez-Pastor et al. 2017; Intihar et al. 2019). Consistent with this view, increased HSF1 levels in the striatum of zQ175; CK2α' +/− mice enhanced the expression of PGC-1α and its target genes (Gomez-Pastor et al. 2017) and demonstrated an interconnection between HSF1 and PGC-1α in HD. An additional regulatory mechanism that can potentially explain PGC-1α downregulation in HD involves p53, which has

been shown to bind to PGC-1α promoter in neuroblastoma cells and regulates its expression (Aquilano et al. 2013). These studies indicate that HSF1, p53, and PGC-1α conform to an intricate pathway that can operate together in controlling mitochondrial function (Fig. 7). Future efforts should focus on elucidating the molecular mechanisms that directly link the interplay between HSF1, p53, and PGC-1α in HD.

7.6 Excitatory Synapse Regulation in HD; Connection Between HSF1 and Mitochondrial Dysfunction

The striatum receives inputs from both the cortex and thalamus and coordinates these inputs to regulate movement and cognition. Among the different chemical synapses that control striatum function, glutamatergic excitatory synapses play a fundamental role. Several lines of evidence suggested that early clinical manifestations of HD may arise from excitatory synaptic dysfunction, including progressive MSNs axonal degeneration (Albin et al. 1992), alteration of neurite outgrowth and maintenance (Trushina et al. 2004), disruption of synaptic gene expression (Luthi-Carter et al. 2003; Manczak and Reddy 2015; Rozas et al. 2010; Smith et al. 2014), collapse in axonal transport of organelles and neurotrophic factors (Gunawardena et al. 2003; McGuire et al. 2006; Trushina et al. 2004), and an imbalance between excitatory and inhibitory systems (Benn et al. 2007; Twelvetrees et al. 2010). Although the exact function of HTT is not known, McKinstry et al. demonstrated the requirement of HTT for normal excitatory synapse development in both cortical and striatal circuits (McKinstry et al. 2014). Several additional studies have shown the specific deterioration in the cortico-striatal circuitry in HD and proposed that these defects may be responsible for striatum degeneration (Bunner and Rebec 2016; Cepeda et al. 2007; Dogan et al. 2015; Rebec 2018). However, other studies in YAC128 and zQ175 HD mouse models have suggested an early dysfunction in the thalamo-striatal circuit, which occurs before an overt HD phenotype (Gomez-Pastor et al. 2017; Kolodziejczyk and Raymond 2016). Interestingly, Gomez-Pastor et al. showed that manipulation of the HSF1 degradation pathway in the zQ175 mice by removing one allele of CK2α’ resulted in increased HSF1 levels and restoration of the thalamo-striatal synapse connectivity (Gomez-Pastor et al. 2017). Unfortunately, the mechanism by which HSF1 regulates this specific circuitry in HD is still unknown.

Previous studies have demonstrated that HSF1 plays a vital role in the regulation of synapse function and formation. Hsf1 deficient mice showed a significant decrease in the number of dendritic spines and dendrite length in the hippocampus, downregulation of polysialylated-neural cell adhesion molecule (PSA-NCAM) required for synapse formation. They decreased expression of the postsynaptic protein PSD-95 needed to anchor NMDARs and AMPARs to the postsynaptic membrane (Uchida et al. 2011). Also, the authors showed that overexpression of a caHSF1 in Hsf1−/−mice rescued PSD-95 expression and improved synapse function (Uchida et al. 2011). Other studies that support the role of HSF1 in the regulation of synaptic genes were conducted in cells and mouse models of AD treated with the Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG) (Chen et al. 2014). In this study, the authors showed that 17-AAG treatment enhanced HSF1 activity and increased the expression of synapsin I and synaptophysin (presynaptic proteins regulating vesicle cycling) and PSD-95, leading to the prevention of amyloid-β-induced memory loss (Chen et al. 2014). Also, the identity of several HSE in different synaptic genes, including DLG4 (encoding PSD-95) and DLG1 (encoding SAP97) (Chen et al. 2014; Ting et al. 2011). These studies suggest that HSF1 plays a direct role in the structural and functional integrity of synaptic connections underpinning learning and memory. However, since the role of HSF1 in synaptic gene expression impairment in HD has not been established yet, other hypotheses may be possible (Fig. 8).

Reports have shown that chaperones exert protective functions at the synapse level, and the sole

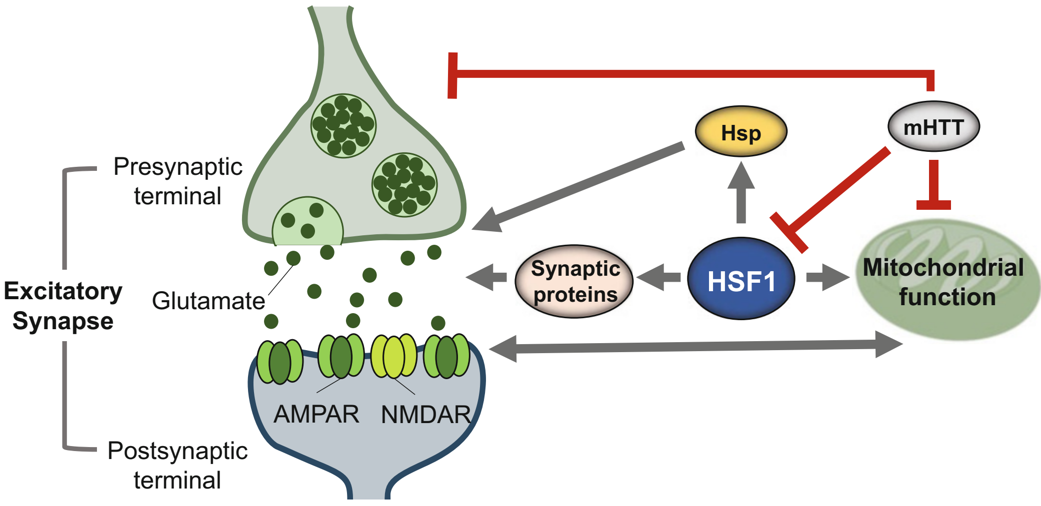

Fig. 8 Working model for the connection between HSF1, mitochondrial impairment, and synapse dysfunction in HD. mHTT alters excitatory synaptic transmission and mitochondrial function by altering Ca2+ buffering capacity and PGC-1α and p53 levels. mHTT is also responsible for promoting HSF1 degradation, which results in downregulation of Hsp and synaptic proteins. The effects caused by HSF1 depletion then further disrupt glutamate release and uptake in neurons, resulting in excitotoxicity. (Reprinted from "Excitatory synapse impairment and mitochondrial dysfunction in Huntington's disease: heat shock factor 1 (HSF1) converging mechanisms" by (Zarate and Gomez-Pastor 2020), with permission from Neural Regeneration Research)

overexpression of Hsp70 in cortical cells provides protection against glutamatergic excitotoxicity and improves glutamatergic synaptic transmission (Mokrushin et al. 2005; Song et al. 2016). Therefore, upregulation of Hsp by HSF1 might impact excitatory synapses in HD by improving protein folding and scaffolding at the synapse zone. An additional mechanism that can explain the restoration of excitatory synapses is an increase in HSF1 levels in the correction of mitochondrial function. Mitochondria are essential in synaptic transmission through ATP production, Ca2+ homeostasis, synthesis of glutamate, synaptic vesicle recycling, and functional maintenance of dendritic spines (Smith et al. 2016; Zeron et al. 2002). As discussed above, HSF1 regulates mitochondrial function and MMP by controlling the expression of PGC-1α in HD cells (Gomez-Pastor et al. 2017; Zarate and Gomez-Pastor 2020). Therefore, HSF1 could modulate excitatory synapses in HD throughout its action on PGC-1α. However, further studies are warranted to ascertain how these mechanisms are selective to thalamo-striatal excitatory synapses, whether HSF1-dependent PGC-1α expression constitutes a causal role in MSNs degeneration in HD, and how HSF1 maintains synapse integrity in the adult brain.

7.7 Role of HSF1 in Other PolyQ-Related Diseases

HD belongs to a family of polyQ-related diseases that include spinal and bulbar muscular atrophy (SBMA), dentatorubral-pallidoluysian atrophy (DRPLA), and several spinocerebellar ataxias (SCAs) (Williams and Paulson 2008). As in HD, the cause of all these diseases is a CAG repeat expansion in the mutated gene. Although it occurs in entirely unrelated genes that affect different cell types and brain regions, all these polyQ diseases share several features. The characterization of PolyQ diseases is nuclear localization of

the toxic protein (Orr 2012; Orr and Zoghbi 2007), the propensity for the mutant proteins to form insoluble aggregates in neurons (Gusella and MacDonald 2000), instability in the CAG repeat number with a tendency for further elongations (Schols et al. 2004), and an inverse correlation between the CAG repeat length and the age of onset (Zoghbi and Orr 2000). Similar to HD, depletion of HSF1 as well as impairment of Hsp expression has been documented in many polyQ diseases and demonstrated that failure in the HSF1-Hsp axis enhances pathogenesis (Evert et al. 2018; Katsuno et al. 2005; Kondo et al. 2013; Tsai et al. 2005). Different studies have also reported the neuroprotective effects of activating HSF1 in many cellular and rodent models of polyQ diseases (Fujimoto et al. 2005; Rimoldi et al. 2001), indicating that failure in the HSR is a common and underlying mechanism in several polyQ-related diseases.

In SBMA, the CAG expansion occurs in the first exon of the androgen receptor (AR) gene (Katsuno et al. 2012; La Spada et al. 1991) and affects males exclusively (Chen et al. 2018; Grunseich et al. 2014). Nuclear accumulation of the polyQ-AR occurs in both neuronal and non-neuronal cells but preferentially affects motor neurons in the brainstem and spinal cord (Adachi et al. 2005; Katsuno et al. 2002). Kondo et al. showed a significant decrease in HSF1 levels in different brain regions of the SBMA mouse model AR-97Q and autopsy specimens from patients with SBMA. The authors also observed that pathogenic polyQ-AR accumulation was greater in tissues where HSF1 levels usually are lower (brain and pancreas). In contrast, tissues with relatively higher HSF1 levels (liver and testis) did not show polyQ-AR accumulation. Interestingly, Hsf1 depletion expanded the distribution of polyQ-AR accumulation to those tissues that are generally not affected, aggravating the SBMA phenotype. On the contrary, lentiviral-mediated overexpression of HSF1 decreased the accumulation of polyQ-AR and neuronal atrophy in the brain of AR-97Q mice (Kondo et al. 2013). Pharmacological activation of HSF1 has also shown beneficial effects on reducing polyQ-AR aggregation and amelioration of SBMA-like phenotypes (Bott et al. 2016; Hargitai et al. 2003). Treatment with arimoclomol, a molecule known to prolong the activation of HSF1, induced chaperones expression and enhanced motor neuron survival, and delayed disease progression in SBMA mice (Bott et al. 2016); (Hargitai et al. 2003).

DRPLA is a rare autosomal dominant polyQ disease and the most HD-like disease among the polyQ disorders. It is clinically characterized by cerebellar ataxia, chorea, myoclonus, epilepsy, dementia, and seizures (Tsuji 2012). The CAG repeat expansion occurs in ATROPHIN-1 (Atro), a transcriptional corepressor (Zhang et al. 2002). Although there are not many studies evaluating the role of HSF1 in this disease, Nisoli and colleagues demonstrated that overexpression of Hsp40 significantly suppressed polyQ-Atro toxicity in a *Drosophila* model of DRPLA (Nisoli et al. 2010). Additional studies in HeLa cells transfected with an expression vector containing 81 CAG repeats of DRPLA cDNA and expressing a dominant active form of HSF1 (Ad-HSF1-ΔRDT) showed decreased polyQ aggregates compared to cells expressing wild-type HSF1 (Fujimoto et al. 2005; Rimoldi et al. 2001). The authors also demonstrated that the effect of Ad-HSF1-ΔRDT on polyQ aggregates suppression was more efficient than overexpression of any of the significant Hsp (Hsp27, Hsp40, Hsp70, and Hsp110). These pieces of evidence suggest a beneficial role of activating HSF1 in DRPLA. However, additional in vivo studies will be necessary to determine the implications of HSF1 in this disease fully.

SCAs are a heterogeneous group of autosomal dominant inherited ataxias that are characterized by degeneration of Purkinje cells in the cerebellum and occasional degeneration in the brainstem, spinal cord, and basal ganglia (Schols et al. 2004; Zoghbi 2000). To date, at least 43 subtypes of SCA have been classified depending on their genetic locus (Klockgether et al. 2019; Sun et al. 2016). Among them, six SCA subtypes, SCA 1, 2, 3, 6, 7, and 17, are the most common ones, and the genes affected are ATAXIN-1 (Banfi et al. 1994), ATAXIN-2 (Satterfield and Pallanck 2006), ATAXIN-3 (Kawaguchi et al. 1994), α1A

subunit of the voltage-dependent calcium channel Cav2.1 (CACNA1A) (Orr 2012), ATAXIN-7 (David et al. 1997), and TATA-box binding protein (TBP) (Koide et al. 1999), respectively. The signs and symptoms may vary between different SCAs, but they also share some phenotypes like an uncoordinated walk (gait), poor hand-eye coordination, and abnormal speech (dysarthria). Interestingly, Ingenwerth and colleagues reported that mice lacking Hsf1 showed gait and other motor symptoms reminiscent of cerebellar ataxia. Also, they showed that CALBINDIN, a calcium-binding protein, was reduced in the cerebellum of Hsf1−/−mice and suggested a role of HSF1 in the regulation of Purkinje cell calcium homeostasis (Ingenwerth et al. 2016). A study conducted in a cell model of SCA6 demonstrated that the levels of HSF1 and Hsp70 were significantly downregulated (Li et al. 2009). Similarly, Tsai et al. showed decreased expression of Hsp27 and Hsp70 in transformed lymphoblastoid cells from patients with SCA7 (Tsai et al. 2005). Conversely, activation of HSF1 by a small molecule HSF1A inhibits the interaction between HSF1 and the chaperonin complex TRiC (Neef et al. 2014), suppressed polyQ aggregation, and ameliorated neurotoxicity in a fly model of SCA3 (Neef et al. 2010). These studies led to pharmacological and genetic manipulations of HSF1 in different SCAs to explore the potential role of HSF1 as a therapeutic target and showed successful induction of Hsp expression and amelioration of polyQ aggregation (Adachi et al. 2003; Chan et al. 2000; Chang et al. 2013; Chen et al. 2010; Cummings et al. 2001; Fujikake et al. 2008; Ghosh and Feany 2004; Gong and Golic 2006; Helmlinger et al. 2004; Katsuno et al. 2005; Kobayashi et al. 2000; Malik et al. 2013; Wang et al. 2013; Warrick et al. 1999b).

Rimoldi et al. utilized a non-neuronal cell system of SCA1 expressing ATAXIN-1 with polyQ tracts of different lengths and determined the effect of de-repressed HSF1 mutant variants on polyQ aggregation (Rimoldi et al. 2001). They showed that HSF1-S303G and S307G, variants unable to be phosphorylated, significantly induced Hsp70 and Hsp40 expression and abolished nuclear polyQ-ATAXIN-1 accumulation. Other pharmacological approaches have used Hsp90 inhibition to activate HSF1. Ding et al. showed that treatment of a neuronal cell model of SCA1 with BIIB021, a synthetic Hsp90 inhibitor, increased the transactivation capacity of HSF1, leading to decreased aggregation and toxicity induced by mutant ATAXIN-1 (Ding et al. 2016). Administration of 17-AAG, another known Hsp90 inhibitor, suppressed inclusion body formation, and eye degeneration in a *Drosophila* model of SCA3 in an HSF1-dependent manner (Fujikake et al. 2008). Alternative treatments with herbal extracts with HSF1 activation properties have also reduced toxic aggregates formation in cells expressing mutant ATAXIN-3 (Chang et al. 2013). The effects of Hsp90 inhibitors in cellular and *Drosophila* SCA models are promising; however further studies are required to determine the effects of these inhibitors in more relevant in vivo models of SCA. This strategy has already been tested in the R6/2 HD mice and showed that pharmacological activation of HSF1 using the Hsp90 inhibitor NVP-Hsp990 failed to maintain long-term benefits in vivo, a problem that may be due to progressive loss of HSF1 protein in HD (Gomez-Pastor et al. 2017). Since previous reports have shown HSF1 depletion in SCAs, further experiments will be necessary to uncover the causes that mediate HSF1-Hsp depletion in the different polyQ diseases and whether this may impact future pharmacological studies.

8 HSF1 as a Therapeutic Target in HD and Other PolyQ Diseases

Over the past decade, there has been a profound advancement in understanding the molecular mechanisms responsible for neuronal death in HD and other polyQ diseases, emphasizing those pathways that lead to protein misfolding and aggregation. This knowledge has resulted in development of several molecules with therapeutic potential (Neef et al. 2011). However, current advances in clinical trials are focused on using antisense oligonucleotides against HTT to lower

HTT expression (Imbert et al. 2019; Lemprière 2019; Tabrizi et al. 2019). This section will discuss the strategies focused on the activation of the HSR as a potential future venue to ameliorate protein aggregation and neurodegeneration in HD and other polyQ diseases.

One of the most exploited strategies to induce the HSR and ameliorate protein aggregation is Hsp90 inhibitors. As discussed earlier, Hsp90 suppresses HSF1 multimerization and transactivation (Bharadwaj et al. 1999; Zou et al. 1998), and therefore pharmacological inhibition of Hsp90 leads to HSF1 activation and Hsp induction. Well-studied Hsp90 inhibitors are geldanamycin, 17-AAG, 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), celastrol, radicicol, and Hsp990. Geldanamycin is a benzoquinone ansamycin antitumor antibiotic, and Sittler and colleagues reported its first therapeutic effect in a cell culture model of HD (Sittler et al. 2001). The authors showed that geldanamycin induces the expression of Hsp40 and Hsp70 and suppresses aggregation of mHTT. Its anti-aggregation activity was subsequently tested in hippocampal slice cultures derived from HD mice (Hay et al. 2004). However, recent reports have shown that geldanamycin has low solubility, inadequate blood-brain-barrier permeability, and toxicity (Bose and Cho 2017). Less toxic derivatives of geldanamycin are 17-AAG and 17-DMAG. Treatment of 17-AAG strikingly decreased degeneration of photoreceptor neurons in *Drosophila* models of both HD and SCA3 (Fujikake et al. 2008). Importantly, 17-AAG benefits were abolished when HSF1 was knocked down (Fujikake et al. 2008). Research has shown that 17-DMAG inhibits the formation of mHTT aggregates with higher efficiency than geldanamycin and 17-AAG (Herbst and Wanker 2007). Celastrol constitutes a new class of Hsp90 inhibitors that, unlike the classical Hsp90 inhibitors (geldanamycin and 17-AAG), does not block ATP binding to Hsp90. Instead, Celastrol inhibits Hsp90 activity by binding to its C-terminus domain and blocking Hsp90 oligomerization (Zhang et al. 2008, 2009). Celastrol contributes to the activation of HSF1 and Hsp induction (Westerheide et al. 2004) and suppresses polyQ-induced aggregation in vitro (Wang et al. 2005; Zhang and Sarge 2007). However, celastrol has shown activity against numerous targets besides HSF1 (Lee et al. 2006; Sreeramulu et al. 2009; Yang et al. 2006; Zhang and Sarge 2007) and therefore, the HSF1-dependency in the effects mediated by celastrol is unclear.

The use of 17-AAG has been explored in mouse models of SBMA and animal models from other proteinopathies such as AD and frontotemporal dementia and demonstrated its benefits in improving synaptic connectivity and behavior (Chen et al. 2014; Ho et al. 2013; Katsuno et al. 2005; Waza et al. 2005). Unfortunately, the effects of 17-AAG and other Hsp90 inhibitors on HSF1 activation in HD progression in the mammalian brain have remained unknown due to their low efficiency in crossing the blood-brain barrier (Ebrahimi-Fakhari et al. 2013; Egorin et al. 2001; Porter et al. 2010). A new Hsp90 inhibitor developed by Novartis and with brain-penetrant properties is Hsp990 (Jackrel and Shorter 2011; Menezes et al. 2012). Oral administration of Hsp990 increased Hsp, reduced aggregate load in the brain, and improved motor symptoms at early stages in R6/2 HD mice (Labbadia et al. 2011). Unfortunately, the use of Hsp990 failed to provide long-term benefits in the R6/2 mice and resulted in the attenuation of the HSR. The authors attributed this defect to an alteration in the chromatin structure on the Hsp promoters that resulted in chronic Hsp downregulation. However, more recent studies suggested that failure to provide long-term benefits upon Hsp90 inhibition in vivo may be due to the pathological degradation of HSF1 reported in HD (Gomez-Pastor et al. 2017). In support of this study, Chafekar and Duennwald reported that STHdhQ111 cells treated with radicicol, another Hsp90 inhibitor, showed very modest or absent effects on the levels of Hsp70 and Hsp27 as well as increased cell sensitivity (Chafekar and Duennwald 2012). They hypothesized that lack of Hsp induction might be due to the low levels of HSF1 observed in those cells compared with wild-type STHdhQ7 cells (Chafekar and Duennwald 2012). Therefore,

these studies suggest that activation of HSF1 via Hsp90 inhibition in HD may not be a successful therapeutic strategy after all.

Due to the toxicity issues of many Hsp90 inhibitors reported in HD, other strategies have investigated alternative ways of inducing the HSR (Calamini et al. 2011). Calamini and colleagues developed a high-throughput screening using HeLa cells stably transfected with a heat shock-inducible reporter containing the proximal human HSPA2 (a gene encoding Hsp70.1) promoter sequence upstream of a luciferase reporter gene and measured the activation of the HSR in more than 900,000 molecules. The authors identified a series of small molecules (PR, proteostasis regulators) that induced HSF1-dependent chaperone expression, did not inhibit Hsp90 activity, and restored protein folding in PC12 cells expressing HTTQ74-GFP and in *C. elegans* expressing YFP-tagged Q35 protein. Besides the activation of HSF1, the study revealed several molecules provided proteome stability by activating other factors, including DAF-16/FOXO and SKN-1/NRF-2, and suggested an activation in HSR via alternative mechanisms. An additional screening conducted by Neef and colleagues used a humanized yeast-based high-throughput screen insensitive to proteotoxic stress and Hsp90 inhibition and identified a small molecule activator of human HSF1, so-called HSF1A (Neef et al. 2010). The study reported that HSF1A repressed aggregation and cytotoxicity of mHTT in a cell culture model of HD and ameliorated polyQ-induced cytotoxicity in a *Drosophila* model of SCA3. Further studies revealed that HSF1A inhibits the repressive interaction between HSF1 and the chaperonin complex TRiC/CCT, consequently increasing HSF1 activity and Hsp expression (Neef et al. 2014). However, the effects of the PRs or HSF1A in mouse models of HD or other polyQ diseases are still unknown.

Instead of disrupting the interaction between HSF1 and its negative regulators, other strategies have searched for molecules that directly enhance HSF1 activity. Bimoclomol and its derivative arimoclomol are non-toxic hydroxylamine derivatives and are co-inducers of the HSR under stress conditions (Kalmar et al. 2002; Vigh et al. 1997). Reports have suggested that bimoclomol directly binds to HSF1, increasing the time and duration of HSF1 binding to the respective target genes and therefore prolonging the activation of HSF1 (Hargitai et al. 2003). The direct binding was analyzed by measuring binding of radiolabeled [3H]bimoclomol to purified proteins by equilibrium dialysis where [3H]bimoclomol showed binding to both recombinant and native HSF1 but not Hsp70 or Hsp90. However, other studies suggested that bimoclomol enhances HSF1 activity indirectly through altering plasma membrane fluidity (Torok et al. 2003). The mechanism of action for prolonged HSF1 activity by bimoclomol or arimoclomol remains unclear. Despite the controversy in the mechanism of action of these molecules, both bimoclomol and arimoclomol have shown cytoprotective value in mitigating motor neuron degeneration in SBMA (Malik et al. 2013) as well as in an array of different diseases such as ALS (Kalmar et al. 2008; Kieran et al. 2004), diabetes (Biro et al. 1997; Erdo and Erdo 1998), cardiac dysfunction (Jednakovits et al. 2000; Lubbers et al. 2002; Polakowski et al. 2002), and cerebrovascular disorders (Melville et al. 1997). Unfortunately, arimoclomol did not improve motor function or rescue of striatal pathology in YAC128 HD mice (Pouladi et al. 2010). An additional therapeutic strategy pursuing prolonged HSF1 DNA binding is the use of SIRT1 agonists. SIRT1 is a deacetylase responsible for deacetylating HSF1-Lys80 in the DBD domain and promoting DNA binding release (Westerheide et al. 2009). Studies in different animal models provided convincing evidence that SIRT1 and different SIRT1 agonists like resveratrol protect neurons in cell and mouse models of HD and *C. elegans*, although there were controversial results reported in a fly model (Duan 2013; Parker et al. 2005). The therapeutic potential of resveratrol is currently being tested in clinical trials for HD (ClinicalTrials.gov identifier: NCT02336633). The study has revealed a reduction in neurodegeneration among HD patients treated with resveratrol by showing an improvement in brain energy profiles measured by 31P-magnetic resonance spectroscopy.

It is essential to remember that HSF1 undergoes a progressive and pathological degradation in HD, and therefore strategies aimed at stimulating HSF1 activity may not be effective in the long-term. Thus, other strategies are investigating the stabilization of HSF1 protein levels rather than activity stimulation as a more attractive strategy to ameliorate long-term protein aggregation in HD. Riluzole, an FDA approved anti-glutamatergic agent for ALS treatment, has shown the ability to increase HSF1 protein levels by disrupting the association between HSF1 and the chaperone-mediated autophagy pathway (Yang et al. 2008). Moreover, riluzole increased the expression of the glutamate transporter GLT1 and abolished NMDA-induced excitotoxicity in an HSF1-dependent manner (Liu et al. 2011). Experiments using animal models of HD and other proteinopathies have determined the neuroprotective effects of riluzole (Douhou et al. 2002; Scherfler et al. 2005; Schiefer et al. 2002). More importantly, riluzole alleviated clinical symptoms by preserving brain structure and increasing neurotrophin production in patients with HD (Bonelli and Hofmann 2007; Squitieri et al. 2009). Although this molecule failed in phase III HD clinical trials in 2007 (ClinicalTrials.gov Identifier: NCT00277602), its efficacy is currently being tested to treat other polyQ disorders such as SCA2 (ClinicalTrials.gov Identifier: NCT03347344). The use of protein kinase CK2 inhibitors (TID43, Emodin, and CX4945) has also shown the ability to increase HSF1 protein levels by preventing Ser303/307-phosphorylation-dependent degradation. CK2 inhibitors increased Hsp expression and decreased mHTT load and cell death in different cellular models of HD. Inhibition of CK2 has been recently proposed as a potential target to treat different neurological and psychiatric disorders due to its ability to modulate HSF1 levels and inflammation (Castello et al. 2017; Gomez-Pastor et al. 2017). Further studies will be necessary to address these inhibitors' potential benefits in animal models of polyQ-related diseases.

Many of the molecules discussed in this section, whether synthetic or natural, have shown some forms of neuroprotection against HD or other polyQ diseases in different cellular models. However, many of them have failed when tested in more relevant and physiological animal models. Many of the studies have also explored the single use of one molecule or another but never a combination of them. Due to the complexity of the HSR, it may be necessary to combine different strategies to obtain a synergistic effect on the activation and stabilization of HSF1 and Hsp expression and, therefore, a more successful strategy towards preventing long-term aggregation and neurodegeneration.

9 Conclusion

Since the discovery of HSF1 in 1984, hundreds of studies have contributed to improving our understanding of this critical transcription factor's protective roles. While HSF1 was initially ascribed to modulate the Hsp in response to heat shock conditions, we know now that HSF1 controls very diverse physiological processes, including cell growth and differentiation, apoptosis, oxidative stress, aging, immune and inflammatory responses, and neuronal development through the regulation of numerous target genes, mainly in a temperature-independent manner. It is not difficult to predict that defects in HSF1 activation at any stage of an organismal life may lead to devastating consequences. The transcriptional program governed by HSF1 allows cells to survive under adverse conditions induced by proteotoxic stress in diverse diseases. Cumulative evidence presented in this book chapter has demonstrated the critical role of HSF1 in different neurological disorders, specifically in HD. Various groundbreaking studies on HSF1 have helped to elucidate the mechanisms by which neurons fail to fight aggregation and succumb to death, contributing to the design and exploration of potential therapeutic strategies to aid HSF1 in the course of HD and other pathologies. While we have made tremendous advancements in understanding HSF1 role in physiology and disease, many questions remain unanswered. To design effective and successful therapies in the future to ameliorate the

negative effects of aging and neurodegeneration, it will be necessary to fully characterize the differential roles of HSF1 in the young vs. adult brain, assess the spatiotemporal effects of altering HSF1 levels in the adult brain, and characterize all the factors that contribute to the HSR impairment in the aging mammalian brain.

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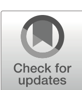

Leveraging Tissue Engineering for Skin Cancer Models

Sumayah Oudda, Abdulla M. Ali, Anna L. Chien, and Seungman Park

Abstract

Bioengineered in vitro three-dimensional (3D) skin model has emerged as a promising tool for recapitulating different types of skin cancer and performing pre-clinical tests. However, a full-thickness 3D model including the epidermis, dermis, and hypodermis layers is scarce despite its significance in human physiology and diverse biological processes. In this book chapter, an attempt has been made to summarize various skin cancer models, including utilized skin layers, materials, cell lines, specific treatments, and fabrication techniques for three types of skin cancer: melanoma, basal cell carcinoma (BCC), and squamous cell carcinoma (SCC). Subsequently, current limitations and future directions of skin cancer models are discussed. The knowledge of the current status of skin cancer models can provide various potential applications in cancer research and thus a more effective way for cancer treatment.

Keywords

Basal cell carcinoma (BCC) · In vitro 3D model · Melanoma · Skin cancer · Squamous cell carcinoma (SCC)

Abbreviations

5-FU	5-Fluorouracil
AK	Actinic Keratosis
ALA	Aminolevulinic Acid
BCC	Basal Cell Carcinoma
BEC	Blood Endothelial Cells
BLM	BRO Lung Metastasis
BM	Basement Membrane
CD31	Cluster of Differentiation 31
cDC2	Type-2 Conventional Dendritic Cells
Cldn4	Claudin 4
CLEC2A	C-Type Lectin Domain Family 2 Member A
cSCC	Cutaneous Squamous Cell Carcinoma
DED	De-Epidermized Dermis
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor

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G-CSF	Granulocyte Colony-Stimulating Factor
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HBL	Human Diffuse Large B-cell Lymphoma
HGF/SF	Hepatocyte Growth Factor/Scatter Factor
HMVEC	Human Microvascular Endothelial Cells
HNSCC	Head and Neck Squamous Cell Carcinoma
HPV	Human Papillomavirus
LEC	Lymphatic Endothelial Cells
LEF-1	Lymphoid Enhancer Binding Factor 1
LOX	Lysyl Oxidase
MAPK	Mitogen-Activated Protein Kinase Pathway
MMP-9	Matrix Metalloproteinase-9
NBCSS	Nevoid Basal Cell Carcinoma Syndrome
NK	Natural Killer
OMA1	Overlapping with the m-AAA Protease 1 Homolog
PDPN	Podoplanin
Ptch1	Patched 1
RDEB	Recessive Dystrophic Epidermolysis Bullosa
RGP	Radial Growth Phase
RNAi	RNA Interference
SCC	Squamous Cell Carcinoma
SHH	Sonic Hedge Hog
SMO	Smoothened
TGFβ	Transforming Growth Factor Beta
TJ	Tight Junction
TNF- α	Tumor Necrosis Factor Alpha
TRAIL	Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand
UV	Ultraviolet
VGP	Vertical Growth Phase

1 Introduction

Skin is commonly described as a protective layer because it is the body's first line of defense against many harmful threats. Over the past century, the increased exposure time to sunlight and UV has drastically increased the incidence of skin cancer (Zarebkohan et al. 2020). In the United States, skin cancer occurs in 5.4 out of every million (Cakir et al. 2012). Skin cancer is classified into three types: melanoma, basal cell carcinoma (BCC), and squamous cell carcinoma (SCC) (Fig. 1). Melanoma is the most dangerous cancer and accounts for 74% of the death toll, even though it makes up only 4% of all skin cancer cases (Dubas and Ingraffea 2013). Non-melanoma cancers, including SCC and BCC, are known to be less fatal than melanoma cancer, taking up 80% and 20%, respectively (Zarebkohan et al. 2020).

Various skin models have been utilized to better understand the molecular and cellular mechanisms involved in the development of cancer and to develop potential therapeutic methods for its treatment. A two-dimensional (2D) cell culture is one of the most common models for skin cancer research as well as many biological studies (Jensen and Teng 2020). However, numerous studies have revealed that there are considerable disparities between 2D and 3D tumor environments in terms of physiology, gene and protein profiles, cell/tissue function, and abnormal reaction toxicants (Carvalho et al. 2015; Duval et al. 2017; Lelièvre et al. 2017). For example, skin fibroblasts in 3D were physiologically relevant, having a dendritic shape, as opposed to in 2D, where they have a higher spreading area and more stress fibers (Park et al. 2015). As such, traditional 2D models have a very low translational potential. To reduce the gap between 2D and 3D in vitro models, animal models have been adopted due to their increased similarity to humans. Murine models were successfully tested to predict the efficacy and pharmacodynamics of anti-cancer drugs (Kuzu et al. 2015). However, there are still some limitations to animal models due to the inherent differences and a very low rate of reproducibility between humans and mice, thus resulting in less than 10% of data which is possible to translate (Carvalho et al. 2018; Mak et al. 2014). In the case of skin tissue, there was a significant difference in the architecture of the hypodermis between the two species

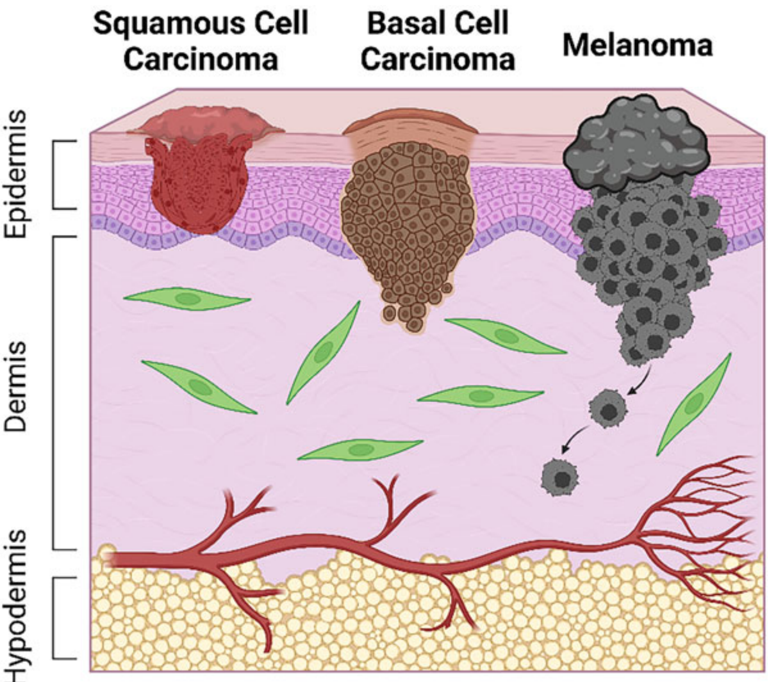

Fig. 1 Types of skin cancer. Squamous cell carcinoma (SCC) starts in the squamous cells of the skin, whereas basal cell carcinoma (BCC) develops in the basal cells. Melanoma, the deadliest form of skin cancer, frequently grows in a mole or is suddenly created as a dark spot, developing from melanocytes

(Khavari 2006). Hence, there is a necessity to develop a more effective system that mimics human physiology (Unnikrishnan et al. 2021).

Recent developments in 3D in vitro cancer models have provided an alternative to traditional approaches to recapitulate the tumor microenvironment with accuracy in a low-cost and simple manner (Li et al. 2019). The most commonly used methods for replicating 3D tumors include in vitro spheroids, microfluidics, assembling, tumor formation in 3D gels, and bioprinting (Asghar et al. 2015; Cui et al. 2017). These 3D in vitro models are more physiologically relevant, but data for studying skin cancer using these models are still scarce. Moreover, most of the 3D skin disease models, including cancer or wound healing, have utilized single- or double-layered tissues composed of epidermis or epidermis/dermis layers, excluding the hypodermis layer, despite its significance in several biological processes (Zimoch et al. 2021). In this review, we summarize various skin tissue models and their applications for cancer research using three different types of cancer: melanoma, SCC, and BCC. Since there are only a few studies using tri-layered skin tissue for skin cancer research in 3D in vitro models, we discuss the future directions regarding the role of adipose tissue in 3D skin cancer research.

2 Skin Cancer Models

2.1 Melanoma Skin Model

Melanoma is the deadliest skin cancer because of its apparent propensity to metastasize despite being the least frequent (Bourland et al. 2018). To mimic the melanoma microenvironment and understand the dynamics of melanoma cancer cells, diverse 2D and 3D melanoma skin models have been developed and utilized (Table 1).

A de-epidermized skin tissue model has been widely used for melanoma research. An organotypic skin melanoma construct consisting of a stratified epidermis, basement membrane, and dermis was fabricated by utilizing a de-epidermized, de-cellularized dermis (DED)

Table 1 Studies using melanoma skin tissue models

Skin layer	Material	Cell type	Fabrication method	Signaling molecule/ protein	Additional component	Reference
Epidermis Basement membrane Dermis	Rat tail collagen type I	Keratinocytes Fibroblasts 451-LU (metastatic melanoma cells)	Manual Hanging drops	Epidermal growth factor	TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) UVB Cisplatin	Vörsmann et al. (2013)
Epidermis Basement membrane Dermis	De-epidermized dermis	Keratinocytes Fibroblasts WM35 (melanoma cell line- radial growth phase) SK-MEL-28 (melanoma cell line-metastatic phase)	Manual			Haridas et al. (2017)
Epidermis Basement membrane Dermis	Rat tail collagen type I	Keratinocytes Fibroblasts Melanocytes WM35 (RGP melanoma cells) WM793 and WM115 (VGP melanoma cells) WM852 (metastatic melanoma cells)	Manual	EGF bFGF/Ad5		Meier et al. (2000)
Epidermis Basement membrane Dermis	Rat tail collagen type I	Keratinocytes Fibroblasts Melanoma cells	Manual		PLX4032 (Vemurafenib)	Lee et al. (2010)
Dermis	Rat tail collagen type I	Fibroblasts 451Lu and SKMel28 melanoma cells	Manual		RAF kinase inhibitor MEK inhibitors PI3K inhibitors	Meier et al. (2007)
Epidermis Basement membrane Dermis	De-epidermized dermis 0.5% collagenase A solution	Keratinocytes Fibroblasts HBL melanoma cells A375-SM melanoma cells C8161 melanoma cells	Manual	TNF-alpha Fibrin clot solution	Fibrin Ibuprofen	Marques and MacNeil (2016)
Epidermis Basement membrane Dermis (without stromal fibroblasts)	De-epidermized dermis	Keratinocytes Adherent melanoma cells (BLM)	Manual	Epidermal growth factor		Van Kilsdonk et al. (2010)
Epidermis Basement membrane Dermis	Collagen matrix	Keratinocytes Fibroblasts A375 melanoma cell line 451Lu melanoma cell line	Model produced by MatTek		Fisetin	Syed et al. (2014)

(continued)

Table 1 (continued)

Skin layer	Material	Cell type	Fabrication method	Signaling molecule/ protein	Additional component	Reference
Epidermis Basement membrane Dermis	Anchor papers	Keratinocytes Fibroblasts Human microvascular endothelial cells (HMVEC: includes blood and lymphatic endothelial cells) A375, Malme 3 M, SK-MEL 28, RPMI 7951, WM983A, and WM983B melanoma cells	Self-assembly method Hanging drops	Epidermal growth factor	Vermurafenib	Bourland et al. (2018)
Epidermis Basement membrane Dermis	De-epidermized dermis	Keratinocytes Fibroblasts BLM, BLM-GFP, Mel624, and A375 melanoma cells cDC2s	Manual Microneedle array system	Epidermal growth factor Keratinocyte growth factor		Di Blasio et al. (2020)

(Di Blasio et al. 2020). Fibroblasts were seeded onto the reticular dermal side of the DED while keratinocytes were later seeded onto the papillary side, on top of the basement membrane. Various melanoma cell lines including BLM, BLM-GFP, Mel624, and A375 were incorporated into the epidermis and dermis. To assess the impact of tumor microenvironment on immune cells, type-2 conventional dendritic cells (cDC2s) were then seeded into the dermis. The 3D skin model in this study demonstrated that the tumor microenvironment transforms wildtype, immunostimulatory cDC2s into immunosuppressive CD14+ dendritic cells that cannot effectively induce T-cell activation. This model highlights one manner in which melanoma cells propagate changes that weaken the immune system.

Kilsdonk et al., similar to the previous study (Di Blasio et al. 2020), also used a de-epidermized dermis to construct an organotypic skin model (Van Kilsdonk et al. 2010). The DED contained the de-cellularized dermal component and the basement membrane (BM), which were taken advantage of by carrying out trials where melanoma cells were either seeded into the reticular dermis or on top of the basement membrane, without keratinocytes included at first. In addition, no fibroblasts were incorporated into the skin construct in order to focus on the cell interactions between melanoma and keratinocytes. When metastatic melanoma cells (BLMs) were seeded onto the basement membrane without keratinocytes, the BM served as a barrier for invasion, and the melanoma cells could not cross into the dermis. In contrast, when keratinocytes were incorporated to form a stratified epidermis conjoined with the dermis, BLM invasion occurred. The experiments supported evidence that cross-talk between keratinocytes and metastatic melanoma cells is required for encouraging BLMs to pass through the BM by activating proteases such as MMP-9.

Marques et al. also utilized a de-epidermized dermis where fibroblasts were seeded onto the reticular dermis, and keratinocytes and melanoma cells were seeded onto the papillary surface (Marques and MacNeil 2016). This study was motivated by the fact that patients experience a local recurrence of melanoma in the wound bed post-surgical excision of the primary tumor. The 3D skin tissue model, complete with a stratified epidermis, basement membrane, and dermis, received a wound by using a scalpel blade to form a cut through the epidermis and into the dermis. The in vitro model demonstrated that inflammation can heighten melanoma invasion but be reduced by anti-inflammatory treatment

using three different melanoma cell lines: A375SM and HBL invasion were increased by wounding and decreased with ibuprofen treatment, while C8161's severe invasion was not impacted by wounding, TNF-α, or ibuprofen. The 3D skin tissue model used in this study has proved to be an immensely useful tool for assessing the behavior of different melanoma cell lines' unique responses to external factors.

Two different melanoma cell lines – WM35 and SK-MEL-28 – were employed to study cell invasion using a 3D skin equivalent model consisting of de-epidermized dermis containing fibroblasts, keratinocytes, and melanoma cells (Haridas et al. 2017). The invasion of the melanoma cell lines was quantified over 3 weeks, and the results demonstrated that SK-MEL-28 melanoma cells invaded the dermis quickly; at a slower rate, WM35 melanoma cells eventually invaded the dermis as well. In addition, as the invasion progressed, the basement membrane lost integrity, being partially absent. This led to the conclusion that interference with the basement membrane is potentially associated with the transition from the radial growth phase (RGP) to the vertical growth phase (VGP).

Lee et al. fabricated a human skin equivalent with a stratified epidermis, basement membrane, and dermis by means of the manual method (Lee et al. 2010). An acellular collagen foundation was set first, followed by a mixture of fibroblasts with rat tail collagen placed on top. Finally, keratinocytes and melanoma cells were seeded last. An inhibitor of BRAF V600E, PLX4032 (vemurafenib), an established drug used in melanoma treatment, was then applied to the skin construct. Results showed that PLX4032 exclusively decreased proliferation and induced apoptosis of melanoma cells with the BRAF V600E mutation. Furthermore, the tumors were diminished, highlighting the aggressive impact of the inhibitor on melanoma cells with the V600E oncogene.

The self-assembly method was leveraged to fabricate an organotypic skin melanoma substitute with a stratified epidermis, basement membrane, dermis, blood vessels, and lymphatic vessels (Bourland et al. 2018). This model, which highly mimics native skin, involved seeding fibroblasts onto three anchor papers to form cell sheets. Human microvascular endothelial cells (HMVEC) including lymphatic endothelial cells (LEC) and blood endothelial cells (BEC) were seeded into two of the cell sheets. On the third cell sheet, melanoma spheroids and keratinocytes were seeded. The three cell sheets were at last layered together to form the human skin substitute. The study exhibited that blood and lymphatic vessels were successfully formulated, distinguished by markers such as CD31 and PDPN; the vessels were also metabolically active, as seen by an increased presence of chemokine ligand 21 and angiopoietin-2. By including capillary networks, the 3D model more accurately represents native skin compared to 2D and 3D models without endothelial cells. The substitute was also assessed when treated with vemurafenib, and results showed that the proliferation of melanoma cells decreased significantly after treatment, although some proliferative cancer cells remained.

Syed et al. utilized a full-thickness skin model with A375 melanoma cells that are commercially produced by MatTek Corporation (Syed et al. 2014). The model includes a differentiated epidermis and dermis alongside the melanoma cells exhibiting RGP, VGP, and metastatic behavior. It should be noted that the in vitro skin equivalent was supplemented with media, both with and without fisetin (i.e., control), a tetrahydroxyfalvone. Results revealed that the fisetin-treated samples had significantly reduced melanoma cells and melanocytic lesions compared to the control. The study also highlighted that the experimental treatment did not cause any notable damage to the keratinocytes or fibroblasts, thus showcasing the advantage of using 3D skin constructs to assess not only the effects of potential drug treatments on melanoma but also on the skin morphology as a whole. Further investigation discovered that fisetin directly interacts with and binds to mTOR and p70S6K, suggesting that fisetin's antiproliferative effect stems from its interference in cell-signaling pathways.

A simple single- or double-layered skin model has also been widely used. A skin equivalent comprised of only the dermis, no basement

membrane nor epidermis present, was constructed using an acellular collagen solution made from rat tail collagen type I (Meier et al. 2007). A collagen matrix with fibroblasts and metastatic melanoma cells (with the V600E mutation) was then placed over the acellular layer. When the matrix was treated with the media containing MEK inhibitors, PI3K inhibitors, and/or RAF kinase inhibitor, the fibroblasts were not impacted by treatment. However, the RAF kinase inhibitor and the PI3K inhibitor, when used individually, decreased the melanoma invasion throughout the dermis. Moreover, using the two types of inhibitors together resulted in complete tumor suppression with only few melanoma cells left in the construct. This study demonstrates the opportunity to use bioengineered skin tissue for pre-clinical, in vitro testing of potential treatments that target cell signaling pathways such as MAPK and AKT. In another study, Meier et al. constructed double-layered skin mimetic tissue using the manual method, producing a stratified epidermis, basement membrane, and dermis. Similarly, a fibroblast-collagen matrix was set onto the acellular foundation and supplemented with medium containing epidermal growth factor. Subsequently, keratinocytes and melanoma cells were seeded. Results showed that basement membrane was successfully synthesized in vitro by fibroblasts and keratinocytes in the skin equivalent. Furthermore, transduction of the bFGF gene into melanoma cells exhibiting radial growth phase (RGP) allowed RGP melanoma cells to invade and nest in the dermis, a characteristic not seen without transduction. This model represents a potential application to study individual genes and their influence on melanoma phenotypes.

2D and 3D melanoma models were tested and compared to explore the effects of a chemotherapy drug and UVB using organotypic skin tissue, including a stratified epidermis, basement membrane, and dermis (Vörsmann et al. 2013). Melanoma spheroids, made of the metastatic 451-LU cell line, were produced by placing the cells using the hanging drop method and were embedded into the fibroblast-collagen matrix. The spheroids were treated with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and ultraviolet-B radiation (UVB), whereas others were treated with TRAIL and cisplatin. The study observed that whereas 2D melanoma models showed tumor cell death for TRAIL +UVB and TRAIL+cisplatin, only the latter caused apoptosis in melanoma spheroids of the 3D skin construct. Thus, UVB lost its ability to render melanoma tumors sensitive to TRAIL once the tumors were in the heterogenous model that better represented native skin. Furthermore, the TRAIL+cisplatin treatment selectively targeted melanoma cells since apoptosis was not induced in fibroblasts or keratinocytes. Overall, this study underscores that 2D results do not necessarily translate to 3D outcomes, reiterating the need to use multilayered 3D skin constructs for pre-clinical research.

2.2 Squamous Cell Carcinoma (SCC) Skin Model

Squamous cell carcinoma (SCC), the second most prevalent type of skin cancer, is characterized by aberrant, accelerated squamous cell proliferation. Similar to the melanoma model, single- or double-layered skin tissues have been commonly available using diverse fabrication methods, including a cutting-edge bioprinting technique (Table 2).

Browning et al. adopted a 3D skin model through a bioprinting method detailed by Derr et al. (Derr et al. 2019), to study cutaneous squamous cell carcinoma, where the model is composed of a stratified epidermis, basement membrane, and dermis (Browning et al. 2020). A commercial bioprinter (REGENHU 3D Discovery Bioprinter) was used to fabricate the dermis. In brief, fibroblasts were loaded into a syringe that was loaded into the bioprinter and then dispensed into the hydrogel consisting of gelatin, fibrinogen stock solution, collagen I, and elastin. A laminin/entactin solution was then hand-pipetted onto the dermal foundation to form the basal layer, followed by the deposition of keratinocytes. Afterward, A431 cSCC spheroids were manually placed onto the top of the skin

Table 2 Studies using SCC skin tissue models

Skin layer	Material	Cell type	Fabrication method	Additional component	Reference
Epidermis Dermis Basement membrane	Gelatin Fibrinogen stock solution Collagen I Elastin Laminin/ entactin	Keratinocytes Fibroblasts A431 cSCC cells	Bioprinting	RegenHU 3DDiscovery bioprinter	Browning et al. (2020)
Epidermis Dermis Basement membrane	Collagen G (collagen I and III)	Keratinocytes Fibroblasts SCC 12 cells	Manual	δ-Aminolevulinic acid (ALA, photosensitizer drug) Visible radiation (545 nm)	Obrigkeit et al. (2009)
Epidermis Dermis Basement membrane	Rat tail collagen I Fibronectin solution	Keratinocytes Fibroblasts SCC-25 cells	Manual		Brauchle et al. (2013)
Epidermis Dermis Basement membrane	Collagen I	Keratinocytes Fibroblasts SCC-12 cells	Manual	Ingenol mebutate gel	Zoschke et al. (2016)
Epidermis Dermis	Collagen I	Fibroblasts SCC12 cells Natural killer cells	Manual	Anti-CLEC2A blocking antibody OMA1	Gonçalves-Maia et al. (2020)
Epidermis Dermis	Rat collagen I	Fibroblasts HNO97, HNO199, HNO136, HNO206 (head and neck squamous cell carcinoma cells)	Manual	G-CSF and GM-CSF antibody against G-CSF and GM-CSF	Gutschalk et al. (2006)
Epidermis Dermis	Rat tail collagen I Matrigel	RDEB fibroblasts RDEB-cSCC keratinocytes (recessive dystrophic epidermolysis bullosa)	Manual	SB505124 PF573228 β-aminopropionitrile LY294002	Mittapalli et al. (2016)
Epidermis Dermis	Viscose fiber fabric	Fibroblasts Head and neck squamous cell carcinoma tissue slices	Manual	Fractionated irradiation	Engelmann et al. (2020)
Epidermis		Ker-CT (keratinocyte cell line)	Manual		Smirnov et al. (2019)
Epidermis Basement membrane Dermis	De-epidermized dermis	Fibroblasts Keratinocytes with recombinant retroviruses	Manual	pLXSN-8E7 retrovirus	Akgül et al. (2005)

model. It was observed that the cSCC cells exhibited radial and vertical growth phase. In addition, the model's integrity was supported by gene expression analysis that agreed with findings from in vivo cSCC studies. Certain groups were treated with 5-fluorouracil (5-FU), a chemotherapeutic drug, and results demonstrated that 5-FU selectively killed cSCC cells with insignificant toxicity to the non-cancerous keratinocytes.

An organotypic skin that consisted of a stratified epidermis, basement membrane, and dermis was created by means of the manual method to study photodynamic therapy, which involves a photosensitizer and light (Obrigkeit et al. 2009). Collagen III as well as Collagen I-containing fibroblasts served as the dermal component, and keratinocytes were seeded onto the dermal layer, followed by SCC 12 cells that were placed onto

the center of the construct. Experimental observations revealed that the cancerous cells in the skin equivalent exhibited characteristics similar to in vivo studies such as irregularly shaped keratinocytes with larger nuclei and the ability to proliferate in the epidermis and eventually the dermis. In addition, the photodynamic experiments consisted of a control group, a group treated solely with a pro-photosensitizer, a group treated with green light, and a group treated with both pro-photosensitizer and green light. The pro-photosensitizer, δ-aminolevulinic acid (ALA), converts to the photosensitizer protoporphyrin when placed in the tissue environment. Only in the experimental group treated with both ALA and green light was there substantial impact: SCC tumor size decreased, SCC apoptosis was induced, and SCC mitotic activity decreased. Since photodynamic therapy had already been integrated as a treatment for patients with SCC prior to these experiments, this research helped validate the use of SCC skin equivalents to reliably study the effects of potential cancer-targeting drugs.

A 3D SCC skin model consisting of a stratified epidermis, basement membrane, and dermis was used to understand the characteristics of SCC cells (Brauchle et al. 2013). Fibroblasts were embedded in rat tail collagen I and fibronectin solution was placed onto the collagen matrix. Then, either keratinocytes or SCC-25 cells were seeded onto the dermal component for a non-cancerous or SCC cancerous model, respectively. The disease model did not exhibit robust spatial organization nor a stratified corneous layer in contrast to the non-cancerous model. The SCC model illustrated characteristics similar to in vivo studies: keratin accumulations were present, and capsular tumor cell nests were formed. Unlike in vivo experiments, SCC-25 cells had not infiltrated the dermis spontaneously. This study also found that SCC-25 cells appeared to have weak cell-cell contacts, as seen by small gaps between the cells in the epidermis. Uniquely, Raman spectroscopy, a non-invasive technique, was utilized to distinguish between non-cancerous and cancerous models using differences in the Raman spectra. This form of spectroscopy proved useful in identifying SCC versus non-SCC skin tissue.

Zoschke et al. also used engineered human skin using the manual fabrication method that resulted in an organotypic model with a stratified epidermis, basement membrane, and dermis (Zoschke et al. 2016). Collagen I formed the foundation and was then layered with further collagen I integrated with fibroblasts. Keratinocytes were seeded onto the dermal layer, and the cancerous models added SCC-12 cells into the epidermis. SCC models were incorporated with different ratios of keratinocytes to SCC-12 cells to represent different stages of SCC (epidermal invasion only or epidermal and dermal invasion). Certain experimental groups received topical treatment of Ingenol mebutate gel, a drug that has been used for patients with non-melanoma skin cancer. Whereas non-cancerous models had a fully stratified epidermis, SCC models did not and were found to have parakeratosis. SCC-12 cells were also found to break down the basal membrane. The stratum spinosum of disease models had “architectural disarray”. Thus, the organotypic models proved useful in assessing a layer-by-layer look at the effect of SCC on skin. In addition, proteins related to the tight junction (TJ) were assessed: in SCC models, certain TJ proteins were reduced, such as Cldn4 and occludin. Numerous other differences were described as well; the stratum corneum of cancer models had lower lipid to protein ratios, lower cholesterol and ceramide levels, and a greater excess in phospholipids than non-cancerous models. Organotypic samples with more invasive SCC had a higher surface pH than normal. To study the permeability of skin tissue, caffeine permeation was assessed, and the SCC epidermis-dermis model had a permeability coefficient more than two-fold of the non-cancerous model. Furthermore, certain experimental groups were treated topically with Ingenol mebutate gel, which resulted in epidermal cell necrosis in both non-cancerous and cancerous models, resembling the outcomes of actinic keratosis (AK) patients who received application of the drug and experienced pain and skin irritation. Overall, these findings suggest that SCC presence in the skin may result in impaired skin barrier function and that organotypic skin is a suitable method of studying topical treatments.

Natural killer (NK) cells were incorporated into organotypic skin tissue, including an epidermis and dermis (Gonçalves-Maia et al. 2020). The NK cells were embedded into a collagen I matrix; fibroblasts in collagen I, seeded above by SCC-12 cells, were then placed onto the NK cell layer. Experimental observation revealed that the NK cells were able to move upward into the dermal component. In addition, this study examined how the interaction between fibroblasts and NK cells through the expression of CLEC2A genes impacts SCC-12 progression. In organotypic models of wild-type fibroblasts that expressed CLEC2A, the immune cells decreased the invasion of SCC-12. However, in models with xeroderma pigmentosum fibroblasts or cancer-associated fibroblasts, both of which do not significantly express CLEC2A, the NK cells did not reduce the cancer's invasion rate. In experiments with wild-type fibroblasts treated with anti-CLEC2A blocking antibody OMA1, SCC-12 invasion was prominent as well. These studies highlight the significance of different cell types and their gene profile to understand how squamous cell carcinoma behaves.

Gutschalk et al. investigated the behavior of head and neck squamous cell carcinoma using a manually produced organotypic model with an epidermis and dermis (Gutschalk et al. 2006). Fibroblasts were combined with rat collagen type I to form the dermal equivalents and various head and neck squamous cell carcinoma (HNSCC) lines were seeded on top for different experimental purposes. Certain trials employed HNSCC cells that secreted granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF). Addition of G-CSF and GM-CSF to cell lines, whether they already had the capacity to secrete them, increased migration and proliferation in the organotypic models. Cell lines that secrete G-CSF and GM-CSF themselves were characterized by invasion into the dermal equivalent; however, when antibodies to the two factors were incorporated, the tumor cells' invasive ability was severely reduced. The study highlighted the need to re-evaluate the potential side effects of using G-CSF and GM-CSF to treat certain secondary effects of cancer treatment.

Engelmann et al. studied the heterogeneous nature of head and neck squamous cell carcinoma (HNSCC) using organotypic skin models, including an epidermis, dermis, and immune cells as well (Engelmann et al. 2020). Rather than using a collagen-based scaffold, the dermal equivalent was created by culturing fibroblasts on a viscose fiber fabric, a technique that allows the fibroblasts to produce the extracellular matrix. To assess characteristics of cancer cell behavior at a patient-specific level, HNSCC samples from patients, which either came from HPV-driven HNSCC or non-HPV-driven HNSCC, were sliced, and the tissue slices were overlayed on top of the dermal equivalent. The organotypic models that utilized tissue samples of non-HPV driven HNSCC had similar morphology to the primary tumors of the patients they were derived from; meanwhile, there were notable deviations between HPV-derived models and their corresponding primary tumors, such as p16INK4a expression later in the trials. Models from different patients could also exhibit different proliferation behaviors: invasive growth pattern (growth into the dermis), expansive growth pattern (horizontal growth in the epidermis), and silent growth pattern (neither invasion nor expansion). Certain experimental groups were treated with fractionated irradiation, and the irradiation caused varying effects on the unique cancer models, mimicking how patients with SCC may respond differently to the same treatment. Overall, this study emphasizes the value of organotypic tissue as a fast and effective way to evaluate a variety of parameters and responses.

An in vitro skin disease model composed of two layers (epidermis and dermis) was utilized to study recessive dystrophic epidermolysis bullosa (RDEB) and associated cutaneous squamous cell carcinoma (cSCC) (Mittapalli et al. 2016). The disease model consisted of a matrix of rat tail collagen I, matrigel, and RDEB fibroblasts. cSCC keratinocytes obtained from RBED samples (RBED-cSCC keratinocytes) were seeded onto the dermal layer to form the epidermis. Inhibitors of TGFβ signaling, lysyl oxidase (LOX), or integrin β1-mediated mechanosignaling were added to the model's culture medium to

observe their effect toward cancerous invasion (inhibitors included SB505124, PF573228, β-aminopropionitrile, LY294002). Experimental models with RBED fibroblasts and RBED-cSCC keratinocytes had cancer cells invading the dermal equivalent, while models with normal human fibroblasts did not. Moreover, organotypic skin with RBED fibroblasts had significantly stiffer matrices than normal fibroblasts. When inhibitors such as SB505124 (TGFβ signaling) or β-aminopropionitrile (LOX inhibitor) were used as treatments, matrix stiffening and collagen fibril thickness decreased, respectively, which subsequently decreased the invasion of cancerous cells. This research underscored how cSCC is invigorated by the extracellular matrix conditions produced by RBED, bringing attention to the relationships between different skin diseases.

Akgül et al. utilized a de-epidermized dermis seeded with fibroblasts within the stroma and keratinocytes on top of the papillary side of the dermis to assess the effect of certain HPV oncogenes on keratinocytes (Akgül et al. 2005). In the disease models, keratinocytes were transduced with pLXSN-8E7 retrovirus, which causes the keratinocytes to produce the E7 protein of HPV type 8. It was observed that in the transduced models, keratinocytes invaded the dermis and horn pearls were formed; the basement membrane (BM) was also disrupted, and collagen VII, a marker of the BM, was reduced. Certain extracellular matrix (ECM) metalloproteinases were upregulated and likely caused the degradation of ECM components that were found to have normal levels in the control groups but decreased in experimental models. These findings highlight HPV's ability to induce the invasive keratinocyte phenotype seen in squamous cell carcinoma.

A 3D organotypic model only composed of the epidermis was used to study keratinocyte differentiation (Smirnov et al. 2019). Ker-CT cells from an immortalized keratinocyte cell line were manually seeded on inserts and placed on culture dishes. The samples were later exposed to air-lifting, and the stratified epidermis then underwent immunofluorescence analysis for ZNF185, a protein proven necessary for keratinocyte differentiation. The organotypic model had observable and abundant ZNF185. In contrast, experiments with RNAi-knockdown of p63, a transcription factor for ZNF185, demonstrated a significant decrease in ZNF185, which disrupted cell-cell adhesion and tissue stability. These results helped confirm that p63 had a direct impact on ZNF185 expression; genetic analysis revealed that p63 serves as a transcription factor that binds to an enhancer of ZNF185. Such results may imply that squamous cell carcinomas with inadequate keratinocytes differentiated could have a loss of ZNF185 expression or function.

2.3 Basal Cell Carcinoma (BCC) Skin Model

Compared to melanoma and SCC, BCC is generally less invasive and metastatic; BCC is thus incorporated into 2D in vitro studies more frequently than 3D organotypic models. Since BCC cells are not propagated ex vivo (Gache et al. 2015), most bioengineered organotypic models used to study BCC employ other keratinocyte cell types, often genetically altered, as seen in the research below (Table 3).

Bigelow et al. manually formulated a 3D organotypic BCC model consisting of a stratified epidermis, basement membrane, and dermis (Bigelow et al. 2005). Fibroblasts were integrated with collagen to serve as the dermal equivalent. HaCaT (human epidermal keratinocytes) cells were transfected with a vector containing shh cDNA, which codes for a protein (shh) active in a cell signaling pathway involved in the development of basal cell carcinoma called the sonic hedgehog (shh) pathway (Dahmane et al. 1997). The shh HaCaT cells were seeded onto the dermal equivalent to form the epidermal layer. Comparison between control HaCaT versus shh HaCaT experiments highlighted a few notable differences: keratinocytes in shh models maintained cuboidal morphology after stratification, whereas control keratinocytes had the expected squamoid shape in superficial layers; the shh model infiltrated the dermal layer, whereas the control did not; the shh model had upregulated matrix metalloproteinase

Table 3 Studies using BCC skin tissue models

Skin layer	Material	Cell type	Fabrication method	Signaling molecule/ protein	Additional component	Reference
Epidermis Basement membrane Dermis	Collagen	HaCaT cells (keratinocytes) transfected with Shh Fibroblasts	Manual	Recombinant EGF protein	Shh cDNA in Bill Neo Vector AG1478 (EGF inhibitor)	Bigelow et al. (2005)
Epidermis Basement membrane Dermis	Chitosan-cross-linked collagen-GAG matrix	Keratinocytes Fibroblasts HaCat cells (keratinocytes) transfected with LOX antisense constructs	Manual	Epidermal growth factor	pcDNA3 and LipofectAMINE (for transfection) β-aminoproprionitrile (inhibitor of LOX activity)	Bouez et al. (2006)
Epidermis Basement membrane Dermis	Type I collagen	Keratinocytes with PATCHED ± phenotype Fibroblasts	Manual			Brellier et al. (2008)
Epidermis Basement membrane Dermis	Matrigel Type I collagen	NTert-1 cells (keratinocytes) transfected with Gli1 or Gli2 Fibroblasts	Manual		pBabePuro and retroviral particles β6 RNAi SU11274 (MET kinase inhibitor)	Marsh et al. (2008)
Epidermis Basement membrane Dermis	Type I bovine collagen gel	Keratinocytes NBCCS (nevoid basal cell carcinoma syndrome) fibroblasts	Manual		Anti-SHH (Sonic hedgehog) 5E1 monoclonal antibody Isotype-matched anti-cMyc 9E10 monoclonal antibody	Gache et al. (2015)
Epidermis Basement membrane Dermis	Matrigel Collagen	Keratinocytes (HaCaT/NEB1/N/Tert) transfected with PTCH1 small hairpin RNA Fibroblasts	Manual		PTCH1 small hairpin RNA SMO RNAi	Rahman (2013)
Epidermal mimic	Laminin-rich extracellular matrix	BCC-1 cells	Manual		Vismodegib Radiation	Hehlgans et al. (2018)

and cytokeratin 14 in contrast to the control. Importantly, the shh model had higher levels of EGF (epidermal growth factor) receptor phosphorylation on serine 845 and 1068, increasing the EGFR signal propagation. When EGF was added to control and shh models, both sets of experiments demonstrated an increase of dermal infiltration relative to their non-EGF-treated counterparts (the control had no invasion prior to EGF addition). When an EGF signaling inhibitor was applied, dermal invasion of the shh model was decreased significantly. Overall, this research elucidated the presence of a shh-EGF signaling pathway relationship that resulted in the basal cell carcinoma phenotype (Bigelow et al. 2005).

Bouez et al. sought to study the importance of lysyl oxidase (LOX) in BCC as well as overall epidermal homeostasis and crafted an organotypic model consisting of a stratified epidermis, basement membrane, and dermis (Bouez et al. 2006). The model was generated manually, first by seeding fibroblasts into a chitosan-cross-linked collagen-GAG matrix to form the dermal equivalent. Then, either keratinocytes, wild-type

HaCaT cells, vehicle vector HaCaT cells, or HaCaT cells transfected with an antisense LOX construct were seeded on top of the dermal layer. Experimental models were treated with β-aminoproprionitrile, an inhibitor of LOX activity. Results showed that LOX was absent from tumor cells of BCC samples from patients but was notably present in the stroma surrounding the cancerous cells. In the organotypic models treated with β-aminoproprionitrile, collagen fibers appeared less regular in their size and shape. Furthermore, the lamina densa, a component of the basement membrane, was disorganized in the presence of the LOX activity inhibitor. β-Aminoproprionitrile was limited in that it did not promote invasion of the keratinocytes into the dermis. In striking contrast, the only experimental model that exhibited invasion into the dermis was the model incorporating HaCaT cells transfected with an antisense LOX construct. Thus, interruption of LOX's expression and not solely its recognized enzymatic activity was required to develop a BCC-infiltration phenotype, opening a line of question to what unknown functions or associations LOX may also have.

Brellier et al. crafted an organotypic skin model manually, using the protocol detailed by Bernerd et al.'s study of epidermal cancer (Bernerd et al. 2001; Brellier et al. 2008). Brellier et al.'s model involved a 3D construct with a stratified epidermis, basement membrane, and dermis. The dermal layer consisted of fibroblasts integrated with type I collagen; the epidermal layer for the experimental models consisted of keratinocytes from patients with a genetic syndrome called nevoid basal cell carcinoma which makes an individual more susceptible to BCC. The patient-derived keratinocytes had a genotype of PATCHED +/− whereas wild-type keratinocytes had a phenotype of PATCHED +/+. The PATCHED +/− model displayed invasion into the dermis, unlike the control. The experimental model also exhibited higher levels of laminin B1 and beta-1 integrin and lower levels of keratin 10 and loricrin. Importantly, the PATCHED +/− model demonstrated a notable increase in cyclin D1 (cell cycle regulator) compared to the control. Interestingly, the amount of PATCHED mRNA in the control and experimental groups were similar, so a discrepancy in PATCHED transcriptional expression did not explain the BCC-like phenotype of the heterozygous model (Brellier et al. 2008).

Marsh et al. similarly created a 3D skin model consisting of an epidermis, basement membrane, and dermis by seeding fibroblasts into a 50:50 matrix of Matrigel and type I collagen (Marsh et al. 2008). Keratinocytes retrovirally transfected with Gli1, a transcription factor associated with BCC development, were then seeded onto the dermal foundation. The research sought to uncover the impact of αvβ6 integrin toward BCC invasion since αvβ6 is overexpressed in a more aggressive subtype of BCC called morphoeic BCC. The Gli1-transfected keratinocytes did indeed express αvβ6. In transwell experiments devoid of fibroblasts, αvβ6 did not supplement the invasion of Gli1 keratinocytes. However, with the combination of results from transwell, co-culture, and organotypic experiments, the indirect relationship between αvβ6 and invasive BCC behavior was uncovered. αvβ6 activated TGF-β1 (transforming growth factor); TGF-β1 caused fibroblasts to transition into myofibroblasts; myofibroblasts increase HGF/SF (hepatocyte growth factor/scatter factor); HGF/SF signalizing promoted the infiltration of transfected keratinocytes. The organotypic skin assisted in understanding this sequence: in the 3D construct where β6 RNAi was included to suppress αvβ6 expression, the transfected keratinocytes had reduced invasion, assumingly because fibroblasts did not become myofibroblasts. In organotypic experiments where SU1224 (a Met kinase inhibitor) was used to disrupt the HGF/SF signaling pathway, reduced invasion was also observed.

Gache et al. developed an organotypic skin model to study dermal-epidermal interactions of nevoid basal cell carcinoma syndrome (NBCCS) (Gache et al. 2015). In the experimental groups, NBCCS fibroblasts were embedded in type I bovine collagen gel to form the dermal equivalent. Wild-type keratinocytes were then seeded onto the dermis. Certain experiments were treated with anti-SHH (Sonic Hedge Hog) 5E1 monoclonal antibody (while the control for this group was treated with the isotype-matched anti-cMyc 9E10

monoclonal antibody). The NBCCS-fibroblasts organotypic models resulted in an epidermis of lower thickness, loss of bona fide cornified layers, parakeratosis, increased deposition of LamininB1 and β1 Integrin, and clefts at the dermo-epidermal junction. Interestingly, the NBCCS models also demonstrated a substantial increase in p53, a key feature in the suppression of keratinocyte proliferation. When the organotypic skin was treated with anti-SHH 5E1 monoclonal antibody, the deviations from wild-type epidermis listed above were mitigated, supporting the notion that NBCCS fibroblasts hyper-secrete SHH and disrupt the epidermis by means of the SHH signaling pathway.

An in vitro organotypic skin model of BCC, where fibroblasts were embedded in a Matrigel-collagen matrix, was created using the manual method to understand PATCHED and other pertinent signaling pathways (Rahman 2013). Various types of immortalized human keratinocytes (HaCaT, NEB1, or N/Tert) were then seeded onto the dermis. The keratinocytes were transfected with PTCH1 short hairpin RNA in order to suppress PATCHED and observe the repercussions. Certain experiments employed SMO RNAi meant to knockdown SMO, a protein downstream of PATCHED in the SHH signaling pathway. Importantly, the 3D model did not fully recreate BCC characteristics seen in in vivo behavior. The study led to multiple conclusions: part of PATCHED is expressed in the nucleus, PATCHED suppression promotes nuclear/perinuclear SMO, and the increase in Gli1 due to PATCHED suppression is not significantly responsive to SMO inhibitors. These findings corroborate that SMO-independent mechanisms are likely involved when PTCH1 is knocked down, resulting in the persistent BCC phenotype despite the presence of pharmaceutical treatments targeting SMO.

Hehlgans et al. tested a 3D colony formation assay that provided BCC-1 cells a more epidermis-like environment compared to 2D plating (Hehlgans et al. 2018). The BCC-1 cells were diluted into a laminin-rich extracellular matrix, and certain experiments were treated with vismodegib, a monotherapy drug that inhibits the Hedgehog signaling pathway, as well as radiation. Unlike the 2D models, the 3D experiments demonstrated that vismodegib significantly sensitizes BCC cells to radiation, causing a higher fraction of dead cells as the concentration of vismodegib increases. The fabricated cancer model proved to be useful in observing and assessing pre-clinical outcomes of treatment combinations.

3 Discussion and Conclusion

So far, various advanced skin tissue models have been developed to study cancer research. However, the majority of these studies have included only one layer (epidermis) or two layers (epidermis and dermis), neglecting the hypodermis despite the significant roles of adipocytes and adipose tissue in skin cancer. For instance, adipocytes have activated the Akt (Ser-473 and Thr-450) signaling pathway of melanoma cells, thereby promoting melanoma growth (Kwan et al. 2014). Another studies showed that de-differentiation of adipocytes into fibroblast-like cells has been able to trigger melanoma cell migration by activating the signaling pathway of Wnt5a, β-catenin, and LEF-1 (Zoico et al. 2018). As such, leveraging a fully functionalized tri-layered skin tissue model is essential to better understand the molecular mechanism and the pathology of skin cancer.

In addition to cancer, adipose tissue has significantly affected skin tissue regeneration and wound healing. For example, a novel functional hypodermal-dermo-epidermal tri-layered skin substitute with vascularization was proposed by Zimoch et al. (2021). It was suggested that adipose mesenchymal stem cell-derived adipocytes and TGF-β1 secreted by these cells in adipose tissue had a profound impact on the maturation and differentiation of keratinocytes and fibroblasts as well as epidermal morphogenesis. Monfort et al. also observed a similar effect with hypodermis components that promote epidermal differentiation (i.e., differentiation of epithelial cells into mature keratinocytes) using a tri-layered fibrin-based skin substitute (Monfort et al. 2013).

There are still some limitations to fabricating a functional adipose tissue – in particular, preadipocyte differentiation. It is substantially difficult to induce preadipocyte differentiation in vivo. This is because primary cells have a limited life span. In addition, the isolation of preadipocytes from fibroblast-like cells is another challenge to overcome (Ntambi and Kim 2000). Hence, adipose precursor cell lines such as pluripotent fibroblasts or unipotent preadipocytes have been adopted for the differentiation of preadipocytes into adipocytes due to the stability and unlimited passage as an in vitro model. However, there is still difficulty differentiating preadipocytes into adipocytes due to the susceptibility of adipocytes to in vitro skin tissue, and inconsistent viability by nature (Zimoch et al. 2021). Forming vascular networks in the engineered tissue is also pivotal for cancer modeling. The absence of vascularization in the tissue is known as a major reason for the failure of the transplant of engineered skin tissue (Miyazaki et al. 2019). However, vascularization is a challenging part in the field of tissue engineering (Auger et al. 2013). In particular, replicating simultaneous vascularization and adipogenesis in 3D scaffolds is extremely difficult. Prior studies showed that human microvascular endothelial cells (HMVEC) or human umbilical vein endothelial cells (HUVEC) could be useful for recapitulating a more complex tumor microenvironment with vasculature (Laschke and Menger 2016). Another recent study by Zimoch et al. showed a successful development of prevascularized tri-layered skin substitutes and implanted them in mice for the first time (Zimoch et al. 2021). Moreover, the majority of research using in vitro tumor models has been focused on the interaction between the tumor and its microenvironment, whereas few studies have been performed on understanding a tissue-specific microenvironment (Bourland et al. 2018). In addition to these limitations, understanding tumor heterogeneity in terms of mutations, and selecting appropriate biomaterials and model designs are enormous hurdles to be addressed (Unnikrishnan et al. 2021).

Over the last decades, skin cancer incidence has rapidly increased by repeated and unprotected skin exposure to ultraviolet (UV) rays from sunlight. As such, developing a functional full-thickness tri-layered 3D skin model is needed to predict more accurate cancer cell behavior as well as drug screening and efficacy. Future treatments for skin cancer may be significantly impacted and developed by the use of these 3D cancer models for personalized medicine.

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Application of Drug Repurposing-Based Precision Medicine Platform for Leukaemia Patient Treatment

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Abstract

Drug resistance in leukaemia is a major problem that needs to be addressed. Precision medicine provides an avenue to reduce drug resistance through a personalised treatment plan. It has helped to better stratify patients based on their molecular profile and therefore improved the sensitivity of patients to a given therapeutic regimen. However, therapeutic options are still limited for patients who have already been subjected to many lines of chemotherapy. The process of designing and developing new drugs requires significant resources, including money and time. Drug repurposing has been explored as an alternative to identify effective drug(s) that could be used to target leukaemia and lessen the burden of drug resistance. The drug repurposing process usually includes preclinical studies with drug screening and clinical trials before approval. Although most of the repurposed drugs that have been identified are generally safe for leukaemia treatment, they seem not to be good candidates for monotherapy but could have value in combination with other drugs, especially for patients who have exhausted therapeutic options. In this review, we highlight precision medicine in leukaemia and the role of drug repurposing. Specifically, we discuss the several screening

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methods via chemoinformatic, in vitro, and ex vivo that have facilitated and accelerated the drug repurposing process.

Keywords

Drug repurposing · Drug screening · Leukaemia · Platforms · Precision medicine

List of Abbreviations

ALL	Acute Lymphocytic Leukaemia
AML	Acute Myeloid Leukaemia
CLL	Chronic Lymphocytic Leukaemia
CML	Chronic Myeloid Leukaemia
QSAR	Quantitative Structure-Activity Relationship
FDA	Food and Drug Administration
WHO	World Health Organisation
FAB	French-American-British Association
BCR	B-cell Receptor
BTK	Bruton Tyrosine Kinase
PIK	Phosphoinositide 3-Kinase

1 Introduction

Blood cancers, like most malignancies, occur from genetic modifications resulting in neoplastic mutations in the DNA causing abnormal cell proliferation. The proliferation of the incompetent blood cells increases vulnerabilities to infections, making hematologic malignancies some of the most challenging cases of cancer to manage. Leukaemia is a blood cancer originating in the bone marrow from lymphoid or myeloid cell modification. Based on the duration of maturation and development of leucocytes, leukaemia is divided into acute and chronic subtypes. The response of patients to standard treatment varies considerably, and the survival rate differs among subtypes. The 5-year survival rate of acute myeloid leukaemia patients is 20%, whereas chronic lymphocytic leukaemia patients experience a better prognosis with an 86% survival rate (Andresen and Gjertsen 2017; Ou et al. 2022). Although these metrics have improved with advances in medicine, many patients will relapse or become refractory to the treatment. The drug resistance phenomenon observed can be explained by the heterogeneous nature of the disease as well as the one-size-fits-all type of treatment options patients follow. Therefore, new drugs or alternative treatments need to be identified to complement the existing ones or the unmet need for effective chemotherapeutic drugs.

Many researchers have evaluated the possibility of redirecting the use of already approved drugs to emerging and heterogeneous diseases. The literature abounds with evidence of drugs successfully repurposed and released in the market with new indications for the prevention (Rothwell et al. 2011), or the treatment of cancer (Jourdan et al. 2020; Latif et al. 2012). The results from virtual, experimental studies and more importantly clinical trials have reported the efficacy of different classes of drugs against leukaemia subtypes. Additionally, many publications emphasise the benefit of using existing drugs for other indications. However, according to some authors, only 75% of drugs can theoretically be repurposed, and the proportion is even smaller in practice (Nosengo 2016). Many FDA-approved drugs for the treatment of cancer have been redirected and shown to have antileukaemic activities. This is obvious as cancers share some similarities, and one anticancer agent indicated in the treatment of a type of cancer is more likely to be active in another cancer type. For example, dasatinib, a kinase inhibitor used to treat certain types of chronic myelogenous leukaemia has been identified as a targeted therapy for T-cell acute lymphoblastic leukaemia (Laukkanen et al. 2017). Another example is gefitinib, approved as an inhibitor of the epidermal growth factor receptor for the treatment of certain lung and breast cancers, has been repurposed for chronic myelogenous leukaemia treatment as a BCR-ABL tyrosine kinase inhibitor (Singh et al. 2017).

2 Characteristics of Leukaemia Subtypes and Influence on Therapy

Leukaemia affects both children and adults, with a predominance in whites and males (Davis 2014). The four major types of leukaemia are acute lymphoblastic leukaemia (ALL), which is more likely to occur in children; chronic lymphocytic leukaemia (CLL); acute myelogenous leukaemia (AML); and chronic myelogenous leukaemia (CML), which is more common in adults. The term acute means that the disease will progress rapidly in the absence of treatment, whereas chronic leukaemia requires observation and is harder to cure. On the other hand, lymphocytic and myelogenous refers to the type of bone marrow cells affected in the cancer type with lymphocytes, for lymphocytic or lymphoblastic leukaemia, and granulocytes and monocytes, for myelogenous leukaemia. The age factor is one of the elements to take into consideration when selecting a treatment for a particular patient. For example, the type of ALL found in children is different from the type that usually presents in adults. Additionally, younger ALL patients have a better prognosis than elderly patients for which the outcome of intensive chemotherapy is poor but with higher toxicity (Fattoum et al. 2015).

Generally, the differences in leukaemia subtypes are found in the clinical, haematological, and molecular characteristics of the disease. Therefore, the subtypes and accompanying characteristics can affect the disease progression and treatment response, indicative of the need for personalised therapy.

2.1 Lymphocytic Leukaemia

In acute lymphocytic leukaemia (ALL), there are three subtypes, namely: L1, L2, and L3. Patients classified as having the L1 subtype have relatively small lymphoblasts compared to those of subtypes L2 and L3. Blasts cells possess specific features, which influence the treatment. For example, blasts with runt-related transcription factor1 are more likely to respond to treatment with purine analogues (Faderl et al. 2010). Patients with hyperdiploidy, who possess lymphoblasts particularly susceptible to cytotoxic drugs, are known to have a better prognosis than those who do not. Hyperdiploidy in ALL is defined as the presence of 51 to 65 chromosomes. This cytogenic abnormality is common in paediatric patients who comprise more than 25% of B lymphocyte-ALL compared to adults at 7–8%, giving children a favourable outcome and a better survival rate. However, some patients of this group may not respond to treatment due to the presence of additional cytogenic aberrations particularly on chromosomes 1q and 17q.

Chronic lymphocytic leukaemia also called chronic lymphatic leukaemia (CLL) is characterised by a high level of expression of BCL-2, an oncoprotein that inhibits apoptosis. Intracellular heterogeneity is also observable in CLL with IGVH mutation status and the 17p and/or 11q deletion and the presence of a molecule called ZAP-70 affecting treatment outcome and overall survival (Montserrat and Dreger 2016). Results of a study in Canada showed that - first-line chemotherapy in CLL patients with 11q deletion is required urgently; however, these group of patients experience a longer overall survival after treatment (Goy et al. 2017).

2.2 Myeloid Leukaemia

There are two classification conventions for AML. The World Health Organisation (WHO) classifies AML in subtypes based on the type of genetic abnormality encountered and the French-American-British (FAB) classification relies on the type of cells affected and their level of maturity (M0-M7) (Walter et al. 2013). Phenotypic variations include AML with a mixed phenotype consisting of two forms of leukaemia combined; AML with minimal differentiation designated AML-M0, in which there is no blasts differentiation and the absence of myeloid markers; and AML with/without maturation (Juliusson and Hough 2016). Myeloblastic leukaemia with maturation (M2) and myelomonocytic (M4) are both the major subtypes of AML with better and average prognosis, respectively.

The common types of genetic abnormalities encountered in acute myeloid leukaemia (AML) are translocations [t(8,21), t(9,11), t(6,9)], and inversions [inv(16), inv. (3)]. AML patients with these abnormalities aged below 60 years old have a better prognosis after treatment with cytarabine with 85% rates of complete remission compared to patients with 20q deletion or patients with abnormalities involving chromosomes 3, 5, or 7 who only attain 30–50% complete remission. Furthermore, the correlation between the level of certain enzymes like lactate dehydrogenase (LDH) in patients' serum and shorter survival time has been demonstrated in a Chinese AML cohort. The study revealed that lactate dehydrogenase level was associated with a high risk for 60-day mortality among AML patients in China, especially for those with a concentration of 570 U/L and greater. In the same study, patients with a distinct subset of AML (secondary AML: AML evolving from prior blood conditions like aplastic anaemia or myelodysplasia) were also observed to have a poor prognosis. On the other hand patients with a normal karyotype were classified as having a standard or intermediate risk with a 50–85% complete remission rate (Xiao et al. 2021).

The major hallmark in chronic myeloid leukaemia (CML) is the Philadelphia (Ph) chromosome, which corresponds to the translocation of chromosomes 9 and 22, and BCR-ABL fusion gene. Approximately 90% of CML patients are Ph-positive, characterised by an increased genetic instability, and only 5–10% are classified as Ph-negative but can possess the BCR-ABL fusion gene and or other additional genetic mutations (Abdulmawjood et al. 2021). Genetic and clinical features in CML phases determine the sensitivity of patients to treatments. For example, the response of Ph + AML patients to standard imatinib is reduced when the disease progresses from the chronic phase (CML-CP) to the accelerated phase (CML-AP) or to the blastic phase (CML-BP), which requires second-generation tyrosine kinase inhibitors (Deininger 2015).

3 The Case for Drug Repurposing

De novo drug discovery is a very risky process that is long, labour-intensive, and highly expensive. It has about a 45% failure rate chiefly due to safety or toxicity issues. The estimated cost for research and development of a new drug ranges from 1 to 2 billion dollars (DiMasi et al. 2016) and could take up to 15–17 years. Comparably, drug repurposing, repositioning, or reprofiling is a cost-efficient strategy to circumvent the difficulties associated with the de novo drug discovery process as it can help to save 4 to 7 years in the development phases with a reduced risk of failure (Xue et al. 2018). Indeed, the average cost to relaunch a drug repositioned is $8.4 million (Bhagat and Butle 2021; Xue et al. 2018).

The concept of drug repurposing is based on two fundamental scientific principles, which stipulate that various diseases share similar targets and that drugs can have pleiotropic effects. Target-based repositioning offers the advantage of facilitating the process of identifying specific targets and pathways using tools, such as molecular docking and binding-site similarity, that can be exploited in the drug development process (Jourdan et al. 2020; Pushpakom et al. 2019). Drug repurposing also offers the possibility to characterise diseases based on their molecular profile and link the profiles to drugs that are not used for the specific disease. In the era of precision medicine, drugs are administered based on the molecular profile of the patient enhancing the effect of chemotherapy, which emphasises the potential of drug reprofiling and repurposing inpatient treatment and management (Nweke and Thimiri Govinda Raj 2021). The drug repurposing process (shown in Fig. 1) involves a major step which is drug sensitivity screening used in determining the effect of the drug(s) on the cancer cells.

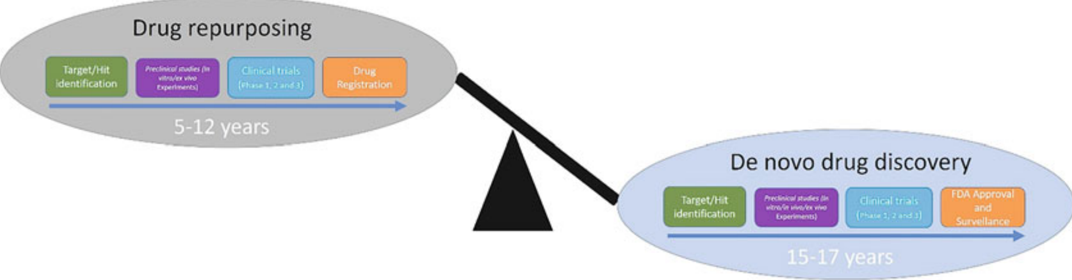

Fig. 1 The process of drug repurposing compared to de novo drug discovery. The drug repurposing process requires a shorter amount of time, lesser money, and fewer regulatory processes compared to de novo drug discovery

4 The Link Between Precision Medicine and Drug Screening

Precision medicine is a treatment strategy that focuses on identifying effective treatment approaches based on patients' genetic, environmental, and lifestyle features. With the increased knowledge in molecular and genetic characterisation of cancer cells and various cell-host interactions, precision medicine has emphasised the need for new drugs in the management of leukaemia. Both precision medicine and drug repurposing are relevant for leukaemia and particularly for patients who have relapsed or are developing drug resistance and do not have any other treatment options (Li and Jones 2012).

Common treatment options for leukaemia are based on cancer-fighting drugs, like chemotherapy, biological, and targeted therapies, and non-drug-based treatments, like radiation therapy and stem cell transplant. Chemotherapy, which is the primary form of therapy, uses drugs infused into the bloodstream. Unfortunately, due to the high heterogeneity of the disease and the specificities of each patient, the need for new drugs has been exacerbated. Therefore, precision medicine is used to reduce the lack of drug efficacy and the development of drug resistance through the amelioration of patients' stratification and their sensitivity to new drugs (Dugger et al. 2018).

Drug screening can play a pivotal role in precision medicine by rapidly providing a drug sensitivity profile for patient-derived cells. This approach can enable the identification of compounds or drugs that may be effective against a patient's tumour.

5 Drug Screening Platforms

Drug screening platforms are an important aspect of the drug discovery and development process. Indeed, the platform developed for drug screening and resistance testing, the methods, and the models selected for the purpose have an impact on the duration, the cost, and the outcomes of the process. Various platforms are used to screen drugs for their pharmacological effects on models of diseases. In oncology, chemosensitivity studies comprise computer-aided screening, in vitro cell lines-based screening, and human primary cancer culture to better predict clinical responses (Fig. 2). Advances in the development of drug screening platforms seek to reproduce the complexity of the interactions within the tumour microenvironment. Some examples of these platforms are described.

5.1 Microfluidic Platforms

Microfluidic platforms for drug screening allow for the accurate processing and control of small volumes of fluid coupled with automated data collection of cell responses. The adoption of microfluidic technology provides significant

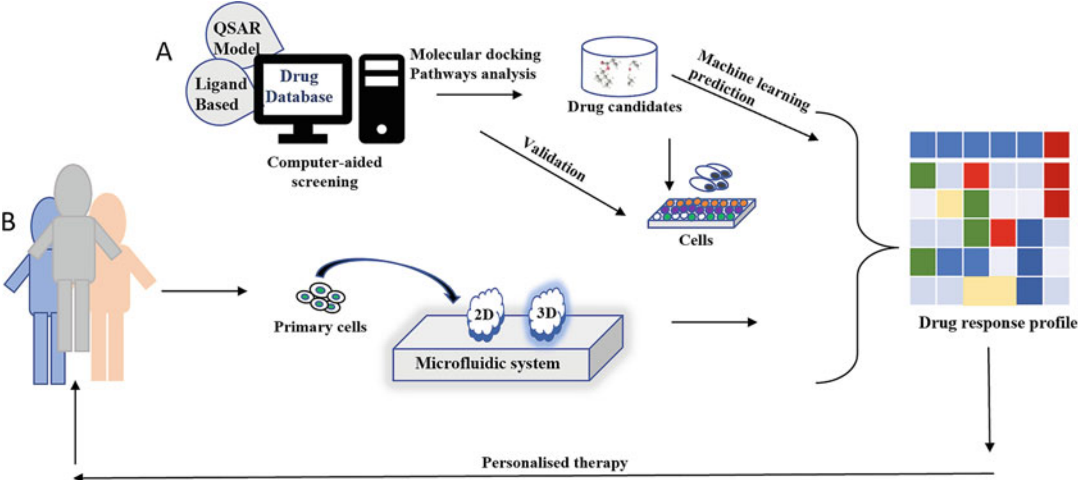

Fig. 2 **Drug screening flatforms**. (**a**) *Computer-aided drug screening platform*: The selected compound from a virtual screening can be validated in vitro using cell lines or primary cells. (**b**) *Microfluidic system*: Use of patient-derived cells to mimic the tumour microenvironment and predict drug response

benefits, such as low reagent consumption, rapid mixing, quick operation and utilisation, and accurate control of the fluid properties. Types of microfluidic platforms used in drug screening include continuous-flow, successive droplet, and droplet array platforms (Serra et al. 2020). For drug evaluation and validation, microfluidic platforms utilise methods such as microfluidic single-cell, microfluidic cell spheroid, single-organ-on-a-chip, and multi-organ-on-a-chip (Liu et al. 2021). These platforms offer the possibility to better understand cell heterogeneity, 3D cell culture that recapitulates cell-cell interactions and mimics organ functions in vitro.

5.2 Virtual Drug Screening Platform

With an appropriate virtual screening platform, researchers can identify potent anticancer hits by screening millions of drugs based on the structure or ligand-based approach. The quantitative structure-activity relationship (QSAR) model uses the chemical structure of compounds in databases to predict their biological and pharmacokinetic activities. Screening databases for the identification of hit/lead compounds is a powerful approach to lower the cost of the drug screening process by identifying viable targets (Sliwoski et al. 2014). The pharmacological, structural, biochemical, toxicological characterisation, and the archiving of key compounds have helped predict the exploitability and the development of new compounds (Rognan 2017). Given the high volume of biological targets and drugs available, setting in vitro drug screening and testing for all drug candidates is nearly impossible. Thus, a virtual platform makes use of computer-aided screening to assess drug banks and molecular target databases to identify the best compound (s), which can further undergo in vitro testing for validation (Singh et al. 2017).

Gorgulla and colleagues have established an open-source drug discovery platform that allows ultra-virtual screening. The highly automated platform called VirtualFlow incorporates each step required for the screening of billions of compounds using various molecular docking techniques with the use of software, such as AutoDock, GOLD, or AutoDock Vina. The virtual platform features include linear scaling behaviour, cloud computing integration, and autonomous deployment for ligand screen pipeline (Gorgulla et al. 2020). Such platforms allow computer-aided drug screening using artificial intelligence, machine learning, and deep learning

applications, which recently are currently being implemented in precision oncology and drug repurposing. Regarding the use of machine learning algorithms to predict repurposing prospects using drug data, various types of machine learning algorithms, such as supervised and unsupervised machine learning, are useful in building and evaluating predictive models by identifying unique patterns in biological data connected to the medications(Vamathevan et al. 2019).

Although virtual screening has some limitations like the environment of drugs screened being different from the microenvironment of a living organism, the virtual screening has led to the identification of compounds of interest for the treatment of leukaemia (Table 1). For example, Sohraby and colleagues used a set of computational analyses to predict the best inhibitor for chronic myelogenous leukaemia (CML) drug-resistant T315I mutant and BCR-ABL1 mutation. The structure-based virtual screen (pharmacophore and E-pharmacophore virtual screening) and molecular dynamics study of 1,590 FDA drugs led to the identification of new ligands. The molecular docking revealed that chlorhexidine has a stronger effect on both T315I mutant and wild-type BCR-ABL1 than ponatinib, the reference drug used in the treatment of CML, (Sohraby et al. 2017). Chlorhexidine is an antibacterial agent with a broad spectrum of antimicrobial activity. It was developed in 1950 and is used as an antiseptic and antibiotic in dermatology and dentistry to disinfect the skin, clean wounds, and treat yeast in the mouth (Mariotto et al. 2020; Rothwell et al. 2011). The antibacterial mechanism of action of chlorhexidine is due to its capacity to disrupt the cell membrane at high concentrations and stop bacteria growth at low concentrations. Other drug groups identified by the authors as tyrosine kinases inhibitors include heavy metal antagonists, aminoglycosides, and protease inhibitors.

Similarly, one study performed virtual screening of 1,424 compounds on a ligand-based virtual screening platform and showed that gefitinib, a compound used in the treatment of breast cancer, is a potential target of the allosteric pocket of ABL kinase (Singh et al. 2017).

5.3 Pre-Clinical Models of Drug Screening

Complimenting virtual drug screening to testing in pre-clinical models enhances the drug repurposing process and can further lead to the identification of novel therapeutic agents that selectively targets cancer cells like leukaemia cells (Table 2). One

Table 1 Examples of compounds with potent antileukaemic activities identified by virtual screening

Drugs	Drug class	Primary indication	New indication	References
Ovoflavin	Vitamins	Prevention of weight loss and skin lesions	BCR kinase inhibitor (CML)	(Natarajan et al. 2019)
Hesperidin	Bioflavonoid	Bioflavonoid		
Butirosin	Aminoglycoside antibiotics	Active against gram bacteria		
Chlorhexidine	Antimicrobial	Antibacterial	Antileukaemic (CML) Tyrosine kinase inhibitor	(Sohraby et al. 2017)
Paromomycin	Aminoglycoside antibiotics	Antibiotic		
Deferoxamine	Heavy metal antagonist	Anti-hemochromatosis		
Ritonavir	Protease inhibitor	Antiretroviral		
Hydrocarpin	Natural product	Flavonoids	Antileukaemia (AML) Inhibits AML receptors BCL-2, FLT3	(Bultum et al. 2022)
Bromocriptine	Dopamine receptor agonist	Hyperprolactinemia Parkinson's disease	Anti-AML: Induces apoptosis and myeloid differentiation	(Lara-Castillo et al. 2016)

Table 2 Examples of compounds from in vitro and ex vivo screening with potent antileukaemic activities, their classes, and indications

Drugs	Drug class	Primary indication	New indication	References
Papaverine	Antispasmodics (alkaloid)	Vasodilator	Antileukaemic (CML) Promotes apoptosis and inhibits BCR-ABL signalling	(Parcha et al. 2021)
Acriflavine	Antibiotic	Antiseptic	Antileukaemic (AML, CML); modulate STAT3/5	(Hallal et al. 2020)
Albendazole	Anthelmintic	Antiparasitic	AML, inhibits colony formation	(Matchett et al. 2017)
Niclosamide	Anthelmintic	Antiparasitic	T-ALL, inhibits GSH synthesis and NFAT signalling	(Hamdoun et al. 2017)
Moxidectin Ivermectin Milbemycin	Anthelmintic (macrocyclic lactone)	Antiparasitic	Relapsed ALL, Induces cell death and synergises with BCL-2 agonists	(Mezzatesta et al. 2020)
Metformin	Biguanides	Antidiabetic (type 2)	Activates p-AMPK, synergises with sorafenib in FLT3-mutated AML	(Shadman et al. 2015; Wang et al. 2015)
Quinacrine	Acridines	Antimalaria	Anti-AML	(Eriksson et al. 2015)

important relevance of the use of pre-clinical models is in demonstrating the synergistic effects of drugs on cancer cells. This combinatorial treatment can lead to enhanced efficacy in the treatment and reduction in toxicity. Importantly, in vitro drug screening offers the advantage that drugs can be tested in an environment that mimics the biological microenvironment. Based on their in silico results, one study undertook an in vitro test to validate the activity of gefitinib on K562 cells (human CML immortalised cell line expressing BCR-ABL RECEPTOR). The combination of gefitinib (5uM) and imatinib revealed a synergistic effect meaning that gefitinib can enhance the imatinib inhibitory effect. Researchers have shown interest in screening drugs for leukaemia in vitro by medium to high-throughput drug screening with encouraging results. Using high-throughput screening in vitro on leukaemic cancer cell lines as well as primary cells led to crucial information necessary to improve the management of leukaemia. It has been reported that the standard dose of ibrutinib and venetoclax can be reduced, thereby lowering costs and reducing toxicity in patients while maintaining efficacy (Skånland et al. 2020).

Ex vivo drug screening on primary human leukaemic cells has led to insights by identifying single or drug combinations. The advantage of ex vivo experiments over in vitro is the actionable or translational results that can match patient profile for personalised diagnosis or treatment. Isoprenaline, a beta-adrenergic receptor inhibitor has demonstrated antileukaemic activity for the first time and has been combined with the purine analogue Fludarabine and with ibrutinib, a Bruton's tyrosine kinase inhibitor. The results showed a synergistic effect of isoprenaline and ibrutinib on freshly isolated CLL primary cells with a better synergistic effect for cells with greater ibrutinib resistance (Nehdi et al. 2021).

6 Clinical Evidence for Drug Repurposing

Virtual and laboratory drug screening are rapid and can lead to the unexpected discovery of new antileukaemic drugs. However, the interaction of the body with the drug cannot be easily assessed ex vivo. Therefore, clinical trials are important to direct the final opinion on the efficacy of the compound being investigated. There is little clinical evidence of new drugs successfully repurposed in leukaemia, and one possible explanation is the cost associated with undertaking clinical studies (Nipp et al. 2015). Another reason might be that a drug that has shown antitumour activity in silico, in vitro, or even in vivo with the

use of animal models can be a failure once assessed clinically because of safety issues or inefficacy (Sun et al. 2022). The complexity of the human organism and the patient's intrinsic and extrinsic features are some important points impacting the response of a patient to a certain treatment.

It should however be noted that there have been some instances resulting in the clinical utility of repurposed drugs in leukaemia treatment. One example is thioridazine, a drug of the phenothiazine family belonging to the class of drugs named antipsychotics. It is a drug previously widely prescribed for the treatment of depressive disorders and schizophrenia before it was withdrawn from the market globally in 2005 because of its adverse effect on cardiac rhythm. Thioridazine was later screened and confirmed to possess anti-inflammatory (Baig et al. 2018), and antibiotics activities (Van den Driessche et al. 2017). It was suggested as a potential drug to tackle drug resistance in acute myelogenous leukaemia (Jourdan et al. 2020; Sachlos et al. 2012). A phase 1 trial completed by Aslostovar et al., evaluating the efficacy of Thioridazine in patients with refractory/relapsed acute myelogenous leukaemia, demonstrated the safety and efficacy of thioridazine in combination with cytarabine (Aslostovar et al. 2018).

Another example is pyrimethamine, an antiparasitic drug indicated for the prevention and treatment of toxoplasmosis infection and malaria. A clinical trial evaluated the efficacy of pyrimethamine in patients with relapsed and refractory chronic lymphocytic leukaemia (CLL) after having demonstrated its capacity to drastically reduce the expression of the STAT 3 gene in vitro. Three cohorts of CLL patients who had received an average of six previous therapies were enrolled in a phase 1 trial. The results show no objective response based on the guidelines of the International Workshop on CLLW-CLL in 2008, but half of the patients achieved stable disease and a median overall survival of 22.2 months was observed. Though the use of pyrimethamine as monotherapy did not lead to convincing results, important insights into the mechanism of action suggest that pyrimethamine can be combined with well-established signalling pathways inhibitors, such as venetoclax, BTK, and PIK inhibitors (Brown et al. 2021).

Other combinational studies have demonstrated encouraging results for new regimens in the treatment of leukaemia. Pravastatin (statins), a lipid-lowering medication, has been combined with idarubicin and cytarabine in newly diagnosed (Shadman et al. 2015) and relapsed (Advani et al. 2014) AML patients, where 54% achieved complete remission.

7 Conclusion and Future Directions

There is a growing trend in drug repurposing, which represents a quicker and cost-effective route to discovering new drugs for treating leukaemia. Several methods of chemoinformatic, in vitro, and ex vivo studies have facilitated and accelerated the identification of compounds with a repurposed potential in leukaemia. The drug repurposing process is a cycle that goes from existing compounds to new disease indications. Although drug repurposing is time and cost-saving compared to de novo drug discovery, the process still requires lots of resources and time. Careful selection of techniques and the models to be utilised is crucial. The combination of chemoinformatic, in vitro, and ex vivo drug sensitivity screening seems to be a less-risky approach to discovering, validating new targets, and repurposing drugs for leukaemia treatment. Though most of the non-cancer repurposed drugs are generally safe, they are not good candidates for monotherapy in the treatment of leukaemia but are shown to synergise with other well-known antileukaemic drugs or other drugs to provide greater clinical efficacy.

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Stem Cell-Derived Extracellular Vesicles as a Potential Therapeutic Tool for Eye Diseases: From Benchtop to Bedside

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Abstract

Stem cell-derived extracellular vesicles (SC-EVs) have remarkably drawn clinicians' attention in treating ocular diseases. As a paracrine factor of stem cells and an appealing alternative for off-the-shelf cell-free therapeutics, SC-EVs can be conveniently applied topically on the ocular surface or introduced to the retina via intravitreal injection, without increasing the risks of immunogenesis or oncogenesis. This chapter aims to assess the potential applications for EV, obtained from various types of stem cells, in myriad eye diseases (traumatic, inflammatory, degenerative, immunological, etc.). To the best of our knowledge, all relevant pre-clinical studies are summarized here. Furthermore, we highlight the up-to-date status of clinical trials in the same realm and emphasize where future research efforts should be directed. For a successful clinical translation, various drawbacks of EVs therapy should be overcome (e.g., contamination, infection, insufficient yield, etc.). Moreover, standardized, and scalable extraction, purification, and characterization protocols are highly suggested to determine the exosome quality before they are offered to patients with ocular disorders.

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Keywords

Exosomes · Extracellular vesicles · Mesenchymal stem cells · Microvesicles · Ocular diseases · Ophthalmic disorders · Stem cells

Abbreviations

AAVR	AAV receptor
ADSC	Adipose tissue stem cells
BMSCs	Bone marrow-derived mesenchymal stem cells
cGVHD	Chronic graft versus host disease
cMSCs	Corneal mesenchymal stem cells
CREB	cAMP response element-binding protein
DED	Dry eye disease
DM-Exos	Diabetic Exos
EAU	Experimental autoimmune uveoretinitis
EndMT	Endothelial–mesenchymal transition
ESCs	Embryonic stem cells
ESEVs	Embryonic stem extracellular vesicles
EVs	Extracellular vesicles
exo-AAV	Exosome-associated adeno-associated virus
Exos	Exosomes
FDA	Food and Drug Administration (in USA)
HG	Hyperglycemia
HMGB1	High mobility group box protein 1
HRMECs	Human retinal microvascular endothelial cells
HSP90	Heat shock protein 90
ITGA1	Integrin subunit α1
LEC	Limbal epithelial cells
lncRNAs	Long non-coding RNAs
LSC	Limbal stromal cell
MCP-1	Monocyte chemotactic protein
MDSCs	Myeloid-derived suppressor cells
MHs	Macular holes
MSCs	Mesenchymal stem cells
MVs	Microvesicles
N-Exos	Normal exosomes
ONC	Optic nerve crush
RD	Retinal degeneration
RGC	Retinal ganglion cells
sEVs	Small extracellular vesicles
SRS	Subretinal space
UC	Umbilical cord
UMSC	Umbilical cord-derived mesenchymal stem cells

1 Background

Extracellular vehicles (EVs) are membrane-bounded structures that represent an essential communicating entity between cells. EVs are diverse and usually with overlapped physical characteristics. Selective packaging and selective delivery for EVs are crucial for functional EVs in an intracellular signaling system (Raposo and Stahl 2019). There are three main forms of EVs as shown in Fig. 1: microvesicles (~20 nm to 1 μm), exosomes (~40 to 120 nm), and apoptotic bodies (~500 nm to 5 μm) (Hauser et al. 2017; Battistelli and Falcieri 2020; Kakarla et al. 2020). Microvesicles are generated by outward budding of the plasma membrane while exosomes are generated by inward budding of endosomal membrane before fusion and release from cell membrane. Furthermore, apoptotic bodies (ApoBDs) appear as membrane sac as a result of plasma membrane blebbing and nuclear fragmentation (Kakarla et al. 2020).

To date, the mechanism of packaging of signaling contents is not fully understood. However, the endosomal sorting complexes required for transport machinery (ESCRT) along with other assembly pathways play a pivotal role in understanding the formation of EVs (Raposo and Stahl 2019).

Due to their different mechanisms of biogenesis, physiological conditions, and cell type, EVs are loaded with various native contents of proteins, lipids, nucleic acids, metabolites, and other biofunction molecules (Abels and Breakefield 2016). In addition, EVs can be modified to carry therapeutic agents such as DNAs, RNAs, polysaccharides, proteins, and various other small molecules (Liu et al. 2022).

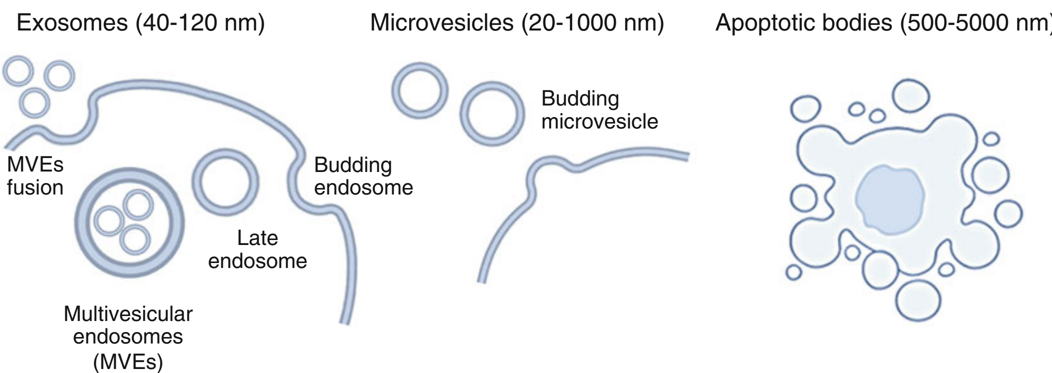

Fig. 1 Schematic representation of the three main forms of EVs according to their size and biogenesis: exosomes, microvesicles, and apoptotic bodies

As gene/drug delivery vehicles, synthetic lipidic or polymeric delivery systems – as liposomes and polymeric nanoparticles – show very significant immunogenicity, short circulation duration, poor tropism, toxicity issues, in addition to physical and chemical instability (Attia et al. 2019). On the other side, EVs are considered as ideal nano-vehicle due to their outstanding stability, biocompatibility, and the diverse bioactive ingredients that can be loaded within the vesicles (Liu et al. 2022). Moreover, EVs can induce therapeutic effects by many mechanisms as targeting, immunoregulation, and signal transduction. Up till now, cell-based therapy (e.g., stem cells and immune cells) carries with it the concerns of tumorgenicity and immunogenicity, among other issues. The unregulated cell growth and activation of immune system can lead to tumor development and formation (Attia et al. 2021a, b). Interestingly, EVs derived from mesenchymal stem cells (MSCs) hold the same regenerative ability and immunomodulatory potential as their parental cells and possibly will overcome the shortcomings of MSCs (Attia and Mashal 2020). The research of various classes of EVs has developed rapidly over time with a diverse biomedical application in the treatment and diagnosis of many diseases such as cancer (Lin et al. 2022), diabetes (Prattichizzo et al. 2021; Soltani et al. 2022), cardiovascular disorders (Burrello et al. 2022), liver diseases (Borrelli et al. 2018), Alzheimer's disease (Kapogiannis et al. 2019), and other many medical disorders. Furthermore, the availability of different routes to introduce/apply EVs (e.g., intraperitoneal, oral, intravenous, intranasal, intraocular, topical, subcutaneous, etc.) has markedly widened their clinical applications. However, their isolation and characterization in terms of yield and purity are still hurdles toward their prosperous clinical applications (Pinheiro et al. 2018).

2 Application of EVs in Ocular Disorders

2.1 Pre-clinical Applications

Many pre-clinical studies have explored the therapeutic potential of SC-EVs as a novel cell-free strategy in myriad eye diseases. Most of these up-to-date studies are summarized in Table 1.

2.1.1 Corneal Disorders

(a) Corneal Epithelial Defect/Wound

One of the leading causes of blindness is corneal scarring, for which corneal transplantation has been the standard therapy. However, due to the limited availability of donors, many studies were conducted using stem cells and their extracellular vesicles as an alternative therapy (Shojaati et al. 2019). Nuzzi et al. reported that BMSC-EVs contain a complex cargo of platelet-derived growth factor (PDGF), organelles, lipids, mRNAs, microRNAs, mitochondria, and cytokines. These contents induced proliferation and inhibited apoptosis of corneal endothelial cells in a corneal

Table 1 Pre-clinical studies using MSC-derived EVs for the treatment of various eye disorders

Study title	Eye disease	Stem cell source	EVs	Mechanism	Ref.
Corneal disorders					
Mesenchymal stem cells reduce corneal fibrosis and inflammation via extracellular vesicle-mediated delivery of miRNA	Corneal wound	cMSCs	EVs	EVs fuse with corneal epithelial and stromal cells and deliver miRNA to block scarring and initiate regeneration of transparent corneal tissue after wounding	Shojaati et al. (2019)
Effect of stem cell-derived extracellular vesicles on damaged human corneal endothelial cell	Corneal wound	BMSCs	EVs	A complex cargo within EVs; platelet-derived growth factor (PDGF), organelles, lipids, mRNAs, microRNAs, mitochondria, and cytokines	Nuzzi et al. (2021)
Effect of human corneal mesenchymal stromal cell-derived exosomes on corneal epithelial wound healing	Corneal epithelial wound	cMSCs	Exos	Encapsulated proteins, messenger RNA, and microRNA	Samaeekia et al. (2018)
Comparison of exosomes derived from induced pluripotent stem cells and mesenchymal stem cells as therapeutic nanoparticles for treatment of corneal epithelial defects	Corneal epithelial defects	iPSC and UMSC	Exos	Upregulating cyclin A and CDK2	Wang et al. (2020)
Mesenchymal stem cell-derived extracellular vesicles protect human corneal endothelial cells from endoplasmic reticulum stress-mediated apoptosis	Corneal endothelial dystrophy	BMSCs	EVs	miRNA transfer is responsible for the reduction of the levels of ER stress and apoptosis	Buono et al. (2021)
Dry eye disorders					
hADSCs-derived extracellular vesicles inhibit NLRP3 inflammasome activation and dry eye	Dry eye	ADSCs	EVs	Suppression of NLRP3 inflammasome formation, caspase-1 activation, and IL-1β maturation.	Xu et al. (2020)
Exosomes derived from mouse adipose-derived mesenchymal stem cells alleviate benzalkonium chloride-induced mouse dry eye model via inhibiting NLRP3 inflammasome	Dry eye	Adipose tissue MSCs	Exos	Inhibiting NLRP3 inflammasome	Wang et al. (2022)
Regulation of human umbilical cord mesenchymal stem cell-derived exosomes on peripheral blood macrophages from rabbit autoimmune dry eye	Autoimmune dry eye	UMSC	Exos	Polarizing peripheral blood macrophages toward immune-suppressive M2-like phenotype	Li et al. (2019)

(continued)

Table 1 (continued)

Study title	Eye disease	Stem cell source	EVs	Mechanism	Ref.
Uveal disorders					
MSC-derived extracellular vesicles attenuate immune responses in two autoimmune murine models: type 1 diabetes and uveoretinitis	Autoimmune uveoretinitis	BMSC	Evs	Inhibiting the activation of antigen-presenting cells and suppressing development of Th1 and Th17 cells	Shigemoto-Kuroda et al. (2017)
Effects of mesenchymal stem cell-derived exosomes on experimental autoimmune uveitis	Experimental autoimmune uveoretinitis	UMSC	Exos	Inhibiting the chemoattractive CCL2 and CCL21	Bai et al. (2017)
Glaucoma					
Mesenchymal stem cell–derived small extracellular vesicles promote neuroprotection in a genetic DBA/2J mouse model of glaucoma	Glaucoma	BMSCs	sEVs	Delivery of multiple miRNAs that interfere with multiple neuroprotective pathways	Mead et al. (2018a)
Mesenchymal stem cell–derived small extracellular vesicles promote neuroprotection in rodent models of glaucoma	Glaucoma	BMSC	sEVs	The neuroprotective effect of sEV, at least partially, is explained by direct action on RGC through miRNA-dependent mechanisms	Mead et al. (2018b)
Retinal disorders					
Bone marrow-derived mesenchymal stem cells-derived exosomes promote survival of retinal ganglion cells through miRNA-dependent mechanisms	Traumatic optic neuropathy (loss of retinal ganglion cells)	BMSCs	Exos	miRNA transfer rather than the protein content of exos	Mead and Tomarev (2017)
Protective effects of intravitreal administration of mesenchymal stem cell-derived exosomes in an experimental model of optic nerve injury	Traumatic optic neuropathy	BMSCs	Exos	MSC-exos attenuate RGCs apoptosis and inflammation partly via the PI3K/AKT pathway	Cui et al. (2021)
UMSC-derived exosomes promote retinal ganglion cells survival in a rat model of optic nerve crush	Traumatic optic neuropathy (retinal ganglion cells loss)	UMSC	Exos	Glia cells activation in retina and optic nerve via miRNA 21 transfer	Pan et al. (2019)
Extracellular vesicles derived from human ES-MSCs protect retinal ganglion cells and preserve retinal function in a rodent model of optic nerve injury	Optic nerve injury	ESCs	EVs	Suppression of the tauopathy process of retinal ganglion cell degeneration	Seyedrazizadeh et al. (2020)
Protective effect of intravitreal administration of exosomes derived from mesenchymal stem cells on retinal ischemia	Oxygen-induced retinopathy	BMSC	Exos	Pro-angiogenic signaling proteins and pro-survival-associated proteins from both the CREB and HSP pathways	Moisseiev et al. (2017)

(continued)

Table 1 (continued)

Study title	Eye disease	Stem cell source	EVs	Mechanism	Ref.
Mesenchymal stem cell-derived extracellular vesicles and retinal ischemia reperfusion	Retinal ischemia	BMSCs	EVs	MSC-EVs endocytosed by retinal cells or retained in vitreous humor decrease neuroinflammation and neuronal apoptosis	Mathew et al. (2019)
UBA2 activates Wnt/β-catenin signaling pathway during protection of R28 retinal precursor cells from hypoxia by extracellular vesicles derived from placental mesenchymal stem cells	Retinal hypoxia	PMSCs	Exos	Re-established the UBA2 function and stimulated the Wnt signaling pathway	Koh et al. (2020)
Exosomes derived from MSCs ameliorate retinal laser injury partially by inhibition of MCP-1	Laser-induced retinal injury	Adipose tissue MSCs or UMSC	Exos	Downregulation of MCP-1 influenced by proteins or RNAs encapsulated in MSC-exos	Yu et al. (2016)
Human embryonic stem cell-derived extracellular vesicles alleviate retinal degeneration by upregulating Oct4 to promote retinal Müller cell retrodifferentiation via HSP90	Retinitis pigmentosa and age-related macular degeneration	ESCs	MVs	Regulating the expression of Oct4 in Müller cells by HSP90 mediation in MVs	Ke et al. (2021)
Mesenchymal stem cell-derived exosomes ameliorate blue light stimulation in retinal pigment epithelium cells and retinal laser injury by VEGF-dependent mechanism	Age-related macular degeneration	UMSC	Exos	Downregulation of VEGF expression influenced by vital proteins or RNAs encapsulated in MSC exos	He et al. (2018)
Therapeutic effects of mesenchymal stem cell-derived exosomes on retinal detachment	Retinal detachment	BMSCs	Exos	Inhibiting inflammatory cytokine induction and anti-apoptotic effects	Ma et al. (2020)
Exosomes derived from neural progenitor cells preserve photoreceptors during retinal degeneration by inactivating microglia	Retinal degeneration	NPCs	Exos	17 miRNAs in NPC-exos that inhibit microglial activation	Bian et al. (2020)
Bone marrow mesenchymal stem cells-induced exosomal microRNA-486-3p protects against diabetic retinopathy through TLR4/NF-κB Axis repression	Diabetic retinopathy	BMSCs	Exos	miR-486-3p via TLR4/NF-κB axis repression	Li et al. (2021)
MSC-derived exosomal lncRNA SNHG7 suppresses endothelial–mesenchymal transition and tube formation in diabetic retinopathy via miR-34a-5p/XBP1 axis	Diabetic retinopathy	BMSCs	Exos	X-box binding protein upregulation and miR-34a-5p downregulation	Cao et al. (2021)

(continued)

Table 1 (continued)

Study title	Eye disease	Stem cell source	EVs	Mechanism	Ref.
Adipose mesenchymal stem cells-secreted extracellular vesicles containing microRNA-192 delays diabetic retinopathy by targeting ITGA1	Diabetic retinopathy	Adipose-derived mesenchymal stem cells	EVs	miR-192-targeted and negatively regulated ITGA1	Gu et al. (2021)
Exosomes derived from mesenchymal stem cells modulate miR-126 to ameliorate hyperglycemia-induced retinal inflammation via targeting HMGB1	Diabetic retinopathy	UMSC	Exos	miR-126-exos decreased HMGB1 expression and inhibited activation of NF-jB/p65	Zhang et al. (2019)
Adipose mesenchymal stem cells-derived exosomes attenuate retina degeneration of streptozotocin-induced diabetes in rabbits	Diabetic retinopathy	Adipose tissue MSCs	Exos	Regenerative retinal changes were associated with exos-shuttled micRNA-222	Safwat et al. (2018)
Extracellular vesicles secreted from mesenchymal stem cells exert anti-apoptotic and anti-inflammatory effects via transmitting microRNA-18b in rats with diabetic retinopathy	Diabetic retinopathy	UCMSCs	sEVs	sEVs-transferred miR-18b regulates the NF-κB pathway by targeting the MAP3K1	Xu et al. (2021)

wound model. It was believed that bone marrow-derived mesenchymal stem cell extracellular vesicles (BMSC-EVs) incorporate mitochondria into the endogenous mitochondria of the injured cell, restoring its homeostasis (Nuzzi et al. 2021). Another studied source for MSCs was corneal stromal stem cells (CSSC) as reported by Shojaati et al. They administered CSSC EV to a model of corneal wound, showing a reduced expression of the fibrotic genes Col3a1 and Acta2. Also, CSSC-EVs are rapidly fused with corneal epithelial and stromal cells and deliver miRNA to block scarring and initiate regeneration of transparent corneal tissue after wounding (Shojaati et al. 2019). These findings were in line with the results of Samaeekia et al. who isolated corneal MSCs (cMSCs) from human cadaver corneas and attributed their effects to the presence of encapsulated proteins, messenger RNA, and microRNA (Samaeekia et al. 2018). In addition, Wang et al. used both MSCs-Exos and iPSCs-Exos as therapeutic strategies in corneal epithelial defect. It was noticed that both iPSCs-Exos upregulated cyclin A and CDK2, thus promoting cell regeneration, along with the inhibition of apoptosis. In the meantime, iPSCs-Exos showed a more prominent effect on corneal epithelium healing (Wang et al. 2020).

(b) Corneal Endothelial Dystrophy

In a study on endothelial corneal dystrophy, exosomes (Exos) were isolated from BMSCs and showed that miRNA transfer from Exos is responsible for the reduction of the levels of ER stress and apoptosis in corneal tissue. This was achieved through downregulation of endoplasmic reticulum stress-related genes in corneal endothelial cells, as well as caspase-3 activation (Buono et al. 2021).

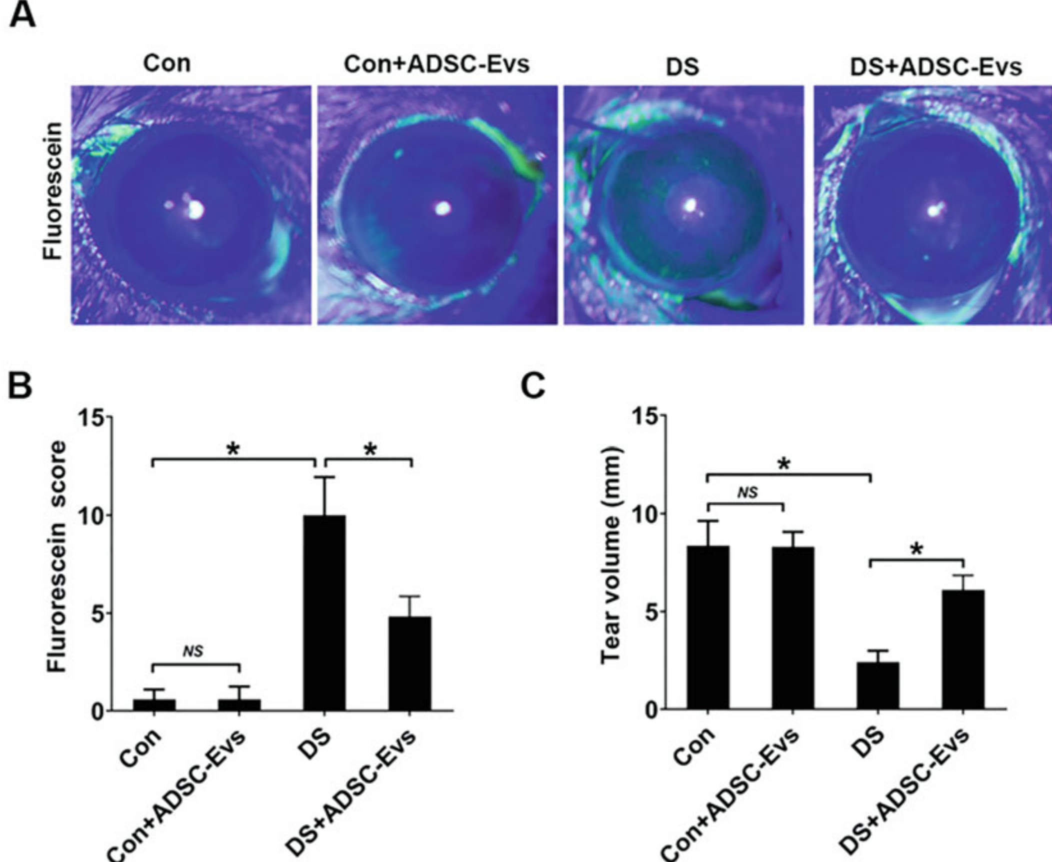

Fig. 2 Effects of topical application of hADSC-EVs on DS-induced ocular surface damage. hADSC-Evs were topically applied four times daily under DS (DS + hADSC-EVs), while mice of DS group (DS) and normal control group (Con) received PBS. (**a**) Representative image of fluorescein sodium staining in cornea. (**b**) The mean score of fluorescein sodium staining. (**c**) Phenol red cotton test for the quantification of tear production. $n = 6$ mice per group. Data was shown as mean ± SEM. $*p < 0.05$ (Xu et al. 2020). (CC BY 4.0 License)

2.1.2 Dry Eye Disease

Dry eye disease (DED) is a common disorder of the ocular surface worldwide, that is characterized by loss of the tear film homeostasis, thus affecting the quality of life. It was reported that adipose tissue stem cells (ADSC)-EVs application alleviated DED progression by blocking NLRP3 upregulation and increased IL-1β maturation. The treated mice showed reduced corneal epithelial defects, decreased goblet cell loss, and reduced inflammatory reaction (Fig. 2) (Xu et al. 2020). This was consistent with Wang et al. who reported that ADSC-Exos reduced the expression of NLRP3, caspase-1, IL-1β, and IL-18 in the conjunctiva of a dry eye model (Wang et al. 2022). As well, in an autoimmune model of dry eye, it was stated that MSC-Exos can regulate peripheral blood macrophages to an immunosuppressive M2-like phenotype, preventing the production of TNF-α and IL-1β, yet increasing the anti-inflammatory IL-10 and TGF-β expression levels (Li et al. 2019).

2.1.3 Uveal Disorders

It was stated that MSC-EVs may be a promising therapy for autoimmune diseases. A study reported their immunosuppressive effects in autoimmune uveitis. MSC-EVs increased IL-10-

expressing regulatory dendritic cells, repressed development of Th1 and Th17cell and inhibited APCs activation, thus preventing experimental autoimmune uveoretinitis (EAU) (Shigemoto-Kuroda et al. 2017). Similarly, Bai et al. tested the effect of local MSC-Exo administration on EAU. A reduction of T cell infiltration in the eyes was noted. This was as a result of inhibiting the CCL2 and CCL21 chemoattractive effects on inflammatory cells, thus hindering the migration of inflammatory cells (Bai et al. 2017).

2.1.4 Glaucoma

In a chronic model of glaucoma, the administration of BMSC small extracellular vesicles (sEV) provided neuroprotection. Around 43 miRNAs were found within sEVs. The transfer of miRNA via sEV to the damaged retinal tissue preserved the number of axons and RGC in the studied model. However, sEV has been effective only in a 6-month-old model not at 9 or 12 months. (Mead et al. 2018a, b).

2.1.5 Retinal Disorders

(a) Retinal Degeneration

Some studies reported the role of EVs in conditions associated with loss of retinal ganglion cells (RGC) such as optic neuropathy and glaucoma. A study tested exosomes from BMSC in an optic nerve crush (ONC) model and demonstrated that exosomes promoted the survival of RGC and axon regeneration. This was achieved through the miRNA delivery to the inner retinal layers. This study did not specify the type of miRNA; however, they mentioned some miRNA variants such as miR-17-92 which suppress phosphatase and tensin homolog expression, along with miR-146a which acts on epidermal growth factor receptor mRNA (Mead and Tomarev 2017). This was in line with the results of Cui et al. who reported that in ONC, MSC-exos reduced the apoptosis of RGCs. That was through endorsing Bcl-2/Bax ratio expression, preventing caspase-3 activation as well as restoring AKT pathway. Also, MSC-exos have an anti-inflammatory role by downregulating pro-inflammatory cytokines and upregulating the expression of anti-inflammatory factors (Cui et al. 2021). In another model of ONC, the transport of UMSC-Exos showed neuroprotective effects. UMSC-Exos promoted RGCs survival with no effect on the axon regeneration. This might be due to glia cells activation in retina and optic nerve besides miRNA 21 transfer, which accounted for 12.5% of the total miRNA in the exosomes (Pan et al. 2019). On the other side, Seyedrazizadeh et al. reported an increase in axon counts in the damaged optic nerve (Fig. 3). They demonstrated that in the early neurodegeneration process, cis p-tau levels are highly elevated, which were suppressed by EV treatment. This tauopathy leads to disruption in the axonal microtubules and apoptosis which is termed “cistauosis” (Seyedrazizadeh et al. 2020).

In an oxygen-induced retinopathy (OIR) model, the transplantation of MSC-Exos showed attenuation of RGCs apoptosis and inflammation. The results indicated that exosomes from MSCs contain pro-survival-associated proteins from the cAMP response element-binding protein (CREB) pathway as well as the shock protein (HSP) pathways. These pathways were affected by retinal ischemia (Moisseiev et al. 2017). Similarly, in a model of oxygen-glucose deprivation, the intravitreal administration of MSC-EVs promoted functional recovery and attenuated inflammation and apoptosis. EVs were internalized by retinal neurons, RGC, and microglia. Mathew et al. (2019) reported the participation of target cell heparin sulfate proteoglycans in EV endocytosis by retinal cells and the binding of EVs to the vitreous proteins (Mathew et al. 2019). This was consistent with a study on the benefits of placenta-derived mesenchymal stem cells (PMSCs) exosomes on retinal hypoxic damage in R28 cells (retinal precursor cells). It reported recovery of regeneration markers expression in R28 cells. This study demonstrated that PMSC-derived exosomes re-established the UBA2 function and stimulated the Wnt signaling pathway (Koh et al. 2020). Furthermore, Yu et al. found that exosomes derived from both adipose tissue and umbilical

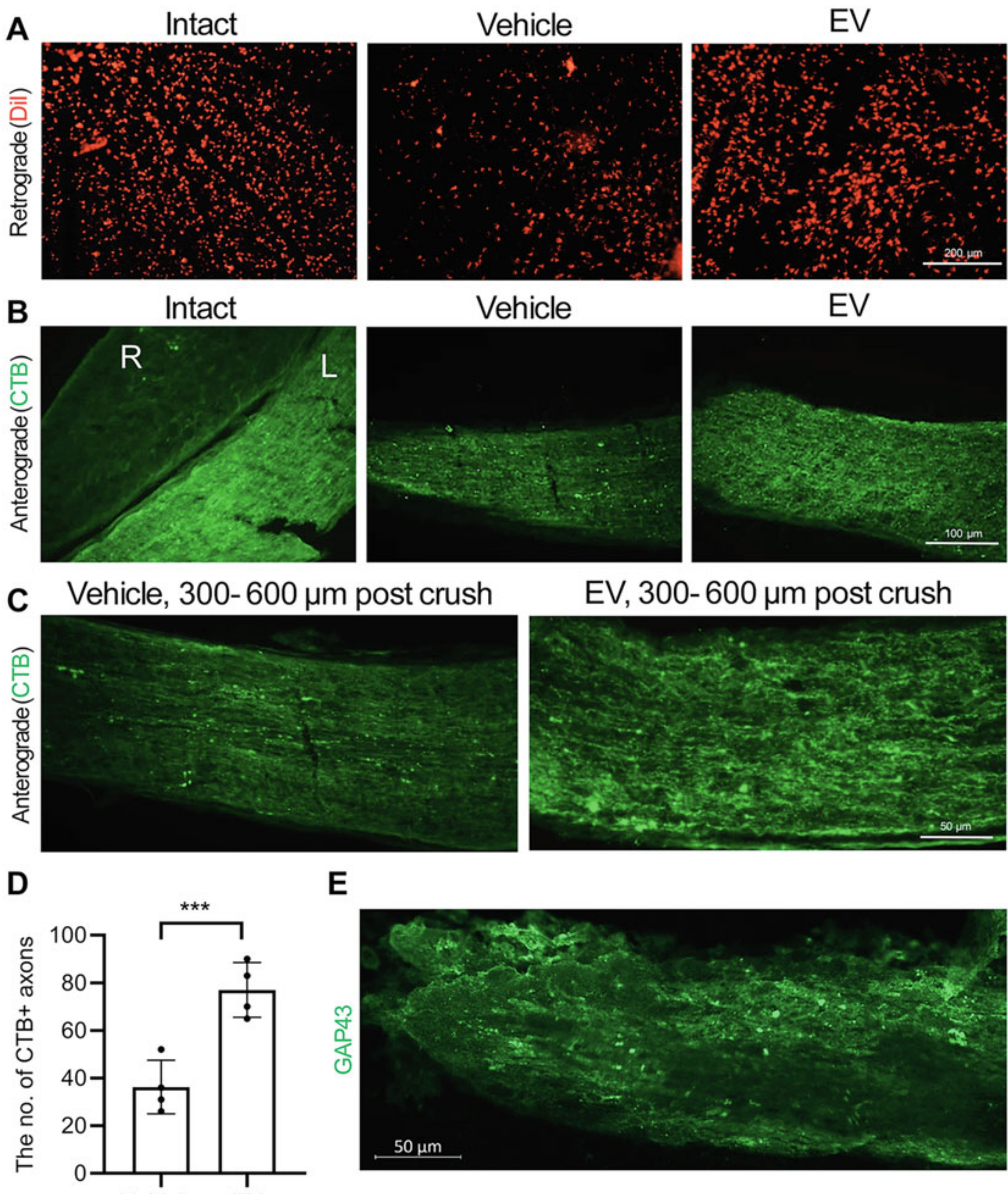

Fig. 3 Retro- and anterograde axonal tracing 60 days post-treatment. (**a**) The intact retinal ganglion cells (RGCs) soma in the vehicle and extracellular vesicle (EV) groups that was retrogradely stained with DiIC18(3) (DiI; red). (**b, c**) Longitudinal cryosections and immunostaining against chlorotoxin B (CTB) in the intact group of the anterograde show the left (L) and right (R) optic nerves. CTB was only injected in the left optic nerve. Data from the anterograde tracing shows that more CTB+ axons extended the length of the optic nerve in the EV group, whereas smaller numbers in the vehicle were seen, even at a distance of 300–600 μm in the distal site of crush. (**d**) Quantification of CTB+ RGCs axons in the optic nerve in (**c**). Data are shown as mean ± SD for four optic nerves per group. The data were analyzed by the unpaired t test. ***$P < 0.001$. (**e**) A longitudinal section of the optic nerve with EV, which shows numerous axons with GAP43 expression (Seyedrazizadeh et al. 2020). (CC BY 4.0 License)

cord MSCs inhibited apoptosis and reduced inflammatory response following laser-induced retinal injury. This was achieved through the downregulation of monocyte chemotactic protein (MCP)-1 in the retina. Studies have reported that retinopathies induce MCP-1 secretion which in turn attracts macrophages and microglial cells, causing further tissue damage. Proteins or RNAs in MSC-Exos might have caused this MCP-1 inhibition (Yu et al. 2016). A study by Ke et al. showed that ESEVs promoted the dedifferentiation and retro-differentiation of Müller cells into retinal progenitor cells. This was confirmed by the presence of CHX10-positive cells in the inner layer of the retina following ESEV injection. HSP90 in MVs rather than in exosomes is believed to be upregulating Oct4 expression in Muller cells facilitating their retro-differentiation (Ke et al. 2021). Another study reported that MSCs-exosomes reduced retinal cell damage through downregulation of VEGF-A. The expression of VEGF was increased following blue light-induced retinal laser injury, then inhibited by MSC-Exos. This might be achieved by proteins or RNAs encapsulated in MSC-Exos (He et al. 2018). Along with the previous studies, MSC-Exos can be used as therapeutic agents in retinal detachment (RD) by reducing photoreceptor cell degeneration. This was evidenced by the reduced expression of TNF-α and IL-1β and decreased cleavage of autophagy-related protein 5 (Atg5). Proteins in MSC-Exos were revealed to have anti-inflammatory, anti-apoptotic, and neuroprotective effects, such as Comp, which can block the activation of caspase-3 and suppress apoptosis and Anxa1, which has anti-inflammatory action. Therefore, MSC-Exo neuroprotection effects count on the actions of different exosome contents rather than a single one (Ma et al. 2020). Additionally, transplantation of neural progenitor cells (NPCs) might be effective in the treatment of RD as demonstrated by Zeng et al. They found that grafted NPCs secreted exosomes in the subretinal space (SRS) in an RD model. In addition, direct administration of NPC-Exos was found to attenuate apoptosis of photoreceptor and conserve visual function. NPC-Exos were internalized by retinal microglia and repressed their activation. Using miRNA profiling, 17 miRNAs were found to be contained in NPC-Exos. They targeted TNF-α, IL-1β, and COX-2 in activated microglia, thus inhibiting inflammatory signal pathways (Bian et al. 2020). To overcome the challenge of gene transfer to the retina, exosome-associated adeno-associated virus (Exo-AAV) has been studied. Exo-AAV showed deep penetration into the layers of the retina, including the inner nuclear and outer plexiform layers, and the outer nuclear layer to a lesser extent. This was explained by the different mechanisms to take up exosomes such as receptor mediated endocytosis, macropinocytosis, or phagocytosis in contrast to AAV, which has a restricted uptake mechanism. Consequently, its association with exosome might help the uptake of AAV to variable cell types. It was found that Exo-AAV particles depend on the universal AAV receptor (AAVR) on the cell surface. Thus, Exo-AAV and AAV might use the same pathways (Wassmer et al. 2017). Moreover, a study suggested that retinal pigment epithelium-derived Exos have a downregulatory immune effect on cultured undifferentiated lymphocytes or monocytes. ARPE-19 cultures were used as a source of Exos, after stimulation with inflammatory cytokines and without stimulation. RPE-derived Exos significantly repressed T-cell proliferation in both cultures. Only in the cytokine-stimulated RPE cells, upregulation of proinflammatory cytokines and monocyte death were triggered by Exos. It was stated that RPE-derived Exos exposed to inflammatory cytokines stimulation can express proapoptotic molecules thus reducing the infiltration of immune cells to RPE and photoreceptors. T-cell suppression in the non-stimulated RPE cells was achieved by the enrichment of CD14+CD16+ monocytes and upregulation of TGF-b1. Meanwhile, Exos from cytokine-stimulated RPE cells possibly hinder T-cell stimulation due to the lack of APC (Knickelbein et al. 2016).

(b) Diabetic Retinopathy

Diabetic retinopathy (DR) is a chronic eye disease, leading to irreversible blindness. Some studies have been conducted to expound the effect of

exosomes on DR. Li et al. showed that BMSC-derived exosomal miR-486-3p had a protective effect and suppressed apoptosis via TLR4/NF-κB repression in mice with DR. BMSC-derived exosomes were transfected with miR-486-3p or TLR4-related oligonucleotides and plasmids. Upregulating miR-486-3p or downregulating TLR4 repressed oxidative stress and apoptosis, and helped proliferation of Muller cells (Li et al. 2021). In addition, a study demonstrated that long non-coding RNAs lncRNAs SNHG7 are transferred by MSC-derived exosomes to human retinal microvascular endothelial cells (HRMECs). They repressed migration, endothelial–mesenchymal transition (EndMT), and tube formation of HRMECs. This was confirmed to be as a result of X-box binding protein upregulation and miR-34a-5p downregulation (Cao et al. 2021). It is notable that integrin subunit α1 (ITGA1) variants are linked to type 2 diabetes. Gu et al. investigated the role of MSC-derived extracellular vesicles in angiogenesis in a diabetic model. They demonstrated that miR-192 in EVs regulated ITGA1, suppressing the inflammatory reaction along with angiogenesis (Gu et al. 2021). In another study, overexpression of miR-126 in MSC-Exos was found to downregulate the high-mobility group box 1 (HMGB1) signaling pathway in diabetic rats, as well as reduce the NLRP3 inflammasome activity in human retinal endothelial cells (Zhang et al. 2019). Moreover, Safwat et al. isolated exosomes from adipose tissue-derived MSCs and injected them into a model of DR. They noticed a significant negative relationship between serum glucose and micRNA-222 expression level in the retina. Following treatment with MSCs-Exos, the expression of micRNA-222 was increased with an observed retinal tissue regeneration. It was found that micRNA-222 can inhibit the neovascularization detected in DR by binding to the c-Kit receptor (Safwat et al. 2018). Another study suggested the anti-inflammatory and anti-apoptotic roles of UMSCs-sEVs in DR, where miR-18b transfer could target mitogen-activated protein kinase 1 and inhibit NF-κB p65 phosphorylation (Xu et al. 2021).

2.2 Clinical Applications

To assess the current situation in terms of clinical trials using stem cell-derived EVs in the treatment of eye diseases, we tried to identify all interventional trials registered at ClinicalTrials.gov, either enrolling patients, active, or completed. Till the date of July 14, 2022, two studies were identified to date (NCT04213248 and NCT03437759). Among patients with chronic graft versus host disease (cGVHD), 60–90% suffer symptoms of dry eye. They mostly present with progressive development of dryness, foreign body sensation, photophobia, eye pain, or even blindness. To date, the first-line treatment of cGVHD remains to be hormonal or combined with an immunosuppressant as cyclosporine. The treatment of dry eye symptoms related to cGVHD is mainly symptomatic (e.g., artificial tears, lacrimal punctum occlusion, local immunosuppressant, blepharoplasty, etc.). Despite the wide variety of new immunosuppressants and monoclonal antibodies used in clinic, their overall efficacy is still unsatisfactory. Moreover, they require long-term treatment which is costly and results in significant side effects. In a trial to seek an alternative treatment, umbilical mesenchymal stem cell-derived exosomes (UMSC-Exo) have been widely studied in diseases affecting several organs (e.g., liver, kidney, skin, etc.). Promising pre-clinical studies found that UMSC-Exo can significantly alleviate the symptoms of dry eye in an animal model. Consequently, a research team in Guangdong, China is currently running an interventional clinical trial (phase 1 and 2) aiming to assess the alleviation of dry eye symptoms in patients with cGVHD after UMSC-Exo treatment by measuring Ocular Surface Index Score (OSDI). The second objective of the study includes the measurement of tear secretion amount, tear break time, the areas stained by fluorescent, ocular redness, tear meniscus, and best corrected visual acuity. Approximately 27 study subjects are recruited. The treatment group receives artificial tears for 2 weeks to normalize the baseline, followed by intervention of UMSC-Exo 10ug/drop, four times a day for 14 days. The follow-up visit will be 12 weeks, progression of dry eye is being measured.

In another clinical trial, the participants with large and longstanding idiopathic macular holes (MHs) underwent vitrectomy, internal limiting membrane peeling, MSC, or MSC-Exo intravitreal injection. UMSC-Exo were isolated from supernatants of MSCs via sequential ultracentrifugation. At the time of study enrollment, physical examinations, best-corrected visual acuity (BCVA), and intraocular pressure were measured and fundoscopy was performed. All diagnoses of MHs were confirmed via spectral-domain optical coherence tomography (OCT), and the minimum linear diameter (MLD) of each MH was measured parallel to the retinal pigment epithelium. The participants are followed up for at least 6 months using the same beforementioned parameters.

Authors reported that the intravitreal injection of MSCs and MSC-Exos at the end of regular PPV might improve the anatomic and visual outcomes of surgery for refractory MHs (Zhang et al. 2018). Interestingly, the MSC-Exo therapy appeared to be safer than MSCs therapy in terms of the risk of cell proliferation. Moreover, it is easier because no additional surgical procedure is needed. Nevertheless, the study had obvious limitations. First of all, a control/Sham group was not included. Therefore, the precise therapeutic effects of the MSC or MSC-Exo treatment in MH closure cannot be estimated. In general, well-designed clinical trials with larger samples and control groups will provide a much better understanding of the true therapeutic potential of MSC-Exos for patients with MHs.

3 Obstacles on the Way

Despite the extensive pre-clinical research on exosomes, a few clinical trials on eye disorders are registered so far. To date, the FDA has not approved the clinical use of exosome products. Patients enrolled in clinical trials should also be aware of potential risks of exosomes. Recently, patients who were treated with products marketed as containing exosomes (from the ReGen Series® distributed by Liveyon, LLC) have experienced serious adverse events, including bacterial infections. Therefore, the CDC advises patients considering stem cell treatments to make sure the product they are considering is on the FDA's approved list of stem cell treatments. In case the stem cell product is not on the approved list, or if they are considering an exosome product, patients are advised to ask the provider to provide them with documentation from the FDA and that they have FDA's permission to research the product, even if the stem cells or exosomes are autologous in origin. This documentation would include an IND application number and acknowledgment communication issued by FDA. The following is considered a short list of the potential risks that might be associated with exosome therapy.

3.1 Contamination

If the exosomes in use are not correctly extracted and sterilized, they might be contaminated with intracellular or extracellular genetic material from the MSCs of origin. Such genetic material could possibly be harmful resulting in unfavorable cell signaling, potentially leading to faulty growth or development of cells at the target site or elsewhere in the body.

3.2 Recycled MSCs

Only new and unused MSCs should be considered as a source for exosomes extraction to ensure their purity and efficacy. Otherwise, the heterogeneity of amount or quality growth factors is inevitable. Moreover, advanced passages of MSCs hold a potential threat of contamination compared to the primary, unpassaged MSCs.

3.3 Insufficient Yield

The therapeutic preparation depends mainly on the huge number of exosomes. Therefore, a sample with significantly fewer exosomes might result in a poor clinical outcome.

3.4 Bacterial Infection

Exosomes are within a much smaller size range compared to bacteria. Nevertheless, inappropriate extraction methods or imperfect sterilization of the exosome extract can allow for contamination of the final therapeutic product. Since some infection cases have been reported of acute bacterial infections from exosome therapy, it is necessary to work with a trusted exosome therapy clinic to ensure safe treatment.

For instance, in the year 2018, an unfortunate incidence took place when bacterial infections were encountered in patients who have received unapproved cell-based therapies claimed to include exosomes from the ReGen Series® (distributed by Liveyon, LLC). Most of these patients developed symptoms such as pain, swelling, or chills within a few days of receiving the therapeutic products which resulted in an immediate withdrawal of these products. Consequently, the Food and Drug Administration (FDA) has inspected the company that processed these products and found problems with the manufacturing process.

4 Concluding Remarks

Due to the true unmet need for an optimal therapeutic avenue to treat or mitigate the progression and manifestation of several degenerative and immunologic eye disorders, stem cell-derived EVs are deemed the next-generation cell-free therapeutics. For instance, intravitreal injection of MSCs and MSC-Exos at the end of regular PPV may improve the anatomic and visual outcomes of surgery for refractory MHs. MSC-Exos therapy is safer than cell therapy with regard to the risk of cell proliferation, and it is easier because no extra surgery is needed. Injecting MSCS-EVs intravitreally does not cause the complications noticed when their cells of origin (MSCs) were injected instead (e.g., vitreous opacity, immunologic rejection, or proliferative vitreous retinopathy) (Zhang et al. 2018). Therefore, they are believed to be a safe, biomimetic alternative to stem cell therapy (Rostom et al. 2020; Attia et al. 2021a, b) with lower oncogenic and immunological risks, and greater targeting specificity (Mathew et al. 2019).

As demonstrated in this chapter, several studies confirmed the critical role of stem cell-derived EVs in treating ophthalmic disorders. Moreover, these nano/microvesicles could be also ideal carriers to deliver drugs thanks to their bi-layer phospholipid membrane structure, high biocompatibility, and optimal size. The lipid bilayer membrane is able to protect the EV cargo from degradation. Therefore, EVs can travel long distances and cross biological barriers to gain access to target cells. Therefore, they are considered as natural delivery vehicles for drug, nucleic acid, or other bioactive substances. Nevertheless, we are still far away from a solid understanding of their exact mechanism(s) of action on target cells. This might explain why clinical studies in this regard are still quite limited. Stem cells, of various types, are a rich source of EVs that depict many beneficial effects for many diseases, are ideal for drug/gene delivery, and are used in studies of many diseases. With such mounting evidence of the therapeutic efficacy of EVs obtained from stem cells, it seems favorable to translate such therapeutic agent into clinic for treating ocular diseases in the near future.

The treatment of many intraocular diseases relies on injection because of the anatomical structure and multiple barriers of the eye. Frequent intraocular injections create heavy burdens to the healthcare system and the lifestyle of patients. Therefore, the use of topical drugs to treat retinal diseases has always been an attractive solution. The use of EVs in the anterior segment is markedly favored by their demonstrated transcellular uptake by corneal epithelium thanks to their structural similarity to their cell membranes and high affinity. From there, EVs could potentially convey messages across long distances in tissues, with the message contained in the lipid layer as packed receptors or ligands, enzymes, or the bioactive lipids themselves (Robciuc et al. 2014). In a recent study, Matthew B. and team injected EVS into the vitreous. Days

later, these EVs were found in astrocytes, microglia, Muller cells, and retinal neurons. The in vivo retention time varied from one retinal cell type to another, with the longest time being 14 days in the retinal ganglion cells with no sign of toxicity (Mathew et al. 2021). The EVs are known to bind to extracellular matrix components as fibronectin, rendering the vitreous body a potential reservoir for EV delivery to the retina. Nevertheless, Matthew B. and colleagues reported that most EVs were rapidly cleared from the vitreous. Such findings could have important implication on therapeutic application in a way that repeated injections might be still required, unless the surface of EVs is altered (e.g., by modifying the EV surface with peptide sequences) to enhance binding to vitreous collagen and other extracellular matrix proteins.

Eventually, the rapid and effective clinical translation of EVs as a therapeutic tool for ocular disorders relies on a thorough understanding of the significance and limitations of pre-clinical models.

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Cannabinoids as Prospective Anti-Cancer Drugs: Mechanism of Action in Healthy and Cancer Cells

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Abstract

Endogenous and exogenous cannabinoids modulate many physiological and pathological processes by binding classical cannabinoid receptors 1 (CB1) or 2 (CB2) or non-cannabinoid receptors. Cannabinoids are known to exert antiproliferative, apoptotic, anti-migratory and anti-invasive effect on cancer cells by inducing or inhibiting various signaling cascades. In this chapter, we specifically emphasize the latest research works about the alterations in endocannabinoid system (ECS) components in malignancies and cancer cell proliferation, migration, invasion, angiogenesis, autophagy, and death by cannabinoid administration, emphasizing their mechanism of action, and give a future perspective for clinical use.

Keywords

Apoptosis · Autophagy · Cancer · Cannabinoid receptors · Cannabinoids · Cell cycle · Invasion · Migration · Proliferation

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Abbreviations

2-AG	2-Arachidonoyl glycerol
AA-5HT	Arachidonoyl serotonin
ABDH6/12	Alpha/beta-hydrolase domain containing 6/12
ACEA	Arachidonyl-2′chloroethylamide
ACF	Aberrant crypt foci
ACPA	Arachidonoyl cyclopropilamide
AEA	Anandamide
AKT	Protein kinase B
AMPK	5’ AMP-activated protein kinase
ANG-2	Angiotensin II
ASD	Autism spectrum disorder
ATF-4	Activating transcription factor-4
BAX	Bcl-2-associated X protein
BCL-2	B-cell lymphoma 2
BDS	Botanical cannabinoid extraction
CAMKKβ	Calcium ions/calmodulin-stimulated protein kinase kinase β
CAMP	Cyclic adenosine monophosphate
CB	Cannabinoid
CB1	Cannabinoid receptor 1
CB2	Cannabinoid receptor 2
CBC	Cannabichromene
CBD	Cannabidiol
CBDA	Cannabidiolic acid
CBDV	Cannabidivarin
CBE	Cannabielsoin
CBG	Cannabigerol
CBL	Cannabicyclol
CBN	Cannabinol

CBND	Cannabinodiol
CBT	Cannabitriol
CHOP	C/EBP homologous protein
COX	Cyclooxygenase
CXCR4	C-X-C chemokine receptor type 4
CYC D1	Cyclin D1
CYP-450	Cytochrome P450
DAGL	Diacylglycerol lipase
DC	Dendritic cell
DS	Dravet syndrome
ECS	Endocannabinoid system
EGF	Epithelial growth factor
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase 1/2
FAAH	Fatty acid amid hydrolase
FAK	Focal adhesion kinase
FDA	Food and Drug Administration
GEM	Gemcitabin
GPR	G-protein coupled receptor
GRP78	Chaperone protein glucose-regulated protein 78
GVHD	Graft versus host disease
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intercellular adhesion molecule-1
IGF-IR	Type 1 insulin-like growth factor receptor
IL6/10	Interleukin 6/10
iNOS	Inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
MAGL	Monoacylglycerol lipase
MAPK	Mitogen-activating protein kinase
MDSC	Myeloid-derived suppressor cell
MMP-2/9	Matrix metalloproteinase 2/9
MS	Multiple sclerosis
MTORC-1/2	Mammalian target of rapamycin C-1/2
NAAA	N-Acylethanolamide-hydrolysing acid amidase
NAPE	N-acyl phosphatidylethanolamine
NAPE-PLD	N-acyl phosphatidylethanolamine phospholipase D
NAT	N-Acyltransferase
NFκB	Nuclear factor kappa B
NOXA	Phorbol-12-myristate-13-acetate-induced protein 1
NSCLC	Non–small cell lung cancer
OEA	Oleoylethanolamide
P21	Cyclin-dependent kinase inhibitor 1
P27	Cyclin-dependent kinase inhibitor 1B
PAI-1	Plasminogen activator inhibitor 1
PAK1	P21-activated kinase 1
PEA	Palmitoylethanolamide
PI3K	Phosphoinositide 3 kinase
PKA	Protein kinase A
PLC	Phospholipase C
PPARα	Peroxisome proliferator–activated receptor α
PPARγ	Peroxisome proliferator–activated receptor γ
ROS	Reactive oxygen species
SMAC	Second mitochondria-derived activator of caspase
STAT3	Signal transducer and activator of transcription 3
TIMP	Tissue inhibitor of metalloproteinase
TNF-α	Tumor necrosis factor-alpha
TRIB3	Tribbles pseudokinase 3
TRPV	Transient receptor potential cation channel subfamily V member
UPA	Urokinase-type plasminogen activator
VCAM1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
XIAP	X-linked inhibitor of apoptosis
βIII Tub	βIII Tubulin
Δ^9-THC	Delta-9-tetrahydracannabinol

1 Introduction

Cannabinoids are terpenophenolic compounds which are classified as plant-derived phytocannabinoids, endocannabinoids produced by humans and animals and synthetic forms produced in laboratory. These compounds have been extensively studied for their biological roles in physiological and pathological processes

(Shah et al. 2021) including cell proliferation (Braile et al. 2021; Daris et al. 2019), migration (Daris et al. 2019; Kovalchuk and Kovalchuk 2020), invasion (Sledzinski et al. 2021; Tomko et al. 2020), angiogenesis (Lee et al. 2021; Wang and Multhoff 2021), autophagy (Hinz and Ramer 2019; Lee et al. 2021), and apoptosis (Leo and Abood 2021; Vecera et al. 2020). In recent years, many clinical studies concerning the cannabinoid management have been conducted on their relieving effect on chemotherapy-related nausea and vomiting, spasms, neuropathic pain, insomnia, and seizures (Mücke et al. 2018; Pauli et al. 2020; Sawtelle and Holle 2021). Therefore, this chapter focuses on the recent preclinical and clinical advances in the fields of cannabinoids and their effects on cellular mechanisms in healthy and cancerous cells.

2 Focus on Cannabinoids

2.1 Phytocannabinoids

Cannabis sativa L. (marijuana) plant comprises more than 100 psychoactive terpenophenolic compounds known as cannabinoids (Abrams and Guzman 2015; Bogdanovic et al. 2017; McAllister et al. 2015). Delta-9-tetrahydracannabinol (Δ^9-THC), cannabidiol (CBD) and cannabigerol (CBG) are known as the major compounds among all phytocannabinoids (Pagano et al. 2021). Cannabinol (CBN), cannabichromene (CBC), cannabidiolic acid (CBDA), cannabinodiol (CBND), cannabielsoin (CBE), cannabicyclol (CBL), cannabitriol (CBT), and cannabidivarin (CBDV) are the other well-known minor phytocannabinoids (Walsh and Holmes 2022). Plasma concentration of Δ^9-THC reaches its highest level at 1–6 h after the cannabis ingestion, and its half-life is approximately 20–30 h (Abrams and Guzman 2015). Maximum concentration of Δ^9-THC reaches in 2–10 min after the cannabis inhalation and the levels decrease rapidly within 30 min (Abrams and Guzman 2015; Baglot et al. 2021).

2.2 The Endocannabinoid System (ECS)

The endocannabinoid system (ECS) comprises endogenous agonists called “endocannabinoids”, enzymes responsible for synthesizing and degrading endocannabinoids, and cannabinoid (CB) receptors (Lu and Mackie 2021; Pertwee 2012). Endocannabinoids are known as natural lipid mediators found in human body (Lu and Mackie 2021), and best characterized endocannabinoids anandamide (N-arachidonoylethanolamine, AEA) and 2-arachidonoyl glycerol (2-AG) generally act through classical CB1 and CB2 receptors (K. A. Johnson and Lovinger 2016; Martinez-Pena et al. 2021; Wu 2019). Besides CB1/2 receptors, both endogenous and exogenous cannabinoids may interact with other G-protein-coupled receptors, GPCR55, GPCR18, GPCR92 or GPCR12 (Biringer 2021; Irving et al. 2017; Pacher et al. 2020; Starowicz et al. 2007); transient receptor potential vanilloid (TRPV) channels TRPV1 or TRPV2 (Martinez-Pena et al. 2021; Petrosino et al. 2016), and nuclear peroxisome proliferator–activated receptor α (PPARα) (P. Morales and Jagerovic 2020; Muller et al. 2018) to regulate various physiological processes involving hemostasis and energy balance (Bellocchio et al. 2008; Martinez-Pena et al. 2021), appetite (Jager and Witkamp 2014; Wu 2019), memory and learning (Wu 2019), and control in nausea and vomiting (Parker et al. 2011; Sharkey et al. 2014). Anandamide is produced with the catalysis of N-acyl phosphatidylethanolamine (NAPE) by N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) (De Petrocellis and Di Marzo 2009; Lu and Mackie 2021; Pyszniak et al. 2016). 2-AG is synthesized by conversion to diacylglycerol by diacylglycerol lipase (DAGL) enzyme, depending on the activation of phospholipase C (PLC) (Battista et al. 2012; De Petrocellis and Di Marzo 2009; Martinez-Pena et al. 2021). Diacylglycerol is generally hydrolyzed with monoacylglycerol lipase (MAGL) or alpha/beta-hydrolase domain containing 6/12 (ABDH6/12) (Grabner et al. 2017; Lu and Mackie 2021; Moreno et al. 2019), and AEA is hydrolyzed by fatty acid

amide hydrolase (FAAH) (De Petrocellis and Di Marzo 2009; Massi et al. 2013; Pyszniak et al. 2016). AEA and 2-AG are also hydrolyzed by cyclooxygenases (COX, e.g. COX-2) (Egmond et al. 2021; Lu and Mackie 2021; Maccarrone 2017; Urquhart et al. 2015), lipoxygenases (LOX, e.g. ALOX isoforms) (Egmond et al. 2021; Maccarrone 2017), cytochrome P450 (CYP-450) or monooxygenases as well (Lu and Mackie 2021; Pyszniak et al. 2016; Zelasko et al. 2015) (Fig. 1). AEA, oleoylethanolamide (OEA), and palmitoylethanolamide (PEA) are also hydrolyzed by N-acylethanolamide-hydrolyzing acid amidase (NAAA) (Lu and Mackie 2021; Pagano et al. 2021; Ramer et al. 2019).

CB1 receptor is predominantly located in synaptic terminals in hippocampus, basal ganglia, cerebellum, and cerebral cortex in central nervous system (Egmond et al. 2021; Lu and Mackie 2021; Pacher et al. 2020; Smiarowska et al. 2022; Wu 2019), bronchial and bronchiolar epithelia in respiratory system (Boyacıoğlu et al. 2021; Smiarowska et al. 2022), uterus, ovary, follicular fluid, embryo and placenta in female reproductive system (Bilgic et al. 2017; Fonseca et al. 2018; Martinez-Pena et al. 2021; Scotchie et al. 2015), testis, vas deferens and prostate in male reproductive system (du Plessis et al. 2015; Walker et al. 2019), and duodenal subepithelial region in digestive system (Health Canada 2018;

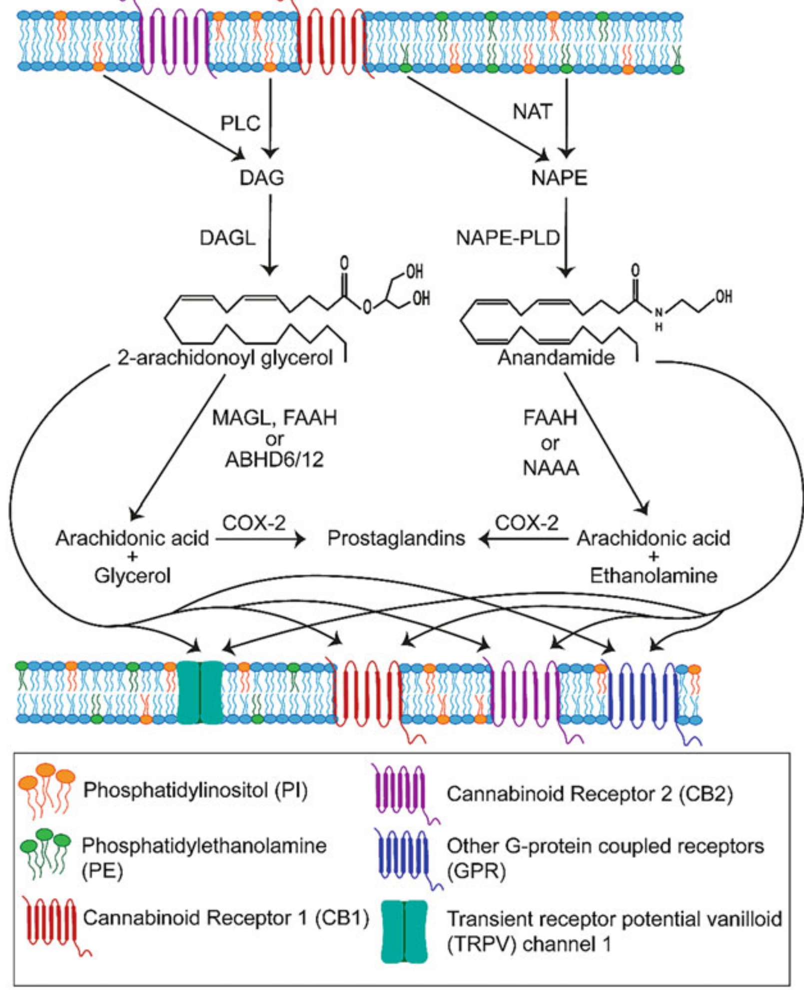

Fig. 1 Schematic representation of endocannabinoid synthesis and breakdown. *ABHD6/12* α/β-hydrolase domain containing protein 6 or 12, *COX-2* Cyclooxygenase 2, *DAG* 1,2 Diacylglycerol, *DAGL* Diacylglycerol lipase, *FAAH* Fatty acid amide hydrolase, *MAGL* Monoacylglycerol lipase, *NAAA* N-Acylethanolamide-hydrolysing acid amidase, *NAPE* *N*-Arachidonoyl phosphatidylethanolamine, *NAPE-PLD* NAPE phospholipase D, *NAT* *N*-Acyltransferase, *PLC* Phospholipase C

Lee et al. 2016; Smiarowska et al. 2022), whereas lymphocytes, monocytes, macrophages, mast cells, and natural killer cells carry CB2 receptor in immune system (Chakravarti et al. 2014; Compagnucci et al. 2013; Lu and Mackie 2021; Martinez-Pena et al. 2021). Our group previously revealed that CB1 and 2 receptors are present in bone marrow mononuclear cells and hematopoietic stem cells (Kose et al. 2018).

CB ligands interact with $G\alpha_{i/o}$ coupled receptors (Nogueras-Ortiz and Yudowski 2016) that in turn inhibit adenylyl cyclase enzyme, decrease cyclic adenosine monophosphate (cAMP) production, and activate the downstream mitogen-activating protein kinase (MAPK)/ phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway, respectively (Abrams and Guzman 2015; Turgeman and Bar-Sela 2019). Those signaling cascades are directly related with cell proliferation, migration, and death balance (Egmond et al. 2021; Howlett 2005). CB1/2 receptor and MAGL gene deletions have been reported to cause a deceleration in the progression of various cancer types, and an increase in their expression might also trigger carcinogenesis (Hinz and Ramer 2019; Moreno et al. 2019) and other pathological conditions such as traumatic brain injury, stroke or drug addiction (D.-j. Chen et al. 2017; Gallego-Landin et al. 2021). CB2 receptor level increases in various neurological diseases such as Alzheimer's disease (Aso and Ferrer 2016), depression (Onaivi et al. 2008), and Parkinson's disease (Concannon et al. 2016) when compared to CB1.

2.3 Synthetic Cannabinoids

Synthetic cannabinoids are manufactured as functional analogues of phytocannabinoids and endocannabinoids not only binding to CB1 or CB2 receptors (Egmond et al. 2021; Lim et al. 2021; Mangal et al. 2021; Smiarowska et al. 2022) but also interacting with intracellular survival or apoptotic molecules (Pyszniak et al. 2016). Synthetic cannabinoids are also known as bioactive compounds when compared to natural cannabinoids (Mangal et al. 2021; Morales and Reggio 2019). Synthetic cannabinoid agonists cannot cross the blood–brain barrier despite Δ^9-THC (Smiarowska et al. 2022). Non-specific CB1/2 agonists such as WIN55–212-2, HU-210, CP55–940, JWH-018 or KM-233; CB1 agonists like arachidonoylcyclopropylamide (ACPA), arachidonyl-2′chloroethylamide (ACEA) and methanandamide; CB1 antagonists such as SR141716 (also known as Rimonabant), and CB2 agonists such as CB65, JWH-133, and JWH-015 (K. A. Johnson and Lovinger 2016; Khan et al. 2016; Ladin et al. 2016; Pyszniak et al. 2016; Sledzinski et al. 2021; Velasco et al. 2016) have been developed to stimulate CB1/2 receptors pharmacologically. Synthetic cannabinoids have been substantially researched in preclinical studies for their antitumor properties involving suppression of proliferation, angiogenesis, invasion, migration and metastasis and stimulation of autophagy and apoptosis, through binding CB1 or CB2 receptors with a higher affinity (Pyszniak et al. 2016; Sledzinski et al. 2021; Velasco et al. 2016).

2.4 Cannabinoids in Healthy Vs Cancer Cell Behavior

Both endogenous and exogenous cannabinoids have crucial biological roles in many physiological and pathological processes (Shah et al. 2021). Cannabinoid agonists provide intracellular Ca^{2+} release for vascular (Howlett and Abood 2017), gastric (Mahavadi et al. 2014), and myometrial (Brighton et al. 2009) smooth muscle contraction via $G_{i/o}$-dependent PI3K, Src kinase, and extracellular signal-regulated kinase 1/2 (ERK1/ 2) activation under the regulation of CB1/2 receptors. Those agonists also regulate the reorganization of actin cytoskeleton through focal adhesion kinase (FAK) phosphorylation and Ras-Raf-MEK-ERK1/2 cascade (Dalton et al. 2013). There have been studies revealing their involvement in learning and memory (Smiarowska et al. 2022), circadian rhythm (Vaseghi et al. 2021), regulation of food intake (Silvestri and Di Marzo 2013; Silvestri et al. 2011), and homeostasis (Klumpers and Thacker

2018) and on-going large-scale studies including the use of cannabinoids for their antinociceptive (Good et al. 2019; Häuser et al. 2018; Lichtman et al. 2018), anti-inflammatory (Turcotte et al. 2015), neuroprotective (Minerbi et al. 2019), immunomodulatory (Das et al. 2019), and antiepileptic (Das et al. 2019; Moreno et al. 2019) properties.

The ECS ligands, AEA and 2-AG, or their metabolites may reach detectable picomolar plasma levels providing an equilibrium between the tissues and the circulation (Röhrig et al. 2019). However, they are known to be unstable in circulating system as being catalyzed by ECS enzymes in plasma under physiological conditions (Lanz et al. 2018). Our group also previously showed the presence and concentration of AEA and 2-AG metabolites in healthy rat plasma samples (Ozdurak et al. 2010). On the contrary, altered ECS components including enzymes and receptors are positively correlated with tumorigenesis (Daris et al. 2019; Drozd et al. 2022; Laezza et al. 2020; Pagano et al. 2021). Elevated CB1/2 receptor levels were demonstrated in breast (Caffarel et al. 2010; Pérez-Gómez et al. 2015), endometrial (Thangesweran Ayakannu et al. 2015; Guida et al. 2010), ovarian (Messalli et al. 2014), prostate (Chung et al. 2009; Cipriano et al. 2013; Singh et al. 2020) and non–small cell lung (NSCLC) cancers (Boyacıoğlu et al. 2021; Preet et al. 2011; Xu et al. 2019), melanoma (Carpi et al. 2017; Zhao et al. 2012), and hepatocellular carcinoma (Mukhopadhyay et al. 2015). Reduced protein expressions of NAPE-PLD, FAAH, and/or MAGL (Ramer et al. 2021) were positively correlated with AEA or 2-AG synthesis in colorectal (Chen et al. 2015; Sun et al. 2013), endometrial (Ayakannu et al. 2019), hepatocellular (Zhu et al. 2016) carcinoma, and glioma (Wu et al. 2012). The effect of cannabinoids on cell proliferation, migration, invasion, angiogenesis, autophagy, and death is schematized in Fig. 2 and will be discussed in detail below.

2.4.1 Cannabinoids in Cell Proliferation

Cannabinoids reduce proliferation of various cancer cells through cannabinoid or non-cannabinoid receptor mechanisms. Δ^9-THC exerts anti-proliferative effect (Fowler 2015) on A549, H460, H1792, and SW-1573 NSCLC (Baram et al. 2019; Milian et al. 2020; Preet et al. 2008; Sarafian et al. 2008), LNCaP, 22RV1, DU-145, and PC-3 prostate cancer (De Petrocellis et al. 2013), Panc1, Capan2, BxPc3, and MiaPaCa2 pancreatic cancer (Carracedo et al. 2006), HeLa cervical cancer (Ramer and Hinz 2008), U266 and RPMI multiple myeloma (Nabissi et al. 2016), MDA-MB231 breast cancer (Hirao-Suzuki et al. 2019), HL60 acute myeloid leukemia (Katherine A. Scott et al. 2017), T98G, U87MG, and GL261 glioma (López-Valero et al. 2018; Scott et al. 2014), D283, D425, and PER547 medulloblastoma (Andradas et al. 2021), IC-1425EPN and DKFZ-EP1NS ependymoma (Andradas et al. 2021), and SF126, U251, and U87 glioblastoma (Marcu et al. 2010; Torres et al. 2011) cell lines through ERK1/2 activation, PI3K/Akt inhibition and Raf-1 translocation. Non-psychoactive natural CBD inhibits the proliferation of A549, H460, and primary NSCLC (Ramer et al. 2013), SKOV-3 ovarian cancer (Fraguas-Sánchez et al. 2020), MDA-MB231 breast cancer (McAllister et al. 2007; Nallathambi et al. 2018), U878MG, U373MG, SF126, U251, and U87 glioblastoma (Marcu et al. 2010; Singer et al. 2015; Torres et al. 2011), T acute lymphoblastic leukemia and Jurkat (Kalenderoglou et al. 2017), SUM159 triple negative breast cancer (Mohamad Elbaz et al. 2015), SK-N-SH neuroblastoma (Fisher et al. 2016), LNCaP and DU-145 prostate cancer (De Petrocellis et al. 2013), D283, D425, and PER547 medulloblastoma (Andradas et al. 2021), IC-1425EPN and DKFZ-EP1NS ependymoma (Andradas et al. 2021), and CaCo-2 and HCT116 colon adenocarcinoma (Aviello et al. 2012) cells by elevating p53, EGFR, ERK1/2, Akt, and C/EBP homologous protein (CHOP) and/or inhibiting transient receptor potential cation channel subfamily M (melastatin) member 8 (TRPM8). 2-AG and methanandamide reduce viability of PC-3 and primary prostate cancer cells by activating caspase-3 and ERK1/2 levels and by reducing Bcl-2 and Akt levels (Orellana-Serradell et al. 2015). CB1 inverse agonist Rimonabant (SR141716) inhibits proliferation of HCT116 and SW48 colon cancer cells by inducing

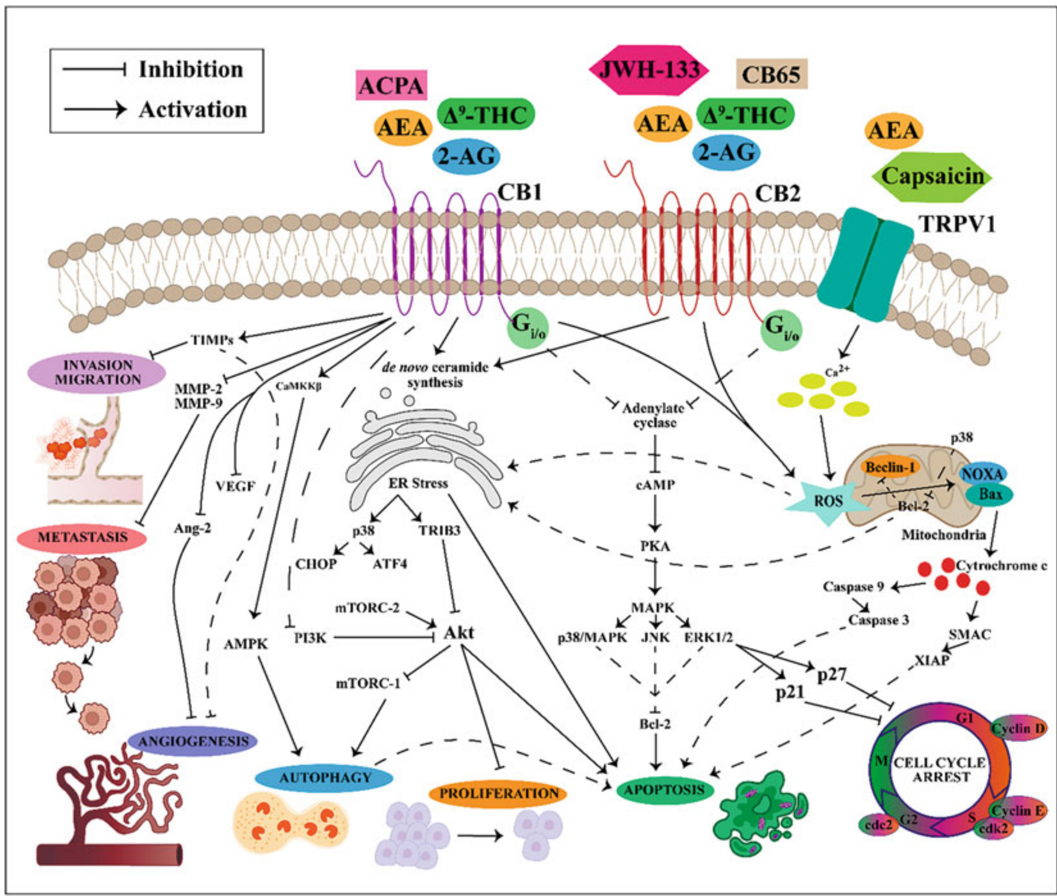

Fig. 2 An overview of downstream signaling pathways in a cancer cell by various exogenous and endogenous cannabinoids via CB1, CB2, and TRPV1 receptors. Activation of those cannabinoid and non-cannabinoid receptors stimulates de novo ceramide synthesis which induces endoplasmic reticulum (ER) stress, p8, TRIB3, CHOP, and ATF-4. Activation of TRIB3 and mTORC-2 and inhibition of p-PI3K lead to prevention of Akt phosphorylation and, therefore, cell proliferation. Inhibited p-Akt also decreases mTORC-1 level and induces autophagy in cancer cell. Cannabinoids induce ERK1/2 which triggers p27 and p21 leading to cyclin D and E, cdc2, and cdk2 reduction and cell cycle arrest. Release of Ca^{2+} stimulates ROS production, activates ER stress, induces NOXA and Bax and mitochondrial cytochrome c release, which activates caspase 9 and 3 leading to apoptosis. Stimulated CB1/2 receptors inhibit invasion and migration by enhancing TIMPs, metastasis by reducing MMP2 and 9, and angiogenesis by inhibiting VEGF and Ang-2. *Δ⁹-THC* Delta-9-tetrahydracannabinol, *2-AG* 2-Arachidonoylglycerol, *ACPA* Arachidonoyl cyclopropilamide, *AEA* Anandamide, *Akt* Protein kinase B, *Ang-2* Angiotensin II, *ATF-4* Activating transcription factor-4, *Bax* Bcl-2-associated X protein, *Bcl-2* B-cell lymphoma 2, *CaMKKβ* Calcium ions/calmodulin-stimulated protein kinase kinase β, *cAMP* Cyclic adenosine monophosphate, *CB1* Cannabinoid receptor 1, *CB2* Cannabinoid receptor 2, *CHOP* C/EBP homologous protein, *ERK1/2* Extracellular signal-regulated kinase 1/2, *JNK* c-Jun N-terminal kinase, *MAPK* Mitogen-activated protein kinase, *MMP-2/9* Matrix metalloproteinase 2/9, *mTORC-1/2* Mammalian target of rapamycin C-1/2, *NOXA* Phorbol-12-myristate-13-acetate-induced protein 1, *p21* Cyclin-dependent kinase inhibitor 1, *p27* Cyclin-dependent kinase inhibitor 1B, *PI3K* Phosphoinositide 3-kinase, *PKA* Protein kinase A, *ROS* Reactive oxygen species, *SMAC* Second mitochondria-derived activator of caspase, *TIMP* Tissue inhibitor of metalloproteinase, *TRIB3* Tribbles pseudokinase 3, *TRPV1* Transient receptor potential cation channel subfamily V member 1, *VEGF* Vascular endothelial growth factor, *XIAP* X-linked inhibitor of apoptosis

cytochrome C release and TRAILR-1, −2, and −3 expressions and downregulating Bcl-2 and XIAP (Proto et al. 2017). Rimonabant also shows Wnt/β-catenin-mediated anti-proliferative effect on primary colon cancer stem cells in vitro (Fiore et al. 2018). Non-selective pan CB agonist WIN55,212–2 or JWH-133 has anti-proliferative effect on T98G, LN18, LN229, U251MG, and U87MG glioma cell lines by inducing intrinsic apoptotic pathway and DNA fragmentation (Ellert-Miklaszewska et al. 2021), LNCaP and PC-3 prostate cancer cells by downregulating PI3K/Akt/mTOR cascade (Morell et al. 2016), A549, SW-1573, A459, CALU1, H460 and H1299 NSCLC cells through PI3K/Akt and JNK pathways (Boyacıoğlu et al. 2021; Preet et al. 2011; Ravi et al. 2014; Vidinsky et al. 2012), and 786-O, SMKTR2, SMKT-R3, Caki-2, RCC-6, 769-P, Caki-1, and ACHN human renal carcinoma lines by stimulating cell cycle arrest at G0/G1 phase (Khan et al. 2018). WIN55,212–2 inhibits BEL7402 hepatocellular carcinoma cell line by inducing p27, downregulating cyclin D1 and, therefore, promoting cell cycle arrest at G0/G1 phase and reducing ERK1/2 protein expression (D. Xu et al. 2015). WIN55,212–2 diminishes the viability of A549 NSCLC cells by increasing DNA fragments in nucleus (Müller et al. 2017). Cannflavin A, a compound of *Cannabis sativa*, reduces the proliferation of T24 and TCCSUP bladder transitional cell carcinoma lines (Andrea M. Tomko et al. 2022). Our group also demonstrated that AEA and 2-AG decrease HEp-2 human laryngeal squamous cancer cell proliferation in vitro (Önay et al. 2022).

2.4.2 Cannabinoids in Cell Migration, Invasion, and Angiogenesis

Tumor growth and expansion are highly dependent on neovascularization, cancer cell migration, and metastasis (Laezza et al. 2020; Wang and Multhoff 2021). Anti-angiogenic, anti-invasive, and anti-metastatic activities of cannabinoids have been extensively tested to block the induction and expansion of tumor growth (Pagano et al. 2021; Ramer et al. 2021; Vecera et al. 2020). Those effects have been associated with various metalloproteinases, inhibitors, and adhesive molecules (Braile et al. 2021; Sledzinski et al. 2018; Wang and Multhoff 2021). JWH-133 prevents angiogenesis and migration of human umbilical vein endothelial cells (HUVECs) by activating tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) besides inducing DNA fragmentation in A549, H460, and/or H358 NSCLC cells (Ramer et al. 2014; Vidinsky et al. 2012). CBD inhibits angiogenesis of HUVECs through vascular endothelial growth factor 1 or 2 (VEGF1/2), angiopoietin-2, urokinase-type plasminogen activator (uPA), and matrix metalloproteinase 2 or − 9 (MMP-2/9) blockage (Solinas et al. 2012). Selective CB1 agonist ACEA and JWH-133 inhibit invasion of U138 glioma cells (Tim Hohmann et al. 2017). Coincubation of selective CB1 receptor antagonist AM281 with ACEA significantly diminishes the invasion of LN229 glioblastoma cell line in vitro (T. Hohmann et al. 2019). CBD inhibits A549, H358, and/or H460 NSCLC cell invasion by decreasing plasminogen activator inhibitor-1 (PAI-1) expression (Ramer et al. 2010b) or by upregulating TIMP-1 (Ramer et al. 2012; Ramer et al. 2010a) or intercellular adhesion molecule-1 (ICAM-1) (Haustein et al. 2014; Ramer et al. 2012) levels. CBD also prevents epithelial growth factor (EGF)-induced migratory ability of 4 T1.2 and SUM159 triple-negative breast cancer by inhibiting MMP-2 and -9 expressions in addition to phosphorylated Akt (p-Akt) and ERK (Elbaz et al. 2015) or Ishikawa, PCEM004a and PCEM004b endometrial cancer lines (Marinelli et al. 2020) in vitro. Anti-invasive and anti-migratory effects of AEA have been revealed in U251 glioma cells in vitro (Ma et al. 2016). FAAH inhibitors arachidonoyl serotonin (AA-5HT) and URB597 diminish A549 cell metastasis and invasion via upregulation of TIMP-1 (Winkler et al. 2016). CB2 receptor agonist JWH-015 reduces migratory and invasive properties of M2-polarized macrophages when co-cultured with A549 NSCLC cells through

inhibition of FAK, vascular cell adhesion molecule 1 (VCAM1) and MMP-2 expressions (Ravi et al. 2016). Anti-metastatic property of CBD, Δ^9-THC, SR141716A, and/or SR144528 (CB2 receptor antagonist) has been established in MDA-MB231 breast cancer cells via Id1 downregulation (McAllister et al. 2011; Murase et al. 2014) and p-ERK and p38/MAPK upregulation (McAllister et al. 2011). WIN-55, 212–2 inhibits migration and metastasis of SGC7901 and AGS gastric cancer cell lines via COX-2, vimentin, and p-Akt downregulation and E-cadherin upregulation (Xian et al. 2016). Δ^9-THC decreases motility of HEC-1B and AN3 CA endometrial cancer cells by inhibiting MMP-9 expression (Zhang et al. 2018) and U266 and RPMI multiple myeloma cells by reducing CXCR4 and CD147 (Nabissi et al. 2016).

2.4.3 Cannabinoids in Cell Autophagy and Death

Cannabinoids activate autophagy and apoptosis through CB1/2 or other non-cannabinoid receptors. Δ^9-THC, WIN-55,212–2, and/or JWH-015 stimulate A549 and SW-1573 NSCLC cell apoptosis by inhibiting EGF-induced p-ERK, p-JNK, and p-Akt (Preet et al. 2008; Preet et al. 2011). Δ^9-THC, JWH-015, CBD, AEA, and/or Met-F-AEA (combined with URB597) induce apoptosis of U87MG, U118MG, and T98G glioblastoma (Ivanov et al. 2020; Ivanov et al. 2017), A549 NSCLC (Ramer et al. 2013; Ravi et al. 2014), Ishikawa, Hec50co, MFE-280, and/or HEC-1a endometrial cancer (Fonseca et al. 2018; Marinelli et al. 2020), and HepG2 and HuH-7 hepatocellular liver carcinoma (Vara et al. 2011) cell lines through JNK, p38-MAPK phosphorylation, p-Akt inhibition, NF-κB phospho-p65 reduction, caspase-3/−7 activation or COX-2, and PPAR-γ upregulation. Cannflavin A promoted apoptosis of T24 bladder transitional cell carcinoma lines through caspase-3 cleavage in vitro (Tomko et al. 2022). We also previously demonstrated that specific CB1 receptor agonist ACPA induces A549, H1299, H358, and H838 NSCLC cell line apoptosis by inhibiting Akt/PI3K pathway, glycolysis, TCA cycle, amino acid synthesis, and urea cycle and by activating JNK cascade (Boyacıoğlu et al. 2021). LV50, a compound having high affinity to CB2 receptor, promotes apoptosis of Jurkat leukemia cells by inducing cleavage of caspase-3/−8 and PARP (Capozzi et al. 2018). CBD induces apoptosis of HCT116 and DLD-1 colorectal cancer cell lines through ROS-dependent Noxa activation (Jeong et al. 2019).

Autophagy is a self-degradative process involving packaging of cytoplasmic organelles called autophagosome (Chang 2020; Pagano et al. 2021). Cannabinoids are known to stimulate autophagy through various cellular mechanisms including ceramide accumulation by hydrolysis of sphingomyelin or de novo ceramide synthesis (Gómez del Pulgar et al. 2002; Lee et al. 2021; Pagano et al. 2021). Newly synthesized ceramide induces expressions of p38, CHOP, ATF-4, and TRIB3 (see Fig. 2), thus inhibiting PI3K/Akt cascade or activating Ca^{2+}/calmodulin-dependent kinase kinase (CaCMKK) through ER stress (Das et al. 2019; Kabir et al. 2019; Ramer et al. 2021). Δ^9-THC stimulates sphingolipid synthesis, dihydroceramide accumulation, and autophagosome and autolysosome production in U87MG glioma cell line (Hernández-Tiedra et al. 2016). CBD activates autophagy in Jurkat, MOLT-3, CCFR-CEM, K562, Reh, and RS4;11 leukemia cell lines via increasing LC3-II expression, damaging permeability of mitochondria and releasing cytochrome c (Olivas-Aguirre et al. 2019). Combined Δ^9-THC, CBD, CBG, and CBN treatment induces autophagy of MCF-10A non-cancerous breast cell line by activating lipid synthesis, lysosomal vacuoles, and ER-stress-related chaperone protein glucose-regulated protein 78 (GRP78) expression (Schoeman et al. 2020). ACPA and CB2 receptor agonist GW405833 stimulates autophagy of Panc1 pancreatic cancer cells through AMPK activation and Akt/c-Myc inhibition (Dando et al. 2013). Δ^9-THC treatment activates autophagy of CHL-1, A375, and SK-MEL-28 melanoma cells by elevating LC3-positive autophagosome and cytochrome-c levels (Armstrong et al. 2015).

2.5 Preclinical In Vivo Studies and Clinical Status of Cannabinoids

Preclinical in vivo studies show that cannabinoid administration leads to decrease in proliferation, migration, invasion, angiogenesis, autophagy, and death in pancreatic (Aizikovich 2020; Carracedo et al. 2006; Donadelli et al. 2011; Sharafi et al. 2019; Yang et al. 2020), lung (Ramer et al. 2012; Ramer et al. 2013; Ravi et al. 2016; Ravi et al. 2014; Winkler et al. 2016; Yasmin-Karim et al. 2018), breast (Elbaz et al. 2017; McAllister et al. 2011; Murase et al. 2014; Nasser et al. 2011), prostate (De Petrocellis et al. 2013; Morales et al. 2013; Morell et al. 2016; Qiu et al. 2019; Roberto et al. 2019), colorectal (Aviello et al. 2012; Borrelli et al. 2014; Deng et al. 2022; Kargl et al. 2013; Martínez-Martínez et al. 2016; Proto et al. 2017; Romano et al. 2014), brain (Gurley et al. 2012; López-Valero et al. 2018; Scott et al. 2014; Singer et al. 2015) and liver (Vara et al. 2013; Vara et al. 2011) cancers, melanoma (Armstrong et al. 2015; Glodde et al. 2015; Kenessey et al. 2012; Simmerman et al. 2019), multiple myeloma (Barbado et al. 2017), and neuroblastoma (Fisher et al. 2016) through PI3K/AKT/mTOR, ERK/MAPK and/or PAI-1 signaling pathways. Recent reports relating to the cannabinoids in various in vivo cancer models are presented in detail (Table 1).

2-AG: 2-Arachidonoyl glycerol; AA-5HT: Arachidonoyl serotonin; ACEA: Arachidonyl-2′ chloroethylamide; ACF: Aberrant crypt foci; Akt: Protein kinase B; AM281: CB1-specific antagonist; AMPK: 5’ AMP-activated protein kinase; Bax: Bcl-2-associated X protein; BDS: Botanical cannabinoid extraction; CBD: Cannabidiol; CBG: Cannabigerol; Cyc D1: Cyclin D1; COX-2: Cyclooxygenase-2; CXCR4: C-X-C chemokine receptor type 4; DC: Dendritic cell; EGFR: Epidermal growth factor receptor; ERK: Extracellular signal-regulated kinase; GEM: Gemcitabin; GW9662: PPARγ antagonist; IGF-IR: Type 1 insulin-like growth factor receptor; IL6/10: Interleukin 6/10; iNOS: Inducible nitric oxide synthase; i.p.: intraperitoneal; i.t.: intratumoral; JWH-015: CB2-specific agonist; JWH-133: CB2-specific agonist; KM-233: Synthetic analogue of THC; MDSC: myeloid-derived suppressor cell; Met-F-AEA: Stable analogue of anandamide; MMP2/9: Matrix metalloproteinase 2/9; NFκB: Nuclear factor kappa B; O-1602: Cannabidiol analogue; O-1663: Resorcinol derivative; PAI-1: Plasminogen activator inhibitor-1; PAK1: P21-activated kinase-1; PM49: Synthetic cannabinoid quinone; p.o.: per oral; PPARγ: Peroxisome proliferator-activated receptor γ; p.t.: peritumoral; s.c.: subcutaneous; SR1 (or SR141716): CB1-specific antagonist (Rimonabant), SR144528: CB2-specific antagonist; STAT3: Signal transducer and activator of transcription 3; TIMP-1: Tissue inhibitor of matrix metalloproteinases-1; TNF-α: Tumor necrosis factor-alpha; URB597: FAAH inhibitor; βIII Tub: βIII Tubulin; Δ^9-THC: Delta-9-tetrahydracannabinol.

Cannabinoid agonists are currently used in the treatment of obesity (Bi et al. 2020; McClements 2020) and as neuroprotective agents for various diseases (Gado et al. 2019) involving Parkinson’s (Celorrio et al. 2016; Cristino et al. 2020) and Alzheimer’s diseases and multiple sclerosis (Black et al. 2019; Novotna et al. 2011) in the clinic. An attention to the use of cannabinoids for medical applications has grown due to their antinociceptive (Brunetti et al. 2020; Bruni et al. 2018; Good et al. 2019; VanDolah et al. 2019) and antiepileptic (Billakota et al. 2019; Brunetti et al. 2020; VanDolah et al. 2019) effects and the modulatory roles in appetite, nausea, and vomiting (Strouse 2016; VanDolah et al. 2019; White 2019). Dronabinol (Abrams and Guzman 2015; Shah et al. 2020) and nabilone oral capsules (Abuhasira et al. 2018; Shah et al. 2020) have equal potency to cure chemotherapy-related nausea and vomiting when compared to the US Food and Drug Administration (FDA)-approved other antiemetic drugs. Clinical studies reveal the relieving effect of nabiximols, oromucosal spray with THC and CBD as active ingredients, on spasms and neuropathic pain in multiple sclerosis (Abuhasira et al. 2018; Lowe et al. 2021)

Table 1 Review of in vivo findings regarding the effects of cannabinoids on different cancer models

Disease model	Cell line	Cannabinoid	Route	Dose	Effect	References
Xenograft pancreas cancer	PaCa44	SR1	i.p.	0.28 mg/kg (with GEM) (2 days/week)	Tumor growth↓	Donadelli et al. (2011)
	Panc-1	ALAM023, ALAM108	p.o.	120.0, 40.0 mg/kg/day	Tumor growth↓	Aizikovich (2020)
	TB33117	CBD:THC (1:1)	p.o.	250 μl/day	Tumor growth↓, PAK1-dependent anti-tumor activity	Yang et al. (2020)
Xenograft lung cancer	A549	CBD	i.p.	5.0 mg/kg (3 days/week)	Tumor growth↓, PAI-1↓	Ramer et al. (2010b)
	A549	JWH-133 and WIN55,212–2 or + SR144528 or AM281	i.p. or p.t.	1.0 (JWH-133), 0.1 (WIN55,212–2), 1.0 (SR144528), 0.1 (AM281) mg/kg/day	Tumor growth↓, Ki-67↓, angiogenesis↓, CD31↓, metastasis↓	Preet et al. (2011)
	A549	CBD	i.p.	5.0 mg/kg (3 days/week)	Tumor growth↓, ICAM-1↑, TIMP-1↑, metastasis↓	Ramer et al. (2012)
	A549	CBD, GW9662	i.p.	5.0, 1.0 mg/kg (3 days/week)	Tumor growth↓, COX-2↑, PPAR-γ↑, angiogenesis↓, CD31↓	Ramer et al. (2013)
	H460	Met-F-AEA, URB597 or met-F-AEA + URB597	–	5.0, 1.0 mg/kg (3 days/week)	Tumor growth↓, Ki-67↓, p-EGFR↓, p-ERK↓, p-Akt↓, MMP-2↓, MMP-9↓	Ravi et al. (2014)
	A549	AA-5HT, URB597	i.p.	5.0, 1.0 mg/kg (3 days/week)	Metastasis↓, TIMP-1↑	Winkler et al. (2016)
	ED1	JWH-015	i.p.	7.5 mg/kg (3 days/week)	Tumor growth↓, Ki-67↓, CD31↓, lung colonization↓	Ravi et al. (2016)
	LLC-1	CBD	i.t.	0.1, 5.0 mg/kg	Slight increase in survival, tumor growth↓	Yasmin-Karim et al. (2018)
Xenograft melanoma	HT168-M1	AEA, ACEA	i.p.	0.24 and 1.2 mg/kg/day	Metastasis↓, liver colonization↓	Kenessey et al. (2012)
	CHL-1	Δ^9-THC-BDS and CBD-BDS	p.o.	7.5 mg/kg THC-BDS + 7.5 mg/kg CBD-BDS	Tumor growth↓, LC3↑	Armstrong et al. (2015)
	B16 or HCmel1	Δ^9-THC	s.c.	5.0 mg/kg/day	No effect on the development of skin tumors	Glodde et al. (2015)
	B16F10	CBD	i.p.	5.0 mg/kg (2 days/week)	Tumor growth↓	Simmerman et al. (2019)
Xenograft breast cancer	4 T1	CBD	i.p.	1.0 mg/kg/day (1.5 μM)	Cell proliferation↓, invasion↓, Id1↓, cells in G0/G1 phase↑, cells in S phase↓	McAllister et al. (2011)
	NT 2.5	JWH-015	p.t.	5 mg/kg/day	Tumor growth↓, cell proliferation↓, Ki-67↓, p-CXCR4↓, p-ERK↓	Nasser et al. (2011)

(continued)

Table 1 (continued)

Disease model	Cell line	Cannabinoid	Route	Dose	Effect	References
	MDA-MB231	CBD or O-1663	i.p.	0.3, 1.0 mg/kg/day	Survival↑, metastasis↓, invasion↓, Id1↓, Ki-67↓	Murase et al. (2014)
	SUM159 or MCF-7	JWH-015	p.t.	10.0 mg/kg	Tumor growth↓, EGFR↓, IGF-IR↓, STAT3↓, AKT↓, ERK↓	Elbaz et al. (2017)
Xenograft multiple myeloma	U266	WIN55,212–2	i.p.	5.0 mg/kg (every day/2 days-week)	Tumor growth↓	Barbado et al. (2017)
Xenograft prostate cancer	LNCaP or PC-3	PM49	i.p.	2.0 mg/kg/day	Tumor growth↓	Morales et al. (2013)
	LNCaP	CBD-BDS	i.p.	1.0 mg/kg/day	Tumor growth↓	De Petrocellis et al. (2013)
	PC-3	WIN55,212–2	s.c.	0.5 mg/kg/day	Tumor growth↓, βIII tub↓	Morell et al. (2016)
	LNCaP and DU-145	WIN55,212–2	i.p.	5.0 mg/kg (3 days/week)	Tumor growth↓, cell proliferation↓	Roberto et al. (2019)
	Panc02	2-AG	i.p.	20.0 mg/kg/day	MHC-class II↑, CD83↑, CD86↑, DC maturation, MDSC expansion	Qiu et al. (2019)
Xenograft colorectal cancer	–	CBD	i.p.	1.0, 5.0 mg/kg (3 days/week)	ACF↓, p-Akt↓, cleaved-caspase 3↑, iNOS↑, COX-2↑	Aviello et al. (2012)
	–	O-1602	i.p.	3 mg/kg (every 2 days)	Cell proliferation↓, BAX↑, p53↑, DNA fragmentation↑, NFκB↑, p65↑, STAT3↑, TNF-α↓	Kargl et al. (2013)
	HCT116	CBG	i.p.	1.0–10.0 mg/kg/day	Tumor growth↓, ACF↓	Borrelli et al. (2014)
	–	CBD-BDS	i.p.	5.0 mg/kg/day	Tumor growth↓, ACF↓	Romano et al. (2014)
	HT29	JWH-133	i.p.	1.0, 5.0 mg/kg/day	Tumor growth↓, p-Akt↑	Martínez-Martínez et al. (2016)
	HCT116	SR141716	p.t.	0.7 mg/kg (3 days/week)	Tumor growth↓, β-catenin in the cytoplasm↑, Cyc D1↓, c-Myc↓	Proto et al. (2017)
	SW480	ACEA	p.t.	1.5 mg/kg/day	Tumor growth↓, IL-6↑, TNF-α↑, IL-10↓, CCL22↓, Arg-1↓, and CD206↓, M2 macrophage differentiation↓	Deng et al. (2022)

(continued)

Table 1 (continued)

Disease model	Cell line	Cannabinoid	Route	Dose	Effect	References
Xenograft brain cancer	U87MG	KM-233	i.p.	2.0, 4.0, 8.0, 12.0 mg/kg (twice daily)	Tumor growth↓	Gurley et al. (2012)
	GL261	Δ^9-THC, CBD	i.p.	2.0 mg/kg (on days 9, 13, and 16)	Tumor growth↓, tumor sensitivity↑	K. A. Scott et al. (2014)
	3,832, 387	CBD	i.p.	15.0 mg/kg (5 days/week)	Tumor growth↓, p-Akt↓, Ki67↓, caspase-3↑	Singer et al. (2015)
	U87MG	Δ^9-THC, CBD (1:1 or 1:5)	p.o.	15.0 mg/kg/day	Tumor growth↓, survival↑	López-Valero et al. (2018)
Xenograft neuroblastoma	SK-N-SH	Δ^9-THC, CBD	i.p.	20.0 mg/kg/day	Tumor growth↓, cleaved-caspase 3↑	Fisher et al. (2016)
Xenograft liver cancer	HepG2 or HuH-7	Δ^9-THC, JWH-015	s.c.	15.0, 1.5 mg/kg/day	Tumor growth↓, p-AMPK↑, p-Akt↓, p-S6↓, LC3-II↑, pro-caspase 3↓	Vara et al. (2011)
	HepG2	Δ^9-THC, JWH-015 or + GW9662	p.t.	15.0, 1.5 mg/kg/day	Tumor growth↓, PPARγ↑	Vara et al. (2013)

and improvement in sleep disorders in patients with insomnia (Klumpers and Thacker 2018). FDA-approved Epidiolex, as CBD active ingredient, is currently used for seizures related to Lennox-Gastaut and Dravet syndromes (Abu-Sawwa and Stehling 2020; Levinsohn and Hill 2020; Steele et al. 2019). A phase I/II trial exploring the immune-modulatory and anti-inflammatory potency of CBD showed that it avoids graft versus host disease (GVHD) incidence when administered in addition to standard GVHD prophylaxis (Yeshurun et al. 2014). It is worth noting that SR141716 as an anorectic agent used for the obesity treatment was banned by the FDA due to its severe side effects (Khan et al. 2016; Shah et al. 2019). Studies consisting of the analgesic and anti-epileptic properties of cannabinoids on various diseases are shown in Table 2.

3 Future Perspectives for Cannabinoids as Prospective Agents for Cancer

Phytocannabinoids and endogenous and synthetic cannabinoids have been examined in preclinical research works and clinical trials to assess the therapeutic potential for various diseases including cancers. One of the key pitfalls occurs in the short half-lives and psychotropicity of cannabinoids. Therefore, it is crucial to use anti-cancer cannabinoids effective in triggering intrinsic apoptotic mechanisms at low doses without reaching central nervous system. Natural cannabis derivatives are clinically used for pain relief but the horizon should be expanded on their application as anti-tumor agents. Still, a major gap remains which needs to be filled by new research works to clarify the effects of cannabinoids on the tumor microenvironment. Moreover, outputs of in vitro molecular tests should be translated to in vivo models, since in vitro data does not precise the possible problems within the diseased animal as a whole. Preclinical randomized studies convey the therapeutic performance of cannabinoids on cellular mechanisms. Clinical trials including phase trials provide the assessment of personalized performance of different cannabinoid system agents before translation to clinic. A literature search of clinicaltrials.gov by September 2022 found 77 completed clinical studies about cannabis/cannabinoid use in mental disorders, psychotic disorders, pain, immune system diseases, gastrointestinal diseases, central nervous system

Table 2 Completed clinical cannabinoid trials with their effects on various diseases and related symptoms

Disease/ symptom	Compound and dose (per day)	Route	Formulation	Total exposure time	Effect	References
MS	Nabiximol (129 mg THC and 120 mg CBD)	Oromucosal	Spray	10 weeks	No significant effect	Kavia et al. (2010)
MS-related spasticity	Nabiximol (2.7: 2.5 mg THC:CBD) (12 doses/day)	Oromucosal	Spray	19 weeks	Spasm frequency↓	Novotna et al. (2011)
Cancer-associated pain	Nabiximol (2.7: 2.5 mg THC:CBD) vs 2.7 mg THC (10 doses/day)	Oromucosal	Spray	2–9 weeks	Analgesic effect in THC:CBD-applied group	Johnson et al. (2010, 2013) and Lichtman et al. (2018)
Neuropathic pain	12 doses of nabiximol or 2.7:2.5 mg THC: CBD (8–24 doses/day)	Oromucosal	Spray	15 weeks - 6 months	Pain↓ Sleep quality↑	Lynch et al. (2014) and Serpell et al. (2014)
Epilepsy	2–5 mg/kg CBD (25–50 mg/kg depending on the intolerance)	Oral	Liquid oil	12 weeks	Anticonvulsant effect Seizures↓	Devinsky et al. (2016)
Epilepsy	5–20 mg/kg CBD or 2 mg/kg TIL-TC150 (50:1 THC:CBD extract), 2–16 mg/kg CBD and 0.04–0.32 mg/kg THC	Oral	Liquid oil	11–20 weeks	Anticonvulsant effect Seizures↓	Devinsky et al. (2017, 2018) and McCoy et al. (2018)
Nausea/ vomiting due to chemotherapy	TN-TC11M (2.5 mg/ 2.5 mg THC:CBD, 1:1) (30 mg/30 mg/day depending on the intolerance)	Oral	Capsule	5 days	Vomiting and nausea↓	Mersiades et al. (2018)
Cancer-associated pain	50–600 mg/ml CBD	Oral	Liquid oil	4 weeks	Analgesic effect	Good et al. (2019)
Hepatic impairment	100 mg/ml CBD (Epidiolex) (single dose of 200 mg)	Oral	Liquid formulation	4 weeks	Effective in low-dose therapy in patients with hepatic impairment	Taylor et al. (2019)
Behavioral problems for ASD	1 mg/kg CBD:THC (20:1) (3 doses/day)	Oral	Liquid oil	3 months	Disruptive behaviors↓	Aran et al. (2021)
DS-associated seizures	100 mg/ml CBD (Epidiolex) (2.5–20 mg/kg/day)	Oral	Liquid formulation	2 weeks	Seizures↓ Adverse effects including diarrhea, pyrexia, somnolence, convulsion, nasopharyngitis, decreased appetite	Scheffer et al. (2021)

MS Multiple sclerosis, *THC* Tetrahydrocannabinol, *CBD* Cannabidiol, *ASD* Autism spectrum disorder, *DS* Dravet syndrome.

diseases and various syndromes including Dravet, Tourette, and Lennox-Gastaut syndromes. In 31 out of 77 (40.26%) studies, cannabinoids were tested for their pain-relieving capability; 24 out of 31 searches have been confirmed in phase II/III clinical trials. Epidiolex, CBD oral solution, was approved by FDA on June 25, 2018, to alleviate the seizures observed in Lennox-Gastaut and Dravet syndromes. On the other hand, depression and suicide in patients caused withdrawal of CB1 antagonist rimonabant from the market. No clinical trial or approval has been reported for cannabinoids as anti-cancer therapeutics. As to future prospects, cannabinoids might be evaluated as potential chemotherapeutic drugs or effective adjunctive therapeutics to be used with chemotherapeutics or other targeted agents. However, further investigations are necessary to clarify the safety and potency of cannabinoids.

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Direct Reprogramming of Somatic Cells into Induced β-Cells: An Overview

Gloria Narayan, Ronima K R, and Rajkumar P. Thummer

Abstract

The persistent shortage of insulin-producing islet mass or β-cells for transplantation in the ever-growing diabetic population worldwide is a matter of concern. To date, permanent cure to this medical complication is not available and soon after the establishment of lineage-specific reprogramming, direct β-cell reprogramming became a viable alternative for β-cell regeneration. Direct reprogramming is a straightforward and powerful technique that can provide an unlimited supply of cells by transdifferentiating terminally differentiated cells toward the desired cell type. This approach has been extensively used by multiple groups to reprogram non–βcells toward insulin-producing β-cells. The β-cell identity has been achieved by various studies via ectopic expression of one or more pancreatic-specific transcription factors in somatic cells, bypassing the pluripotent state. This work highlights the importance of the direct reprogramming approaches (both integrative and non-integrative) in generating autologous β-cells for various applications. An in-depth understanding of the strategies and cell sources could prove beneficial for the efficient generation of integration-free functional insulin-producing β-cells for diabetic patients lacking endogenous β-cells.

Keywords

Acinar cells · β-cells · Direct reprogramming · Ductal cells · Endocrine cells · Exocrine cells · Liver cells

Abbreviations

Ad	Adenovirus
ESCs	Embryonic stem cells
GLP1	Glucagon-like peptide 1
GLUT2	Glucose transporter 2
GTPase	Guanosine triphosphate binding protein
HNF-4α	Hepatocyte nuclear factor-4 α
IFN-γ	Interferon-γ
IL-1β	Interleukin-1β
MAPK	Mitogen-activated protein kinase
NOD-SCID	Nonobese diabetic-severe combined immunodeficiency
PI3K	Phosphoinositide 3-kinase/ phosphatidylinositol 3-kinase
PP	Pancreatic polypeptide
STAT	Signal transducer and activator of transcription

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TGFβ Transforming growth factor β
TNFα Tumor necrosis factor-α
VP16 Virus protein 16

1 Introduction

Diabetes mellitus is a chronic metabolic disorder characterized by high blood glucose levels that pose serious damage to the blood vessels, nerves, eyes, heart, and kidneys over time. It is also one of the leading causes of death globally, accounting for 536.6 million lives affected worldwide in 2021 (Sun et al. 2022). This number is projected to increase to 783.2 million in 2045 if no preventive measures are taken (Sun et al. 2022). Although multiple drugs are available for the effective management of diabetes mellitus, this only provides a temporary solution to the ever-growing global condition.

Various strategies have been developed to date or under investigation for the treatment of diabetes mellitus (Fig. 1). The general approach to replenish β-cells in a diabetic patient is either to transplant β-like cells derived via stem cell differentiation or healthy β-cells obtained from cadaveric/brain-dead donors (Agrawal et al. 2021; Shapiro et al. 2000). Another viable alternative was the development of islet transplantation; however, ethical concerns, porcine to human virus transmission (in the case of porcine islet transplantation), immune rejection, and shortage of cadaveric donors made it unsuccessful (Merani and Shapiro 2006; Pellegrini et al. 2016). To overcome the immune rejection during transplantation, immunosuppressive drugs were used; however, the occurrence of serious opportunistic infections acted as a roadblock to this approach (Harlan et al. 2009). Insulin therapy is the life-saving practical option for the treatment of type 1 diabetes mellitus (since the onset of the disease) and type 2 diabetes mellitus (later stage) to date. The high risk associated with insulin therapy is that it may precipitate acute comatose hypoglycemia if there is no proper control over its dosage and time of treatment. Another drawback of insulin is gastric acid lability; therefore, it has to be administered through a subcutaneous or intramuscular route (Crasto et al. 2016). Furthermore, insulin treatment necessitates multiple insulin shots (after every carbohydrate-rich diet) in a day, thus making it a cumbersome, painful, and impractical long-term solution. Moreover, weight gain upon long-term insulin therapy markedly increases the chances of cardiovascular risks (Cichosz et al. 2016). These above challenges

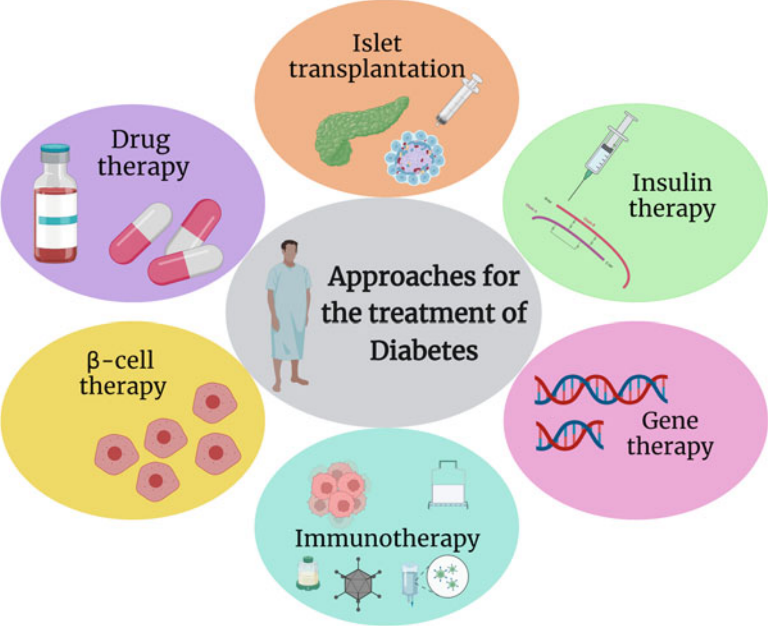

Fig. 1 A pictorial representation of various approaches for the treatment of diabetes mellitus. This includes islet transplantation, insulin therapy, drug therapy, β-cell therapy, gene therapy, and immunotherapy

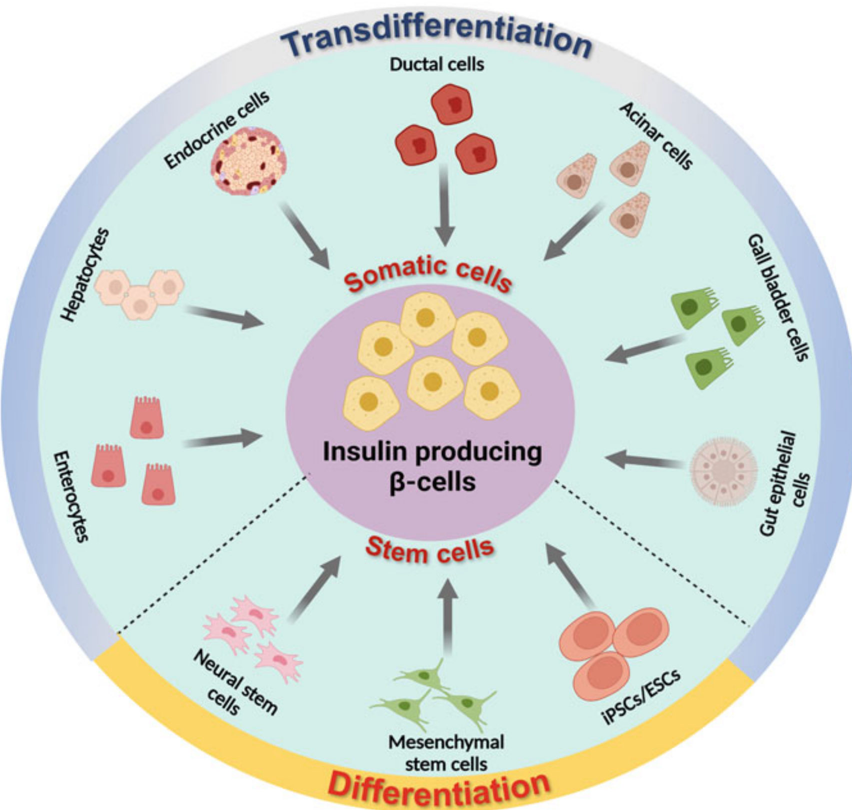

Fig. 2 Various adult cells that can be reprogrammed to insulin-producing β-cells. A representation of various terminally differentiated somatic cells that can be transdifferentiated to insulin-producing β-cells is depicted. This includes enterocytes, hepatocytes, endocrine cells, ductal cells, acinar cells, gall bladder cells, and gut epithelial cells. The stem cells that can be differentiated into insulin-producing β-cells such as neural stem cells, mesenchymal stem cells, and iPSCs/ESCs are also represented (iPSCs: induced pluripotent stem cells; ESCs: embryonic stem cells)

necessitate exploring novel strategies that can cure diabetes mellitus permanently.

Direct reprogramming of various terminally differentiated somatic cells to another cell type bypassing the stem cell state has proved to be a promising approach in the field of regenerative medicine (Ferber et al. 2000; Ieda et al. 2010). This approach saves us from the arduous and lengthy procedure taken in the case of stem cell-based therapy. Similarly, direct reprogramming has also been investigated to generate β-cells from a variety of different cell sources/types (Fig. 2) by various labs. Direct reprogramming involves ectopic expression of key transcription factors in terminally differentiated cells in the presence of essential growth factors and small molecules under defined culture conditions to generate the desired cell type. This review deals with the various adult cell types explored by different studies to generate functional β-cells for prospective biomedical applications (Fig. 2).

2 Liver Cells to β-Cells

In embryonic development, the liver and pancreas share a developmental history, and it has been proposed that these organs develop from the same precursor cells (Cerdá-Esteban et al. 2017; Sumazaki et al. 2004; Xiao et al. 2018). These

organs contain a variety of different cell types, which are of endodermal origin as they arise from adjacent regions of the anterior endoderm during embryogenesis (Cerdá-Esteban et al. 2017; Sumazaki et al. 2004; Xiao et al. 2018). Therefore, liver cells have been the first choice for direct reprogramming to give rise to insulin-producing β-cells (Fig. 3). During early 2000, various in vivo as well as in vitro studies demonstrated the potency of hepatocytes for their efficient conversion to pancreatic cells (Cavelti-Weder et al. 2015). Studies have also reported that the expression of a single pancreatic-specific gene was sufficient to bring about the direct conversion of hepatocytes to β-cells (Ferber et al. 2000; Horb et al. 2003; Jarikji et al. 2007; Sumazaki et al. 2004). Pdx1 is the most explored transcription factor in this aspect and has been used by various studies to convert hepatocytes to insulin-secreting β-like-cells (Ferber et al. 2000; Horb et al. 2003; Kojima et al. 2002; Sapir et al. 2005; Zalzman et al. 2003). Forced expression of this homeodomain transcription factor in mouse liver cells (Ferber et al. 2000), rat enterocytes (Kojima et al. 2002), human hepatocytes (Sapir et al. 2005; Zalzman et al. 2003), and *Xenopus laevis* (Horb et al. 2003; Jarikji et al. 2007) resulted in the upregulation of multiple pancreatic-specific genes like glucagon, pancreatic polypeptide, and elastase, including insulin (Zalzman et al. 2003). The reprogrammed cells stored and secreted insulin from defined granules in a glucose-regulated manner. When Pdx1 was retrovirally transduced in mouse liver cells, it activated insulin 1, insulin 2, and prohormone convertase 1/3 genes (Ferber et al. 2000). In addition, it ameliorated streptozotocin-induced diabetes; however, the effect of ectopic Pdx1 on insulin secretion was monitored for only 2 weeks (Ferber et al. 2000). Pdx1, when tagged to VP16 (a transcriptional activation domain from herpes simplex virus) in transgenic *Xenopus laevis* tadpoles as well as human and murine hepatocytes, converted a specific region of the liver to the endocrine pancreas and reversed streptozotocin-induced hyperglycemia with the substantial increase in blood insulin levels (Horb et al. 2003; Imai et al. 2005; Kaneto et al. 2005). The converted cells secreted either insulin or amylase, which is a key exocrine enzyme (Horb et al. 2003), indicating the formation of both pancreatic endocrine and exocrine cells. Notably, it has been observed that liver-specific gene expression is turned off from the regions converted to the pancreas (Horb et al. 2003). In contrast, Imai and group reported no expression of amylase in the converted

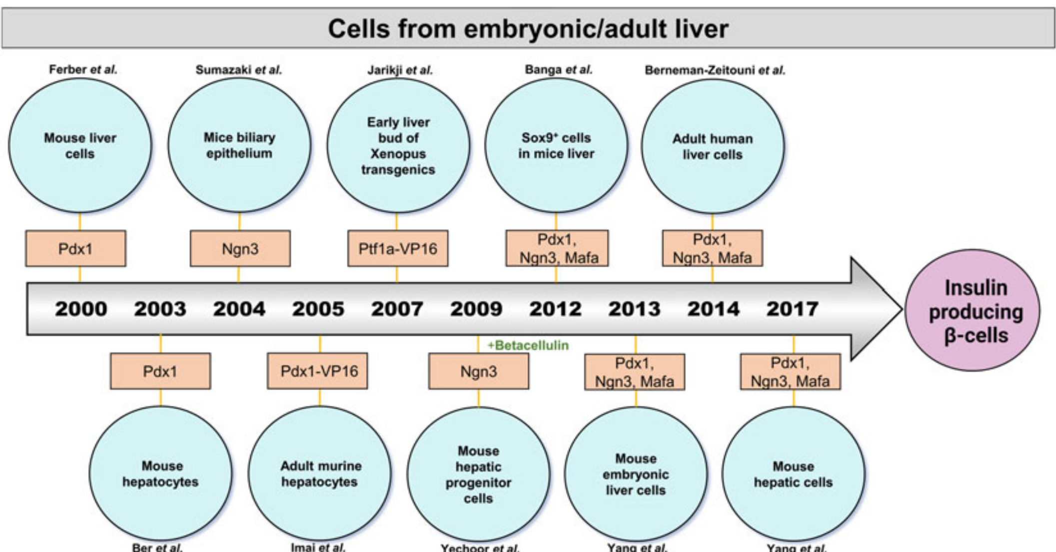

Fig. 3 A timeline depicting different cells from embryonic or adult liver that can be reprogrammed to insulin-producing β-cells using various reprogramming factors

cells; however, the cells continued to express albumin, transferrin, and hepatocyte markers in insulin-producing cells, suggesting incomplete transdifferentiation of adult murine liver cells (Imai et al. 2005). Moreover, when these groups compared the transdifferentiation capability of Pdx1-VP16 to its unmodified variant in *Xenopus leavis* tadpoles and mammalian cells, it failed to reprogram liver cells to β-cells (Horb et al. 2003; Imai et al. 2005). It was speculated that wild-type Pdx1 required specific coactivators like histone acetyltransferases (originally not expressed in the liver) for transdifferentiation; therefore, the fusion of VP16 to PDX1 helped to overcome the absence of these coactivators to enable transdifferentiation (Horb et al. 2003). Furthermore, cells treated with Pdx1 along with Activin A in serum-free conditions showed upregulation of β-cell markers like NeuroD and Nkx2.2 and downregulation of liver and other pancreatic-specific markers like pancreatic polypeptide and glucagon (Zalzman et al. 2005). Additionally, the inclusion of other pivotal β-cell transcription factors like NeuroD1 and Ngn3 along with Pdx1-VP16 has been shown to significantly improve insulin biosynthesis and ameliorate glucose tolerance (Kaneto et al. 2005). To further investigate, other groups also transdifferentiated liver cells to pancreatic endocrine and exocrine cells by ectopically overexpressing Pdx1 and observed the upregulation of endocrine-specific hormones like insulin, glucagon, somatostatin, and pancreatic polypeptide (Ber et al. 2003; Miyatsuka et al. 2003). In a study, Ber and colleagues suggested that Pdx1 induces its own expression and confers the "long-lasting" effect to the converted cells (Ber et al. 2003). However, a wide repertoire of pancreatic genes like glucagon, somatostatin, etc., are activated during the process and the mice transplanted with generated β-like cells suffered from hyperbilirubinemia (Ber et al. 2003; Miyatsuka et al. 2003). In 2004, Sumazaki and co-workers converted cells of the biliary system (epithelium) to pancreatic tissue in *Hes1*-deficient mice (Sumazaki et al. 2004). The reason to choose *Hes1*-deficient mice is because *Hes1* gene is known to encode for Hes1 protein, which negatively regulates endodermal endocrine differentiation by repressing Ngn3 during biliary organogenesis (Sasai et al. 1992). The group noticed that biliary epithelium in *Hes1*$^{-/-}$ mice expressed Ngn3 and the cells differentiated into endocrine and exocrine cells forming acini or islet-like structures (Sumazaki et al. 2004). Similar to Pdx1, Ptfa also has the capacity to convert early liver buds to pancreatic cells when tagged to VP16 in *Xenopus laevis* tadpoles (Jarikji et al. 2007). It was observed that wild-type Ptf1a protein failed to convert liver cells to pancreatic cells; however, it succeeded in converting stomach and duodenum cells to insulin-producing cells (Jarikji et al. 2007). It is noteworthy that Ptf1a-VP16-treated liver cells mimicked acinar cells with abundant expression of only amylase enzyme and no detection of insulin or somatostatin, suggesting its role in promoting acinar fate (Jarikji et al. 2007). On the other hand, ectopic overexpression of wild-type Ptf1a in stomach and duodenum cells promoted both exocrine as well as endocrine fate (Jarikji et al. 2007). Furthermore, three-dimensional culture conditions not only improved the viability of the reprogrammed cells but also promoted the expansion of the starting cell type (Nagaya et al. 2009). When intrahepatic biliary epithelial adult mouse cells were cultured in a two-dimensional culture condition, the cells died within 2 weeks, whereas cells cultured in a three-dimensional condition could sustain cell growth and promote expansion with improved cell viability (Nagaya et al. 2009). These cells were positive for mature β-cell markers like Ins1, Ins2, and prohormone convertase 1 and 2 (Nagaya et al. 2009). However, faint expression of amylase was also observed in the differentiated cells (Nagaya et al. 2009), indicating the presence of pancreatic exocrine cells in the transdifferentiated cells. It is also worth mentioning that only a few cells released insulin after 7 days of transduction of Ad-Pdx1-VP16 adenoviral vector and the inclusion of multiple growth factors and small molecules like nicotinamide, Activin A, betacellulin, hepatocyte growth factor, and exendin-4 could not improve the differentiation efficiency significantly (Nagaya et al. 2009). Furthermore, a different group claimed that overexpression of Ngn3 using a helper-dependent adenoviral vector along with betacellulin is sufficient to switch cellular fate from hepatic-progenitor cells to glucose-responsive insulin-producing cells

(Yechoor et al. 2009). However, these cells showed abnormal transcriptional cascade and eventually lost the capability of producing insulin due to failure in expressing other β-cell markers like Pax4, Nkx2.2, and Nkx6.1. A combination of Ngn3 along with Pdx1 and MafA secreted ten-fold higher insulin protein and prolonged the glucose responsiveness for up to 4 months in streptozotocin-treated NOD-SCID mice, proving the greater effectiveness of the combination compared to a single factor (Ackermann et al. 2016). However, the converted cells failed to express insulin exclusively and stained positive for other pancreatic markers like glucagon and somatostatin, proving mixed phenotypes. The cells also showed lower levels of *Nkx2.2* and *Nkx6.1* (Ackermann et al. 2016), which are crucial for β-cell development, proliferation, and maintenance (Aigha and Abdelalim 2020; Doyle and Sussel 2007). The same group also reprogrammed mouse embryonic liver cells, and their findings were in line with the previous results (Yang et al. 2013). However, the insulin content in these cells was as low as 3% of the mature β-cells and lacked glucose responsiveness showing the immature phenotype of the reprogrammed cells (Yang et al. 2013). In the following year, Berneman-Zeitouni and group shed light on the temporal and hierarchical control of the transcription factors imparting on the reprogramming process (Berneman-Zeitouni et al. 2014). The study explored the role of three pivotal transcription factors, PDX1, PAX4, and MAFA, which control three stages of pancreatic development in the human liver to pancreatic transdifferentiation (Berneman-Zeitouni et al. 2014). The combined effect of these factors substantially increased the number of insulin-positive cells. Moreover, the reprogrammed cells displayed mature β-cell characteristics like glucose-stimulated insulin secretion (Berneman-Zeitouni et al. 2014). The aforementioned studies used viral gene delivery methods, which can result in random viral integration causing insertional mutagenesis and tumorigenicity (Borgohain et al. 2019; Dey et al. 2021; Haridhasapavalan et al. 2019). Although these studies elucidated the transcriptional landscape in pancreatogenesis, the reprogrammed cells hugely failed for clinical applications and, thus, required a non-genetic approach. In 2017, Yang and group used a hydrodynamic-based gene delivery method to introduce Pdx1, Ngn3, and MafA into mouse hepatocytes and reprogrammed them into insulin-producing cells (Cavelti-Weder et al. 2015). Multiple hydrodynamic injections successfully reversed hyperglycemic conditions in diabetic mice along with the expression of multiple pancreatic-specific developmental genes like *Nkx6.1, NeuroD1, Pax4*, and *Isl1* (Cavelti-Weder et al. 2015). Thus, multiple studies have proved that liver cells or hepatocytes are a preferable option in direct reprogramming to β-cells. However, the safest method for transdifferentiation remains in question and requires further detailed investigation.

3 Endocrine Cells to β-Cells

Endocrine pancreatic cells are arranged in small cluster of cells and are called islets of Langerhans or simply islets. Each cluster/islet comprises majorly of β-cells (around 90%), which secretes insulin. It also contains α-cells, δ-cells, ε-cells, and PP-cells (around 10%) that secretes glucagon, somatostatin, ghrelin, and pancreatic polypeptide, respectively. Insulin secreted by β-cells helps to maintain normoglycemic conditions. A number of studies have used endocrine cells as the starting cell source (Fig. 4) (Thorel et al. 2010; Bramswig et al. 2013; Chera et al. 2014; Collombat et al. 2009; Sangan et al. 2015; Yang et al. 2011) largely because of the developmental similarities. Among the various endocrine cell types available, α-cells are the most widely used due to various reasons such as (i) their inherent plasticity, (ii) sharing developmental similarity with β-cells that enables reprogramming, (iii) α-cell hyperplasia observed in diabetic patients, which in turn, provides an abundant source for reprogramming, and (iv) easily accessible α-cell genome showed by assay for transposase-accessible chromatin with high-throughput sequencing, making these cells amenable for transdifferentiation (Ackermann et al. 2016; Chakravarthy et al. 2017; Xiao et al. 2018; Ye et al. 2015). The plasticity of α-cells was demonstrated by Thorel and co-workers through cell lineage-tracing

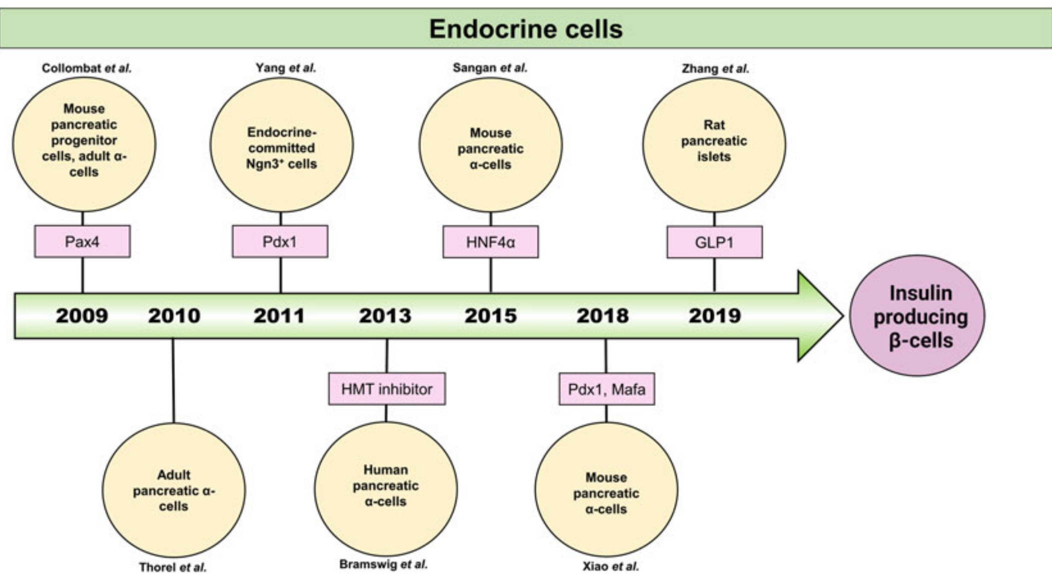

Fig. 4 A timeline depicting endocrine cells from different sources (both mouse and human) that can be reprogrammed to insulin-producing β-cells using various reprogramming factors. (HMT: histone methyltransferase; HNF4α: hepatocyte nuclear factor 4 alpha)

experiments in vivo (Thorel et al. 2010). This study showed that α-cells got converted to β-cells spontaneously upon near-total β-cell ablation and insulin treatment, demonstrating the occurrence of inter-endocrine cell plasticity (Thorel et al. 2010). This study speculated that a near-total ablation of β-cells released some form of signals, which resulted in the physiological need to replenish β-cells that allowed its regeneration from α-cells (Thorel et al. 2010). This study also observed that the regeneration efficiency hugely varied between 32% and 81%, even when the ablation degree was similar (Thorel et al. 2010). Later the same group reported that the conversion of α-cells to β-cells was age-independent (Chera et al. 2014). The study showed that senescence did not alter α-cell plasticity, and these cells continued to convert to β-cells from puberty to adulthood upon near-total β-cell loss. However, before the rise of puberty, no detectable conversion of α-cells to β-cells was observed, and it was in juveniles that δ-cells got converted to β-cells to replenish the lost cells, with a remarkable decrease in somatostatin levels post-conversion (Chera et al. 2014). This was clearly demonstrated by cell lineage tracing experiments, which revealed that regenerated insulin$^+$ cells were from dedifferentiated δ-cells (Chera et al. 2014). However, deeper insights into the transcriptional network and epigenetic regulation are required to understand the underlying mechanisms involved in converting multiple endocrine cell lineages, such as α-cells and δ-cells to β-cells.

Various studies have primarily focused on the forced expression of a particular key β-cell transcription factor and elucidated how each of these factors bestows β-cell phenotype to non-β-cells. One of the key transcription factors, Pax4, is known to favor β-cell fate and identity during endocrine morphogenesis (Mellado-Gil et al. 2016). Thus, Collambat and co-workers explored the potentiality of Pax4 in the conversion of mouse pancreatic progenitor cells and adult α-cells to insulin-producing β-cells (Collombat et al. 2009). From this study, it was worth noticing that post-partum, an increase in the islet size was observed upon ectopic Pax4 overexpression, and this increase was primarily due to insulin$^+$/Pax4$^+$ cells (Collombat et al. 2009). Markedly, the number of glucagon$^+$ cells was reduced by 77%, while the population of other islet-specific

cell types was unaltered (Collombat et al. 2009). In addition, there was an increase in islet size and the number of insulin-producing cells, suggesting the role of Pax4 in continuously converting glucagon+ cells to β-cell phenotype (Collombat et al. 2009). Also, it was highlighted that the reactivation of Ngn3 upon Pax4 overexpression is very crucial to tackle the hypoglucagonemia condition, which activated various compensatory mechanisms, provoking progenitor cells to convert first to α-cells and then to β-cells. These converted β-cells are functional at an early age and repopulate the islets of diabetic mice, and could maintain normoglycemia (Collombat et al. 2009). Another common and remarkable transcription factor that has been employed to convert different endocrine-specific cell lineages to β-cells is Pdx1. Pdx1 is the "master regulator" in β-cell maturation, proliferation, and function as its homozygous mutation led to hyperglycemic condition (Gannon et al. 2001; Gao et al. 2014). When Pdx1 expression was enforced in endocrine-committed Ngn3+ cells during embryonic development, a slight increase in the number of β-cells was observed with the subsequent decrease in α-cells, while postnatally, this conversion continued at a higher rate, leading to the complete absence of α-cells (Cavelti-Weder et al. 2015). This study revealed the single-handed role played by Pdx1 as a potent reprogramming factor (Cavelti-Weder et al. 2015). Pdx1, when combined with MafA, successfully reversed diabetes in alloxan-treated NOD/SCID mice within 2 weeks, restoring euglycemia (Xiao et al. 2018). Moreover, significant improvement in the glucose response was also observed in mice, along with a substantial increase in β-cell mass (Xiao et al. 2018). Sangan et al. explored the role of the HNF4α transcription factor in transdifferentiation and demonstrated that this factor could also induce β-cell phenotype in α-cells (Sangan et al. 2015). When HNF4α was ectopically expressed in αTC1-9 cells, a commonly used murine α-cell line, it converted α-cells to β-cells by suppressing glucagon expression (Sangan et al. 2015). The reprogrammed cells were of increased size and positive for β-cell-specific markers like insulin, C-peptide, GLUT2, glucokinase, and Pax4, enabling the cells to secrete insulin in a glucose-regulated manner (Sangan et al. 2015). However, the conversion was incomplete as the cells lacked the expression of key factors like Pdx1 (Sangan et al. 2015). Moreover, ectopic expression of Pax4 alone in αTC1-9 cells showed no effect on gene expression in vitro, which contradicted the previous in vivo findings by Collombat and group (Collombat et al. 2009; Sangan et al. 2015), indicating that additional factors are essential for the transdifferentiation of α-cells to β-cells. Glucagon-like peptide 1 (GLP1) is one such potential factor that reversed hyperglycemia in diabetic animal models and restored the β-cell population (Zalzman et al. 2003). This study demonstrated that GLP1, when infused in streptozotocin-induced rat pancreatic islets, promoted the endogenous neogenesis of β-cells, possibly by regulating GLP1 receptor and its downstream signaling pathway (PI3K/AKT/FOXO1) (Zalzman et al. 2003). This regulation resulted in the enhanced Pdx1 and MafA mRNA expression and reduced MafB levels (Zalzman et al. 2003), suggesting that GLP1 promoted β-cell function and suppressed α-cell identity.

Apart from pancreatic-specific genes, epigenetic markers also play a pivotal role in reprogramming α-cells into β-cells. Bramswig and group elucidated the epigenetic landscape involved in α-cells as well as exocrine cell transition to β-cells (Bramswig et al. 2013). The group discovered that α-cells displayed multiple bivalent marks on the development genes and showed similar bivalent modification profiles to ESCs. This similarity indicates that α-cells have a plastic epigenomic state, explaining the relative ease of reprogramming α-cells to β-cells (Bramswig et al. 2013).

4 Exocrine Cells to β-Cells

4.1 Acinar Cells

The exocrine portion of the pancreas comprises acinar and ductal cells that contribute to 95% of the total mass of the pancreas (Das 2014). It also

shares developmental similarities with the endocrine pancreas, which makes these cells a preferred choice for dedifferentiation into β-cells. It has been observed that exocrine cells such as acinar cells can turn on endocrine machinery when cultured in specific media conditions in vitro (Fig. 5) (Baeyens et al. 2005; Minami et al. 2005). This conversion was shown by merely treating acinar cells with Activin A and betacellulin to generate insulin-producing cells (Mashima 1996). However, the cells stained positive for the pancreatic polypeptide as well (Mashima 1996), indicating incomplete transdifferentiation of acinar cells. Later, two different groups attempted to convert pancreatic exocrine cells to insulin-producing cells (Baeyens et al. 2005; Minami et al. 2005). These groups used rat or mouse exocrine cells and treated the cells with different growth factors and small molecules. The resulting cells were identical to endogenous β-cells with insulin-secretory granules (Baeyens et al. 2005; Minami et al. 2005). Furthermore, the cells showed glucose responsiveness, maintained normoglycemia, and stained positive for key β-cell markers like C-peptide, GLUT2, etc. (Baeyens et al. 2005; Minami et al. 2005). These findings were further validated using cell lineage tracing experiments, which revealed that amylase^{+}/elastase^{+} acinar cells activated epidermal growth factor signaling that favored the transition (Minami et al. 2005). Although these studies claimed to have generated mature β-cells, some of the reprogrammed colonies stained positive for pre-endocrine or early endocrine markers like protein gene product 9.5. This proved that the cells belonged to a transitional state where it was insulin and protein gene product 9.5 double positive (Minami et al. 2005). Therefore, subsequent studies modified the dedifferentiation protocols to include key β-cell-specific transcription factors for efficient direct conversion.

In 2008, Zhou and group screened a total of 9 (Pdx1, Ngn3, NeuroD, Nkx2.2, Nkx6.1, Pax4, Pax6, Isl1, and MafA) transcription factors that were reported to have a pivotal role in reprogramming exocrine cells to β-cells (Zhou et al. 2008). Among these factors, Ngn3, Pdx1, and MafA showed the most promising results. The differentiated cells closely resembled the endogenous β-cells in size and ultrastructure. Moreover, the cells ameliorated hyperglycemia, remodeled local vasculature, and expressed mature β-cell markers (Zhou et al. 2008). However, the

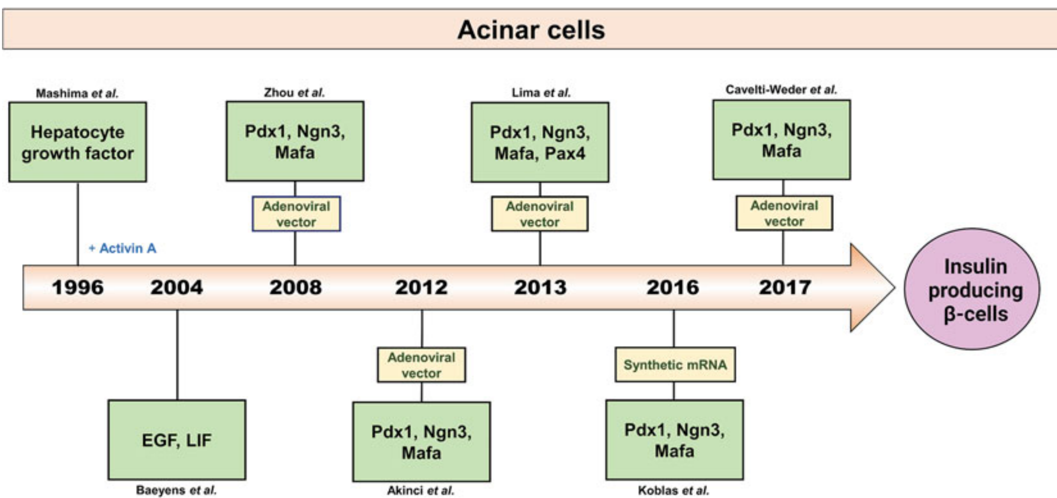

Fig. 5 A timeline indicating different factors that can be used for reprogramming acinar cells to insulin-producing β-cells. Growth factors like hepatocyte growth factor, epidermal growth factor, leukemia inhibitory factor, and reprogramming factors like Pdx1, Ngn3, and MafA are mostly used. Adenoviral transduction is the most common gene delivery method used by different studies. Other methods used for the delivery of reprogramming factors are also mentioned

number of induced β-cells declined over the next 2 months and became undetectable after 7 months of transduction (W. Li et al. 2014). So, it was speculated that the reprogramming efficiency could be improved if the factors could be introduced in a single polycistronic construct to induce efficient direct lineage conversion. Using this strategy, the researchers observed enhanced insulin production that ameliorated diabetes for up to 13 months (Li et al. 2014). A similar approach was used to generate β-cells from acinar cells using a polycistronic vector containing Pdx1, Ngn3, and MafA and performing their in vivo transduction in adult mice (Cavelti-Weder et al. 2017). Using the same set of transcription factors, Akinci and co-workers delivered these factors through an adenoviral approach into rat pancreatic acinar cells (AR42j-B13 cell line) (Akinci et al. 2012). The study observed clear expression of *Ins1* and *Ins2* genes at the RNA level, and the transduced cells stained positive for insulin and C-peptide at the protein level, and could cure diabetes in streptozotocin-induced mice (Akinci et al. 2012). However, the major limitation was the cells were not glucose-responsive and secreted insulin in an unregulated manner (Akinci et al. 2012). Furthermore, it was shown that the inhibition of epithelial-to-mesenchymal transition along with the addition of Rho-associated kinase inhibitor, Y27632, and TGFβ inhibitor reprogrammed human exocrine cells to behave like β-cells (Lima et al. 2013). The target cells, when dedifferentiated in vitro, tend to grow in monolayers of mesenchymal cells, facilitating lineage conversion of acinar cells to β-cells. The group further demonstrated that the transdifferentiated cells secreted insulin upon glucose stimulation and could normalize blood glucose levels in the streptozotocin-diabetic NOD/SCID mice model (Lima et al. 2013). All these studies thus proved the capability of human as well as murine exocrine cells to convert to β-cells and secrete insulin in a glucose-responsive manner. However, the major shortcoming in each of these studies was the use of viral methods to bring about the transition. Thus, to avoid the possibility of tumorigenic transformation associated with integrating viral methods and enhance the safety of the generated β-cells, Koblas and group attempted to reprogram rat pancreatic exocrine cells to insulin-producing β-cells using synthetic mRNAs of the core reprogramming factors, Pdx1, Ngn3, and MafA (Koblas et al. 2016). When mRNAs of these transcription factors were introduced into AR42J cells, the treated cells could process proinsulin to insulin and its byproduct C-peptide (Koblas et al. 2016). Additionally, the reprogrammed cells showed the upregulation of multiple key β-cell genes like *Insulin, Sur1, Kir6.2, Pcsk1*, and *Pcsk2*, along with limited insulin secretion similar to immature β-cells (Koblas et al. 2016). The study further demonstrated that pretreatment of cells with 5-Aza-2′-deoxycytidine, a DNA-hypomethylating agent, followed by transfection with mRNAs improved the reprogramming efficiency from 3.5% to 14.3% (Koblas et al. 2016). Moreover, only 5-Aza-2′-deoxycytidine pretreated cells could secrete insulin successfully in the presence of high glucose. However, in spite of the improved reprogramming efficiency, the insulin content was significantly lower than the rat pancreatic islets used as a control (Koblas et al. 2016). The study speculated that epigenetic modulators and additional transcription factors like Nkx6.1, Pax6, and Isl1 that could positively impact insulin gene expression might be required to attain a mature β-cell phenotype (Koblas et al. 2016). Therefore, further optimization and addition of factors are needed to reprogram human or murine pancreatic exocrine cells to β-cells.

4.2 Ductal Cells

It has been proved that ductal cells have the capacity for endocrine differentiation due to the presence of ductal stem cells in the fetus as well as in neonates (Solar et al. 2009). This capability to expand and differentiate is expected to continue even after the perinatal period. Under certain severe conditions, such as pancreatectomy in rats, the cells have been observed to grow and multiply. This opens up a whole new avenue to study islet neoformation involving ductal cells. To test the hypothesis, different groups have isolated human and mouse ductal cells and

attempted to reprogram them to hormone-secreting islet cells (Fig. 6). In early 2000, Bonner-Weir and colleagues cultured pancreatic tissue from eight individuals (Bonner-Weir et al. 2000). The cells were grown in monolayers and overlaid with a film of matrigel. This alteration helped the cells to acquire cyst-like structures that eventually gave rise to islet-like buds. Subsequent culturing of these cells increased the overall insulin content by 10- to 15-fold (Bonner-Weir et al. 2000). However, the islet cells stained double positive for cytokeratin-19 (ductal marker) as well as insulin, indicating the immature nature of the transdifferentiated cells (Bonner-Weir et al. 2000).

In the subsequent years, multiple studies have explored the potential of pancreatic ductal cells to differentiate into endocrine and exocrine cells upon deliberate pancreatic injury or pancreatic ductal ligation in both neonatal and adult animals (Inada et al. 2008; Li et al. 2010; Van de Casteele et al. 2013; Xu et al. 2008). The studies proved that, during pancreatic ductal ligation, β-cell progenitors which resided in the ductal lining got activated and compensated for the β-cell loss. These cells were $Ngn3^+$ and gave rise to all islet cell types, including proliferative glucose-responsive cells both ex vivo and in situ (Van de Casteele et al. 2013; Xu et al. 2008). Interestingly, the conversion of non-β to β-cells improved at higher levels of Ngn3 (Van de Casteele et al. 2013; Xu et al. 2008). It was suggested that multipotent progenitor cells come to play autonomously to elevate β-cell mass rather than self-duplicating the existing β-cells (Van de Casteele et al. 2013; Xu et al. 2008). Moreover, the ablation of $Ngn3^+$ cells led to the depletion of β-cell expansion, suggesting the role of $Ngn3^+$ $insulin^-$ cells in β-cell proliferation (Van de Casteele et al. 2013). These observations were supported by different groups where the ductal cells were specifically marked using the Cre-loxP system under the human carbonic anhydrous II promoter (Inada et al. 2008). These carbonic anhydrous II^+ cells gave rise to both new islets as well as acini cells (Inada et al. 2008). Additionally, the expression of Hnf6 was originally lost in mature ductal cells; however, during pancreatic ductal ligation, its expression reappeared in proliferative ductules, which further differentiated into pancreatic lobes (Li et al. 2010). Upon ligation, the cells also started expressing MafA-like mature β-cells,

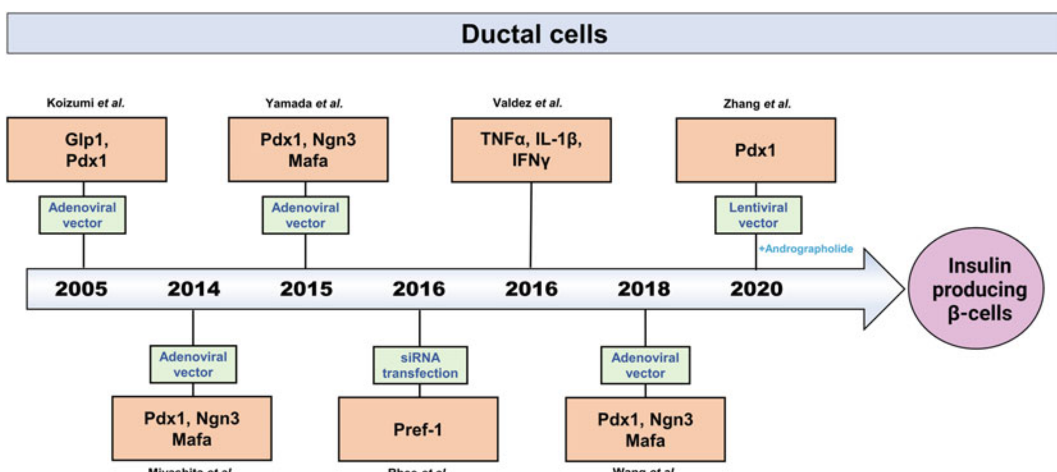

Fig. 6 A timeline indicating different factors that can be used for reprogramming ductal cells to insulin-producing β-cells. Proinflammatory cytokines such as TNFα, IL-1β, IFN-γ, and reprogramming factors such as Glp1, Pdx1, Ngn3, MafA, and Pref-1 are commonly used. Adenoviral transduction is the most common gene delivery method used to date by different studies. Other methods used for the delivery of reprogramming factors are also mentioned. (TNFα: Tumor necrosis factor alpha, IFN-γ: Interferon-gamma, IL-1β: Interleukin 1 beta)

which further supported that pancreatic ductal cells serve as the starting cell source for mature insulin-producing β-cells (Li et al. 2010). In addition, the treatment of ductal cells with gastrin and epidermal growth factor further led to the rise in β-cell number upon β-cell ablation (Rooman and Bouwens 2004). Glp1, in conjunction with Pdx1, also reprogrammed immortalized pancreatic epithelial cells of a ductal origin to insulin-producing cells (Koizumi et al. 2005).

Duct-to-endocrine shift can also be enhanced by inhibiting Delta-Notch signaling and Fbw7, the recognition receptor of the SCF FBW7 E3 ubiquitin ligase, and by co-expressing Myt1 (Sancho et al. 2014; Valdez et al. 2016). In line with this, the loss of Fbw7 resulted in the stabilization of Ngn3 and promoted the endocrine developmental differentiation program (Sancho et al. 2014). Apart from these, activation of MAPK and Akt signaling by overexpression of preadipocyte factor 1 directed human pancreatic ductal cells toward β-like-cell fate by improving glucose homeostasis and facilitating insulin secretion (Rhee et al. 2016). The study reported that MAPK signaling increased insulin secretion by nucleocytoplasmic translocation of two factors, namely FOXO1 and PDX1 (Rhee et al. 2016). Further, it was reported that Akt signaling activated its downstream targets like Rab43, a GTPase-activating protein, which in turn, enhanced glucose-stimulated insulin secretion (Rhee et al. 2016). In addition, inflammatory cytokines like TNFα, IL-1β, and IFN-γ also enabled ductal-to-endocrine cell reprogramming via STAT-dependent NGN3 activation in human ductal epithelial cells, PANC-1 (Valdez et al. 2016). Similarly, the treatment of PANC-1 cells with andrographolide, which is a chemical compound extracted from *Andrographis paniculata*, induced pancreatic ductal cell differentiation to insulin-producing cells by stimulating PDX1 expression at both mRNA as well as protein levels (Zhang et al. 2020). However, the plasticity of these ductal cells still remains a question since different groups reported that the differentiation capacity of these cells is restrained after birth and postnatally (Kopp et al. 2011; Solar et al. 2009). Also, during pancreatic ductal ligation, the ductal epithelium had an insignificant contribution to the acinar and endocrine cell population due to its restricted plasticity (Kopp et al. 2011; Solar et al. 2009). The study supported its findings using lineage-tracing experiments, which revealed that treatment with gastrin and epidermal growth factor failed to reprogram ductal cells to β-cells during pancreatic ductal ligation (Solar et al. 2009). Also, $Sox9^+$ ductal cells had an insignificant contribution toward β-cell neogenesis in the early postnatal stage and no contribution in adulthood (Kopp et al. 2011). Nevertheless, exogenous overexpression of pancreatic-specific key transcription factors, PDX1, Ngn3, and MafA, has been reported to augment the reprogramming efficiency of pancreatic ductal cells to insulin-producing cells by expressing β-cell-specific genes, and also corrected hyperglycemia in the diabetic mouse model (Miyashita et al. 2014; Van de Casteele et al. 2013; Yamada et al. 2015). Sequential administration of these defined factors in the required dosage can further improve the reprogramming efficiency (Miyashita et al. 2014). It has been observed that excessive expression of MafA along with Pdx1 and Ngn3 resulted in an inhibitory effect in the reprogramming process (Miyashita et al. 2014). However, overexpression of Pdx1 along with Ngn3 and MafA enhanced the reprogramming efficiency (Miyashita et al. 2014). These findings established the importance of the dosage of application in the reprogramming paradigm.

5 Conclusion

There are multiple potential approaches to generate β-cells from various cell sources/types for the treatment of diabetes mellitus. β-cells can be derived from adult stem cells, ESCs, or induced pluripotent stem cells that require reprogramming of somatic cells to a pluripotent/multipotent state and their subsequent differentiation to a desired cell type. These approaches provide an unlimited source of cell type of choice, but require time and effort. While immense efforts continue in multiple directions, direct reprogramming proves to be a promising alternative strategy for β-cell regeneration and has been employed by different groups using various starting cell sources/types as

Table 1 Summary of reprogramming studies generating insulin-producing β-like-cells from various cell types/sources

Cell type/ source	Model	Genes	Small molecules/ growth factors	In vivo	Major limitation(s)	Reference
Immature enterocytes	Rat	Pdx1, Isl1	Betacellulin	Yes	Glucose-stimulated insulin secretion was not observed	Kojima et al. (2002)
Intestinal epithelial cells	Rat	Pdx1	Betacellulin	Yes	Insulin was secreted regardless of glucose concentration	Yoshida et al. (2002)
Intestinal epithelial cells	Mice	GLP1	NA	Yes	Differentiation efficiency was very low	Suzuki et al. (2003)
Bone marrow-derived cells	Mouse	NA	NA	Yes	Cells did not secrete insulin upon glucose stimulation	Tang et al. (2004)
Adult bone marrow-derived cells	Rat	NA	Defined culture condition (1% DMSO + high glucose)	Yes	Immature β-cells formed	Oh et al. (2004)
Neural progenitor cells	Human	NA	Defined culture condition	Yes	Immature β-cells formed, did not express β-cell markers like *Nkx6.1*	Hori et al. (2005)
Mesenchymal cells	Rat	NA	Rat pancreatic extract	No	Insulin release from generated cells was low compared to pancreatic islets	Choi et al. (2005)
Intestinal epithelial cells	Rat	MafA	NA	Yes	Insulin^{+} cells were not observed 2 weeks post-infection	Nomura et al. (2006)
Stomach, duodenum, liver	*Xenopus laevis*	Ptf1a, Ptf1a-VP16	NA	Yes	Unmodified Ptf1a had no effect on liver cells, Ptf1a-VP16 was capable of only promoting acinar cell fate	Jarikji et al. (2007)
Gut	Mice	*Foxo1* ablation	NA	Yes	Did not form islet-like structures, did not form gut insulin^{+} cells	Talchai et al. (2012)
Gall bladder	Mouse	Pdx1, Ngn3, MafA	Retinoic acid, dibenzazepine	No	Reprogrammed cells were immature and polyhormonal in nature, did not respond to glucose stimulation, and failed to reverse hyperglycemia	Hickey et al. (2013)
Intestinal epithelial cells	Human	Pdx1, Ngn3, MafA	NA	Yes	Neo-islet cells gradually disappeared within 2–3 weeks	Chen et al. (2014)
Gut organoids-derived from iPSCs	Human	FOXO1 inhibition	NA	Not mentioned	Not mentioned	Bouchi et al. (2014)
Gall bladder	Human	Pdx1, Ngn3, MafA, Pax6	NA	Yes	β-cells formed were immature and the reprogramming efficiency was low	Galivo et al. (2017)

discussed above and listed in Table 1. This approach has multiple advantages like it bypasses the pluripotent state and thereby also avoids the limitations posed by pluripotent cells like inducing tumorigenicity. Direct reprogramming also has faster kinetics as it allows complete control over time and dosage of application of reprogramming factors, growth factors, small molecules, etc. However, this approach comes with numerous challenges that need to be addressed in future studies. Multiple studies have reported the low efficiency of the converted cells as one of the major shortcomings (Galivo et al. 2017; Hori et al. 2005; Suzuki et al. 2003). This challenge can be overcome by the inclusion of other key pancreatic-specific transcription factors along with growth factors and small molecules. 5-Aza-2′-deoxycytidine, a DNA demethylating agent, is one of the small molecules/chemical compounds that has been used in multiple studies to reprogram terminally differentiated cells to β-cells (Koblas et al. 2016; Lefebvre et al. 2010; Lima et al. 2013). However, this molecule is carcinogenic in nature and may introduce mutations in the reprogrammed cells and thus screening of further non-carcinogenic molecules is required for safer reprogramming (Carr et al. 1984, 1988).

Moreover, it has also been observed that the generated reprogrammed cells are immature in nature and do not express the key mature β-cell-specific markers like MafA and Nkx6.1 (Cerdá-Esteban et al. 2017; Galivo et al. 2017; Hickey et al. 2013; Hori et al. 2005). Apart from this, the converted cells are also polyhormonal that do not stain exclusively for insulin, demonstrating heterogenous population post-reprogramming (Aigha and Abdelalim 2020; Chakravarthy et al. 2017; Galivo et al. 2017; Hickey et al. 2013). These cells also have inconsistent glucose responsiveness and it has been observed that reprogrammed cells release insulin invariably (Ackermann et al. 2016; Akinci et al. 2012; Kojima et al. 2002; Yang et al. 2013; Yoshida et al. 2002). Therefore, further refinement in the reprogramming protocols and incorporation of additional factors and small molecules are required to formulate a reliable and reproducible method that can generate autologous transplantable β-cells.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Research Involving Human Participants and/or Animals None.

Availability of Data and Materials Not applicable.

Code Availability Not Applicable.

Author Contribution **GN** was responsible for conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, and final approval of the manuscript; **RKR** was responsible for conception and design, collection and/or assembly of data, and final approval of the manuscript; **RPT** was responsible for conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final editing and approval of the manuscript and financial support. All the authors gave consent for publication.

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