STEMedicine

Vol 1 • Issue 3 • Jul 2020
ISSN 2705-1188

The progress of CAR-T therapy in cancer and beyond

STEMedicine
stemedicine.org

REVIEW ARTICLE

The progress of CAR-T therapy in cancer and beyond

Yehong HUANG, Lulu LI, Wenzhuo LIU, Tao TANG, Liming CHEN*

Department of Biochemistry, School of Life Sciences, Nanjing Normal University, Nanjing 210023, China.

*Correspondence: chenliming1981@njnu.edu.cn
https://doi.org/10.37175/stemedicine.v1i3.47

ABSTRACT

Chimeric antigen receptor T (CAR-T) technology is the pinnacle of modern immunology, system biology, synthetic biology and cellular engineering. It is the powerful new player of cancer therapy since the concept of adoptive cell therapy. Clinical success of CAR-T cells targeting antigen unique to B cell leukemia has made it the focus of new development in cancer therapy. The latest success is reported in a clinical case of using CAR-T cells to treat pediatric acute lymphoblastic leukemia. However, the major challenges are to make CAR-T cells a reliable, controllable, safe and effective platform that could apply to diverse cancer types including solid tumors. In this review we summarize the recent research progress to tackle the challenges and discuss the broader application of CAR-T cells beyond cancer in the context of genome editing era.

Keywords: Chimeric antigen receptor · Cancer · Cell therapy · Immunology

Introduction

Since the "War on Cancer" was launched by US President Richard Nixon in 1971, at the time when human had successfully landed on the moon, however, people are frustrated to see the uprising of cancer incidences worldwide and cancer still remains the top mortality cause in the world (1). The doubt looms in the minds of people whether we could ever win the war. Fortunately, we become so confident in recent years with the advance of new technologies. We have seen the booming of immunotherapy for cancer since it was named the number one breakthrough of the year in 2013 by *Science* journal (2). The success of immunotherapy in clinics has won the scientists behind the discovery the Nobel Prize in 2018. A new technology of immunotherapy: chimeric antigen receptor T cell (CAR-T) therapy was awarded the Advance of the Year 2018 by the American Society of Clinical Oncology. It holds the hope for the final resolution for cancer, the grail therapy. The latest success of using CAR-T technology to combat cancer was reported in a clinical case in Singapore to treat acute lymphoblastic leukemia after the chemotherapy and stem cell transplant failed to deliver the outcome (3,4). No doubt it will be the star of Cancer Moonshot task force. In this article, we will briefly introduce the background, the progress and the challenges of CAR-T cell therapy.

CAR-T cell therapy: the next generation of medicine

Modern medicine was for a long time the extract of natural product or small chemical compound obtained by chemical synthesis. The key technology is analytical and synthetic chemistry. They still remain the mainstay of drugs today and the first pillar of modern medicine. With the development of molecular biology and bioengineering, recombinant proteins or macromolecules became the second pillar of medicine, with Genentech as the herald and model of biotech pharma. Recombinant bio-products like insulin and monoclonal antibodies stand for a substantial proportion of drug market. Now we have the third pillar of medicine, cell therapies like CAR-T cells which have been approved by the Food and Drug Administration (FDA) to treat certain leukemia (5). The therapeutic capabilities of cell entities are distinct. So far, they are the first kind of "live" medicine, which possess unique traits never seen in previous types of medicine. They can grow, proliferate, and move actively towards the drug target. They serve as a platform to equip with multiple armors to tackle the tumor cells and escape the suppressive control of microenvironment (6). They are complex system and subject to manipulation by the theory of control through positive or negative feedback mechanism. The design and manufacture of these agent are

Received: Apr 20, 2020; Accepted: May 31, 2020.

totally different from previous generations of drugs and are more challenging. The foundation comprises of cellular system and synthetic biology, gene transfer technique, monoclonal antibody technology, cell engineering method, and the latest genome editing toolkits. Nonetheless, the corner stone for CAR-T therapy is the deep understanding of immunology and the concept of immunotherapy of cancer.

The foundation of immunotherapy

Immune checkpoint therapy and cell-based therapy like CAR-T are all dependent on the function of immune system: to recognize the danger and clear the threat, either from the outside world or inside the cells of the organism. Immune system in mammals comprises the innate and adaptive branches of immunity. Innate immunity is found in most animals and plants, it is responsible for the first-line defense of pathogens through pattern recognition of different types of foreign molecules, which is usually non-specific and antigen-independent (7). Adaptive immunity is only found in vertebrates and it has evolved to provide delicately regulated repertoire of recognition for both self and non-self antigens through complex network between different cell types, especially between antigen-presenting cells (APC) and T/B lymphocytes which involve the formation of immunological synapse consisting of B or T cell receptors (BCR or TCR) and their cognate ligand with antigen and co-stimulatory molecules. The recognition of different antigens in specific circumstances leads to pathogen-specific effector T cell activation and elimination of pathogens or diseased cells, generation of memory T or B cells, or immune tolerance of self-antigen, collectively regulating the homeostasis of the immune system (8).

Before we introduce the structure of chimeric antigen receptor, we first briefly talk about the natural TCR. The TCR is normally a heterodimer composed of two different subunits selected from the four different types of polypeptides: α, β, γ, δ (**Figure 1**). In human T cells, 95% of the TCR consists of an α and β chain, the rest 5% TCR is composed by γ and δ chains. TCR is a member of immunoglobulin superfamily and resembles the structure of a half antibody (light chain and heavy chain without the Fc region). The TCR module is like an antibody anchored on T cell membrane and responsible for recognizing antigens which are presented by major histocompatibility complex (MHC) molecules on the surface of APCs or tumor cells. However, it has no signaling function on its own as its cytoplasmic tail is very short. The TCR heterodimer then forms a complex with Cluster of Differentiation (CD)3 and ζ chain which can transduce the signal into different pathways such as nuclear factor kappa B (NFκB), phosphoinositide 3-kinase (PI3K), mitogen activated protein kinase (MAPK) through recruitment of tyrosine kinases lymphocyte-specific protein tyrosine kinase (Lck), tyrosine protein kinase Fyn, zeta-chain associated protein kinase 70 (Zap70), subsequently promoting the proliferation and differentiation of cells (9). The TCR complex also recruits or interacts with co-receptors like CD4, CD8, and co-stimulatory

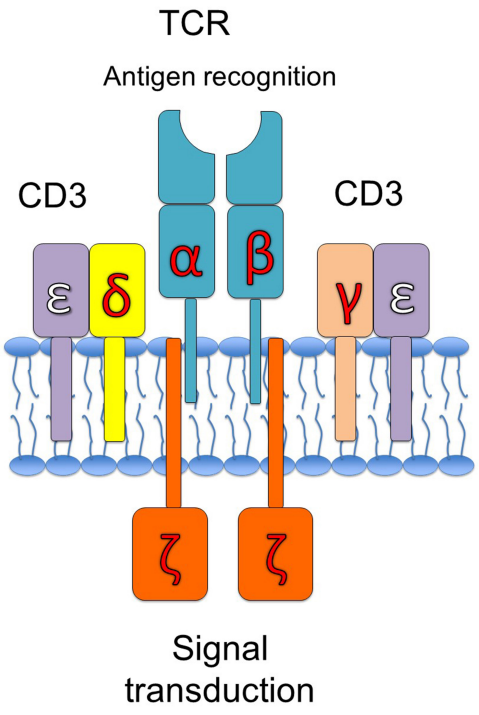

Figure 1. The diagram of TCR structure.

molecules CD28, Inducible T-cell COStimulator (ICOS), OX40, and 4-1BB for optimal activation upon MHC engagement. Upon binding of its ligands CD80/CD86, CD28 is activated and helps amplifying the TCR signaling which turns on cytokine production, cell cycle progression and metabolism reprogramming. 4-1BB belongs to TNFR superfamily and can be induced by TCR and CD28 signaling in both CD4+ and CD8+ cells (10). When engaged with 4-1BB ligand, 4-1BB transduces signal to enhance T cell proliferation, cytokine secretion and cytolytic potential, and more importantly, to reduce the sensitivity of cells to inhibition by transforming growth factor-β (TGFβ) (11). T cells are also under negative regulation of inhibitory molecules like Programmed cell death 1 (PD1), Cytotoxic T lymphocyte antigen-4 (CTLA-4), Lymphocyte activation gene 3 (LAG3) and T cell immunoglobulin-3 (TIM-3) which are the targets of immune check point therapy (12).

Many different subtypes of T cells are identified to distinguish their diverse functions in immune response: CD8+ cytotoxic T cell, CD4+ T helper cells, memory T cells, regulatory T cells, and innate-like T cells including natural kill T cells and gamma delta T cells (13-15). Once activated, cytotoxic T cells are found to have the direct killing ability to eliminate cancer cells, infected or damaged cells by releasing cytotoxins perforin, granzymes and granulysin, or through FAS ligand mediated apoptosis. The targeting of cytotoxic T cells was tightly regulated by MHC-I complex with antigen presented by APCs or tumor cells. This unique trait makes it a potential therapeutic agent for cancers (16).

The hint from adoptive cell therapy

More than a century ago, pioneer immunologist Paul Ehrlich has proposed the term of "magic bullet" which means to harness the power of immune system for disease intervention (17). His scientific achievements won him the Nobel Prize in 1908. The bullet is to specifically target diseased tissue/cells and leave healthy tissue intact. With scientific breakthrough in the biomedical field, people have seen antibiotics, antibodies and targeted small chemical compounds as the examples of magic bullets at different stages. Now we have the latest version, CAR-T cells, also the most advanced generation.

The use of T cells in cancer treatment, adoptive cell therapy, can trace back to more than 20 years ago, when non-genetically modified T cells were expanded in ex-vivo and infused back to melanoma patients to show good safety and achieve objective tumor regression in 50% of the patients (18,19). Similar treatment was used on infectious disease such as CMV and HIV virus infected patients (20,21). Autologous tumor-infiltrating lymphocytes (TILs) are the most effective source of adoptive cell treatment so far. However, there are several limitations for this therapy. Firstly, the isolation and preparation of this kind of TIL are labor-intensive and complex. Secondly, activation of these TILs is dependent on the native TCR and optimal engagement with MHC matching to each patient, which is a daunting task given the enormous pool of MHC alleles. Thirdly, the affinity between TCR and tumor antigen is roughly low, in micromolar range, compared to the high affinity between viral peptide and TCR at nanomolar range. This will likely limit the recognition and antigen-specific activation of cytotoxic T cells.

To overcome the limitations of endogenous TILs used in adoptive cell therapy, researchers developed the chimeric antigen receptor T cells.

The concept of CAR-T design and evolution

In a word, the design of CAR-T is following the principles governing the behaviors of endogenous T cell in the body. Firstly, T cells need to move towards the target via the leukocyte trafficking system. They are mobilized by chemokine signaling and leukocyte recruitment pathway. Secondly, they can recognize the target. The recognition of antigen is assisted by APC via processing of antigen peptides and presenting on the surface by the MHC complex. It has super flexibility and enormous potential rendered by TCR rearrangement and MHC diversity. This is critical for the organism to respond to numerous antigens in a well-controlled manner. However, the delicate system is highly prone to being hijacked by tumors and becomes a weakness for immune defense. Thirdly, T cells need to be activated and able to proliferate under tight control to avoid the self-damage of normal tissues. When the threat is cleared, T cells need to return to dormant or inactive state, but possess the ability to revive when the old threat is detected again. Lastly, T cells need to survive in or overcome the suppressive tumor environment, which is frequently associated with inert of T cells seen in various cancers especially in solid tumors.

The key innovation of CAR-T cells is to replace the variable region of TCR heterodimer with the counterpart from another antibody, both of which are responsible for antigen recognition and are structurally similar. The concept that CAR could recognize target independent of the MHC complex and co-stimulation signal was successfully validated in late 1980s by Kuwana and Eshhar group (22,23). Another key innovation is to simplify intracellular signaling domain into a single chain receptor comprising of CD8 extracellular and transmembrane domain and CD3ζ cytoplasmic domain. The chimeric receptor can express independently of TCR and transduce signal like the native TCR complex (24). These scientific innovations paved the road for the application of CAR-T cells in clinics. It takes more than 20 years from basic concept to actual clinical approval.

In the first-generation CAR-T cells, the sensor domain of conventional TCR was replaced by a single chain variable fragment (scFv) which mimicked the antigen recognition structure of anti-CD19 antibody (**Figure 2**). This design significantly reduced the complexity of heterodimer TCR chains and it enabled the convenient expansion to various targets by switching the scFv module to desired antigen binding domain. Target selection is key to the success of CAR-T and will be discussed further in the later section. Then the extracellular antigen binding domain was fused through a hinge and transmembrane domain with intracellular signaling module CD3 ζ chain. In this way a single chain chimeric receptor could execute the core functions of the native TCR complex composed of more than eight subunits, even in the absence of the MHC complex. This is a great leap of progress in the design of CAR.

To enhance the activation of CAR-T cells, a co-stimulatory domain from CD28 or 4-1BB was inserted between transmembrane domain and the TCR signaling motif CD3 ζ domain. This is the design of the second-generation CAR (**Figure 2**). There was some difference between CD28 and 4-1BB-based CAR co-stimulation effect: CD28-based CAR showed enhanced proliferation and effector functions, while 4-1BB-based CAR induced more pronounced progressive T cell accumulation which may compensate for less immediate potency (11). Other co-stimulation domains from CD27, OX40, ICOS and RIAD were also tested in various settings.

The first FDA approved CAR-T therapy Kymriah is the third generation of CAR-T therapy. The upgrade from the second generation is to insert both CD28 and 4-1BB co-stimulatory domains in tandem before the CD3 ζ domain, which will further enhance the activation of T cells (**Figure 2**). Moreover, this combination of co-stimulatory domains can boost the efficacy of CAR with low binding affinity, expanding the choice of antigen recognition motifs (25). There was also different combination of co-stimulation domains like CD28 plus OX40 in the third generation CAR-T which showed largely similar functionality and persistence. However, some unique trait was found in CAR-T with different combinations. In a neuroblastoma

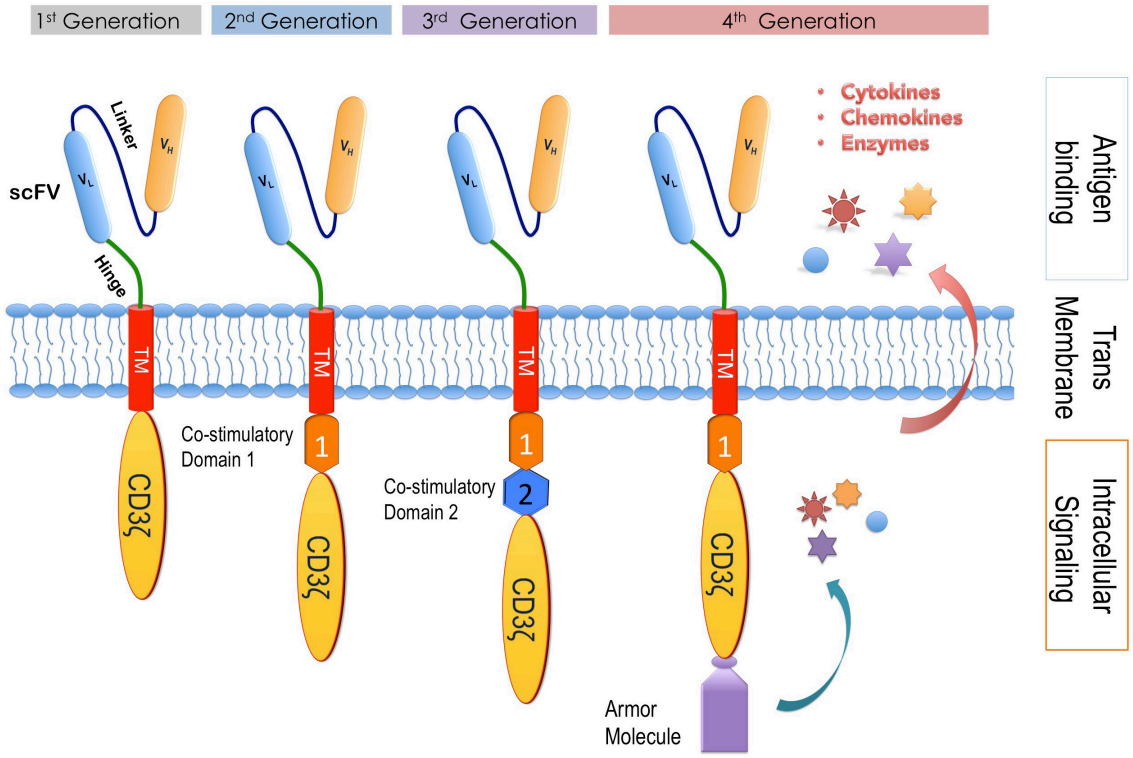

Figure 2. The evolution of CAR. The figure illustrates the different designs of CAR with the addition of various functional modules.

tumor cell model, preclinical results demonstrated that CD28/4-1BB combination outperformed the CD28/OX40 combination in terms of several CAR-T cell properties such as T cell persistence, basal T cell activation and alleviation of T cell exhaustion (26).

Of note, the precise molecular mechanism of CAR-mediated tumor killing has not been well elucidated. The anti-cancer capability may be mediated by direct tumor cytolysis and cytokine secretion. Interestingly, CD4+ CAR-T cells possess the comparable cytolytic ability with CD8+ CAR-T cells. Cytokines like TNFα and IFNγ produced by CAR-T cells may contribute to the damage of tumor microenvironment and growth arrest of tumor cells (10,11).

On the basis of the third generation, new design of CAR-T focuses on the addition of other co-expressed molecules as "armors" that serve various purposes: i) enhance the cytotoxic potential and leukocyte trafficking ability to target site, ii) prolong the cell survival in the suppressive environment, iii) put CAR-T cells under specific control. They are classified as the fourth generation (**Figure 2**). There could be diverse combinations of different "armors" in the final design for the optimal clinical result in particular cancer types. For instance, the CAR-T cells against mesothelin were engineered to co-express cytokine interleukin (IL)-7 and chemokine CCL19 on the backbone of the third generation design, which could significantly enhance the T cell proliferation and survival in solid tumor animal model (27).

The application of CAR-T in blood cancer

Clinical study summary

CAR-T targeting CD19 was the paradigm for the design of CAR-T and achieved remarkable success in B cell leukemia and lymphoma. Based on the robust clinical results, FDA has approved two products. Many CAR-T therapies in B cell lymphoma showed extraordinary clinical outcomes. Some examples were listed in **Table 1**.

The mechanism/reason for success

The success of CD19 CAR-T could be primarily accounted by the following factors: i) the specific target chosen for B cell malignancy. CD19 is almost the perfect target which displays the broad expression at high level in B cell malignancy. The expression of CD19 is tightly restricted to B cell lineage. ii) The on-target off-tumor side effect is very mild. Some patients often showed profound B cell aplasia and concurrently many of them often showed complete remission of disease. The loss of B cells can be effectively managed by replacement therapy with the infusion of immunoglobulin. iii) The acceptable control of severe adverse effects like cytokine release syndrome (CRS) and brain edema even without complete understanding of the mechanism. Currently anti-IL6 antibody, JAK inhibitors and corticosteroids are used for CRS. iv) The easy penetration of CAR-T cells to the target cells and relatively friendly and simple microenvironment for blood malignancy.

Table 1: The clinical result of CD19 CAR-T in B cell leukemia.

Sponsor	CAR co-stimulatory domain	Year	Patients	ORS (%)	Notes	Ref
SCH	4-1BB	2016	36	91%	91% MRD negative CR	[26]
MSKCC	CD28	2014	44	84%	84% CR; MRD after CAR-T treatment is negatively correlated with survival	[27, 28]
NCI	CD28	2015	39	61%	61% CR; a dose-escalation trial	[29, 30]
		2016	5	80%	80% MRD negative CR; first allogeneic CAR without causing GVHD	[31, 32]
UPENN	4-1BB	2014	59	93%	93% CR	[33]
BCM	CD28	2013	4	25%	First allogeneic CAR	[34]
FHCRC	CD28	2016	33	94%	First CAR with defined CD4+ and CD8+ T-cell subsets	[35]

BCM, Baylor College of Medicine; CAR, chimeric antigen receptor; FHCRC, Fred Hutchinson Cancer Research Center; ORS, objective responses; MRD, minimal residual disease; MSKCC, Memorial Sloan-Kettering Cancer Center; NCI, National Cancer Institute; SCH, Seattle Children's Hospital; UPENN, University of Pennsylvania.

Challenge in B cell leukemia

Some form of resistance of CD19 CAR-T has emerged in B cell leukemia under treatment. The loss of CD19 antigen epitope appears to be the primary mechanism of tumor escape in acute leukemia. This is also the common observation for TCR-based cell therapy which depends on the efficient presentation of tumor antigen peptide on the surface of APCs by the MHC complex. About 28% of the patients in a trial with young adults and pediatric patients with acute leukemia were reported to lose the expression of CD19 epitope (35). The potential solution to this problem is to develop dual targeting CAR-T against CD19 and CD22 which are also restricted to B cell lineage (38).

Whereas in patients with chronic lymphocytic leukemia, the resistance to CAR-T therapy was largely due to the insufficient proliferation of CAR-T cells (39). The lack of persistence of CAR-T cells could be solved by better understanding the stimulatory signaling domains (40) or using different type of T cells like sorted memory T cells or stem cells (41). Another challenge is the development of idiotypic antibody against murine scFv region, which could be solved by the use of humanized scFv (42).

The mechanism of and the relationship between CRS and cerebral edema are still elusive. Brain edema could be the consequence of CRS and the extreme manifestation of CRS in neurological system, or totally independent process. It is speculated to be related to inflammatory cytokines. Further study in proper animal model is warranted to elucidate the details.

Expansion of CAR-T beyond B cells and challenge in solid tumors

The success of CAR-T in B cell leukemia has inspired the application into other cancers including melanoma, synovial sarcoma, prostate cancer, colon cancer, kidney cancer and lung cancer (43). However, there are some lessons learned from the CAR-T experiments in solid tumors, which remain as challenges to solve.

Tumor recognition

The crucial issue is the specificity of target molecule. This determines the cross-reactivity toxicity of T cells, either on-target or off-target. It is challenging to find an ideal target antigen on solid tumors. As a restriction of CAR-T therapy, the target must be on the cell surface. Very often the tumor associated antigen (TAA) is enriched on tumor surface, but it is also expressed at low level on normal tissues. So far, this problem is seen on the targets tested in clinical trials like carcinoembryonic antigen (CEA), melanoma antigen (MAGE-A3), folate receptor 1, ganglioside 2 (GD2), human epidermal growth factor receptor 2 (ERBB2), mesothelin, intracellular adhesion molecule 1 (ICAM-1), carbonic anhydrase IX (CAIX), prostate stem cell antigen (PSMA) and mucin 1 (MUC1) (44). The tiny amount of TAA expressed on normal tissues could cause catastrophic consequence in patients with CAR-T treatment (45-48). The higher affinity of CAR to the target is not necessarily correlated with the better efficacy, although the binding affinity is related to safety. The in vivo study with CAR-T against ICAM-1 demonstrated that lower affinity CAR showed better safety and efficacy with enhanced proliferation and less exhaustion when compared to the higher affinity CAR-T cells (49,50). There are new developments with system engineering concept to create more sophisticated recognition circuits. For example, in addition to the single TAA targeting strategy, dual targeting CAR-T cells are also under testing. They have two receptors which constitute the AND-gate, NOT-gate and OR-gate circuits: the function of CAR-T cells is specifically determined by the combination of different targets. The general strategy is to construct two separate CARs against two targets, respectively, and one receptor bears the CD3 ζ chain and the other receptor harbors the co-stimulatory domains (51,52). The upgrade version for AND-gate recognition involves the two receptors in a setting that activation of the first receptor will turn on the expression of the second receptor

that is responsible for cytotoxic function (53,54). The NOT-gate design usually incorporates the signaling motif of inhibitory molecules like PD-1 receptor into the cytoplasmic tail of the CAR which recognizes the antigen expressed on normal tissues. This inhibitory CAR will override the activation CAR which targets TAA (55). In a similar manner, the OR-gate CARs involve two different receptors recognizing two TAAs to refine the targeting and reduce tumor escape, such as CD19/CD22 or CD19/CD20 dual targeting CAR-T cells for B cell leukemia. The clinical result suggested these CAR-T cells were less sensitive to the resistance due to CD19 loss in patients (56,57). These novel designs greatly improve the ability of CAR-T cells to specifically recognize tumor cells yet reduce the collateral damage on normal tissue. The progress of neo-antigen research has found a new treasure of TAA pools which will be the focus of future development of MHC-restricted CAR/TCR design. Recently, a CAR/TCR chimera design targeting the intracellular antigen NY-ESO-1 with the help of HLA-A2 showed promising result (58).

To better handle the potential on-target off-tumor side effect of CAR-T cells, researchers have developed the suicide gene cassette into CAR-T design (59). Inducible caspase 9 could trigger apoptosis when the core fragment of caspase 9 fused to FK506 binding protein (FKBP12) is activated by dimerization induced by FK506 small compound analogue AP1903 (60,61). Another control measure is to co-express the truncated epidermal growth factor receptor (EGFR) without intracellular domain in the CAR-T cells which can be eliminated by EGFR antibody cetuximab-mediated antibody-dependent cellular cytotoxicity (62). In case of unexpected severe adverse event, CAR-T cells could be removed in a controllable manner by these suicide gene designs.

To treat solid tumors effectively, CAR-T cells need to conquer the other challenges: how to find and penetrate into tumor tissues, how to survive and proliferate in the suppressive environment. Next, we will discuss the solutions to tackle the above problems.

Trafficking

T cells need to first move to the site of tumor. This is generally not a big issue for blood malignancies, however, it will take considerable effort for CAR-T cells to do so in solid tumors which are generally more fibrotic and not easy to penetrate. Given the important role of chemokine signaling in leukocyte trafficking, CAR-T cells were engineered to co-express chemokines or chemokine receptors, such as CCR2 and CCL19, to enhance the homing and migration ability of T cells. A case of mesothelin CAR with CCR2 expression showed more than 12-fold increase of T cell trafficking and tumor regression in subcutaneous malignant pleural mesotheliomas tumors. Another GD2 targeting CAR-T cells with CCR2b expression had higher than 10-fold T cell homing in neuroblastoma tumor model (63,64).

Instead of systemic administration of CAR-T cells, local delivery of CAR-T cell is an alternative approach which has been tested in pre-clinical studies with some promising results. In a mouse xenograft study, a breast cancer model with brain metastasis was established, when the CAR-T cells targeting HER2 were injected to tumor intracranially, the tumor was eradicated completely and 100% survival of animals was observed even with re-challenge of tumor cells (65). Another study demonstrated that local administration of HER2-BB ζ CAR-T cells eliminated cancer with much lower dose compared to intravenous delivery (66).

Survive in tumor microenvironment

Many solid tumors are notorious for the suppressive microenvironment which comprises of inhibitory small molecules, suppressive stromal cells and immune cells. Due to the defect in vascular system, tumor cells prefer glycolytic metabolism and further render the environment hypoxic, acidic, oxidative and nutrient deprived. Inhibitory molecules like PD-1 and Galectin-9 are up-regulated in the inflammatory environment and bind to the receptor on T cells and suppress their function. In addition to the combinatory therapy with the checkpoint antibodies which have been the most popular and successful strategy to prevent T cell exhaustion/inhibition, new CAR-T cells with expression of checkpoint inhibitor antibodies have shown promising results in preclinical studies. For example, anti-CAIX CAR-T cells with secretion ability of anti-PD-1 antibody showed substantially enhanced activity against tumor cells compared to normal CAR-T cells, in terms of increased cytokine secretion and immune cell recruitment in human clear cell renal carcinoma mouse model (67). Similar results were observed on CD19 CAR-T with anti-PD-1 antibody in CD19+ lung cancer xenograft model (68).

The tumor cells and their associated stromal and immune cells secrete soluble factors like TGFβ, vascular endothelial growth factor, adenosine, lactate, reactive oxygen species (ROS), prostaglandin E2 and soluble Fas ligand, which contribute to the inflammation related polarization of tumor associated macrophages, abnormal tumor vasculature and suppression of T cell immune responses (69-71). One strategy for CAR-T design is to incorporate the antagonizing enzyme or neutralizing receptor onto cell surface. For instance, a type of CAR-T cells was engineered with catalase, the enzyme for hydrogen peroxide hydrolysis, and showed reduced oxidative stress and improved proliferation and cytotoxicity against tumor cells, compared to the original CAR without catalase (72). TGFβ has been reported to downregulate the secretion of critical Th1 cytokine like IFNγ and impair the cytolytic function of T cells (73). To alleviate the suppressive role of TGFβ, a dominant negative receptor of TGFβRII was co-expressed in T cells targeting EBV, and promoted the persistence and proliferation of such T cells which showed expected result in EBV positive cancers like lymphoma and nasopharyngeal carcinoma (74). Recently, a fusion protein of PD-L1 antibody and TGFβRII truncate was engineered to express on the surface of CAR-T cells and enabled the

dual resistance to both PD-L1 and TGFβ inhibitory signaling (75). To cope with the hypoxic environment of tumors, a CAR was engineered with oxygen-sensing domain of hypoxia-inducible factor (HIF) 1α which is the critical transcription factor stabilized in hypoxia. In this way the CAR will be regulated by HIF1α to express at very low level in normal oxygen level, but it will be highly induced in hypoxia condition (76).

As discussed in previous section, the co-stimulatory domains of different factors are evaluated in CARs. The combination of different co-stimulatory domains plays an important and distinct role to promote T cell activation and persistence in tumor microenvironment. Many studies have suggested that 4-1BB is superior to CD28 in terms of promoting differentiation and persistence. Mechanistically, 4-1BB signaling could reprogram the metabolism of T cells to greater level of fatty acid oxidation and mitochondria generation than CD28, which preferentially turns on the glycolysis pathway (11,77). In addition, the co-expression of cytokines and receptors vital for T cell proliferation is another effective approach. The cytokines include IL-4, IL-7, IL-15, IL-12 and IL-18 (78-80). A CAR targeting prostate stem cell antigen was engineered with a chimera cytokine receptor composed of the extracellular domain of IL-4 receptor and the intracellular domain of IL-7. This kind of cells showed enhanced proliferation and good anti-tumor ability against pancreatic cancer (81).

The new direction of CAR

The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) technology has been the most exciting progress in biotechnology. Coincidentally it was listed as the runner-up of the Top Ten Advances of the Year 2013 in Science journal in which the champion is immunotherapy for cancer. Simply put, CRISPR can be combined with CAR-T therapy to make possible the following innovative design: i) precise editing of T cells to remove the inhibitory signaling receptor like PD-1 pathway (82,83); ii) precise insertion of CAR construct into specific locus of genome, such as TCR promoter region (84); iii) using CRISPR or other genome editing tool to knockout HLA and TCR to generate the off-the-shelf CAR-T for universal application (85-87).

The application of CAR-T therapy has expanded beyond cancer, recently CD19 CAR-T is also tested in autoimmune diseases like systemic lupus erythematosus, as B cells play an essential role in the pathogenesis (88). CAR targeting myelin oligodendrocyte glycoprotein was also tested in multiple sclerosis model. More of this topic is reviewed in this article (4). CAR-T engineered with

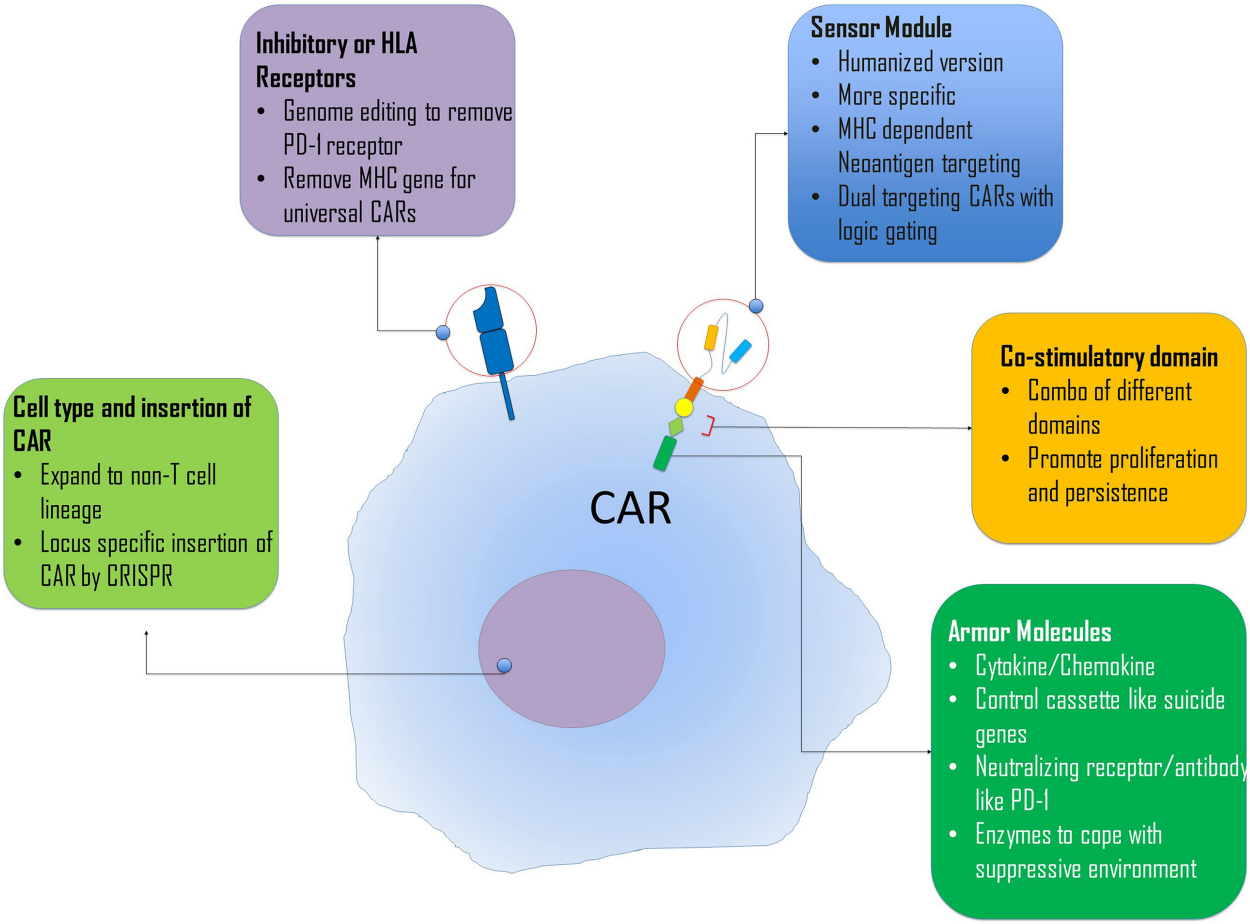

Figure 3. The summary of CAR design listing the current focus of various CAR designs.

broadly effective neutralizing antibody also showed enhanced elimination of HIV-infected cells (89).

Conclusion

As the continuation of the concept of adoptive cell therapy, CAR-T technology has been the ensemble of the magic bullet dream for cancer therapy (**Figure 3**). It represents the future generation of medicine as a living platform integrated with sophisticated system biology and engineering modules with expandability, flexibility and remarkable controllability (6,90). Recent success in treating acute lymphoblastic leukemia when conventional treatments failed to deliver continues to inspire the academics and the public (3). Combination of CAR with latest immunotherapy knowledge and cutting-edge genome editing tools will make cancer a chronic disease in the near future. And this technology can also apply to cell lineages other than T cells (91) and it will be the hope for other lingering threats for human health like autoimmune and infectious diseases.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015;136:E359-86.
2. Couzin-Frankel J. Breakthrough of the year 2013. Cancer immunotherapy. Science 2013;342:1432-3.
3. M Y. No cancer cells detected: first major step to recovery for British boy in Singapore for experimental treatment. Channel News Asia 2020 Jan 16.
4. Maldini CR, Ellis GI, Riley JL. CAR T cells for infection, autoimmunity and allotransplantation. Nat Rev Immunol 2018;18:605-16.
5. Seimetz D, Heller K, Richter J. Approval of first CAR-Ts: have we solved all hurdles for ATMPs? Cell Med 2019;11:2155179018822781.
6. Fischbach MA, Bluestone JA, Lim WA. Cell-based therapeutics: the next pillar of medicine. Sci Transl Med 2013;5:179ps7.
7. Brubaker SW, Bonham KS, Zanoni I, Kagan JC. Innate immune pattern recognition: a cell biological perspective. Annu Rev Immunol 2015;33:257-90.
8. Cooper MD, Alder MN. The evolution of adaptive immune systems. Cell 2006;124:815-22.
9. Germain RN, Stefanova I. The dynamics of T cell receptor signaling: complex orchestration and the key roles of tempo and cooperation. Annu Rev Immunol 1999;17:467-522.
10. Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. Nat Rev Immunol 2013;13:227-42.
11. van der Stegen SJ, Hamieh M, Sadelain M. The pharmacology of second-generation chimeric antigen receptors. Nat Rev Drug Discov 2015;14:499-509.
12. Burugu S, Dancsok AR, Nielsen TO. Emerging targets in cancer immunotherapy. Semin Cancer Biol 2018;52:39-52.
13. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 1986;136:2348-57.
14. Steinman L. A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. Nat Med 2007;13:139-45.
15. Chatila TA. Role of regulatory T cells in human diseases. J Allergy Clin Immunol 2005;116:949-59; quiz 60.
16. Dustin ML. The cellular context of T cell signaling. Immunity 2009;30:482-92.
17. Valent P, Groner B, Schumacher U, Superti-Furga G, Busslinger M, Kralovics R, et al. Paul Ehrlich (1854-1915) and his contributions to the foundation and birth of translational medicine. J Innate Immun 2016;8:111-20.
18. Rosenberg SA, Packard BS, Aebersold PM, Solomon D, Topalian SL, Toy ST, et al. Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. A preliminary report. N Engl J Med 1988;319:1676-80.
19. Rosenberg SA, Restifo NP, Yang JC, Morgan RA, Dudley ME. Adoptive cell transfer: a clinical path to effective cancer immunotherapy. Nat Rev Cancer 2008;8:299-308.
20. Levine BL, Bernstein WB, Aronson NE, Schlienger K, Cotte J, Perfetto S, et al. Adoptive transfer of costimulated CD4+ T cells induces expansion of peripheral T cells and decreased CCR5 expression in HIV infection. Nat Med 2002;8:47-53.
21. Riddell SR, Watanabe KS, Goodrich JM, Li CR, Agha ME, Greenberg PD. Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. Science 1992;257:238-41.
22. Gross G, Waks T, Eshhar Z. Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity. Proc Natl Acad Sci U S A 1989;86:10024-8.
23. Kuwana Y, Asakura Y, Utsunomiya N, Nakanishi M, Arata Y, Itoh S, et al. Expression of chimeric receptor composed of immunoglobulin-derived V regions and T-cell receptor-derived C regions. Biochem Biophys Res Commun 1987;149:960-8.
24. Irving BA, Weiss A. The cytoplasmic domain of the T cell receptor zeta chain is sufficient to couple to receptor-associated signal transduction pathways. Cell 1991;64:891-901.
25. Drent E, Poels R, Ruiter R, van de Donk N, Zweegman S, Yuan H, et al. Combined CD28 and 4-1BB costimulation potentiates affinity-tuned chimeric antigen receptor-engineered T cells. Clin Cancer Res 2019;25:4014-25.
26. Quintarelli C, Orlando D, Boffa I, Guercio M, Polito VA, Petretto A, et al. Choice of costimulatory domains and of cytokines determines CAR T-cell activity in neuroblastoma. Oncoimmunology 2018;7:e1433518.
27. Adachi K, Kano Y, Nagai T, Okuyama N, Sakoda Y, Tamada K. IL-7 and CCL19 expression in CAR-T cells improves immune cell infiltration and CAR-T cell survival in the tumor. Nat Biotechnol 2018;36:346-51.
28. Gardner RA, Finney O, Smithers H, Leger K, Annesley CE, Summers C, et al. Prolonged functional persistence of CD19 CAR t cell products of defined CD4:CD8 composition and transgene expression determines durability of MRD-negative ALL remission. J Clin Oncol 2016;34:3048.
29. Davila ML, Riviere I, Wang X, Bartido S, Park J, Curran K, et al. Efficacy and toxicity management of 19-28z CAR T cell therapy in B cell acute lymphoblastic leukemia. Sci Transl Med 2014;6:224ra25.
30. Brentjens RJ, Davila ML, Riviere I, Park J, Wang X, Cowell LG, et al. CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. Sci Transl Med 2013;5:177ra38.
31. Lee DW, Gardner R, Porter DL, Louis CU, Ahmed N, Jensen M, et al. Current concepts in the diagnosis and management of cytokine release syndrome. Blood 2014;124:188-95.
32. Lee DW, Kochenderfer JN, Stetler-Stevenson M, Cui YK, Delbrook C, Feldman SA, et al. T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. Lancet 2015;385:517-28.

33. Brudno JN, Kochenderfer JN. Toxicities of chimeric antigen receptor T cells: recognition and management. Blood 2016;127:3321-30.
34. Brudno JN, Somerville RP, Shi V, Rose JJ, Halverson DC, Fowler DH, et al. Allogeneic T cells that express an anti-CD19 chimeric antigen receptor induce remissions of B-cell malignancies that progress after allogeneic hematopoietic stem-cell transplantation without causing graft-versus-host disease. J Clin Oncol 2016;34:1112-21.
35. Maude SL, Frey N, Shaw PA, Aplenc R, Barrett DM, Bunin NJ, et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. N Engl J Med 2014;371:1507-17.
36. Cruz CR, Micklethwaite KP, Savoldo B, Ramos CA, Lam S, Ku S, et al. Infusion of donor-derived CD19-redirected virus-specific T cells for B-cell malignancies relapsed after allogeneic stem cell transplant: a phase 1 study. Blood 2013;122:2965-73.
37. Turtle CJ, Hanafi LA, Berger C, Gooley TA, Cherian S, Hudecek M, et al. CD19 CAR-T cells of defined CD4+:CD8+ composition in adult B cell ALL patients. J Clin Invest 2016;126:2123-38.
38. Fry TJ, Shah NN, Orentas RJ, Stetler-Stevenson M, Yuan CM, Ramakrishna S, et al. CD22-targeted CAR T cells induce remission in B-ALL that is naive or resistant to CD19-targeted CAR immunotherapy. Nat Med 2018;24:20-8.
39. Porter DL, Hwang WT, Frey NV, Lacey SF, Shaw PA, Loren AW, et al. Chimeric antigen receptor T cells persist and induce sustained remissions in relapsed refractory chronic lymphocytic leukemia. Sci Transl Med 2015;7:303ra139.
40. Milone MC, Fish JD, Carpenito C, Carroll RG, Binder GK, Teachey D, et al. Chimeric receptors containing CD137 signal transduction domains mediate enhanced survival of T cells and increased antileukemic efficacy in vivo. Mol Ther 2009;17:1453-64.
41. Postow MA, Sidlow R, Hellmann MD. Immune-related adverse events associated with immune checkpoint blockade. N Engl J Med 2018;378:158-68.
42. Sommermeyer D, Hill T, Shamah SM, Salter AI, Chen Y, Mohler KM, et al. Fully human CD19-specific chimeric antigen receptors for T-cell therapy. Leukemia 2017;31:2191-9.
43. Li J, Li W, Huang K, Zhang Y, Kupfer G, Zhao Q. Chimeric antigen receptor T cell (CAR-T) immunotherapy for solid tumors: lessons learned and strategies for moving forward. J Hematol Oncol 2018;11:22.
44. Martinez M, Moon EK. CAR T cells for solid tumors: new strategies for finding, infiltrating, and surviving in the tumor microenvironment. Front Immunol 2019;10:128.
45. Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. Mol Ther 2010;18:843-51.
46. Richman SA, Nunez-Cruz S, Moghimi B, Li LZ, Gershenson ZT, Mourelatos Z, et al. High-affinity GD2-specific CAR T cells induce fatal encephalitis in a preclinical neuroblastoma model. Cancer Immunol Res 2018;6:36-46.
47. Chinnasamy N, Wargo JA, Yu Z, Rao M, Frankel TL, Riley JP, et al. A TCR targeting the HLA-A*0201-restricted epitope of MAGE-A3 recognizes multiple epitopes of the MAGE-A antigen superfamily in several types of cancer. J Immunol 2011;186:685-96.
48. Morgan RA, Chinnasamy N, Abate-Daga D, Gros A, Robbins PF, Zheng Z, et al. Cancer regression and neurological toxicity following anti-MAGE-A3 TCR gene therapy. J Immunother 2013;36:133-51.
49. Min IM, Shevlin E, Vedvyas Y, Zaman M, Wyrwas B, Scognamiglio T, et al. CAR T therapy targeting ICAM-1 eliminates advanced human thyroid tumors. Clin Cancer Res 2017;23:7569-83.
50. Park S, Shevlin E, Vedvyas Y, Zaman M, Park S, Hsu YS, et al. Micromolar affinity CAR T cells to ICAM-1 achieves rapid tumor elimination while avoiding systemic toxicity. Sci Rep 2017;7:14366.
51. Kloss CC, Condomines M, Cartellieri M, Bachmann M, Sadelain M. Combinatorial antigen recognition with balanced signaling promotes selective tumor eradication by engineered T cells. Nat Biotechnol 2013;31:71-5.
52. Lanitis E, Poussin M, Klattenhoff AW, Song D, Sandaltzopoulos R, June CH, et al. Chimeric antigen receptor T Cells with dissociated signaling domains exhibit focused antitumor activity with reduced potential for toxicity in vivo. Cancer Immunol Res 2013;1:43-53.
53. Morsut L, Roybal KT, Xiong X, Gordley RM, Coyle SM, Thomson M, et al. Engineering customized cell sensing and response behaviors using synthetic Notch receptors. Cell 2016;164:780-91.
54. Roybal KT, Rupp LJ, Morsut L, Walker WJ, McNally KA, Park JS, et al. Precision tumor recognition by T cells with combinatorial antigen-sensing circuits. Cell 2016;164:770-9.
55. Fedorov VD, Themeli M, Sadelain M. PD-1- and CTLA-4-based inhibitory chimeric antigen receptors (iCARs) divert off-target immunotherapy responses. Sci Transl Med 2013;5:215ra172.
56. Grada Z, Hegde M, Byrd T, Shaffer DR, Ghazi A, Brawley VS, et al. TanCAR: a novel bispecific chimeric antigen receptor for cancer immunotherapy. Mol Ther Nucleic Acids 2013;2:e105.
57. Zah E, Lin MY, Silva-Benedict A, Jensen MC, Chen YY. T cells expressing CD19/CD20 bispecific chimeric antigen receptors prevent antigen escape by malignant B cells. Cancer Immunol Res 2016;4:498-508.
58. Patel K, Olivares S, Singh H, Hurton LV, Huls MH, Qazilbash MH, et al. Combination immunotherapy with NY-ESO-1-specific CAR⁺ T cells with T-cell vaccine improves anti-myeloma effect. Blood 2016;128:3366.
59. Jones BS, Lamb LS, Goldman F, Di Stasi A. Improving the safety of cell therapy products by suicide gene transfer. Front Pharmacol 2014;5:254.
60. Straathof KC, Pule MA, Yotnda P, Dotti G, Vanin EF, Brenner MK, et al. An inducible caspase 9 safety switch for T-cell therapy. Blood 2005;105:4247-54.
61. Di Stasi A, Tey SK, Dotti G, Fujita Y, Kennedy-Nasser A, Martinez C, et al. Inducible apoptosis as a safety switch for adoptive cell therapy. N Engl J Med 2011;365:1673-83.
62. Wang X, Chang WC, Wong CW, Colcher D, Sherman M, Ostberg JR, et al. A transgene-encoded cell surface polypeptide for selection, in vivo tracking, and ablation of engineered cells. Blood 2011;118:1255-63.
63. Craddock JA, Lu A, Bear A, Pule M, Brenner MK, Rooney CM, et al. Enhanced tumor trafficking of GD2 chimeric antigen receptor T cells by expression of the chemokine receptor CCR2b. J Immunother 2010;33:780-8.
64. Moon EK, Carpenito C, Sun J, Wang LC, Kapoor V, Predina J, et al. Expression of a functional CCR2 receptor enhances tumor localization and tumor eradication by retargeted human T cells expressing a mesothelin-specific chimeric antibody receptor. Clin Cancer Res 2011;17:4719-30.
65. Priceman SJ, Tilakawardane D, Jeang B, Aguilar B, Murad JP, Park AK, et al. Regional delivery of chimeric antigen receptor-engineered T cells effectively targets HER2(+) breast cancer metastasis to the brain. Clin Cancer Res 2018;24:95-105.
66. Nellan A, Rota C, Majzner R, Lester-McCully CM, Griesinger AM, Mulcahy Levy JM, et al. Durable regression of Medulloblastoma after regional and intravenous delivery of anti-HER2 chimeric antigen receptor T cells. J Immunother Cancer 2018;6:30.
67. Suarez ER, Chang de K, Sun J, Sui J, Freeman GJ, Signoretti S, et al. Chimeric antigen receptor T cells secreting anti-PD-L1 antibodies more effectively regress renal cell carcinoma in a humanized mouse model. Oncotarget 2016;7:34341-55.

68. Li S, Siriwon N, Zhang X, Yang S, Jin T, He F, et al. Enhanced cancer immunotherapy by chimeric antigen receptor-modified T cells engineered to secrete checkpoint inhibitors. Clin Cancer Res 2017;23:6982-92.
69. Morrot A, da Fonseca LM, Salustiano EJ, Gentile LB, Conde L, Filardy AA, et al. Metabolic symbiosis and immunomodulation: how tumor cell-derived lactate may disturb innate and adaptive immune responses. Front Oncol 2018;8:81.
70. Ohta A. A Metabolic Immune checkpoint: adenosine in tumor microenvironment. Front Immunol 2016;7:109.
71. Poggi A, Varesano S, Zocchi MR. How to hit mesenchymal stromal cells and make the tumor microenvironment immunostimulant rather than immunosuppressive. Front Immunol 2018;9:262.
72. Ligtenberg MA, Mougiakakos D, Mukhopadhyay M, Witt K, Lladser A, Chmielewski M, et al. Coexpressed catalase protects chimeric antigen receptor-redirected T cells as well as bystander cells from oxidative stress-induced loss of antitumor activity. J Immunol 2016;196:759-66.
73. Lohr J, Ratliff T, Huppertz A, Ge Y, Dictus C, Ahmadi R, et al. Effector T-cell infiltration positively impacts survival of glioblastoma patients and is impaired by tumor-derived TGF-beta. Clin Cancer Res 2011;17:4296-308.
74. Bollard CM, Tripic T, Cruz CR, Dotti G, Gottschalk S, Torrano V, et al. Tumor-specific T-cells engineered to overcome tumor immune evasion induce clinical responses in patients with relapsed Hodgkin lymphoma. J Clin Oncol 2018;36:1128-39.
75. Grenga I, Donahue RN, Gargulak ML, Lepone LM, Roselli M, Bilusic M, et al. Anti-PD-L1/TGFbetaR2 (M7824) fusion protein induces immunogenic modulation of human urothelial carcinoma cell lines, rendering them more susceptible to immune-mediated recognition and lysis. Urol Oncol 2018;36:93.e1-.e11.
76. Juillerat A, Marechal A, Filhol JM, Valogne Y, Valton J, Duclert A, et al. An oxygen sensitive self-decision making engineered CAR T-cell. Sci Rep 2017;7:39833.
77. Kawalekar OU, O'Connor RS, Fraietta JA, Guo L, McGettigan SE, Posey AD, Jr., et al. Distinct signaling of coreceptors regulates specific metabolism pathways and impacts memory development in CAR T cells. Immunity 2016;44:380-90.
78. Gargett T, Brown MP. Different cytokine and stimulation conditions influence the expansion and immune phenotype of third-generation chimeric antigen receptor T cells specific for tumor antigen GD2. Cytotherapy 2015;17:487-95.
79. Hoyos V, Savoldo B, Quintarelli C, Mahendravada A, Zhang M, Vera J, et al. Engineering CD19-specific T lymphocytes with interleukin-15 and a suicide gene to enhance their anti-lymphoma/leukemia effects and safety. Leukemia 2010;24:1160-70.
80. Zhang L, Morgan RA, Beane JD, Zheng Z, Dudley ME, Kassim SH, et al. Tumor-infiltrating lymphocytes genetically engineered with an inducible gene encoding interleukin-12 for the immunotherapy of metastatic melanoma. Clin Cancer Res 2015;21:2278-88.
81. Mohammed S, Sukumaran S, Bajgain P, Watanabe N, Heslop HE, Rooney CM, et al. Improving chimeric antigen receptor-modified T cell function by reversing the immunosuppressive tumor microenvironment of pancreatic cancer. Mol Ther 2017;25:249-58.
82. Ren J, Liu X, Fang C, Jiang S, June CH, Zhao Y. Multiplex Genome Editing to Generate Universal CAR T Cells Resistant to PD1 Inhibition. Clin Cancer Res 2017;23:2255-66
83. Schumann K, Lin S, Boyer E, Simeonov DR, Subramaniam M, Gate RE, et al. Generation of knock-in primary human T cells using Cas9 ribonucleoproteins. Proc Natl Acad Sci U S A 2015;112:10437-42.
84. Eyquem J, Mansilla-Soto J, Giavridis T, van der Stegen SJ, Hamieh M, Cunanan KM, et al. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. Nature 2017;543:113-7.
85. Ren J, Zhang X, Liu X, Fang C, Jiang S, June CH, et al. A versatile system for rapid multiplex genome-edited CAR T cell generation. Oncotarget 2017;8:17002-11.
86. Ruella M, Kenderian SS. Next-generation chimeric antigen receptor T-cell therapy: going off the shelf. BioDrugs 2017;31:473-81.
87. Torikai H, Reik A, Soldner F, Warren EH, Yuen C, Zhou Y, et al. Toward eliminating HLA class I expression to generate universal cells from allogeneic donors. Blood 2013;122:1341-9.
88. Kansal R, Richardson N, Neeli I, Khawaja S, Chamberlain D, Ghani M, et al. Sustained B cell depletion by CD19-targeted CAR T cells is a highly effective treatment for murine lupus. Sci Transl Med 2019;1.1.
89. Ali A, Kitchen SG, Chen ISY, Ng HL, Zack JA, Yang OO. HIV-1-Specific Chimeric Antigen Receptors Based on Broadly Neutralizing Antibodies. J Virol 2016;90:6999-7006
90. Lim WA, June CH. The principles of engineering immune cells to treat cancer. Cell 2017;168:724-40.
91. Patel S, Burga RA, Powell AB, Chorvinsky EA, Hoq N, McCormack SE, et al. Beyond CAR T cells: other cell-based immunotherapeutic strategies against cancer. Front Oncol 2019;9:196.

REVIEW ARTICLE

Ubiquitination in cancer stem cell: roles and targeted cancer therapy

Liu LIU, Shasha YIN, Charles BROBBEY, Wenjian GAN*

Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, SC 29425, USA.

*Correspondence: ganw@musc.edu
https://doi.org/10.37175/stemedicine.v1i3.37

ABSTRACT

Cancer stem cells (CSCs) are a small subset of stem-like cells inside tumors, which possess abilities of unlimited self-renewal, differentiation and proliferation. Extensive studies have suggested that CSCs are one of the major drivers of tumor initiation, metastasis, relapse and therapeutic resistance. Several regulatory networks including transcriptional programs and various signaling pathways tightly control the stemness, proliferation and differentiation of CSCs. Emerging evidence has indicated that post-translational modifications, especially ubiquitination, play a critical role in maintenance of CSC properties. In this review, we summarize current understandings on E3 ubiquitin ligase-mediated regulation of transcription factors and key signaling pathways involved in the regulation of CSCs, and discuss the strategy to target CSCs and E3 ubiquitin ligases for combating cancers.

Keywords: Cancer stem cell · Ubiquitination · E3 ligase · Cell signaling · Transcription factor ·

Introduction

Tumor heterogeneity is a well-known phenomenon that tumor cells derived from different tumors or the same tumor exhibit distinct genotypes and phenotypes, which increases the complexity of cancer diagnosis and treatment (1). Over the past several decades, a few models have been brought up to explain tumor heterogeneity including the predominant cancer stem cell (CSC) model (2), which states that among masses of cells inside a tumor, only a small portion of cells exhibit tumor initiation power (also termed tumor-initiating cells) (3).

In supporting of the CSC model, as early as 1800s, Virchow and Cohnheim postulated that tumors would be rooted from the embryonic cells in the body of "embryonic rests" (4). In 1997, Bonnet and Dick provided the first evidence to demonstrate that CSCs exist in acute myeloid leukemia (AML). They found that a subset of patient-derived AML cells were capable of initiating AML in immuno-suppressed mice (5). To date, CSCs have been isolated from breast, colon, ovary and many other solid tumors (6). Currently, it is broadly acknowledged that CSCs play critical roles in tumor initiation, metastasis, relapse and especially therapy resistance (7). CSCs could promote radioresistance and chemotherapy resistance via various mechanisms in different cancers, which provide CSCs with a survival advantage (8). Therefore, better understanding in CSC biology will facilitate targeting CSCs as a novel approach to combat cancers.

Cancer Stem Cells

CSCs are defined as a minority subset of cells within tumors, which have similar features as normal stem cells including self-renewal and differentiation, plus ability to form tumors (**Figure 1**) (9). CSCs may be originated from normal stem cells through accumulations of genetic alterations, which results in aberrant signaling and enables normal stem cells to obtain constitutively proliferative ability, leading to tumorigenesis (10). For example, introduction of the mutant *p53* in breast cancer mouse model enhances breast cancer progression largely in part because of the expansion of mammary stem cells (11). CSCs may also arise from transformation of somatic cells through reprogramming network controlled by transcription factors. One of milestone findings in the stem cell research field is the generation of induced pluripotent stem cells (iPSCs) by Takahashi and Yamanaka in 2006. They found that over-expression of transcription factors Oct3/4, Sox2, c-Myc and Klf4 is sufficient to convert the mouse adult somatic cells into pluripotent embryonic-like cells under embryonic stem cell (ESC) culture conditions (12). Later studies have identified more critical pluripotency factors that can generate human iPSCs

Received: Feb 12, 2020; Accepted: Mar 3, 2020.

including Nanog (13, 14), LIN28 (15) and Glis1 (16) (**Figure 1**). These proteins are aberrantly activated in many cancers and CSCs. Notably, a recent study showed that over-expression of SOX2, POU3F2, OLIG2 and SALL2 transcription factors could convert the differentiated glioblastoma cells into fully tumorigenic CSCs (17).

Importantly, several core stemness signaling pathways including Notch, Wnt/β-Catenin, Hedgehog, JAK/STAT and NF-κB pathways (**Figure 1**) are involved in the regulation of CSC properties (18). These pathways are aberrantly activated in CSCs and associated with CSC-mediated tumorigenesis including leukemia, breast cancer, lung cancer and other solid tumors. For example, the activated form of STAT3 was significantly upregulated in breast CSC-like cells and inhibition of STAT3 resulted in decreased breast CSC proliferation and clonogenicity (19, 20). These stemness pathways cooperate with pluripotency factors to maintain CSC properties. Interestingly, the JAK/STAT3 signaling and OCT4 have a positive feedback loop: activation of STAT3 upregulates the mRNA levels of OCT4, while OCT4 could boost the activation of the JAK/STAT3 pathway (21, 22).

Ubiquitination System

Post-translational modifications (PTMs) are the key contributors to proteome diversity by conferring various functions on proteins. Ubiquitination is one of the most studied PTMs, which covalently conjugates the small protein ubiquitin (Ub) to the lysine residues (23). Ubiquitination process is a sequential enzymatic cascade consisting of three types of enzymes: ubiquitin-activating enzymes (termed E1s), ubiquitin-conjugating enzymes (termed E2s) and E3 ubiquitin ligases (termed E3s) (**Figure 2**) (24). The E3s are the critical components responsible for the recognition of substrates and determination of substrate specificity. It is predicted that there are more than 600 E3s in human, which can be classified into three major subfamilies: the RING (really interesting new gene) E3s, the HECT (homologous to the E6AP carboxyl terminus domain) E3s, and the RBR (RING-between-RING) E3s (25). These E3s are frequently deregulated in various human diseases and are emerging as attractive therapeutic targets (26).

Ubiquitination pathway regulates protein functions in many ways: marking them for proteasomal-mediated degradation, alteration of their cellular locations, and modulation of protein interactions (25). Ubiquitin can form seven types of poly-ubiquitin linkages on substrates through seven lysine residues (K6, K11, K27, K29, K33, K48 and K63), which serves as different signals to control protein functions. It is widely accepted that K11- and K48-poly-ubiquitin linkages are the proteasome degradation markers, while K63-poly-ubiquitin linkage serves as a non-proteolytic modification in regulating protein activity, localization and signaling transduction (27). Therefore, ubiquitination pathway controls many fundamental biological processes such as replication, transcription and cell signaling transduction that regulate cell proliferation, apoptosis and tumorigenesis. In addition, ubiquitination pathway is a critical determinant of CSC cell fate, which regulates the activation of pluripotency factors and stemness signaling pathways (28).

Regulation of CSC-Related Factors by Ubiquitination

As the abundance of pluripotency factors is the key decider of cell fate, the expression of these factors may be regulated at DNA, RNA and protein levels. Notably, more than 80% of

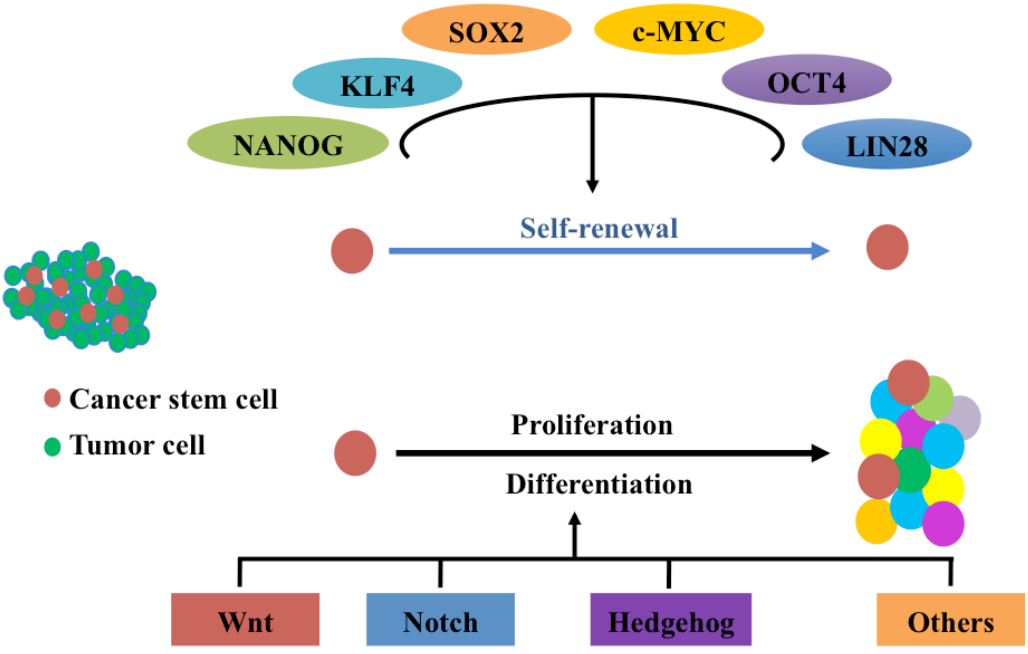

Figure 1. A schematic representation of key transcription factors and molecular signaling pathways involved in CSCs.

proteins in cells are degraded by the ubiquitin-proteasome system (UPS) (29). A mass of evidences suggests that the UPS plays a critical role in controlling CSC properties via regulating the abundance of CSC-related proteins (**Table 1**).

Oct3/4 (POU5F1)

The proper protein levels of Oct3/4 determine distinct cell fate of ESCs. A study has found that down-regulation of Oct3/4 leads to loss of pluripotency, whereas less than two-folds up-regulation of Oct3/4 causes differentiation (30). Similarly, higher Oct4 expression promotes CSC expansion and tumorigenesis in breast cancer mouse model (31). In bladder cancer patient samples, higher expression levels of Oct4 are observed in more advanced cancers and contribute to poor survival (32). A few E3 ubiquitin ligases have been reported to control the Oct4 protein stability. WWP2, a HECT-type E3, interacts with and ubiquitinates Oct4 for 26S proteasomal degradation upon the differentiation of ESCs (33). Itch, another HECT-type E3, catalyzes K63-linked poly-ubiquitination of Oct4, which enhances Oct4 protein stability. Depletion of *Itch* decreases Oct4 expression and significantly suppresses ESCs and iPSCs (34). A recent study has also found that the E3 ligase CHIP (carboxy terminus of HSP70-interacting protein) is a novel partner of Oct4, which promotes Oct4 ubiquitination and degradation via the 26S proteasome. Depletion of *CHIP* promotes breast CSCs, tumorigenesis and tumor metastasis in breast cancer mouse model. Importantly, a reverse correlation between the expression of CHIP and Oct4 was observed in breast cancer patients (35).

Sox2

The stem cell-related transcription factor Sox2 has participated in the maintenance of CSCs in a variety of cancers, including skin and breast cancers. Overexpression of Sox2 enhances tumor initiation and metastasis (36). The E3 ligase WWP2 could target methylated Sox2 for ubiquitination and degradation, leading to cell differentiation (37). Interestingly, the Ube2s, an E2 ubiquitin-conjugating enzyme, mediates the K11-linked poly-ubiquitination of Sox2, resulting in its degradation by proteasome (38). More recently, $CUL4A^{DET1\text{-}COP1}$, belonging to the Cullin-RING finger E3 family, was reported to catalyze Sox2 poly-ubiquitination and degradation upon neural progenitor differentiation (39). These studies indicate that the E3 ligases of Sox2 may govern cancer progression through regulating CSC functions.

KLF4

The role of the Krüppel-like factor (4KLF4) in cancers is context-dependent. It is a tumor suppressor and down-regulated in gastric cancer, liver cancer and lung cancer, whereas it is upregulated in breast cancer and osteosarcoma (40). CSC-enriched spheroid breast cancer cells display higher expression of KLF4. Consistently, overexpression of KLF4 increases CSC population and tumorigenesis in breast cancer (41). The abundance of KLF4 can be regulated by several E3 ubiquitin ligases. FBXO32, a member of SCF E3 ligase subfamily, suppresses breast tumorigenesis by promoting ubiquitination and degradation of KLF4 (42). Mule (Mcl-1 ubiquitin ligase E3), a HECT-type E3, could target KLF4 for degradation to promote entry into S phase and enhance proliferation of T cells (43). Interestingly, the protein levels of TRAF7 (tumor necrosis factor receptor-associated factor 7) are elevated in liver cancer, which is inversely correlated with the KLF4 expression. Further

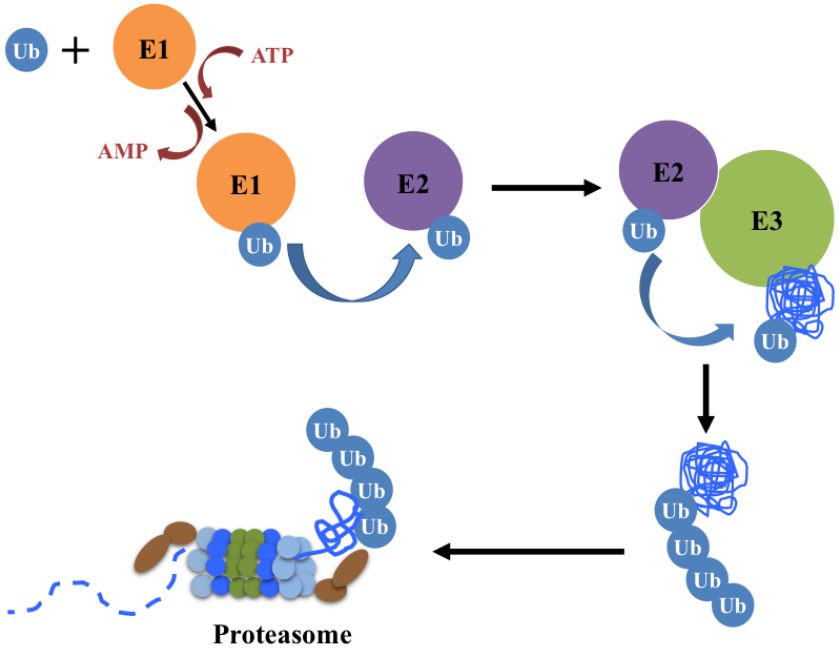

Figure 2. A schematic representation of the ubiquitin-proteasome system.

Table 1: The summary of E3 ligases in regulation of CSC-related proteins

CSC-related protein	E3 ligase	Effect	Reference
Transcription Factors			
Oct3/4 (POU5F1)	WWP2	Degradation	33
	ITCH	Degradation	34
	CHIP	Degradation	35
Sox2	WWP2	Degradation	37
	COP1	Degradation	39
KLF4	FBXO32	Degradation	42
	Mule	Degradation	43
	TRAF7	Degradation	44
c-Myc	Fbw7	Degradation	48-51
	β-TRCP	Stabilization	52
	HectH9	Enhanced activity	53
	KCTD2	Degradation	54
Nanog	SPOP	Degradation	57
	FBXW8	Degradation	58
LIN28	TRIM71	Degradation	61
Notch Signaling Pathway			
Notch	Fbw7	Degradation	64, 66
	Itch	Degradation	67
DLL1, DLL4, JAG1, JAG2	MIB1/MIB2	Degradation	68
	NEUR1/NEUR2	Degradation	68
Wnt Signaling Pathway			
FZD, LRP6	ZNRF3, RNF43	Degradation	71, 72
DVL	Itch	Degradation	74
	NEDD4L	Degradation	75
β-catenin	β-TRCP	Degradation	76
	RNF146	Degradation	77
Axin	Smurf1	Inactivation	78
	SIAH1	Degradation	79
APC	RNF61	Degradation	80
Hedgehog (Hh) Signaling Pathway			
PTCH1	Smurf1, Smurf2		84
	Itch		85
SMO	Unknown	Degradation	86, 87
SuFu	Fbx117	Degradation	88
	Itch/β-arrestin2	Inactivation	89
GLI1	β-TRCP	Degradation	93
GLI2/3	β-TRCP	Partial degradation	90, 91
	SPOP	Degradation	92

study demonstrates that TRAF7 functions as an E3 ligase of KLF4 to promote KLF4 degradation and enhance cancer progression (44). Therefore, these E3 ligases may be responsible for the deregulation of KLF4 in various cancers.

c-Myc

The transcription factor c-Myc is a well-known oncogene that is overexpressed in more than 40% of human cancers. It controls all hallmarks of cancer including genome instability and sustaining proliferation (45). Extensive

studies demonstrate that c-Myc serves as a key factor in the maintenance of CSCs. Inhibition of c-Myc leads to a decrease in CSC population by inducing senescence (46). UPS-mediated degradation of c-Myc represents a main mechanism for controlling its abundance. c-Myc has a short protein half-life, approximately 20-30 minutes (47). There are several E3 ubiquitin ligases responsible for the regulation of c-Myc expression. The E3 ligase Fbw7 (F-box and WD repeat domain-containing 7) could promote c-Myc degradation, which requires prior phosphorylation by glycogen synthase kinase 3 (GSK3) (48). In a chronic myeloid leukemia mouse model, knockout of *Fbw7* elevates c-Myc protein to re-initiate the cell cycle in leukemia-initiating cells (49-51). Interestingly, another F-box E3 ligase β-TRCP catalyzes K63-linked poly-ubiquitination on c-Myc, which stabilizes c-Myc protein by inhibiting Fbw7-mediated degradation (52). The HECT-domain E3 ligase HectH9 also catalyzes poly-ubiquitination of c-Myc with K63 linkage and consequently enhances c-Myc protein stability, promoting cell proliferation (53). The KCTD2 (potassium channel tetramerization domain-containing 2), a Cullin3-based E3, was also reported to promote degradation of c-Myc. Deletion of *KCTD2* elevates c-Myc protein levels and confers CSC properties to glioma cells (54). Other E3 ligases including Skp2, TRIM32, Fbx29 and CHIP also control c-Myc stability.

Nanog

Nanog is upregulated in various cancers and CSCs and correlates with the stage and prognosis of cancers (55). Overexpression of Nanog enhances pluripotency and unlimited proliferation of CSCs (56). Recent studies have revealed that Nanog can be ubiquitinated and subsequently degraded by SPOP, a Cullin 3-based E3, leading to stemness loss of prostate cancer cells (57). The FBXW8 (F-box and WD40 domain-containing protein 8) induces stem cell differentiation by targeting Nanog for degradation (58).

LIN28

LIN28 is another reprogramming factor that can promote pluripotency by suppressing expression of microRNA let-7. LIN28 is an evolutionarily conserved RNA-binding protein that is highly expressed in ESCs and CSCs. It plays a critical role in the regulation of CSC pluripotency and is considered as a marker of CSCs. Depletion of *LIN28* eradicates CSCs in ovarian cancer. Aberrant expression of LIN28A/LIN28B is observed in more aggressive cancers, and contributes to poor prognosis and drug resistance in certain cancer types (59, 60). TRIM71, a member of the tripartite-motif (TRIM) E3 family, negatively regulates LIN28B protein stability via ubiquitin-mediated proteasomal degradation, which leads to tumor suppression (61). However, it is largely unknown how LIN28 protein stability is regulated by other E3 ligases.

Regulation of Stemness Signaling Pathways by Ubiquitination

PTMs are the heart of the signaling transduction, which can confer distinct functions to proteins in response to various environment changes (62). Ubiquitination, one of the most common PTMs, is a key player in controlling the activation of core stemness signaling pathways (**Table 1**).

Notch Signaling Pathway

The Notch signaling pathway is evolutionarily conserved from Drosophila to human. It has important roles in dictating development, tissue renewal, tumor initiation and metastasis. Canonical Notch signaling involves two adjacent cells expressing the Notch receptors and the ligands. Four Notch receptor paralogues (Notch1-4) and five Notch ligands (DLL1, DLL3, DLL4, JAG1 and JAG2) were identified in mammals (63). Both Notch receptors and ligands can be regulated by ubiquitination.

The Notch intracellular domain (NICD) contains a PEST domain (rich in proline, aspartic acid, serine and threonine residues) that can be recognized by E3 ligases. Upon activation, the NICD is promptly ubiquitinated and degraded by the E3 ubiquitin ligase Fbw7 in mammals and its ortholog SEL-10 in *Caenorhabditis elegans* (64). Constitutively active form of Notch with deletion of the PEST domain has been observed in some T-cell acute lymphoblastic leukemia (65). Moreover, loss of *Fbw7* in neural stem cells (NSCs) elevates Notch protein levels, leading to imbalance between self-renewal and differentiation, and finally aberrant brain development (66). Interestingly, the non-activated Notch is ubiquitinated with K29-linkage by Itch/AIP4 E3 ubiquitin ligase and subsequently subjected for lysosomal degradation (67). Studies have also showed that DLL1, DLL4, JAG1 and JAG2 undergo ubiquitination mediated by the RING family E3 ligases, MIB1/MIB2 and NEUR1/NEUR2, which trigger ligand endocytosis (68). Despite advances in understanding the roles of ubiquitination in Notch signaling, it is unclear how these events contribute to CSC and cancer progression.

Wnt Signaling Pathway

Similar to the Notch pathway, the Wnt signaling pathway is another key cascade in controlling stemness and malignant growth. It is hyper-activated in different types of cancers particularly colorectal cancer. Notably, high Wnt activity is considered as a marker of colon cancer stem cells and promotes CSC expansion through up-regulation of its downstream targets including CCND1, FOXM1, MYC and YAP/TAZ (69). The core components of canonical Wnt signaling pathway include receptor Frizzled (FZD), co-receptors LRP5/6, the scaffolding protein Dishevelled (DVL), the major effector β-catenin and destruction complex containing Axin, APC and GSK3β and casein kinase (CK1α) (70). These components can be regulated by the ubiquitination system, which contribute to the temporal and spatial regulation of Wnt signaling pathway activation.

Studies have showed that the zinc and ring finger 3 (ZNRF3) and ring finger 43 (RNF43) E3s target FZD

and LRP6 for ubiquitination-dependent lysosomal degradation, leading to a decrease of FZD receptor at the cell surface (71, 72).

Multiple E3s are involved in regulation of the DVL protein stability. The Cullin-3 based E3 ligase, KLHL12, promotes DVL poly-ubiquitination and degradation in the absence of Wnt (73). Itch, a HECT-type E3, promotes ubiquitination and degradation of phosphorylated DVL depending on the PPXY motif and the DEP domain of DVL (74). The NEDD4L catalyzes the K6-, K27- and K29-linked atypical ubiquitin chains for targeting DVL degradation (75). Without the Wnt ligands, β-catenin is phosphorylated by the destruction complex and subsequently recognized and ubiquitinated by β-TRCP (76).

As a key determinant of the destruction complex, the expression of Axin is tightly controlled. Poly-ADP-ribosylated Axin can be recognized and ubiquitinated by the RING E3 ligase RNF146, leading to Axin degradation (77). Smurf1, a HECT-type E3, catalyzes non-proteolytic K29-linked ubiquitin chains on Axin and consequently impairs Axin interaction with LRP5/6, leading to shutdown of the Wnt signaling pathway. Interestingly, Itch-mediated ubiquitination of Axin is cell-cycle-dependent (78). More recently, a study has found that in the presence of Wnt stimulation, the seven in absentia homolog 1 (SIAH1) competes with GSK to bind and degrade Axin, providing a positive feedback activation of the Wnt signaling (79). Ubiquitination also governs the protein levels of APC to control the function of the destruction complex. Overexpression of MKRN1 E3 ligase induces ubiquitination and degradation of APC. In contrast, knockout of MKRN1 leads to accumulation of APC, which suppresses Wnt pathway activation and cell migration (80).

In addition to the ubiquitination-mediated protein turnover, APC and DVL also undergo K63-linked non-proteolytic poly-ubiquitination, while Axin can form K29-linked poly-ubiquitination, all of which are important for the activation of Wnt signaling (81). As most of these E3 ubiquitin ligases are deregulated in cancers, these studies offer a possible explanation for the aberrant activation of Wnt signaling in CSCs and various cancers.

Hedgehog (Hh) Signaling Pathway

The controlled Hh signaling pathway is crucial for embryogenesis and proper organ growth. Its aberrant activation may promote tumorigenesis, tumor metastasis and drug resistance, which has been documented in leukemia, pancreatic cancer and many other solid tumors (82). Accumulating evidence demonstrates that the Hh pathway is critical for the maintenance and expansion of CSCs. The expression of the core Hh pathway components, including SMO, PTCH1, GLI2/3 and SuFu, is significantly up-regulated in CSCs (83). Growing evidence suggests that deregulation of ubiquitination on these components is a predominate cause for the aberrancy of the Hh signaling pathway.

PTCH1 contains two PPXY motifs in the cytoplasmic C-tail, which mediates its interaction with Smurf1/2, Nedd4, WWP2 and Itch that are HECT-type E3s. Upon Shh stimulation, the expression of Smurf1/2 is up-regulated and targets PTCH1 for degradation by catalyzing poly-ubiquitin chains with K48 and K63 linkages. Knockout of *Smurf1*/*Smurf2* in mice impairs Shh-induced cerebellar organogenesis (84). In the absence of Hh signaling, Itch targets PTCH1 for ubiquitination and degradation (85). Although Nedd4 and WWP2 interact with PTCH1, they do not regulate PTCH1 stability.

SMO can be poly/mono-ubiquitinated, resulting in its degradation by lysosome or 26S proteasome, which is inhibited by Hh stimulation (86, 87). However, the E3 ligases of SMO have not been identified yet.

Sufu is a tumor suppressor and a negative regulator of the Hh signaling pathway by sequestering GLI transcription factors in the cytoplasm. In response to Shh ligand, Sufu is ubiquitinated and degraded by E3 ligase Fbxl17 (F-box and leucine-rich repeat protein 17), leading to the activation of Hh signaling. Knockdown of Fbxl17 leads to the accumulation of Sufu protein and reduction of tumor growth (88). SuFu also undergoes non-proteolytic K63-linked poly-ubiquitination catalyzing by the Itch/β-arrestin2 complex, which is inhibited by the Hh signaling. This event enhances SuFu interaction with GLI3 and keeps Hh signaling off, contributing to tumor suppression (89).

The ubiquitination modification of GLI transcription factors negatively regulates Hh pathway activation. In the absence of Hh, E3 ligase β-TRCP binds and ubiquitinates phosphorylated GLI2/3 that is mediated by kinases PKA, GSK3β and CK1. As a result, GLI2/3 are partially degraded to generate the repressor form (90, 91). In the presence of Hh, Cul3-based E3 ligase SPOP could target the activated full-length form of GLI2/3 for ubiquitination-mediated proteasomal degradation, which serves as a negative feedback regulation of Hh pathway activation (92). Interestingly, β-TRCP also targets GLI1 for complete proteolysis, without generation of the repressor form (93).

Other Stemness Signaling Pathways

The ubiquitination modification also has important functions in governing the activation of other stemness pathways including the NF-κB, JAK/STAT and PI3K/AKT pathways, which has been well discussed in other reviews (94-96).

CSC-Targeting Therapies

As CSCs are a key factor conferring drug-resistance, tumor recurrence and metastasis, targeting CSCs is becoming a potential and promising therapeutic approach. Growing evidence indicates that inactivation of CSC-related transcription factors or signaling pathways can significantly suppress cancer progression and increase the cellular sensitivity to chemotherapy and radiotherapy in preclinical studies. To this end, many CSC-targeted agents have been developed and entered clinical trials (**Table 2**).

Targeting Stemness Pathways

Aberrant activation of stemness controlling pathways

Table 2: The summary of drugs targeting the CSC-related proteins

CSC-related proteins	Compound	Development phase	Reference
Oct3/4 (POU5F1)	KRIBB53	Preclinical	110
c-Myc	MYCi361	Preclinical	111
	10058-F4	Preclinical	112
	GSK525762	Phase I, II	113
LIN28	1632	Preclinical	114
Notch	LY3039478	Phase I	115
	MK0752	Phase I	116
	AL101	Phase I, II	117, 118
FZD,	OMP-18R5 (Vantictumab)	Phase I	119
DVL	NSC668036	Preclinical	120
β-catenin	PRI-724	Phase I, II	121
	E7368	Phase I	122
	BC-2059	Phase I	123
Axin	IWR-1-endo	Preclinical	124
SMO	Vismodegib	FDA approved	125
	Patidegib	Phase III	126
	Taladegib	Phase I, II	127

leads to the unlimited self-renewal and proliferation of CSCs, eventually tumorigenesis and drug resistance. Therefore, targeting these pathways might be a promising strategy to abrogate CSCs and cancers. One of the major Notch pathway inhibitors is the γ-secretase inhibitor (GSI), which comprises the formation of matured NICD by blocking proteolytic cleavages of Notch receptors. GSI has demonstrated strong anti-tumor activity in part by inducing apoptosis of CSCs (97). Combination of GSI with 5-fluorouracil enhances the inhibition on clonogenicity and tumorigenicity of CSCs (98). The Hh inhibitor vismodegib that targets SMO was used to clinically treat basal cell carcinoma and approved by the US Food and Drug Administration in 2012 (99). Many Wnt pathway inhibitors targeting FZD receptors, DVL and β-catenin are in early clinical trials (100).

Targeting CSC-related Transcription Factors

It is a historical challenge to directly target transcription factors for cancer therapy because the inhibitors targeting protein-DNA interaction are difficult to develop as drug-like properties (101). However, emerging research evidence demonstrates that targeting the epigenetic signaling has the potential to be an effective approach for diminishing CSCs (102). Overexpression of JMJD3, a histone H3K27me3 demethylase, decreases OCT4 expression, which results in diminished CSCs and restarted tumor growth in breast cancer (103). BET inhibitor, JQ1, which competes BRD4 binding with acetylated histones at the enhancer of *c-Myc*, markedly decreases its expression, resulting in suppression of tumor growth in multiple cancer models (104, 105). Thus, inhibitors of epigenetic programming that suppress the expression of CSC-related transcription factors, might overcome drug resistance by abrogating CSCs.

Targeting CSC-related E3 Ubiquitin Ligases

As most of CSC-related E3 ubiquitin ligases are frequently defective in cancers, small molecules are needed to restore their expression and function. The milestone for targeting E3 ligases is the development of proteolysis-targeting chimeras (PROTACs) technology. Mechanically, PROTAC is a bifunctional molecule that bridges an E3 ubiquitin ligase and a target protein, promoting ubiquitination and degradation of the target protein by the hijacked E3 ligase (106). Notably, PROTACs including dBET1 and ARV-825 could hijack the E3 ubiquitin ligase cereblon to bind BRD4, resulting in more robust degradation of c-Myc, apoptosis induction and tumor growth compared to BET inhibitors (107, 108). It was also reported that oridonin, a natural diterpenoid compound, could induce Fbw7 expression and GSK-3 activation, resulting in degradation of c-Myc (109). Therefore, reactivation of defective E3 ubiquitin ligases by either PROTAC possesses great potential for pursuing effective therapeutics.

Conclusions and Perspectives

All of the above illustrative examples highlight the role of E3s in control of CSC features and functions on cancer progression. However, knowledge on the ubiquitination and CSCs is far away to be completed. For example, E3 ubiquitin ligases controlling the stability of many CSC-related proteins have not been identified or limited, including Nanog, LIN28 and FZD. Moreover, whether and how the non-proteolytic ubiquitination regulates

CSC biology remains largely unknown. Protein-protein interaction analyses and genome-wide CRISPR screen will contribute to address these questions.

A growing amount of evidence suggests that E3s are potential targets for cancer therapy. Although it is at the early stage for the exploration of inhibitors or activators targeting E3 ubiquitin ligases, the results from preclinical studies and clinical trials thus far are highly promising and encouraging. Particularly, PROTACs offer an excellent opportunity to restore the function of E3 ligases for degrading many undruggable oncoprotein targets including transcription factors. For example, the cancer-derived SPOP mutants fail to bind its substrates, such as Nanog and GLI2/3. Developing PROTACs for SPOP might restore its ability to target these oncoproteins. Therefore, better understanding the E3 ubiquitin ligases and CSCs will facilitate identification of novel therapeutic targets and approaches to combat cancers.

Acknowledgements

This work was supported in part by pilot research funding from Hollings Cancer Center's Cancer Center Support Grant P30 CA138313 at the Medical University of South Carolina, and by the grant R00CA207867 (W.G.) from National Cancer Institute.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Fisher R, Pusztai L, Swanton C. Cancer heterogeneity: implications for targeted therapeutics. Br J Cancer. 2013;108(3):479-85.
2. Marusyk A, Polyak K. Tumor heterogeneity: causes and consequences. Biochim Biophys Acta. 2010;1805(1):105-17.
3. Kreso A, Dick JE. Evolution of the cancer stem cell model. Cell Stem Cell. 2014;14(3):275-91.
4. Capp JP. Cancer stem cells: from historical roots to a new perspective. J Oncol. 2019;2019:5189232.
5. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med. 1997;3(7):730-7.
6. Bighetti-Trevisan RL, Sousa LO, Castilho RM, Almeida LO. Cancer stem cells: powerful targets to improve current anticancer therapeutics. Stem Cells Int. 2019;2019:9618065.
7. Ayob AZ, Ramasamy TS. Cancer stem cells as key drivers of tumour progression. J Biomed Sci. 2018;25(1):20.
8. Phi LTH, Sari IN, Yang YG, Lee SH, Jun N, Kim KS, et al. Cancer stem cells (CSCs) in drug eesistance and their therapeutic implications in cancer treatment. Stem Cells Int. 2018;2018:5416923.
9. Batlle E, Clevers H. Cancer stem cells revisited. Nat Med. 2017;23(10):1124-34.
10. Plaks V, Kong N, Werb Z. The cancer stem cell niche: how essential is the niche in regulating stemness of tumor cells? Cell Stem Cell. 2015;16(3):225-38.
11. Shetzer Y, Molchadsky A, Rotter V. Oncogenic mutant p53 gain of function nourishes the vicious cycle of tumor development and cancer stem-cell formation. Cold Spring Harb Perspect Med. 2016;6(10).
12. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006;126(4):663-76.
13. Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, et al. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell. 2003;113(5):643-55.
14. Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, et al. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell. 2003;113(5):631-42.
15. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, et al. Induced pluripotent stem cell lines derived from human somatic cells. Science. 2007;318(5858):1917-20.
16. Maekawa M, Yamaguchi K, Nakamura T, Shibukawa R, Kodanaka I, Ichisaka T, et al. Direct reprogramming of somatic cells is promoted by maternal transcription factor Glis1. Nature. 2011;474(7350):225-9.
17. Suva ML, Rheinbay E, Gillespie SM, Patel AP, Wakimoto H, Rabkin SD, et al. Reconstructing and reprogramming the tumor-propagating potential of glioblastoma stem-like cells. Cell. 2014;157(3):580-94.
18. Matsui WH. Cancer stem cell signaling pathways. Medicine (Baltimore). 2016;95(1 Suppl 1):S8-S19.
19. Zhou J, Wulfkuhle J, Zhang H, Gu P, Yang Y, Deng J, et al. Activation of the PTEN/mTOR/STAT3 pathway in breast cancer stem-like cells is required for viability and maintenance. Proc Natl Acad Sci U S A. 2007;104(41):16158-63.
20. Sherry MM, Reeves A, Wu JK, Cochran BH. STAT3 is required for proliferation and maintenance of multipotency in glioblastoma stem cells. Stem Cells. 2009;27(10):2383-92.
21. Yin X, Zhang BH, Zheng SS, Gao DM, Qiu SJ, Wu WZ, et al. Coexpression of gene Oct4 and Nanog initiates stem cell characteristics in hepatocellular carcinoma and promotes epithelial-mesenchymal transition through activation of Stat3/Snail signaling. J Hematol Oncol. 2015;8:23.
22. Do DV, Ueda J, Messerschmidt DM, Lorthongpanich C, Zhou Y, Feng B, et al. A genetic and developmental pathway from STAT3 to the OCT4-NANOG circuit is essential for maintenance of ICM lineages in vivo. Genes Dev. 2013;27(12):1378-90.
23. Hershko A, Ciechanover A. The ubiquitin system. Annu Rev Biochem. 1998;67:425-79.
24. Skaar JR, Pagan JK, Pagano M. SCF ubiquitin ligase-targeted therapies. Nat Rev Drug Discov. 2014;13(12):889-903.
25. Zheng N, Shabek N. Ubiquitin Ligases: Structure, Function, and Regulation. Annu Rev Biochem. 2017;86:129-57.
26. Wang D, Ma L, Wang B, Liu J, Wei W. E3 ubiquitin ligases in cancer and implications for therapies. Cancer Metastasis Rev. 2017;36(4):683-702.
27. Akutsu M, Dikic I, Bremm A. Ubiquitin chain diversity at a glance. J Cell Sci. 2016;129(5):875-80.
28. Kang B, Sun XH. Regulation of cancer stem cells by RING finger ubiquitin ligases. Stem Cell Investig. 2014;1:5.
29. Amm I, Sommer T, Wolf DH. Protein quality control and elimination of protein waste: the role of the ubiquitin-proteasome system. Biochim Biophys Acta. 2014;1843(1):182-96.
30. Niwa H, Miyazaki J, Smith AG. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat Genet. 2000;24(4):372-6.
31. Kim RJ, Nam JS. OCT4 expression enhances features of cancer stem cells in a mouse model of breast cancer. Lab Anim Res. 2011;27(2):147-52.
32. Chang CC, Shieh GS, Wu P, Lin CC, Shiau AL, Wu CL. Oct-3/4 expression reflects tumor progression and regulates motility of bladder cancer cells. Cancer Res. 2008;68(15):6281-91.
33. Xu H, Wang W, Li C, Yu H, Yang A, Wang B, et al. WWP2 promotes degradation of transcription factor OCT4 in human embryonic stem cells. Cell Res. 2009;19(5):561-73.
34. Liao B, Zhong X, Xu H, Xiao F, Fang Z, Gu J, et al. Itch, an E3 ligase of Oct4, is required for embryonic stem cell self-renewal and pluripotency induction. J Cell Physiol. 2013;228(7):1443-51.
35. Cho Y, Kang HG, Kim SJ, Lee S, Jee S, Ahn SG, et al. Post-translational modification of OCT4 in breast cancer

tumorigenesis. Cell Death Differ. 2018;25(10):1781-95.
36. Mamun MA, Mannoor K, Cao J, Qadri F, Song X. SOX2 in cancer stemness: tumor malignancy and therapeutic potentials. J Mol Cell Biol. 2020;12(2):85-98.
37. Fang L, Zhang L, Wei W, Jin X, Wang P, Tong Y, et al. A methylation-phosphorylation switch determines Sox2 stability and function in ESC maintenance or differentiation. Mol Cell. 2014;55(4):537-51.
38. Wang J, Zhang Y, Hou J, Qian X, Zhang H, Zhang Z, et al. Ube2s regulates Sox2 stability and mouse ES cell maintenance. Cell Death Differ. 2016;23(3):393-404.
39. Cui CP, Zhang Y, Wang C, Yuan F, Li H, Yao Y, et al. Dynamic ubiquitylation of Sox2 regulates proteostasis and governs neural progenitor cell differentiation. Nat Commun. 2018;9(1):4648.
40. Wang L, Shen F, Stroehlein JR, Wei D. Context-dependent functions of KLF4 in cancers: could alternative splicing isoforms be the key? Cancer Lett. 2018;438:10-6.
41. Yu F, Li J, Chen H, Fu J, Ray S, Huang S, et al. Kruppel-like factor 4 (KLF4) is required for maintenance of breast cancer stem cells and for cell migration and invasion. Oncogene. 2011;30(18):2161-72.
42. Zhou H, Liu Y, Zhu R, Ding F, Wan Y, Li Y, et al. FBXO32 suppresses breast cancer tumorigenesis through targeting KLF4 to proteasomal degradation. Oncogene. 2017;36(23):3312-21.
43. Hao Z, Sheng Y, Duncan GS, Li WY, Dominguez C, Sylvester J, et al. K48-linked KLF4 ubiquitination by E3 ligase Mule controls T-cell proliferation and cell cycle progression. Nat Commun. 2017;8:14003.
44. He H, Wu Z, Li S, Chen K, Wang D, Zou H, et al. TRAF7 enhances ubiquitin-degradation of KLF4 to promote hepatocellular carcinoma progression. Cancer Lett. 2020;469:380-9.
45. Chen H, Liu H, Qing G. Targeting oncogenic Myc as a strategy for cancer treatment. Signal Transduct Target Ther. 2018;3:5.
46. Yang A, Qin S, Schulte BA, Ethier SP, Tew KD, Wang GY. MYC Inhibition Depletes Cancer Stem-like Cells in Triple-Negative Breast Cancer. Cancer Res. 2017;77(23):6641-50.
47. Gregory MA, Hann SR. c-Myc proteolysis by the ubiquitin-proteasome pathway: stabilization of c-Myc in Burkitt's lymphoma cells. Mol Cell Biol. 2000;20(7):2423-35.
48. King B, Trimarchi T, Reavie L, Xu L, Mullenders J, Ntziachristos P, et al. The ubiquitin ligase FBXW7 modulates leukemia-initiating cell activity by regulating MYC stability. Cell. 2013;153(7):1552-66.
49. Thompson BJ, Jankovic V, Gao J, Buonamici S, Vest A, Lee JM, et al. Control of hematopoietic stem cell quiescence by the E3 ubiquitin ligase Fbw7. J Exp Med. 2008;205(6):1395-408.
50. Reavie L, Della Gatta G, Crusio K, Aranda-Orgilles B, Buckley SM, Thompson B, et al. Regulation of hematopoietic stem cell differentiation by a single ubiquitin ligase-substrate complex. Nat Immunol. 2010;11(3):207-15.
51. Takeishi S, Matsumoto A, Onoyama I, Naka K, Hirao A, Nakayama KI. Ablation of Fbxw7 eliminates leukemia-initiating cells by preventing quiescence. Cancer Cell. 2013;23(3):347-61.
52. Popov N, Schulein C, Jaenicke LA, Eilers M. Ubiquitylation of the amino terminus of Myc by SCF(beta-TrCP) antagonizes SCF(Fbw7)-mediated turnover. Nat Cell Biol. 2010;12(10):973-81.
53. Adhikary S, Marinoni F, Hock A, Hulleman E, Popov N, Beier R, et al. The ubiquitin ligase HectH9 regulates transcriptional activation by Myc and is essential for tumor cell proliferation. Cell. 2005;123(3):409-21.
54. Kim EJ, Kim SH, Jin X, Jin X, Kim H. KCTD2, an adaptor of Cullin3 E3 ubiquitin ligase, suppresses gliomagenesis by destabilizing c-Myc. Cell Death Differ. 2017;24(4):649-59.
55. Meng HM, Zheng P, Wang XY, Liu C, Sui HM, Wu SJ, et al. Over-expression of Nanog predicts tumor progression and poor prognosis in colorectal cancer. Cancer Biol Ther. 2010;9(4):295-302.
56. Wang ML, Chiou SH, Wu CW. Targeting cancer stem cells: emerging role of Nanog transcription factor. Onco Targets Ther. 2013;6:1207-20.
57. Zhang J, Chen M, Zhu Y, Dai X, Dang F, Ren J, et al. SPOP Promotes Nanog Destruction to Suppress Stem Cell Traits and Prostate Cancer Progression. Dev Cell. 2019;48(3):329-44 e5.
58. Kim SH, Kim MO, Cho YY, Yao K, Kim DJ, Jeong CH, et al. ERK1 phosphorylates Nanog to regulate protein stability and stem cell self-renewal. Stem Cell Res. 2014;13(1):1-11.
59. Balzeau J, Menezes MR, Cao S, Hagan JP. The LIN28/let-7 Pathway in Cancer. Front Genet. 2017;8:31.
60. Zhou J, Ng SB, Chng WJ. LIN28/LIN28B: an emerging oncogenic driver in cancer stem cells. Int J Biochem Cell Biol. 2013;45(5):973-8.
61. Lee SH, Cho S, Kim MS, Choi K, Cho JY, Gwak HS, et al. The ubiquitin ligase human TRIM71 regulates let-7 microRNA biogenesis via modulation of Lin28B protein. Biochim Biophys Acta. 2014;1839(5):374-86.
62. Karve TM, Cheema AK. Small changes huge impact: the role of protein posttranslational modifications in cellular homeostasis and disease. J Amino Acids. 2011;2011:207691.
63. Guruharsha KG, Kankel MW, Artavanis-Tsakonas S. The Notch signalling system: recent insights into the complexity of a conserved pathway. Nat Rev Genet. 2012;13(9):654-66.
64. Fryer CJ, White JB, Jones KA. Mastermind recruits CycC:CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover. Mol Cell. 2004;16(4):509-20.
65. Haydu JE, De Keersmaecker K, Duff MK, Paietta E, Racevskis J, Wiernik PH, et al. An activating intragenic deletion in NOTCH1 in human T-ALL. Blood. 2012;119(22):5211-4.
66. Matsumoto A, Onoyama I, Sunabori T, Kageyama R, Okano H, Nakayama KI. Fbxw7-dependent degradation of Notch is required for control of "stemness" and neuronal-glial differentiation in neural stem cells. J Biol Chem. 2011;286(15):13754-64.
67. Qiu L, Joazeiro C, Fang N, Wang HY, Elly C, Altman Y, et al. Recognition and ubiquitination of Notch by Itch, a hect-type E3 ubiquitin ligase. J Biol Chem. 2000;275(46):35734-7.
68. Weinmaster G, Fischer JA. Notch ligand ubiquitylation: what is it good for? Dev Cell. 2011;21(1):134-44.
69. de Sousa EMF, Vermeulen L. Wnt signaling in cancer stem cell biology. Cancers (Basel). 2016;8(7).
70. Komiya Y, Habas R. Wnt signal transduction pathways. Organogenesis. 2008;4(2):68-75.
71. Hao HX, Xie Y, Zhang Y, Charlat O, Oster E, Avello M, et al. ZNRF3 promotes Wnt receptor turnover in an R-spondin-sensitive manner. Nature. 2012;485(7397):195-200.
72. Koo BK, Spit M, Jordens I, Low TY, Stange DE, van de Wetering M, et al. Tumour suppressor RNF43 is a stem-cell E3 ligase that induces endocytosis of Wnt receptors. Nature. 2012;488(7413):665-9.
73. Funato Y, Terabayashi T, Sakamoto R, Okuzaki D, Ichise H, Nojima H, et al. Nucleoredoxin sustains Wnt/beta-catenin signaling by retaining a pool of inactive dishevelled protein. Curr Biol. 2010;20(21):1945-52.
74. Wei W, Li M, Wang J, Nie F, Li L. The E3 ubiquitin ligase ITCH negatively regulates canonical Wnt signaling by targeting dishevelled protein. Mol Cell Biol. 2012;32(19):3903-12.
75. Ding Y, Zhang Y, Xu C, Tao QH, Chen YG. HECT domain-containing E3 ubiquitin ligase NEDD4L negatively regulates Wnt signaling by targeting dishevelled for proteasomal degradation. J Biol Chem. 2013;288(12):8289-98.
76. Liu C, Kato Y, Zhang Z, Do VM, Yankner BA, He X. beta-Trcp couples beta-catenin phosphorylation-degradation and regulates Xenopus axis formation. Proc Natl Acad Sci U S A. 1999;96(11):6273-8.

77. Zhang Y, Liu S, Mickanin C, Feng Y, Charlat O, Michaud GA, et al. RNF146 is a poly(ADP-ribose)-directed E3 ligase that regulates axin degradation and Wnt signalling. Nat Cell Biol. 2011;13(5):623-9.
78. Fei C, He X, Xie S, Miao H, Zhou Z, Li L. Smurf1-mediated axin ubiquitination requires Smurf1 C2 domain and is cell cycle-dependent. J Biol Chem. 2014;289(20):14170-7.
79. Ji L, Jiang B, Jiang X, Charlat O, Chen A, Mickanin C, et al. The SIAH E3 ubiquitin ligases promote Wnt/beta-catenin signaling through mediating Wnt-induced Axin degradation. Genes Dev. 2017;31(9):904-15.
80. Lee HK, Lee EW, Seo J, Jeong M, Lee SH, Kim SY, et al. Ubiquitylation and degradation of adenomatous polyposis coli by MKRN1 enhances Wnt/beta-catenin signaling. Oncogene. 2018;37(31):4273-86.
81. Tauriello DV, Maurice MM. The various roles of ubiquitin in Wnt pathway regulation. Cell Cycle. 2010;9(18):3700-9.
82. Wicking C, McGlinn E. The role of hedgehog signalling in tumorigenesis. Cancer Lett. 2001;173(1):1-7.
83. Cochrane CR, Szczepny A, Watkins DN, Cain JE. Hedgehog Signaling in the Maintenance of Cancer Stem Cells. Cancers (Basel). 2015;7(3):1554-85.
84. Yue S, Tang LY, Tang Y, Tang Y, Shen QH, Ding J, et al. Requirement of Smurf-mediated endocytosis of Patched1 in sonic hedgehog signal reception. Elife. 2014;3.
85. Chen XL, Chinchilla P, Fombonne J, Ho L, Guix C, Keen JH, et al. Patched-1 proapoptotic activity is downregulated by modification of K1413 by the E3 ubiquitin-protein ligase Itchy homolog. Mol Cell Biol. 2014;34(20):3855-66.
86. Xia R, Jia H, Fan J, Liu Y, Jia J. USP8 promotes smoothened signaling by preventing its ubiquitination and changing its subcellular localization. PLoS Biol. 2012;10(1):e1001238.
87. Li S, Chen Y, Shi Q, Yue T, Wang B, Jiang J. Hedgehog-regulated ubiquitination controls smoothened trafficking and cell surface expression in Drosophila. PLoS Biol. 2012;10(1):e1001239.
88. Raducu M, Fung E, Serres S, Infante P, Barberis A, Fischer R, et al. SCF (Fbxl17) ubiquitylation of Sufu regulates Hedgehog signaling and medulloblastoma development. EMBO J. 2016;35(13):1400-16.
89. Infante P, Faedda R, Bernardi F, Bufalieri F, Lospinoso Severini L, Alfonsi R, et al. Itch/beta-arrestin2-dependent non-proteolytic ubiquitylation of SuFu controls Hedgehog signalling and medulloblastoma tumorigenesis. Nat Commun. 2018;9(1):976.
90. Bhatia N, Thiyagarajan S, Elcheva I, Saleem M, Dlugosz A, Mukhtar H, et al. Gli2 is targeted for ubiquitination and degradation by beta-TrCP ubiquitin ligase. J Biol Chem. 2006;281(28):19320-6.
91. Wang B, Li Y. Evidence for the direct involvement of {beta} TrCP in Gli3 protein processing. Proc Natl Acad Sci U S A. 2006;103(1):33-8.
92. Zhang Q, Shi Q, Chen Y, Yue T, Li S, Wang B, et al. Multiple Ser/Thr-rich degrons mediate the degradation of Ci/Gli by the Cul3-HIB/SPOP E3 ubiquitin ligase. Proc Natl Acad Sci U S A. 2009;106(50):21191-6.
93. Deng W, Vanderbilt DB, Lin CC, Martin KH, Brundage KM, Ruppert JM. SOX9 inhibits beta-TrCP-mediated protein degradation to promote nuclear GLI1 expression and cancer stem cell properties. J Cell Sci. 2015;128(6):1123-38.
94. Hatakeyama S. Ubiquitin-mediated regulation of JAK-STAT signaling in embryonic stem cells. JAKSTAT. 2012;1(3):168-75.
95. Yang WL, Wu CY, Wu J, Lin HK. Regulation of Akt signaling activation by ubiquitination. Cell Cycle. 2010;9(3):487-97.
96. Chen ZJ. Ubiquitin signalling in the NF-kappaB pathway. Nat Cell Biol. 2005;7(8):758-65.
97. Shih Ie M, Wang TL. Notch signaling, gamma-secretase inhibitors, and cancer therapy. Cancer Res. 2007;67(5):1879-82.
98. Capodanno Y, Buishand FO, Pang LY, Kirpensteijn J, Mol JA, Argyle DJ. Notch pathway inhibition targets chemoresistant insulinoma cancer stem cells. Endocr Relat Cancer. 2018;25(2):131-44.
99. Proctor AE, Thompson LA, O'Bryant CL. Vismodegib: an inhibitor of the Hedgehog signaling pathway in the treatment of basal cell carcinoma. Ann Pharmacother. 2014;48(1):99-106.
100. Krishnamurthy N, Kurzrock R. Targeting the Wnt/beta-catenin pathway in cancer: Update on effectors and inhibitors. Cancer Treat Rev. 2018;62:50-60.
101. Darnell JE, Jr. Transcription factors as targets for cancer therapy. Nat Rev Cancer. 2002;2(10):740-9.
102. Toh TB, Lim JJ, Chow EK. Epigenetics in cancer stem cells. Mol Cancer. 2017;16(1):29.
103. Xun J, Wang D, Shen L, Gong J, Gao R, Du L, et al. JMJD3 suppresses stem cell-like characteristics in breast cancer cells by downregulation of Oct4 independently of its demethylase activity. Oncotarget. 2017;8(13):21918-29.
104. Delmore JE, Issa GC, Lemieux ME, Rahl PB, Shi J, Jacobs HM, et al. BET bromodomain inhibition as a therapeutic strategy to target c-Myc. Cell. 2011;146(6):904-17.
105. Mertz JA, Conery AR, Bryant BM, Sandy P, Balasubramanian S, Mele DA, et al. Targeting MYC dependence in cancer by inhibiting BET bromodomains. Proc Natl Acad Sci U S A. 2011;108(40):16669-74.
106. Paiva SL, Crews CM. Targeted protein degradation: elements of PROTAC design. Curr Opin Chem Biol. 2019;50:111-9.
107. Winter GE, Buckley DL, Paulk J, Roberts JM, Souza A, Dhe-Paganon S, et al. DRUG DEVELOPMENT. Phthalimide conjugation as a strategy for in vivo target protein degradation. Science. 2015;348(6241):1376-81.
108. Lu J, Qian Y, Altieri M, Dong H, Wang J, Raina K, et al. Hijacking the E3 Ubiquitin Ligase Cereblon to Efficiently Target BRD4. Chem Biol. 2015;22(6):755-63.
109. Huang HL, Weng HY, Wang LQ, Yu CH, Huang QJ, Zhao PP, et al. Triggering Fbw7-mediated proteasomal degradation of c-Myc by oridonin induces cell growth inhibition and apoptosis. Mol Cancer Ther. 2012;11(5):1155-65.
110. Jung J, Kim Y, Song J, Yoon YJ, Kim DE, Kim JA, et al. KRIBB53 binds to OCT4 and enhances its degradation through the proteasome, causing apoptotic cell death of OCT4-positive testicular germ cell tumors. Carcinogenesis. 2018;39(6):838-49.
111. Han H, Jain AD, Truica MI, Izquierdo-Ferrer J, Anker JF, Lysy B, et al. Small-Molecule MYC Inhibitors Suppress Tumor Growth and Enhance Immunotherapy. Cancer Cell. 2019;36(5):483-97 e15.
112. Huang MJ, Cheng YC, Liu CR, Lin S, Liu HE. A small-molecule c-Myc inhibitor, 10058-F4, induces cell-cycle arrest, apoptosis, and myeloid differentiation of human acute myeloid leukemia. Exp Hematol. 2006;34(11):1480-9.
113. Matkar S, Sharma P, Gao S, Gurung B, Katona BW, Liao J, et al. An epigenetic pathway regulates sensitivity of breast cancer cells to HER2 inhibition via FOXO/c-Myc axis. Cancer Cell. 2015;28(4):472-85.
114. Roos M, Pradere U, Ngondo RP, Behera A, Allegrini S, Civenni G, et al. A small-molecule inhibitor of Lin28. ACS Chem Biol. 2016;11(10):2773-81.
115. Massard C, Azaro A, Soria JC, Lassen U, Le Tourneau C, Sarker D, et al. First-in-human study of LY3039478, an oral Notch signaling inhibitor in advanced or metastatic cancer. Ann Oncol. 2018;29(9):1911-7.
116. Cook N, Basu B, Smith DM, Gopinathan A, Evans J, Steward WP, et al. A phase I trial of the gamma-secretase inhibitor MK-0752 in combination with gemcitabine in patients with pancreatic ductal adenocarcinoma. Br J Cancer. 2018;118(6):793-801.
117. A phase I study of AL101, a pan-NOTCH inhibitor, in patients (pts) with locally advanced or metastatic solid tumors J Clin Oncol. 2018;36:15_suppl,2515.
118. ACCURACY: phase (P) 2 trial of AL101, a pan-Notch inhibitor,

inhibitor, in patients (pts) with recurrent/metastatic (R/M) adenoid cystic carcinoma (ACC) with Notch activating mutations (Notchact mut). J Clin Oncol. 2019;37:15_suppl,TPS6098.
119. Smith DC, Rosen LS, Chugh R, et al. First-in-human evaluation of the human monoclonal antibody vantictumab (OMP-18r5; anti-Frizzled) targeting the WNT pathway in a phase I study for patients with advanced solid tumors. J Clin Oncol. 2013;31:15_suppl,2540.
120. Shan J, Shi DL, Wang J, Zheng J. Identification of a specific inhibitor of the dishevelled PDZ domain. Biochemistry. 2005;44(47):15495-503.
121. Kimura K, Ikoma A, Shibakawa M, Shimoda S, Harada K, Saio M, et al. Safety, tolerability, and preliminary efficacy of the anti-fibrotic small molecule PRI-724, a CBP/beta-catenin inhibitor, in patients with hepatitis C virus-related virrhosis: a single-center, open-label, dose escalation phase 1 trial. EBioMedicine. 2017;23:79-87.
122. A study of E-7386 in participants with advanced solid tumor including colorectal cancer (CRC) (NCT03833700, 2019). Available online at: https://clinicaltrials.gov/ct2/show/NCT03833700
123. Phase I, open-label, non-randomized study to evaluate safety of BC2059 (NCT03459469, 2018). Available online at: https://clinicaltrials.gov/ct2/show/NCT03459469
124. Lu J, Ma Z, Hsieh JC, Fan CW, Chen B, Longgood JC, et al. Structure-activity relationship studies of small-molecule inhibitors of Wnt response. Bioorg Med Chem Lett. 2009;19(14):3825-7.
125. Axelson M, Liu K, Jiang X, He K, Wang J, Zhao H, et al. U.S. Food and drug administration approval: vismodegib for recurrent, locally advanced, or metastatic basal cell carcinoma. Clin Cancer Res. 2013;19(9):2289-93.
126. Study of patidegib topical gel, 2%, for the reduction of disease burden of persistently developing basal cell carcinomas (BCCs) in subjects with basal cell nevus syndrome (Gorlin Syndrome) (NCT03703310, 2018). Available online at: https://clinicaltrials.gov/ct2/show/NCT03703310
127. Taladegib, paclitaxel, carboplatin, and radiation therapy in treating patients with localized esophageal or gastroesophageal junction cancer (NCT02530437, 2015). Available online at: https://clinicaltrials.gov/ct2/show/NCT02530437.

REVIEW ARTICLE

Endoplasmic reticulum stress as target for treatment of hearing loss

Yanfei WANG, Zhigang XU*

Shandong Provincial Key Laboratory of Animal Cell and Developmental Biology, School of Life Sciences, Shandong University, Qingdao, Shandong 266237, China

*Correspondence: xuzg@sdu.edu.cn
https://doi.org/10.37175/stemedicine.v1i3.21

ABSTRACT

The endoplasmic reticulum (ER) plays pivotal roles in coordinating protein biosynthesis and processing. Under ER stress, when excessive misfolded or unfolded proteins are accumulated in the ER, the unfolded protein response (UPR) is activated. The UPR blocks global protein synthesis while activates chaperone expression, eventually leading to the alleviation of ER stress. However, prolonged UPR induces cell death. ER stress has been associated with various types of diseases. Recently, increasing evidences suggest that ER stress and UPR are also involved in hearing loss. In the present review, we will discuss the role of ER stress in hereditary hearing loss as well as acquired hearing loss. Moreover, we will discuss the emerging ER stress-based treatment of hearing loss. Further investigations are warranted to understand the mechanisms in detail how ER stress contributes to hearing loss, which will help us develop better ER stress-related treatments.

Keywords: ER stress · Unfolded protein response (UPR) · Hearing loss · Inner ear · Cochlea

1. Introduction

The endoplasmic reticulum (ER) is a highly dynamic organelle in eukaryotic cells, playing important roles in protein synthesis, processing, folding, and transportation, as well as lipid synthesis and calcium homeostasis. Newly synthesized transmembrane and secretory proteins must be folded and processed in the ER before being targeted to their final destinations. Misfolded proteins will be folded into correct conformations in the ER with the help of chaperon proteins such as binding immunoglobulin protein (BiP)/glucose regulated protein 78 (GRP78) (1). Alternatively, misfolded proteins are subjected to degradation through the proteasome-dependent ER-associated protein degradation (ERAD) pathway (2). Physiological or pathological conditions such as hypoxia, acidosis, or calcium fluxes can disturb ER homeostasis and result in an accumulation of unfolded or misfolded proteins in the ER, commonly referred to as ER stress. To alleviate ER stress, the so-called unfolded protein response (UPR) is activated, which blocks protein synthesis and activates chaperone gene expression (3).

Three main UPR pathways have been identified so far, which are mediated by ER stress sensors that reside on the ER membranes, namely the inositol-requiring enzyme 1α (IRE1α), the PKR-like ER kinase (PERK), and the activating transcription factor 6α (ATF6α) (**Figure 1**). These sensors are all transmembrane proteins that are normally inactivated by BiP binding at the ER lumen side. Under ER stress, accumulated unfolded proteins sequester BiP from the ER stress sensors and activate UPR through three independent pathways: (i) Upon release from BiP, IRE1α oligomerizes and trans-autophosphorylates itself, which then activates its endoribonuclease activity. Activated IRE1α catalyzes the splicing of *XBP1* mRNA into *XBP1s* that encodes an active transcription factor. XBP1s then enters the nucleus and activates gene expression involved in ER membrane biogenesis and protein folding. (ii) Similar to IRE1α, PERK obtains its kinase activity through oligomerization and trans-autophosphorylation after being released from BiP. After activation, PERK phosphorylates the α subunit of eukaryotic translational initiation factor 2α (eIF2α) on Ser51. Phosphorylated eIF2α attenuates global protein synthesis to reduce the ER protein-folding load. Meanwhile, it enhances the translation of certain proteins such as activating transcription factor 4 (ATF4). ATF4 enters the nucleus and induces gene expression that are involved in ER function and reactive oxygen species (ROS) production. (iii) Upon release from BiP, ATF6α translocates from the ER to the Golgi apparatus. At the

Received: Oct 28, 2019; Accepted: Dec 29, 2019.

Golgi, site-1 protease (S1P) and site-2 protease (S2P) sequentially cleaves ATF6α, releasing the transcription-activating form of ATF6α that enters the nucleus and induces ER chaperon gene expression.

The activation of UPR pathways usually leads to the clearance of unfolded proteins and the restoration of ER homeostasis through reducing global protein synthesis and increasing chaperon protein expression. However, cell death occurs under prolonged or excessive ER stress when the ER protein load greatly exceeds its folding capacity (4). One of the UPR target genes encodes C/EBP homologous protein (CHOP)/growth arrest and DNA damage-inducible gene 153 (GADD153) (5). As a transcription factor, CHOP activates pro-apoptotic gene expression encoding growth arrest and DNA damage-inducible 34 (GADD34), death receptor 5 (DR5), endoplasmic reticulum oxidoreductase-1 (Ero1α), and Bim (6). CHOP also represses anti-apoptotic gene *Bcl-2* expression (7). Other possible cell death pathways induced by ER stress include activation of apoptotic-signaling kinase-1 (ASK1) and p38 MAPK downstream of IRE1α (6). Currently, one intriguing question that requires further investigation is what determines the pro-survival versus pro-death role of ER stress.

ER stress is involved in various diseases, ranging from cancer, diabetes, metabolic syndromes, to neurodegenerative diseases (8). Recently, the role of ER stress in hearing loss has attracted increasing research attention. Hearing loss (deafness) is the most prevalent sensory impairment in humans, affecting around 466 million worldwide (9). Both genetic and environmental factors contribute to hearing loss. Hereditary hearing loss is clinically divided into non-syndromic hearing loss and syndromic hearing loss, depending on the presence of other symptoms besides deafness. Mutations in more than 100 genes have been identified to be responsible for non-syndromic hearing loss, and it is estimated that additional hundreds of genes are involved in hereditary hearing loss. Environmental factors, such as exposure to ototoxic chemicals, noise and ageing, lead to drug-induced hearing loss (DIHL), noise-induced hearing loss (NIHL) and age-related hearing loss (ARHL), respectively. ARHL (also referred to as presbycusis) is especially important today, affecting nearly one-third of individuals over 65 years of age (9). In this review we will discuss the role of ER stress and its therapeutic potentials in both hereditary and acquired hearing loss.

2. ER stress and hereditary hearing loss

2.1 Wolfram syndrome 1 (WFS1)

WFS1, also called wolframin, is a transmembrane protein

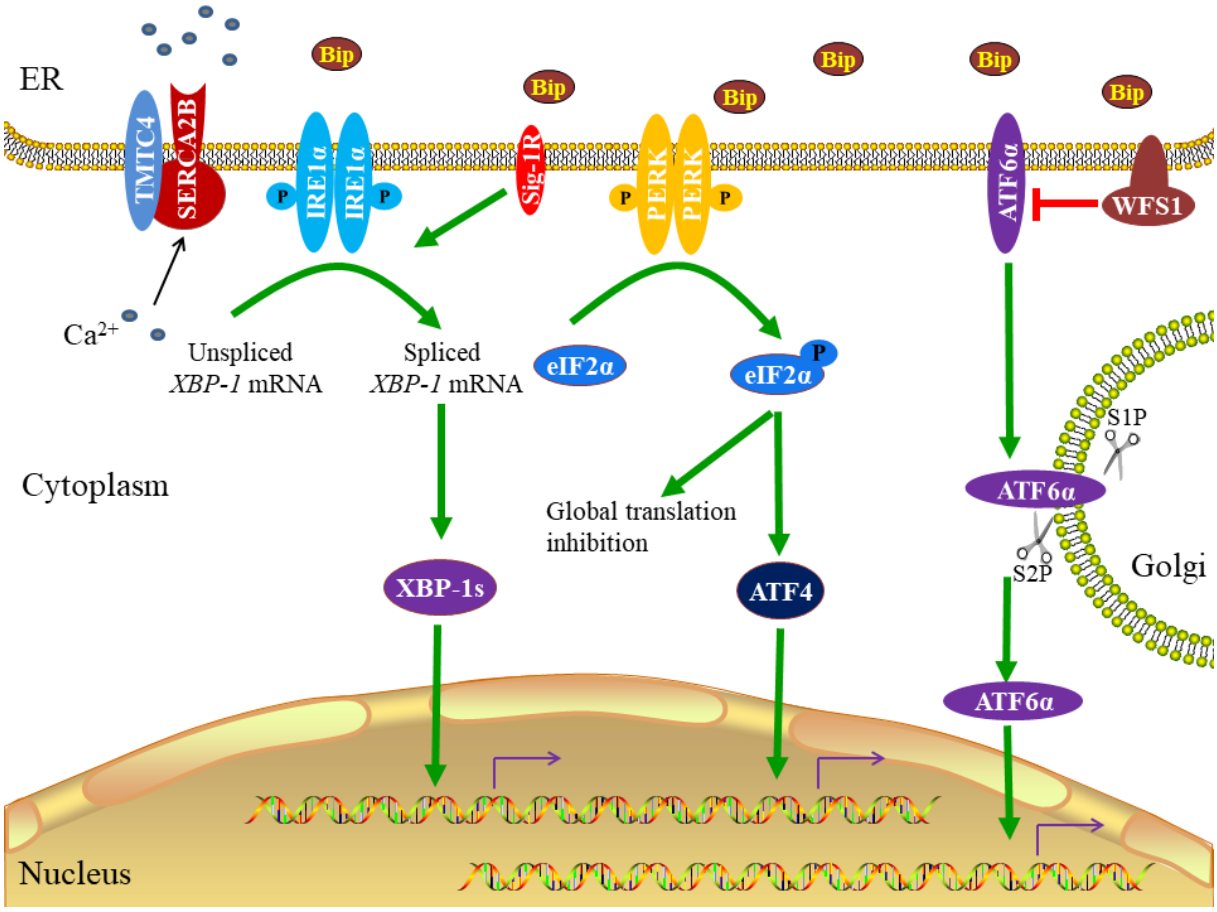

Figure 1. Endoplasmic reticulum (ER) stress and unfolded protein response (UPR) in mammals. See main text for details.

that localizes on the ER membrane. WFS1 interacts with the ER-localized vacuolar-type H^+-ATPase V1A subunit (ATP6V1A), Na^+/K^+ ATPase 1 subunit (ATP1B1), and calmodulin (CaM), suggesting that it might play important roles in ER function and/or homeostasis (10-12). Under ER stress, IRE1α and PERK pathways induce the expression of WFS1, which in turn recruits ATF6α to E3 ligase HMG-CoA reductase degradation protein 1 (HRD1) for proteasomal degradation, therefore provides a negative feedback loop of UPR (13, 14).

Mutations in the *WFS1* gene cause syndromic deafness Wolfram syndrome (WS) or non-syndromic deafness DFNA6/14/38 (15-18). WS is characterized by diabetes insipidus, diabetes mellitus, psychiatric illness, optic atrophy, and hearing loss, and is mostly caused by recessive *WFS1* mutations (15, 16). In contrast, DFNA6/14/38 is caused by dominant *WFS1* mutations (17, 18). *In vitro* studies showed that WS-associated WFS1 dominant mutants induce constitutive ER stress (19, 20). Moreover, in *Wfs1* knockout mice or rats, elevated ER stress could be detected in pancreatic β-cells and retinal cells, consistent with its negative regulatory role in ER stress (21-24).

In the mouse inner ear, WFS1 is widely expressed in hair cells (HCs), spiral ganglion cells (SGCs), supporting cells (SCs), and stria vascularis marginal cells (25). Although *Wfs1* knockout mice or rats develop diabetes and optic atrophy, hearing phenotypes have not been reported in these animals. Recently, WFS1 expression was examined in the cochlea of marmoset (*Callithrix jacchus*), which is a nonhuman primate (26). The results showed that besides those cell types reported in the mouse study, WFS1 immunoreactivity was also detected in the stria vascularis basal cells. The differential expression pattern of WFS1 might help to explain the different hearing phenotypes in *WFS1* deficient rodents and primates. Development of primate models would be helpful for understanding the role of WFS1 in hearing and deafness.

A smaller portion of WS is caused by recessive mutations in the gene that encodes CDGSH iron-sulfur domain-containing protein 2 (CISD2) (27). CISD2, also named as ERIS or Miner1, is an integral membrane protein and localizes on the mitochondria-associated ER membranes (MAMs). Unlike WFS1, the function of CISD2 still remains poorly defined. Nevertheless, CISD2 has been suggested to regulate Ca^{2+} homeostasis and ER stress. *Cisd2*$^{-/-}$ mouse embryonic fibroblasts (MEFs) show dysregulated Ca^{2+} homeostasis and elevated ER stress (28). Similarly, fibroblasts from a WS patient with homozygous *CISD2* mutation (Asn72Ser) show disturbed cellular Ca^{2+} homeostasis and expanded ER compartment, although no overt ER stress phenotype (29). Further investigations are warranted to understand the role of CISD2 in ER stress as well as hearing loss in more detail.

2.2 Transmembrane and tetratricopeptide repeat containing 4 (TMTC4)

TMTC4 is an ER transmembrane protein and is suggested to play important roles in regulating Ca^{2+} dynamics (30). TMTC4 interacts with the Ca^{2+} pump SERCA2B and is involved in maintaining the Ca^{2+} gradient between the cytoplasm and the ER. Inactivation of *Tmtc4* in mice causes increased ER stress and UPR, possibly through dysregulation of ER Ca^{2+} level (30).

TMTC4 is expressed in the HCs and various SCs in the mouse cochlea (30). Mutation in the *TMTC4* gene has not been associated with any diseases including hearing loss in human. However, *Tmtc4* knockout mice show progressive HC loss that leads to early onset hearing loss (30). Consistent with the proposed role of TMTC4 in Ca^{2+} homeostasis and ER stress, cochlear cells of *Tmtc4* knockout mice show enhanced sensitivity to ER-induced apoptosis. In line with this, disruption of *Chop* gene partially improves the auditory function of *Tmtc4* knockout mice (30).

2.3 Cadherin 23 (CDH23)

CDH23 is a large atypical cadherin, consisting of 27 extracellular cadherin repeats, a single transmembrane domain, and a short cytoplasmic part. *CDH23* gene mutations cause syndromic hearing loss Usher syndrome (USH) 1D or non-syndromic hearing loss DFNB12 (31-33). USH is the most common inherited deaf-blindness, and so far ten genes have been associated with USH (34). These genes encode the so-called USH proteins, which bind to each other and play pivotal roles in the stereocilia and ribbon synapses of the HCs.

Evidences suggest that before being transported to the plasma membrane, CDH23 is preassembled into a complex with other USH proteins harmonin and MYO7A at the ER in zebrafish HCs (35). Disruption of the complex induces ER stress characterized by expanded ER membrane and elevated BiP expression, which eventually leads to HC apoptosis (35). These results led to the hypothesis that USH proteins are transported from the ER to their destinations as a protein complex. When one USH protein is defective or missing, other complex components are exposed abnormally and recognized as misfolded proteins, hence triggering ER stress (35).

The involvement of deficient CDH23 in ER stress is further supported by a mutant *Cdh23* mouse line *erlong* (*erl*) with a point mutation T208C. The *erl* mice suffer HC loss that eventually leads to early-onset progressive deafness (36). Further investigation showed that the mutant CDH23 failed to reach the stereocilia. Instead, it colocalized with BiP in the subapical regions of HCs (37). This may activate the PERK-eIF2α-ATF4-CHOP pathway, which eventually leads to HC apoptosis (37). Inactivation of the *Chop* gene, treatment with ER stress modulator salubrinal, chemical chaperone 4-phenylbutyrate (PBA) or tauroursodeoxycholic acid (TUDCA) preserves HCs and delays the progression of hearing loss in the *erl* mice (37-39).

2.4 Connexins

Connexins are a family of membrane-spanning proteins, acting as the building blocks of gap junctions. By connecting the cytoplasm of adjacent animal cells,

gap junctions provide direct intercellular communication for exchange of ions, metabolites, and second messengers. More than 20 mammalian connexin genes have been identified, whose mutations are responsible for various diseases such as peripheral neuropathy, skin disease, cataracts, and hearing loss (40).

In the cochlea, gap junctions are broadly present in the non-sensory cells including the SCs, the stria vascularis, the spiral limbus, and the spiral ligament (41). Mutations in the *GJB2* gene that encodes connexin 26 (Cx26) are responsible for ~50% of non-syndromic hearing loss (42, 43). Moreover, mutations in *GJB6* (Cx30) and *GJB3* (Cx31) are also associated with non-syndromic hearing loss (44-46). Other deafness-related connexins include Cx29 (GJC3) and Cx43 (GJA1). Although mutations in *GJC3* and *GJA1* have not been clearly associated with hearing loss in humans, auditory function is affected in *Cx29* or *Cx43* mutant mice (47-49).

A possible link has been proposed connecting connexin expression and/or function with ER stress. Treating cultured mesangial cells with ER stress inducers leads to decreased Cx43 expression and reduced gap junctional intercellular communication (50). Therefore, gap junctions might protect cells under ER stress by preventing 'stress' signals from transmitting to adjacent cells (50). On the other hand, connexin gene mutations could induce ER stress. When overexpressed in cultured cells, several Cx26, Cx30 and Cx31 deafness-associated mutants are trapped in the ER instead of being localized on the plasma membrane (51-53). Moreover, Cx31R180X- and Cx31E183K-overexpressing cells show elevated BiP/GRP78 expression, indicating elevated ER stress (51). However, ER stress is not elevated in cultured cells overexpressing deafness-associated Cx31 (66delD), suggesting that ER stress is not the sole underlying mechanism of mutant Cx31-associated cell death (54).

2.5 Elongator acetyltransferase complex subunit 3 (ELP3)

Elongator complex plays a pivotal role in regulating protein translation efficiency through modifying the wobble uridine (U34) in the anticodon of various tRNAs (55). This protein complex consists of two sets of six subunits ELP1-ELP6, among which ELP3 acts as the enzymatic core (56). Elongator-mediated tRNA modifications ensure fidelity and efficiency of protein translation, which are essential to normal proteostasis (57, 58). Elongator complex also plays important roles in α-tubulin acetylation, transcriptional elongation, actin organization, kinase signaling, etc., and dysfunction of this complex is associated with various neurological diseases (59).

Conditional knockout of *Elp3* gene in the cortical neurons decreases translation rates and activates the PERK-eIF2α-ATF4 pathway, eventually leading to microcephaly (60). In the cochlea, ELP3 is abundantly expressed in the SGNs and nascent HCs (61). Conditional knockout of *Elp3* gene in the cochlea causes protein misfolding and aggregation, resulting in apoptosis of SGNs and defects in cochlear planar cell polarity (PCP), and eventually leading to severe hearing loss (61). Activation of ER stress was not examined in the cochlea of *Elp3* knockout mice. Nevertheless, chemical chaperone PBA treatment alleviates protein aggregation and PCP deficits in *Elp3* knockout mice, implying that ER stress is likely involved in ELP3-associated hearing loss (61).

3. ER stress and acquired hearing loss

3.1 ER stress and DIHL

Ototoxic chemicals could lead to 'drug-induced' hearing loss (62). Studies in animals and cultured cells suggest that ER stress could be induced by ototoxic chemicals such as aminoglycosides, cisplatin, N-acetyl-para-aminophenol (APAP), 3-nitropropionic acid (3-NP), and N-acetyl-p-benzoquinoneimine (NAPQI) (63-67). Additionally, ER stress activator tunicamycin treatment causes profound hearing loss in rats (68). Further investigations suggest that these chemicals affect different cell types in the cochlea. For example, aminoglycoside gentamicin induces ER stress in SGCs but not HCs, whereas tunicamycin induces ER stress in both HCs and SGCs (65, 68).

Calreticulin (CRT) is an ER-residing chaperone induced under ER stress (69). In the inner ear, CRT is expressed in the HCs and the strial marginal cells, and could bind aminoglycosides such as gentamicin (70), which inhibits the chaperon activity of CRT (71). *Crt* knockout or knockdown MEFs are more susceptible to gentamicin treatment, consistent with a protective role of CRT against gentamicin-induced cytotoxicity (70). CRT has been identified as one of the cisplatin-binding proteins in a screen, which also identified GRP78/BiP albeit at relatively low abundance (72). The significance of CRT and GRP78/BiP binding to gentamicin and cisplatin in hearing and deafness requires further investigation.

Consistent with the potential role of ER stress in DIHL, $Xbp1^{+/-}$ mice are more susceptible to aminoglycoside-induced hearing loss compared to wild-type mice (65). Furthermore, chemical chaperone TUDCA attenuates aminoglycoside-induced hearing loss in $Xbp1^{+/-}$ mice (65). In addition, it was recently shown that TUDCA treatment also exerted a protective effect on cisplatin-induced hearing loss (66).

3.2 ER stress and NIHL

High-intensity noise could cause HCs and SGNs death, which eventually leads to the so-called NIHL (73). Expression levels of BiP/GRP78, XBP1s, CHOP/GADD153, and caspase-3 are elevated in the cochlea of guinea pigs or mice during NIHL, suggesting that ER stress might play a role in this process (30, 74). Integrated stress response inhibitor (ISRIB) inhibits the PERK-eIF2α-ATF4 pathway through activating the guanine nucleotide exchange factor (GEF) eIF2B (75, 76). Treatment of mice with ISRIB prior to noise exposure preserves HCs and improves hearing (30).

Sigma-1 receptor (Sig-1R) interacts with BiP at the MAMs,

and regulates Ca^{2+} signaling and cell survival (77). Under ER stress, Sig-1R expression is increased via the PERK-eIF2α-ATF4 pathway and inhibits cell apoptosis (78). Further investigations have shown that Sig-1R executes protective function through activating the IRE1α-XBP1 pathway and inhibiting CHOP expression (79, 80). Consistent with the protective role of Sig-1R in hearing loss, noise-induced cell death and hearing loss are significantly reduced in mice by treatment with Sig-1R agonist cutamesine (SA4503) (81).

Glucocorticoid-induced leucine zipper (GILZ) is a transcription factor that has been shown to protect cells from apoptosis (82-84). Under ER stress, overexpression of GILZ up-regulates BiP and down-regulates CHOP, ATF4, and XBP1s, and protects cells from apoptosis through a pathway involving mitochondrial function and oxidative phosphorylation (OXPHOS) (85). Recently, it was suggested that GILZ had similar protective effect in NIHL. Overexpression of GILZ protects rats from NIHL through increasing BiP and Bcl-xL, and decreasing CHOP, Bax, and cleaved caspase-3, whereas GILZ silencing has the opposite effect (86).

3.3 ER stress and ARHL

ARHL, also called presbycusis, is more and more common nowadays, affecting nearly one-third of individuals over 65 years of age (9). There are evidences suggesting that ER stress is also involved in ARHL. For example, BiP/GRP78 expression is decreased, whereas CHOP expression is increased in the cochlea of aged mice (87). Consistently, cleaved caspase-3 and caspase-9, but not caspase-12, are elevated in the cochlea of aged mice, indicating the activation of apoptosis (87).

Geranylgeranylacetone (GGA) is a nontoxic acyclic isoprenoid compound with protective function through increasing the expression of HSP70 (88). It has been shown that GGA treatment attenuates ARHL in mice (89). Moreover, GGA could ameliorate 3-NP-induced deafness as well as NIHL (90, 91). The protection of auditory function by GGA has been attributed to its activity as a HSP70 inducer. However, GGA could also induce BiP expression and enhance ER stress (92-94). The potential role of ER stress in GGA-mediated auditory protection awaits further examination.

4. ER stress-based treatment of hearing loss

Many small molecules are able to interfere with ER stress and provide protection for cells, while only a few of them have been tested in treatment of hearing loss. We will discuss these potential ER stress-based treatment of hearing loss in three categories: (1) restoring ER homeostasis; (2) modulating the PERK-eIF2α-ATF4 pathway; and (3) modulating the IRE1α-XBP1 pathway. As mentioned above, HSP70 inducer GGA could also induce BiP expression and enhance ER stress, and has been tested in hearing loss treatment in several animal experiments. At present, the specific target of GGA in ER stress remains elusive.

4.1 Restoring ER homeostasis with chemical chaperones

Chemical chaperones are small chemical compounds that could improve ER folding capacity and restore ER homeostasis, hence are extensively used to reduce ER stress. Unfolded or misfolded proteins are kept from aggregation by chemical chaperones through the interaction between the hydrophobic regions of each other (95). TUDCA and PBA are two most commonly used chemical chaperones. They could reduce aggregate accumulation and revert ER stress, and have been approved by the Food and Drug Administration (FDA) for clinical uses.

TUDCA is a taurine-conjugated derivative of ursodeoxycholic acid (UDCA), which used to be isolated from black bear gallbladders but can now be synthesized chemically. TUDCA has been widely used in experimental and clinical treatments of diabetes, liver disease, and neurodegenerative diseases (95). Recently, it was also tested in treatment of hearing loss. TUDCA treatment preserves HCs and delays the progression of hearing loss in *Cdh23* mutant mice *erl* (38). Moreover, TUDCA treatment also shows protective effects against cisplatin- or aminoglycoside-induced hearing loss in rodents (65, 66, 96).

First synthesized a century ago, PBA has been approved by the FDA in the treatment of urea cycle disease (97). Moreover, it has potential benefits for cancer, diabetes, thalassemia, cystic fibrosis, spinal muscular atrophy, and neurodegenerative diseases (95). PBA can also inhibit histone deacetylase (HDAC) and stimulate gene transcription. The protective effect of PBA on ER stress mainly involves its chaperone activity, given that removal of HDAC inhibitory activity does not affect its protective effect (98). Recently it was shown that PBA treatment preserved HCs and delayed hearing loss progression in *Cdh23* mutant mice *erl* (39). The protective effect of PBA in hearing is further supported by a report showing that PBA could alleviate protein aggregation and hair cell deficits in *Elp3* knockout mice (61).

4.2 Modulating the PERK-eIF2α-ATF4 pathway

ISRIB was identified as an inhibitor of PERK signaling in a cell-based screen (75). It was then shown that ISRIB inhibited the PERK-eIF2α-ATF4 pathway through activating the GEF eIF2B (76). ISRIB treatment shows protective effects in neurogenerative diseases (99, 100). Consistently, treatment with ISRIB preserves HCs and protects mice from NIHL (30). However, ISRIB treatment needs to be applied with caution. Besides inhibiting the PERK signaling, ISRIB could also inhibit stress granule (SG) formation induced by eIF2α phosphorylation (101). It was recently reported that inhibition of SG formation by ISRIB increases HC death in cochlear explants during ototoxicity (102). The controversial effects of ISRIB on HC survival in these two studies require further investigation.

Salubrinal is a selective inhibitor of eIF2α phosphatase complexes GADD34-PP1C, and could prevent ER stress-induced cell death (103, 104). However, its protective effect seems to be cell- and context-dependent. For

example, salubrinal has been shown to enhance fatty acid-induced ER stress and increase rat pancreatic β-cell apoptosis (105). Salubrinal treatment preserves HCs and delays hearing loss progression in the *erl* mice, indicating a protective effect of salubrinal in hearing (37). Taken together, both PERK inhibitor ISRIB and PERK enhancer salubrinal show protective roles in treatment of hearing loss, suggesting that PERK signaling has a dual role in hearing that might be context-dependent.

4.3 Modulating the IRE1α-XBP1 pathway

As mentioned above, Sig-1R is an important ER membrane protein that interacts with BiP at the MAMs and regulates Ca^{2+} signaling (77). Sig-1R stabilizes IRE1α at the MAMs and prolongs IRE1α's activity under ER stress (79, 80). Sig-1R agonists have protective effects in various neurodegenerative diseases (106). Consistently, treatment of Sig-1R agonist SA4503 in mice significantly reduces noise-induced cell death and hearing loss, whereas shows no effect on ARHL (81).

5. Perspectives

ER stress has attracted more and more attentions in recent years. As discussed above, ER stress plays important roles in hearing loss, and could act as an effective target for deafness treatment (**Table 1**). Animal experiments showed that chemicals such as TUDCA, PBA, ISRIB, salubrinal, SA4503, and GGA have protective effects on both hereditary and acquired hearing loss through modulating ER stress. Meanwhile, deletion or overexpression of ER stress-related genes such as *Chop* and *Gilz* also show protective effect on hearing loss. These results suggest that ER stress could act as an effective and promising target for treatment of hearing loss.

However, ER stress-based treatment of hearing loss is still very limited at present. Among the small molecules that have been successfully used clinically or preclinically in treatments of diseases (such as neurodegenerative diseases), only a few have been tested in animal experiments for treatment of hearing loss. Many promising ER stress-related small molecules await further testing in deafness treatment in the future. Meanwhile, cautions must be taken because we now know that ER stress might play different roles in different types of hearing loss, and that the ER stress-targeted drugs might not be that specific. Moreover, ER stress play important roles in various cell types and organs, hence long-term administration of ER stress-targeted drugs might lead to serious adverse effects. Local drug delivery into the inner ear might help to solve the last problem.

Besides reducing side effects, local drug delivery can also help to bypass the blood labyrinth barrier (BLB) that prevents effective drug delivery into the inner ear by systemic administration. The above-mentioned studies delivered drugs systematically via intraperitoneal injection, subcutaneous injection, or oral administration, which are less effective compared with local drug delivery. At present, two main local drug delivery routes are employed in the inner ear, which are intratympanic administration and intracochlear administration (107). In the less invasive intratympanic administration, a drug is delivered to the middle ear through the tympanic membrane, followed by diffusion into the inner ear through the round window. In the rather invasive intracochlear administration, a drug is applied directly to the cochlea, which is more efficient but has a significant risk of damaging the delicate cochlea. Further investigations are warranted to develop non-invasive or minimally invasive local delivery methods for deafness treatment (108). At present, local drug delivery is more frequently used in gene therapy, which is recently emerging as a promising alternative to small molecules in disease treatment.

Local delivery of viruses that encode XBP1s or BiP were shown to improve neuron survival in animal models of neurological diseases (109). Lentivirus-mediated

Table 1. Potential ER stress-base treatment of deafness.

Deafness	Models	ER stress-related treatment	References
DIHL	mice, rats, guinea pigs	GGA, TUDCA	(65, 66, 90, 96)
NIHL	mice, guinea pigs	GGA, ISRIB, SA4503	(30, 81, 91)
	rats	GILZ overexpression	(86)
ARHL	mice	GGA	(89)
HHL	*Cdh23* mutant mice (erl)	TUDCA, PBA, salubrinal	(37-39)
	Cdh23 mutant mice (erl)	*Chop* gene deletion	(37)
	Tmtc4 ko mice	*Chop* gene deletion	(30)
	Elp3 cko mice	PBA	(61)

overexpression of GILZ has been shown to be protective in NIHL (86). However, the specific target cells of lentivirus are unclear in this study. Moreover, lentivirus has long-term safety concerns and is not commonly used in clinical trials (110). Recently, adeno-associated viruses (AAVs) become increasingly used in gene therapy because of its excellent safety and high efficiency (111). Several AAVs have been developed to efficiently deliver genes into HCs or SCs (112, 113). Hence it will be interesting to examine the therapeutic effect of AAV-mediated delivery of XBP1s, BiP, or GILZ into the cochlea.

As mentioned above, *Chop* gene deletion shows a protective effect in *Cdh23* mutant mice and *Tmtc4* knockout mice (30, 37). AAV-mediated RNA interference (RNAi) against dominant deafness-associated *Tmc1* mutation has been shown to improve HC survival and prevent hearing loss (114). It will be interesting to test whether AAV-mediated RNAi against CHOP also has a protective effect in hearing. As an attractive alternative to RNAi, CRISPR/Cas9-mediated genome editing has been employed to disrupt the dominant deafness-associated allele in the *Tmc1* mutant mice (115). In that study, Cas9-sgRNA complex was delivered via cationic lipid, which pointed out a new direction of developing a DNA- and virus-free treatment of hearing loss (115, 116).

Funding body

Our laboratory is supported by grants from the National Natural Science Foundation of China (81771001), Shandong Provincial Key Laboratory of Animal Cell and Developmental Biology (SPKLACDB-2019000), and the Fundamental Research Funds of Shandong University (2018JC025).

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Lee AS. The ER chaperone and signaling regulator GRP78/BiP as a monitor of endoplasmic reticulum stress. Methods. 2005;35(4):373-81.
2. Bernasconi R, Molinari M. ERAD and ERAD tuning: Disposal of cargo and of ERAD regulators from the mammalian ER. Curr Opin Cell Biol. 2011;23(2):176-83.
3. Mori K. Tripartite management of unfolded proteins in the endoplasmic reticulum. Cell. 2000;101(5):451-4.
4. Iurlaro R, Munoz-Pinedo C. Cell death induced by endoplasmic reticulum stress. FEBS J. 2016;283(14):2640-52.
5. Oyadomari S, Mori M. Roles of CHOP/GADD153 in endoplasmic reticulum stress. Cell Death Differ. 2004;11(4):381-9.
6. Sano R, Reed JC. ER stress-induced cell death mechanisms. Biochim Biophys Acta. 2013;1833(12):3460-70.
7. McCullough KD, Martindale JL, Klotz LO, Aw TY, Holbrook NJ. Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. Mol Cell Biol. 2001;21(4):1249-59.
8. Kaneko M, Imaizumi K, Saito A, Kanemoto S, Asada R, Matsuhisa K, et al. ER stress and disease: toward prevention and treatment. Biol Pharm Bull. 2017;40(9):1337-43.
9. Global estimates on prevalence of hearing loss. Geneva: World Health Organization; 2018. Available from: http://wwwwhoint/pbd/deafness/estimates/en/ [cited 2018 Jan 27].
10. Zatyka M, Ricketts C, da Silva Xavier G, Minton J, Fenton S, Hofmann-Thiel S, et al. Sodium-potassium ATPase 1 subunit is a molecular partner of Wolframin, an endoplasmic reticulum protein involved in ER stress. Hum Mol Genet. 2008;17(2):190-200.
11. Yurimoto S, Hatano N, Tsuchiya M, Kato K, Fujimoto T, Masaki T, et al. Identification and characterization of wolframin, the product of the wolfram syndrome gene (WFS1), as a novel calmodulin-binding protein. Biochemistry. 2009;48(18):3946-55.
12. Gharanei S, Zatyka M, Astuti D, Fenton J, Sik A, Nagy Z, et al. Vacuolar-type H+-ATPase V1A subunit is a molecular partner of Wolfram syndrome 1 (WFS1) protein, which regulates its expression and stability. Hum Mol Genet. 2013;22(2):203-17.
13. Fonseca SG, Fukuma M, Lipson KL, Nguyen LX, Allen JR, Oka Y, et al. WFS1 is a novel component of the unfolded protein response and maintains homeostasis of the endoplasmic reticulum in pancreatic beta-cells. J Biol Chem. 2005;280(47):39609-15.
14. Fonseca SG, Ishigaki S, Oslowski CM, Lu S, Lipson KL, Ghosh R, et al. Wolfram syndrome 1 gene negatively regulates ER stress signaling in rodent and human cells. J Clin Investig. 2010;120(3):744-55.
15. Inoue H, Tanizawa Y, Wasson J, Behn P, Kalidas K, Bernal-Mizrachi E, et al. A gene encoding a transmembrane protein is mutated in patients with diabetes mellitus and optic atrophy (Wolfram syndrome). Nat Genet. 1998;20(2):143-8.
16. Strom TM, Hortnagel K, Hofmann S, Gekeler F, Scharfe C, Rabl W, et al. Diabetes insipidus, diabetes mellitus, optic atrophy and deafness (DIDMOAD) caused by mutations in a novel gene (wolframin) coding for a predicted transmembrane protein. Hum Mol Genet. 1998;7(13):2021-8.
17. Bespalova IN, Van Camp G, Bom SJ, Brown DJ, Cryns K, DeWan AT, et al. Mutations in the Wolfram syndrome 1 gene (WFS1) are a common cause of low frequency sensorineural hearing loss. Hum Mol Genet. 2001;10(22):2501-8.
18. Young TL, Ives E, Lynch E, Person R, Snook S, MacLaren L, et al. Non-syndromic progressive hearing loss DFNA38 is caused by heterozygous missense mutation in the Wolfram syndrome gene WFS1. Hum Mol Genet. 2001;10(22):2509-14.
19. De Franco E, Flanagan SE, Yagi T, Abreu D, Mahadevan J, Johnson MB, et al. Dominant ER stress-inducing WFS1 mutations underlie a genetic syndrome of neonatal/infancy-onset diabetes, congenital sensorineural deafness, and congenital cataracts. Diabetes. 2017;66(7):2044-53.
20. Morikawa S, Tajima T, Nakamura A, Ishizu K, Ariga T. A novel heterozygous mutation of the WFS1 gene leading to constitutive endoplasmic reticulum stress is the cause of Wolfram syndrome. Pediatr Diabetes. 2017;18(8):934-41.
21. Ishihara H, Takeda S, Tamura A, Takahashi R, Yamaguchi S, Takei D, et al. Disruption of the WFS1 gene in mice causes progressive beta-cell loss and impaired stimulus-secretion coupling in insulin secretion. Hum Mol Genet. 2004;13(11):1159-70.
22. Yamada T, Ishihara H, Tamura A, Takahashi R, Yamaguchi S, Takei D, et al. WFS1-deficiency increases endoplasmic reticulum stress, impairs cell cycle progression and triggers the apoptotic pathway specifically in pancreatic beta-cells. Hum Mol Genet. 2006;15(10):1600-9.
23. Bonnet Wersinger D, Benkafadar N, Jagodzinska J, Hamel C, Tanizawa Y, Lenaers G, et al. Impairment of visual function and retinal ER stress activation in Wfs1-deficient mice. PLoS One. 2014;9(5):e97222.
24. Plaas M, Seppa K, Reimets R, Jagomae T, Toots M, Koppel T, et al. Wfs1- deficient rats develop primary symptoms of Wolfram syndrome: insulin-dependent diabetes, optic nerve atrophy and medullary degeneration. Sci Rep. 2017;7(1):10220.
25. Cryns K, Thys S, Van Laer L, Oka Y, Pfister M, Van Nassauw L, et al. The WFS1 gene, responsible for low frequencysensorineural hearing loss and Wolfram syndrome, is expressed in a variety of inner ear cells. Histochem Cell

Biol. 2003;119(3):247-56.
26. Suzuki N, Hosoya M, Oishi N, Okano H, Fujioka M, Ogawa K. Expression pattern of wolframin, the WFS1 (Wolfram syndrome-1 gene) product, in common marmoset (Callithrix jacchus) cochlea. Neuroreport. 2016;27(11):833-6.
27. Amr S, Heisey C, Zhang M, Xia XJ, Shows KH, Ajlouni K, et al. A homozygous mutation in a novel zinc-finger protein, ERIS, is responsible for Wolfram syndrome 2. Am J Hum Genet. 2007;81(4):673-83.
28. Wiley SE, Andreyev AY, Divakaruni AS, Karisch R, Perkins G, Wall EA, et al. Wolfram Syndrome protein, Miner1, regulates sulphydryl redox status, the unfolded protein response, and Ca^{2+} homeostasis. EMBO Mol Med. 2013;5(6):904-18.
29. Rouzier C, Moore D, Delorme C, Lacas-Gervais S, Ait-El-Mkadem S, Fragaki K, et al. A novel CISD2 mutation associated with a classical Wolfram syndrome phenotype alters Ca^{2+} homeostasis and ER-mitochondria interactions. Hum Mol Genet. 2017;26(9):1599-611.
30. Li J, Akil O, Rouse SL, McLaughlin CW, Matthews IR, Lustig LR, et al. Deletion of Tmtc4 activates the unfolded protein response and causes postnatal hearing loss. J Clin Investig. 2018;128(11):5150-62.
31. Bork JM, Peters LM, Riazuddin S, Bernstein SL, Ahmed ZM, Ness SL, et al. Usher syndrome 1D and nonsyndromic autosomal recessive deafness DFNB12 are caused by allelic mutations of the novel cadherin-like gene CDH23. Am J Hum Genet. 2001;68(1):26-37.
32. Di Palma F, Holme RH, Bryda EC, Belyantseva IA, Pellegrino R, Kachar B, et al. Mutations in Cdh23, encoding a new type of cadherin, cause stereocilia disorganization in waltzer, the mouse model for Usher syndrome type 1D. Nature Genetics. 2001;27(1):103-7.
33. Bolz H, von Brederlow B, Ramirez A, Bryda EC, Kutsche K, Nothwang HG, et al. Mutation of CDH23, encoding a new member of the cadherin gene family, causes Usher syndrome type 1D. Nat Genet. 2001;27(1):108-12.
34. Mathur P, Yang J. Usher syndrome: Hearing loss, retinal degeneration and associated abnormalities. Biochim Biophys Acta. 2015;1852(3):406-20.
35. Blanco-Sanchez B, Clement A, Fierro J, Jr., Washbourne P, Westerfield M. Complexes of Usher proteins preassemble at the endoplasmic reticulum and are required for trafficking and ER homeostasis. Dis Model Mech. 2014;7(5):547-59.
36. Han F, Yu H, Tian C, Chen HE, Benedict-Alderfer C, Zheng Y, et al. A new mouse mutant of the Cdh23 gene with early-onset hearing loss facilitates evaluation of otoprotection drugs. Pharmacogenomics J. 2012;12(1):30-44.
37. Hu J, Li B, Apisa L, Yu H, Entenman S, Xu M, et al. ER stress inhibitor attenuates hearing loss and hair cell death in Cdh23erl/erl mutant mice. Cell Death Dis. 2016;7(11):e2485.
38. Hu J, Xu M, Yuan J, Li B, Entenman S, Yu H, et al. Tauroursodeoxycholic acid prevents hearing loss and hair cell death in $Cdh23^{erl/erl}$ mice. Neuroscience. 2016;316:311-20.
39. Li B, Zheng T, Yan C, Wang W, Zhang J, Zhang L, et al. Chemical chaperone 4-phenylbutyrate prevents hearing loss and cochlear hair cell death in $Cdh23^{erl/erl}$ mutant mice. Neuroreport. 2019;30(3):145-50.
40. Srinivas M, Verselis VK, White TW. Human diseases associated with connexin mutations. Biochim Biophys Acta. 2018;1860(1):192-201.
41. Wingard JC, Zhao HB. Cellular and deafness mechanisms underlying connexin mutation-induced hearing loss - A common hereditary deafness. Frontiers in Cellular Neuroscience. 2015;9:202.
42. Kelsell DP, Dunlop J, Stevens HP, Lench NJ, Liang JN, Parry G, et al. Connexin 26 mutations in hereditary non-syndromic sensorineural deafness. Nature. 1997;387(6628):80-3.
43. Zelante L, Gasparini P, Estivill X, Melchionda S, D'Agruma L, Govea N, et al. Connexin26 mutations associated with the most common form of non-syndromic neurosensory autosomal recessive deafness (DFNB1) in Mediterraneans. Hum Mol Genet. 1997;6(9):1605-9.
44. Grifa A, Wagner CA, D'Ambrosio L, Melchionda S, Bernardi F, Lopez-Bigas N, et al. Mutations in GJB6 cause nonsyndromic autosomal dominant deafness at DFNA3 locus. Nat Genet. 1999;23(1):16-8.
45. Liu XZ, Xia XJ, Xu LR, Pandya A, Liang CY, Blanton SH, et al. Mutations in connexin31 underlie recessive as well as dominant non-syndromic hearing loss. Hum Mol Genet. 2000;9(1):63-7.
46. del Castillo I, Villamar M, Moreno-Pelayo MA, del Castillo FJ, Alvarez A, Telleria D, et al. A deletion involving the connexin 30 gene in nonsyndromic hearing impairment. NEJM. 2002;346(4):243-9.
47. Tang W, Zhang Y, Chang Q, Ahmad S, Dahlke I, Yi H, et al. Connexin29 is highly expressed in cochlear Schwann cells, and it is required for the normal development and function of the auditory nerve of mice. J Neurosci. 2006;26(7):1991-9.
48. Kim AH, Nahm E, Sollas A, Mattiace L, Rozental R. Connexin 43 and hearing: possible implications for retrocochlear auditory processing. Laryngoscope. 2013;123(12):3185-93.
49. Abitbol JM, Kelly JJ, Barr KJ, Allman BL, Laird DW. Mice harbouring an oculodentodigital dysplasia-linked Cx43 G60S mutation have severe hearing loss. J Cell Sci. 2018;131(9):jcs214635.
50. Huang T, Wan Y, Zhu Y, Fang X, Hiramatsu N, Hayakawa K, et al. Downregulation of gap junction expression and function by endoplasmic reticulum stress. J Biol Chem. 2009;107(5):973-83.
51. Xia K, Ma H, Xiong H, Pan Q, Huang L, Wang D, et al. Trafficking abnormality and ER stress underlie functional deficiency of hearing impairment-associated connexin-31 mutants. Protein Cell. 2010;1(10):935-43.
52. Zhang Y, Wang J, Li L, Sun Y, Feng B. Three common GJB2 mutations causing nonsyndromic hearing loss in Chinese populations are retained in the endoplasmic reticulum. Acta Oto-Laryngologica. 2010;130(7):799-803.
53. Berger AC, Kelly JJ, Lajoie P, Shao Q, Laird DW. Mutations in Cx30 that are linked to skin disease and non-syndromic hearing loss exhibit several distinct cellular pathologies. J Cell Sci. 2014;127(Pt 8):1751-64.
54. Tattersall D, Scott CA, Gray C, Zicha D, Kelsell DP. EKV mutant connexin 31 associated cell death is mediated by ER stress. Hum Mol Genet. 2009;18(24):4734-45.
55. Huang B, Johansson MJ, Bystrom AS. An early step in wobble uridine tRNA modification requires the Elongator complex. RNA. 2005;11(4):424-36.
56. Wittschieben BO, Otero G, de Bizemont T, Fellows J, Erdjument-Bromage H, Ohba R, et al. A novel histone acetyltransferase is an integral subunit of elongating RNA polymerase II holoenzyme. Mol Cell. 1999;4(1):123-8.
57. Johansson MJ, Esberg A, Huang B, Bjork GR, Bystrom AS. Eukaryotic wobble uridine modifications promote a functionally redundant decoding system. Mol Cell Bio. 2008;28(10):3301-12.
58. Nedialkova DD, Leidel SA. Optimization of codon translation rates via tRNA modifications maintains proteome integrity. Cell. 2015;161(7):1606-18.
59. Kojic M, Wainwright B. The many faces of Elongator in neurodevelopment and disease. Front Mol Neurosci. 2016;9:115.
60. Laguesse S, Creppe C, Nedialkova DD, Prevot PP, Borgs L, Huysseune S, et al. A dynamic unfolded protein response contributes to the control of cortical neurogenesis. Dev Cell. 2015;35(5):553-67.
61. Freeman S, Mateo Sanchez S, Pouyo R, Van Lerberghe PB, Hanon K, Thelen N, et al. Proteostasis is essential during cochlear development for neuron survival and hair cell polarity. EMBO Rep. 2019;20(9):e47097.
62. Lanvers-Kaminsky C, Zehnhoff-Dinnesen AA, Parfitt R, Ciarimboli G. Drug-induced ototoxicity: mechanisms,

pharmacogenetics, and protective strategies. Clin Pharmacol Ther. 2017;101(4):491-500.
63. Fujinami Y, Mutai H, Kamiya K, Mizutari K, Fujii M, Matsunaga T. Enhanced expression of C/EBP homologous protein (CHOP) precedes degeneration of fibrocytes in the lateral wall after acute cochlear mitochondrial dysfunction induced by 3-nitropropionic acid. Neurochem Int. 2010;56(3):487-94.
64. Kalinec GM, Thein P, Parsa A, Yorgason J, Luxford W, Urrutia R, et al. Acetaminophen and NAPQI are toxic to auditory cells via oxidative and endoplasmic reticulum stress-dependent pathways. Hearing Res. 2014;313:26-37.
65. Oishi N, Duscha S, Boukari H, Meyer M, Xie J, Wei G, et al. XBP1 mitigates aminoglycoside-induced endoplasmic reticulum stress and neuronal cell death. Cell Death Dis. 2015;6:e1763.
66. Zong S, Liu T, Wan F, Chen P, Luo P, Xiao H. Endoplasmic reticulum stress is involved in cochlear cell apoptosis in a cisplatin-induced ototoxicity rat model. Audiol Neuro-Otol. 2017;22(3):160-8.
67. Tu Y, Fan G, Sun H, Cai X, Kong W. Endoplasmic reticulum stress is involved in spiral ganglion neuron apoptosis following chronic kanamycin-induced deafness. Biosci Rep. 2019;39(2):BSR20181749.
68. Fujinami Y, Mutai H, Mizutari K, Nakagawa S, Matsunaga T. A novel animal model of hearing loss caused by acute endoplasmic reticulum stress in the cochlea. J Pharmacol Sci. 2012;118(3):363-72.
69. Yang Y, Ma F, Liu Z, Su Q, Liu Y, Liu Z, et al. The ER-localized Ca^{2+}-binding protein calreticulin couples ER stress to autophagy by associating with microtubule-associated protein 1A/1B light chain 3. J Biol Chem. 2019;294(3):772-82.
70. Karasawa T, Wang Q, David LL, Steyger PS. Calreticulin binds to gentamicin and reduces drug-induced ototoxicity. Toxicol Sci. 2011;124(2):378-87.
71. Horibe T, Matsui H, Tanaka M, Nagai H, Yamaguchi Y, Kato K, et al. Gentamicin binds to the lectin site of calreticulin and inhibits its chaperone activity. Biochem Biophys Res Commun. 2004;323(1):281-7.
72. Karasawa T, Sibrian-Vazquez M, Strongin RM, Steyger PS. Identification of cisplatin-binding proteins using agarose conjugates of platinum compounds. PLoS One. 2013;8(6):e66220.
73. Liberman MC. Noise-induced hearing loss: Permanent versus temporary threshold shifts and the effects of hair cell versus neuronal degeneration. Adv Exp Med Biol. 2016;875:1-7.
74. Xue Q, Li C, Chen J, Guo H, Li D, Wu X. The Protective effect of the endoplasmic reticulum stress-related factors BiP/GRP78 and CHOP/Gadd153 on noise-induced hearing loss in guinea pigs. Noise Health. 2016;18(84):247-55.
75. Sidrauski C, Acosta-Alvear D, Khoutorsky A, Vedantham P, Hearn BR, Li H, et al. Pharmacological brake-release of mRNA translation enhances cognitive memory. eLife. 2013;2:e00498.
76. Sidrauski C, Tsai JC, Kampmann M, Hearn BR, Vedantham P, Jaishankar P, et al. Pharmacological dimerization and activation of the exchange factor eIF2B antagonizes the integrated stress response. eLife. 2015;4:e07314.
77. Hayashi T, Su TP. Sigma-1 receptor chaperones at the ER-mitochondrion interface regulate Ca^{2+} signaling and cell survival. Cell. 2007;131(3):596-610.
78. Mitsuda T, Omi T, Tanimukai H, Sakagami Y, Tagami S, Okochi M, et al. Sigma-1Rs are upregulated via PERK/eIF2alpha/ATF4 pathway and execute protective function in ER stress. Biochem Biophys Res Commun. 2011;415(3):519-25.
79. Mori T, Hayashi T, Hayashi E, Su TP. Sigma-1 receptor chaperone at the ER-mitochondrion interface mediates the mitochondrion-ER-nucleus signaling for cellular survival. PLoS One. 2013;8(10):e76941.
80. Alam S, Abdullah CS, Aishwarya R, Orr AW, Traylor J, Miriyala S, et al. Sigmar1 regulates endoplasmic reticulum stress-induced C/EBP-homologous protein expression in cardiomyocytes. Biosci Rep. 2017;37(4):BSR20170898.
81. Yamashita D, Sun GW, Cui Y, Mita S, Otsuki N, Kanzaki S, et al. Neuroprotective effects of cutamesine, a ligand of the sigma-1 receptor chaperone, against noise-induced hearing loss. J Neurosci Res. 2015;93(5):788-95.
82. D'Adamio F, Zollo O, Moraca R, Ayroldi E, Bruscoli S, Bartoli A, et al. A new dexamethasone-induced gene of the leucine zipper family protects T lymphocytes from TCR/CD3-activated cell death. Immunity. 1997;7(6):803-12.
83. Asselin-Labat ML, David M, Biola-Vidamment A, Lecoeuche D, Zennaro MC, Bertoglio J, et al. GILZ, a new target for the transcription factor FoxO3, protects T lymphocytes from interleukin-2 withdrawal-induced apoptosis. Blood. 2004;104(1):215-23.
84. Delfino DV, Agostini M, Spinicelli S, Vito P, Riccardi C. Decrease of Bcl-xL and augmentation of thymocyte apoptosis in GILZ overexpressing transgenic mice. Blood. 2004;104(13):4134-41.
85. Andre F, Corazao-Rozas P, Idziorek T, Quesnel B, Kluza J, Marchetti P. GILZ overexpression attenuates endoplasmic reticulum stress-mediated cell death via the activation of mitochondrial oxidative phosphorylation. Biochem Biophys Res Commun. 2016;478(2):513-20.
86. Jia H, Yu Z, Ge X, Chen Z, Huang X, Wei Y. Glucocorticoid-induced leucine zipper protects noise-induced apoptosis in cochlear cells by inhibiting endoplasmic reticulum stress in rats. Med Mol Morphol. 2019; doi: 10.1007/s00795-019-00232-7. [Epub ahead of print]
87. Wang W, Sun Y, Chen S, Zhou X, Wu X, Kong W, et al. Impaired unfolded protein response in the degeneration of cochlea cells in a mouse model of age-related hearing loss. Exp Gerontol. 2015;70:61-70.
88. Patury S, Miyata Y, Gestwicki JE. Pharmacological targeting of the Hsp70 chaperone. Curr Top Med Chem. 2009;9(15):1337-51.
89. Mikuriya T, Sugahara K, Sugimoto K, Fujimoto M, Takemoto T, Hashimoto M, et al. Attenuation of progressive hearing loss in a model of age-related hearing loss by a heat shock protein inducer, geranylgeranylacetone. Brain Res. 2008;1212:9-17.
90. Kim YH, Song JJ, Kim YC, Park KT, Lee JH, Choi JM, et al. Geranylgeranylacetone ameliorates acute cochlear damage caused by 3-nitropropionic acid. Neurotoxicology. 2010;31(3):317-25.
91. Mikuriya T, Sugahara K, Takemoto T, Tanaka K, Takeno K, Shimogori H, et al. Geranylgeranylacetone, a heat shock protein inducer, prevents acoustic injury in the guinea pig. Brain Res. 2005;1065(1-2):107-14.
92. Endo S, Hiramatsu N, Hayakawa K, Okamura M, Kasai A, Tagawa Y, et al. Geranylgeranylacetone, an inducer of the 70-kDa heat shock protein (HSP70), elicits unfolded protein response and coordinates cellular fate independently of HSP70. Mol Pharmacol. 2007;72(5):1337-48.
93. Hayakawa K, Hiramatsu N, Okamura M, Yao J, Paton AW, Paton JC, et al. Blunted activation of NF-kappaB and NF-kappaB-dependent gene expression by geranylgeranylacetone: involvement of unfolded protein response. Biochem Biophys Res Commun. 2008;365(1):47-53.
94. Kanemura H, Kusumoto K, Miyake H, Tashiro S, Rokutan K, Shimada M. Geranylgeranylacetone prevents acute liver damage after massive hepatectomy in rats through suppression of a CXC chemokine GRO1 and induction of heat shock proteins. J Gastrointest Surg. 2009;13(1):66-73.
95. Cortez L, Sim V. The therapeutic potential of chemical chaperones in protein folding diseases. Prion. 2014;8(2):28938.
96. Jia Z, He Q, Shan C, Li F. Tauroursodeoxycholic acidattenuates gentamicin-induced cochlear hair cell death in vitro. Toxicol Lett. 2018;294:20-6.

97. Brusilow SW, Maestri NE. Urea cycle disorders: diagnosis, pathophysiology, and therapy. Adv Pediatr. 1996;43:127-70.
98. Mimori S, Ohtaka H, Koshikawa Y, Kawada K, Kaneko M, Okuma Y, et al. 4-Phenylbutyric acid protects against neuronal cell death by primarily acting as a chemical chaperone rather than histone deacetylase inhibitor. Bioorganic Med Chem Lett. 2013;23(21):6015-8.
99. Halliday M, Radford H, Sekine Y, Moreno J, Verity N, le Quesne J, et al. Partial restoration of protein synthesis rates by the small molecule ISRIB prevents neurodegeneration without pancreatic toxicity. Cell Death Dis. 2015;6:e1672.
100. Hosoi T, Kakimoto M, Tanaka K, Nomura J, Ozawa K. Unique pharmacological property of ISRIB in inhibition of Aβ-induced neuronal cell death. J Pharmacol Sci. 2016;131(4):292-5.
101. Sidrauski C, McGeachy AM, Ingolia NT, Walter P. The small molecule ISRIB reverses the effects of eIF2α phosphorylation on translation and stress granule assembly. eLife. 2015;4:05033.
102. Goncalves AC, Towers ER, Haq N, Porco JA, Jr., Pelletier J, Dawson SJ, et al. Drug-induced stress granule formation protects sensory hair cells in mouse cochlear explants during ototoxicity. Sci Rep. 2019;9(1):12501.
103. Boyce M, Bryant KF, Jousse C, Long K, Harding HP, Scheuner D, et al. A selective inhibitor of eIF2alpha dephosphorylation protects cells from ER stress. Science. 2005;307(5711):935-9.
104. Sokka AL, Putkonen N, Mudo G, Pryazhnikov E, Reijonen S, Khiroug L, et al. Endoplasmic reticulum stress inhibition protects against excitotoxic neuronal injury in the rat brain. J Neurosci. 2007;27(4):901-8.
105. Cnop M, Ladriere L, Hekerman P, Ortis F, Cardozo AK, Dogusan Z, et al. Selective inhibition of eukaryotic translation initiation factor 2 alpha dephosphorylation potentiates fatty acid-induced endoplasmic reticulum stress and causes pancreatic beta-cell dysfunction and apoptosis. J Biol Chem. 2007;282(6):3989-97.
106. Ryskamp DA, Korban S, Zhemkov V, Kraskovskaya N, Bezprozvanny I. Neuronal sigma-1 receptors: Signaling functions and protective roles in neurodegenerative diseases. Front Neurosci. 2019;13:862.
107. El Kechai N, Agnely F, Mamelle E, Nguyen Y, Ferrary E, Bochot A. Recent advances in local drug delivery to the inner ear. Int J Pharm. 2015;494(1):83-101.
108. Hao J, Li SK. Inner ear drug delivery: Recent advances, challenges, and perspective. Eur J Pharm Sci. 2019;126:82-92.
109. Valenzuela V, Martinez G, Duran-Aniotz C, Hetz C. Gene therapy to target ER stress in brain diseases. Brain Res. 2016;1648(Pt B):561-70.
110. Sacheli R, Delacroix L, Vandenackerveken P, Nguyen L, Malgrange B. Gene transfer in inner ear cells: a challenging race. Gene Ther. 2013;20(3):237-47.
111. Colella P, Ronzitti G, Mingozzi F. Emerging issues in AAV-mediated in vivo gene therapy. Mol Ther Methods Clin Dev. 2018;8:87-104.
112. Landegger LD, Pan B, Askew C, Wassmer SJ, Gluck SD, Galvin A, et al. A synthetic AAV vector enables safe and efficient gene transfer to the mammalian inner ear. Nat Biotechnol. 2017;35(3):280-4.
113. Tan F, Chu C, Qi J, Li W, You D, Li K, et al. AAV-ie enables safe and efficient gene transfer to inner ear cells. Nat Commun. 2019;10(1):3733.
114. Shibata SB, Ranum PT, Moteki H, Pan B, Goodwin AT, Goodman SS, et al. RNA interference prevents autosomal-dominant hearing loss. Am J Hum Genet. 2016;98(6):1101-13.
115. Gao X, Tao Y, Lamas V, Huang M, Yeh WH, Pan B, et al. Treatment of autosomal dominant hearing loss by in vivo delivery of genome editing agents. Nature. 2018;553(7687):217-21.
116. Zuris JA, Thompson DB, Shu Y, Guilinger JP, Bessen JL, Hu JH, et al. Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo. Nat Biotechnol. 2015;33(1):73-80.

REVIEW ARTICLE

Optogenetics and photopharmacology in pain research and therapeutics

Federico ISEPPON[a,b*], and Manuel ARCANGELETTI[a]

[a] *Molecular Nociception Group, Wolfson Institute for Biomedical Research, University College London, Gower Street, WC1E 6BT, London, UK.*
[b] *Discovery UK, Neuroscience, Biopharmaceuticals R&D, AstraZeneca, Cambridge, UK.*

*Correspondence: f.iseppon@ucl.ac.uk
https://doi.org/10.37175/stemedicine.v1i3.43

ABSTRACT

Pain afflicts billions of people worldwide, who suffer especially from long-term chronic pain. This gruelling condition affects the nervous system at all levels: from the brain to the spinal cord, the Dorsal Root Ganglia and the peripheral fibres innervating the skin. The nature of the different molecular and cellular components of the somatosensory modalities, as well as the complexity of the peripheral and central circuitry are yet poorly understood. Light-based techniques such as optogenetics, in concert with the recent advances in single-cell genetic profiling, can help to elucidate the role of diverse neuronal sub-populations in the encoding of different sensory and painful stimuli by switching these neurons on and off via optically active proteins, namely opsins. Recently, photopharmacology has emerged from the efforts made to advance optogenetics. The introduction of azo-benzene-based light-sensitive molecular switches has been applied to a plethora of molecular targets, from ion channels and receptors to transporters, enzymes and many more, some of which are paramount for pain research and therapy.

In this review, we summarise the past and ongoing research in the fields of optogenetics and photopharmacology and we discuss the use of light-based techniques for the investigation of acute and chronic pain physiology, besides their potential for future therapeutic use to improve pain treatment.

Keywords: Optogenetics · Photopharmacology · Pain · Phototherapy

Introduction

Pain, according to the International Association for the Study of Pain, is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (1). It is a condition that torments more than 1.5 billion people globally, who suffer especially from long-term chronic pain (2). Chronic pain indeed affects an estimated 20% of adults in Europe and U.S., and the current available treatments produce limited reliefs and moderate to severe side effects (2,3).

In contrast to many neurological disorders, pain affects the nervous system at all levels: from brain regions to spinal cord, Dorsal Root Ganglia (DRGs) and peripheral fibres that innervate the skin and the organs (4). Noxious sensation is mediated through the transmission of sensory inputs from the periphery to the spinal cord via modality-specific afferents that reside in the DRGs and discriminate between the different tissue damaging stimuli (4,5). Furthermore, the different nature of pain sensations (mechanical, thermal, chemical) is also dependent on the integration of the sensory inputs in the dorsal horn of the spinal cord, and abnormalities at any level lead to several pathological conditions, including chronic pain (6-9).

Albeit in the last few years technological advances have shed new light on the different molecular and cellular components of painful sensation, the precise circuitry, as well as the changes that occur in pathological conditions, remain not fully understood.

Genetic profiling of single neurons in the peripheral and central nervous systems has allowed the distinction of different sub-populations of sensory neurons based on specific molecular and cellular markers and may serve as a catalogue of the molecular and chemical bases of somatic sensation and pain (10).

Recently, the development and use of light-based approaches that aim to modulate these neurons and dissect the role of each sub-population in the encoding

Received: Apr 20, 2020; Accepted: Jun 12, 2020.

of the different painful stimuli grew exponentially. Optogenetics offers powerful genetic tools to analyse the function of these distinct cellular circuits (11), while photopharmacology is focused on the modulation of channels and receptors that are differentially expressed throughout the nervous system and paramount for pain input transmission with precise spatial and temporal resolution (12,13).

This article aims to review the recent literature on light-based techniques and their applications for research on acute and chronic pain physiology.

Origin and development of light-based pharmacological approaches

Optogenetics and photopharmacology are techniques that enable precise spatial and temporal control of the activity of specific sub-population of neurons. Optogenetics involves the use of genetically encoded light-sensitive ion channels whose sensitivity is dependent on chromophores of natural origin, such as retinal or flavins, in order to modulate cellular activity within specific cell types (14). Photopharmacology, on the other hand, adopts entirely synthetic photoswitches, that are exogenous and need to be specifically delivered to control the function of native biological targets (15,16). Such compounds need to have the capability to undergo a conformational change upon the delivery of a light stimulus and the physiological activities of these two forms must differ (17).

The development of both optogenetics and photopharmacology is inevitably linked: the first step in the development of optogenetics was the discovery by Stoeckenius and Oesterhelt, in 1971, of the light-sensitive ion channel bacteriorhodopsin. Bacteriorhodopsin is a proton pump driven by green light (maximum activation at 568 nm wavelength) that is used for photosynthesis in archaeon *Halobacterium halobium* (18). Six years later, in 1977, halorhodopsin (HR), an inhibitory, yellow light-sensitive chloride channel was discovered by Matsuno-Yagi and Mukohata (19). However, optogenetics as biotechnology was not established until 2002, when Hegemann and Nagel discovered in green algae the channelrhodopsin (ChR), an excitatory cation channel activated by blue light (20). Concurrently, in a paper published in 2002, Miesenbock showed that light could be used as a tool to stimulate action potential discharge in genetically localised neuron subpopulations (21). Later, in 2005, it was then demonstrated by the same group that light-driven activation of diverse circuits in the brain had a direct effect on animal behaviour in *Drosophila melanogaster* (22). In 2004 Kramer, Trauner and Isacoff applied a chemical optogenetic approach to render voltage-gated potassium channels responsive to light and thus controlling the on-off activity of neurons in culture (23). In 2005, ChR was then used to evoke action potentials in mammalian neurons (11) and from 2007 scientists started to use optogenetics as a tool in live, freely-moving animals (24). Successively, from 2012 onwards, a series of important advancements were made in this field: firstly, the design of red-shifted opsins allowed to use red light wavelengths to reduce scattering in tissues and improve both the efficiency and the spatial depth of the excitation (25,26). Secondly, in 2014, Berndt and colleagues engineered an inhibitory isoform of channelrhodopsin-2 (ChR2), capable of conducting chloride anions instead of monovalent cations (27). Thirdly, extremely relevant for the purpose of this review was the development in 2016 of a bi-stable variant, step-waveform inhibitory channelrhodopsin (SwiChR): this isoform is capable of long-lasting activation upon a brief exposure to blue light and deactivates promptly when illuminated with red light (28,29). Besides ion channels, the continuous improvement of the optogenetic tools has brought to the engineering of chimeric light-sensitive G Protein Coupled Receptors (GPCR) called OptoXRs, that are capable, upon light exposure, of activating the intracellular signalling pathways as efficiently as their endogenous versions (30). Moreover, other components of subcellular signalling have been made light-sensitive: enzymes such as photoactivated adenylyl cyclase, light-oxygen-voltage sensors that facilitate protein-protein interactions, and finally gene expression factors such as photoactivatable Cre recombinase (14,31). These advancements greatly expand the complexity of intracellular modulation beyond the simple on-off switch of the first rhodopsin-based opsins (32).

Photopharmacology originated as an effort to provide more reliable tools to optogenetics and in the last few years has grown noticeably due to its applicability in living systems and its role in complementing the conventional optogenetic techniques. The first breakthrough in this area dated as early as the 1960s, when Erlanger and Nachmansohn investigated azobenzene-based inhibitors of acetylcholinesterase (33,34). However, it was only back in 2012 that Trauner and Kramer matured the idea of developing drugs containing synthetic light-switching molecules. The molecule they synthesized, specifically, was a diethylamine-azobenzene-quaternary ammonium able to replicate the light switching function of opsins by blocking the cell potassium-ion channels when activated by light and unblocking the channels in the dark (35,36). Since then, chemistry in couple with biology have offered a wide variety of synthetic photoswitches with highly convertible properties targeted to ion channels, GPCRs, transporters, enzymes, cytoskeleton proteins and lipids, just to name some (15,37).

Designing probes for light-based research and therapy

Optogenetics

Optogenetics, as mentioned before, is a technique that mainly exploits light-sensitive ion channels, the so-called opsins, to modulate neuronal activity with high spatial and temporal resolution (38). Excitatory opsins, like ChR2, are cation selective channels that cause cation influx and photo-controlled neuron depolarisation when illuminated at blue wavelengths (**Figure 1A**) (11,20,39).

Inhibitory opsins, like Archaeorhodopsin (Arch) or HR, provoke either proton efflux or chloride influx respectively to drive an outward photocurrent that generates hyperpolarisation and promptly inhibits neuronal activity (**Figure 1B**) (40-42). In recent years, the endeavour in genome screening and molecular engineering to expand the optogenetics toolbox has generated faster recovery variant for high-speed imaging (43,44), red-shifted opsins to improve the depth of the light penetration (45,46), and bi-stable opsin variants to induce long-lasting changes in neuronal activity. These latter variants are particularly interesting from a therapeutic point of view, since their capability to induce chronic effects with minimal light delivery would reduce both the need for constant light treatment and the risk of long-term phototoxicity (47).

Moreover, recent works focused on the modulation of intracellular signalling cascades with the engineering of photo-activatable cell-surfaced GPCRs for adrenergic, serotoninergic, dopaminergic, adenosine, glutamate (metabotropic) and μ-opioid receptors (30,48-52). These new OptoXR probes, as they are called, generate the same signalling cascade as the endogenous receptors, whilst they can be triggered with a spatio-temporal precision that is not achievable with traditional pharmacological approaches, thus bringing great advantage in the study of relevant targets in defined regions of the body (**Figure 1C**). This level of precise spatio-temporal control, particularly in the case of μ-opioid receptors, is fundamental in dissecting the opioid contribution in peripheral and central nociceptive circuits (53).

The investigation of somatosensation and pain with optogenetics goes unavoidably in pair with the possibility to deliver the opsins to defined neuronal sub-populations in the central and peripheral nervous systems. Two approaches have

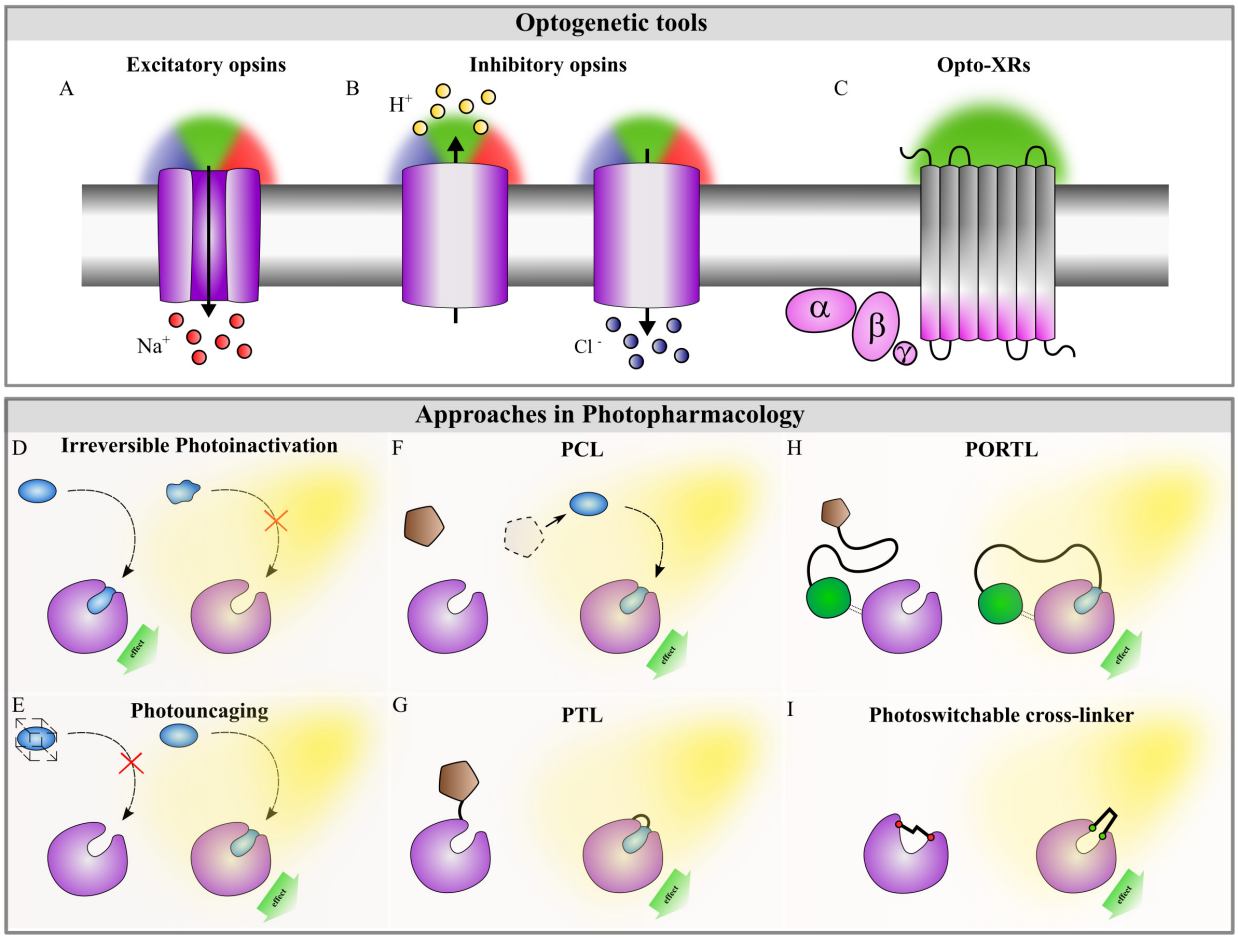

Figure 1. Optogenetic toolbox and Photopharmacological approaches. (A-C) Principal opsins used as optogenetic tools are illustrated. Arrows indicate direction of ion flux. **(A)** Excitatory opsins (*ChR2*) are non-specific cation channels that depolarize neurons when stimulated by light. **(B)** Inhibitory opsins elicit either chloride influx (*iC1C2*, *SwiChR, ChloC, HR*, *Jaws*) or proton efflux (*Arch*) to silence the neuronal activity when illuminated by light. **(C)** Chimeric light-sensitive G-protein coupled receptors (*optoXRs*), consisting of the extracellular and transmembrane domains of light-sensitive rhodopsins with the intracellular regions of a GPCR of interest. **(D-I)** Diverse Photopharmacological approaches are illustrated. Red crosses indicate the switch to the inactive conformation of the photomodulators. **(D)** Irreversible photoinactivation. **(E)** Irreversible photoactivation (*photouncaging*). **(F)** Reversible photoactivation/ inactivation using a PhotoChromic diffusible Ligand (PCL) that upon irradiation switches between an inactive (brown pentagon) and an active (blue ellipse) form, modulating the activity of the target. **(G)** Photo-switchable (closely) Tethered Ligand (PTL) - the photoswitch is, in this case, covalently bound to the target and in close proximity of it. **(H)** Photo-switchable Orthogonal Remotely Tethered Ligand (PORTL) - As for the PTL, the photoswitch is connected to the target but is not in proximity of it. **(I)** Photo-switchable cross-linker - the photoswitch is conjugated on both sides to the target and usually prevents the activity of the target in one of its conformations.

been the most utilised in tackling this issue: viral vectors and opsin-expressing transgenic mice (54-56). The specificity of the viral transgene delivery can be obtained mainly via incorporation of endogenous promoters (57) or recombinase-dependent expression (58,59). Given the experimental problems that can arise with the former method, as well as its partial lack of specificity, the most widely used method for opsin gene delivery is the Cre/Lox-P mediated recombination and conditional expression of transgenes delivered by Adeno-Associated Viruses (AAVs) (60-62). These viruses are injected locally into transgenic mice in which the Cre recombinase expression is restricted to specific neuronal sub-populations (62-64). Conversely, crossing Cre-expressing mice with opsin-expressing lines gives yet another possibility to manipulate molecularly defined sets of neuronal and non-neuronal cells (65-67). These strategies are very advantageous in the study of large cell populations, that however can still comprise heterogeneous sub-populations with different functions within them. Thus, a novel approach called INTRSECT that uses multiple recombinase steps to further refine the specificity of selected subpopulations offers new advantages and great prospect for the study of neuronal circuits underlying specific roles in somatosensation and pain at all levels in the nervous system (68-71).

Photopharmacology

One of the main principles at the basis of photopharmacology is the ability to modulate the pharmacokinetic or pharmacodynamic properties of synthetic molecules by using light. This can be achieved, in most of the cases, with the alteration of a functional group of the drug with a photolysable element (72-74). The main benefit of using this technique is that it permits to reduce the off-target and systemic side effects and to decrease the drug resistance in comparison to a standard drug delivery method (15). Photopharmacological tools have been previously applied to study cancer, diabetes, microbial infections and neurology (15,16,75-81). The importance of this method derives from the fact that potentially every kind of molecule, even with very different range of sizes, can be optically-controlled and thus allowing a fine temporal and spatial control over intracellular or extracellular targets (82).

The effect that the light exerts on its target can be classified into two modalities: reversible and irreversible; each of them have been employed in biology (83).

Irreversible photoinactivation is realized when a freely diffusible compound is irreversibly modified by irradiation and has been mainly used to probe the functional role of a biological target (**Figure 1D**) (84). Also, caged compounds belong to this first category of molecules: they can only be activated once and the chemical strategy approach to gain photocontrol of a target by using these molecules is called *photouncaging*. Technically, a photocage is a chemical group that converts the energy of a photon into energy that is then used to disrupt a chemical bond, strategically placed in a position in which it can modulate the activity of a bioactive molecule (74). Irradiation promotes a reaction that causes the removal of the photocage, triggering the release of the biologically active molecule, switching on (or off) the targeted process (**Figure 1E**) (85). To date, this is the most broadly used photopharmacological approach, and several new photocages continue to appear (73,74,86,87). The other approaches worth mentioning are the recent development of the so-called Photobody (87), that uses the specificity of an antibody fragment to selectively bind and modulate the activity of the desired target, and the family of BODIPY-derived photocages (86); the latter are caged compounds that can be activated with the highest known wavelengths of light through a mechanism that involves a single-photon-release.

As mentioned before, the major drawback of this technique is that the photouncaging process is irreversible and allows to control the properties of a pharmacological compound just once.

Reversible photoswitches, on the basis of the position relative to their target, can be classified into those that interact with their targets through noncovalent interactions (photo-chromic ligands - PCLs) and the ones in which the formation of a covalent bond is involved for the connection to the target (photo-switchable tethered ligands - PTLs, photo-switchable orthogonal remotely tethered ligands - PORTLs). There is also another class of reversible photoswitches, called cross-linkers, that rely on the aid of bioconjugation motifs at both sides of an optically active molecule.

PCLs are freely diffusible molecules in which the irradiation triggers the switch between two different isomeric conformations. As already mentioned, the switch into two different isoforms confers each of them different affinity and/or efficacy, diverse pharmacodynamics properties and may also affect the pharmacokinetics properties. (**Figure 1F**) (13).

A second class of reversible photoswitches includes ligands that are covalently attached to the target through a connection that can be either through a native or an engineered residue. Major advantages of this approach include the ability to accelerate the response by increasing the local concentration of the switches, the ability of the ligand to remain in the proximity of the target and the loss of the need for reapplication of the drug. On the other hand, this approach requires genetic encoding for its full applicability (88).

As mentioned before, tethered ligands can be sub-classified into **(1)** Photo-switchable Tethered Ligands (PTLs) and **(2)** Photo-switchable Orthogonal Remotely Tethered Ligands (PORTLs), depending on the length of the covalent attachment with respect to the ligand binding site.

In respect of PTLs, the photoswitch is attached close to the binding site and the tether is mainly constituted by the photoswitch itself. The switch between the different isomers mainly modifies the concentration of the pharmacophore in the near proximity of the target. They are ideally built as if in one configuration the ligand is physically impeded to reach the binding site while in the other it can exert its function. It requires small bioconjugation molecules, like cysteines (**Figure 1G**).

Conversely, in a PORTL, the tether is much longer, bringing the photoswitch far from the binding site. In this way, the light-induced conformational change affects the efficacy of the tethered ligand rather than its local concentration near the target (**Figure 1H**) (89).

Another class is constituted by light-responsive cross-linkers in which the photoswitch is attached by a covalent bond on both its ends to the target. This method requires the presence of two conjugation motifs on the biomolecule. Upon irradiation, the photoswitch modifies its conformation, triggering then a change in the activity and conformation of the target itself (**Figure 1I**) (13,16,75).

Further considerations on designing photoswitches

An ideal photoswitch must fulfil several requirements to be used in an in vivo model: it should have favourable pharmacokinetics and should be metabolically stable in a given environment. Phototoxicity is an important parameter to bear in mind and, in addition, the photoswitch should have useful photophysical properties, such as high absorbance and quantum yields, and useful thermal relaxation rates (13). A wide range of photoswitches have been used in the last few years but one of the most encouraging one, in terms of its properties, is the reversible molecule called azobenzene. Azobenzene is constituted by a diazo bond (***N*==*N***) that is linked to two phenyl rings. It can adopt the *trans*- or *cis*- conformation: in the former, the phenyl rings are on the opposite sides while in the latter, they are on the same side. UV light triggers the swap between the two isomers of which the *trans*- one is thermodynamically more stable. This process is reversible and can be inverted using heat or by using visible light irradiation (13,35,36,90).

Biological targets in pain research

Pain is an extremely intricate disease which can progress into severe conditions. The effective treatment of pain often lacks the desired level of efficacy, tolerability and target specificity. Optogenetics in the last two decades had a pivotal role in the investigation of pain physiology both in the central and peripheral nervous systems (84). Photopharmacology emerged in the recent years as a potential new approach to be applied in pain research and treatment (91). In this section, we pass into exam all the development in pain research and the potential biological targets that have been unravelled with the aid of these approaches.

Within few years of demonstrating optical control of neuronal cells via ChR2, optogenetic probes were applied *in vivo*, together with surgically implanted optical fibres, to control and study different neural circuits within the brain. This idea has been recently implemented also in the investigation of the central circuits of both the sensory and the affective components of nociception (92,93). In the cortico-limbic networks, the BasoLateral Amygdala (BLA) has been revealed to have a prominent role in the encoding of the 'unpleasantness' of pain (94). The sensory information from the BLA is transmitted to the medial PreFrontal Cortex (mPFC). ChR2 injection in the BLA revealed direct connectivity that was input-specific, and the stimulation of these neurons in rodent models of chronic pain revealed increased feed-forward inhibition by mPFC GABAergic neurons (95). Moreover, the activation of the parvalbumin-positive GABAergic interneurons of the mPFC exacerbated pain responses after peripheral nerve injury, and conversely their inhibition alleviated these responses (96). These data reveal that persistent chronic pain states, provoked by peripheral nerve injuries, lead to a selective activation of BLA inputs on specific mPFC GABAergic interneurons, that in turn inhibit projection neurons in the ventro-lateral PeriAqueductal Gray area (vlPAG): this alteration produces a serial dysfunction of the inhibitory tone of the circuit itself, reducing the strength of serotoninergic and noradrenergic descending pathways involved in pain modulation (**Figure 2A**) (96,97).

Anatomical and physiological evidence has been collected to demonstrate the presence of a circuit between ParaBrachial Nucleus (PBN) and the Central nucleus of the Amygdala (CeA) and its role in the affective dimension of pain (98-100). Excitatory synapses within this circuit are potentiated in various chronic pain models (99,101-103), and direct excitation of CeA neurons with ChR2 induced visceral hyperalgesia after bladder distension (102). Moreover, the investigation of the mechanisms involved in neuropathic pain revealed the presence of a complex modulation (both excitation and inhibition) of the neurons within this circuit, based both on specific molecular identity of the neurons and on their location within different sub-regions of the CeA (**Figure 2A**) (103). Together, these results offer a minor but precise overview of some of the complexity of the circuits that process both the sensory and affective component of pain within the brain, and how paramount is optogenetics to elucidate the role of single projections and specific neuronal subpopulations in the central processing of nociceptive information.

On the other hand, pain perception, as well as the processing of pain information, starts from the periphery, with the nociceptive stimuli travelling through a plethora of sub-populations of sensory neurons in the DRGs to the substantia gelatinosa (laminae I and II) of the dorsal horn (**Figure 2B**) (5,104). A recent article identified 11 neuronal sub-populations by single-cell RNA sequencing, highlighting the complexity of the peripheral coding of multi-modal somatosensation (10). Optogenetics is therefore extremely useful in dissecting the role of these different populations in the coding of sensory and nociceptive inputs (105).

The first peripheral neurons targeted with ChR2 excitatory opsin were Mas-related G-protein coupled receptor member D (Mrgprd)-positive nociceptive neurons: their photo-stimulation revealed the circuitry of their connections to most known classes of lamina II spinal cord neurons (106). Light-dependent activation of Advillin-positive, Transient Receptor Potential Vanilloid 1 (TRPV1)-positive and NaV1.8-positive neurons selectively expressing ChR2 elicited strong nociceptive behaviours, which could be blocked by analgesics administration, indicating that a direct activation of these neuronal sub-populations is

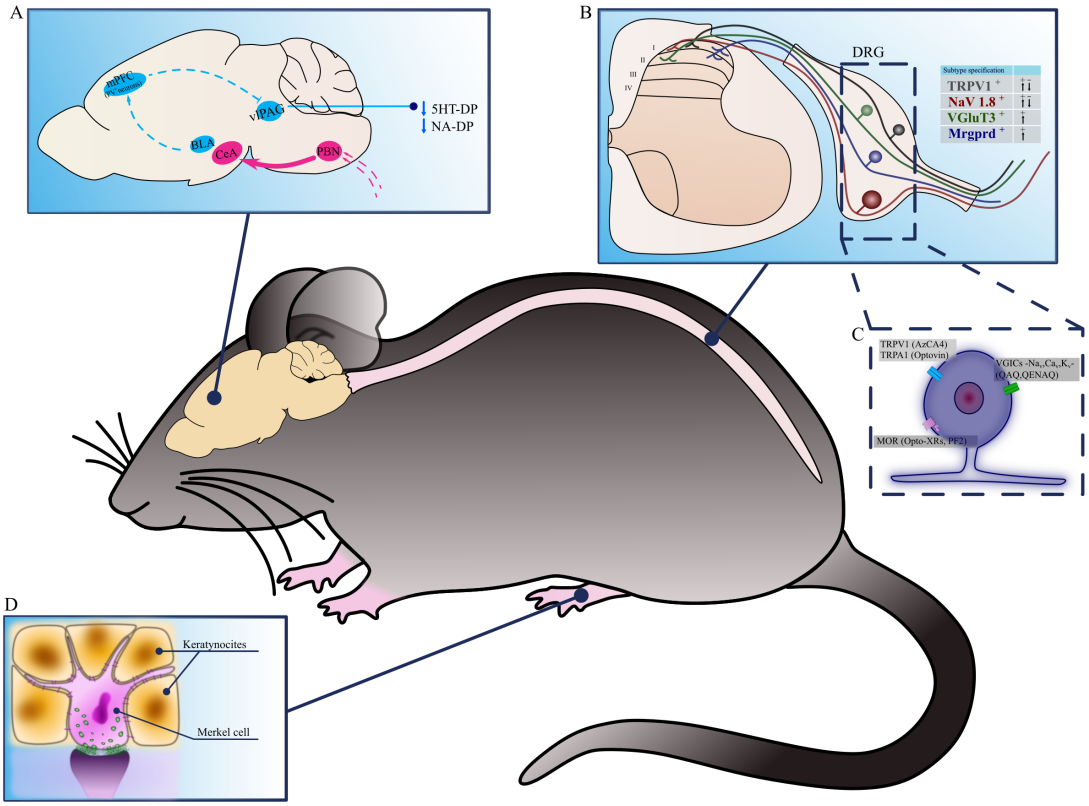

Figure 2. Biological targets of optogenetics and photopharmacology in pain research. Cartoon illustrating some of the targets of interest in the Central and Peripheral Nervous Systems, discussed in this review. **(A)** Overview of some neural circuits of pain within the brain. In light blue it is shown the pathway involving the basolateral amygdala (*BLA*), the medial prefrontal cortex (*mPFC*) and the ventro-lateral PeriAqueductal Gray area (*vlPAG*). The use of optogenetic tools has demonstrated that the modification of the inhibitory tone circuit deeply affects pain modulation during persistent chronic pain states, induced by peripheral nerve injuries. In pink it is shown the circuit between the ParaBrachial Nucleus (*PBN*) and the Central nucleus of the Amygdala (*CeA*) that has a prominent role in the affective dimension of pain. Optogenetics has begun to unravel the profound complexity of this circuit and of the specific sub-populations of neurons involved. **(B)** Diverse sub-populations of sensory neurons in the DRGs form connections at different levels of the substantia gelatinosa (laminae I and II) of the dorsal horn. A list of sub-populations of nociceptive neurons that have a crucial role in pain perception and have been investigated by using optogenetic tools are also shown in the picture (*Mrgprd*$^+$, *TRPV1*$^+$, *NaV1.8*$^+$, *VGLUT3*$^+$). **(C)** Enlargement of a representative neuron in the DRG, showing the main molecular targets involved in nociception that can be currently targeted by specific photo-controllable drugs (*TRP channels in light blue, VGICs in green and MOR in light pink*). **(D)** Optogenetic tools have also been used to elicit responses in peripheral mechano-sensitive cells (*Merkel cells and keratinocytes*) in the epidermis to investigate the role of non-neuronal cells in the perception of innocuous and painful mechanical stimuli.

sufficient to elicit a painful response (62,63,65,107–109). Moreover, prolonged activation of NaV1.8-positive and TRPV1-positive neurons caused a hypersensitivity that lasted long after the stimulus was removed (110,111). Interestingly, the selective activation of the Vescicular GLUtammate Transporter type 3 (VGLUT3)-positive primary afferents elicited only very mild nocifensive behaviours but exacerbated nociceptive responses in a model of chemotherapy-induced neuropathic pain through the Transient Receptor Potential Melastatin 8 (TRPM8) ion channel (112). Conversely, inhibition of the same neuronal populations (NaV1.8-positive, TRPV1-positive) with Arch or HR optogenetic probes alleviates pain behaviours in naïve mice together with murine models of inflammatory and neuropathic pain (**Figure 2B**) (62,111,113). These results are particularly important in pioneering the use of light as an analgesic, opening to the possibility of the use of optogenetics to treat chronic pain. Furthermore, the combination of optogenetic and chemogenetic techniques refines the selection and classification of neuronal sub-populations that have not been specifically genetically identified yet: the use of resiniferatoxin to ablate TRPV1-positive fibres in a transgenic mice expressing ChR2 in Calcitonin related polypeptide 1 (Calca)-positive neurons has brought to the identification of a novel, specific population of High-Threshold MechanoReceptors (HTMR) with unique endings that can be activated by the pulling of a single hair (114).

Furthermore, optogenetic manipulations are not restricted to neuronal cells: in several recent studies peripheral mechano-sensitive cells in the epidermis have been infected to express excitatory and inhibitory opsins. Activation of Merkel cells and keratinocytes is sufficient to elicit action potential discharge in different types of primary afferents, whereas silencing of these cells decreases the spiking of peripheral sensory neurons in response to natural stimuli, as well as ATP release and

nocifensive responses to mechanical painful stimuli (**Figure 2D**) (67,115,116).

Despite revolving mainly around the on/off modulation of whole cell populations, optogenetics has been a keystone in the study of pain circuits, and together with other genetic, electrophysiological and molecular techniques led to the discovery of many important molecular targets for the modulation of pain perception. A more advanced, photopharmacological approach can then be exploited to increase the complexity and capability of research to devise novel approaches to pain modulation and analgesia that can then be translated into therapeutics. To date, only few photo-switchable regulators of nociception have been developed and even less have been described in an *in vivo* system (13,91). In terms of potential targets involved in the pain pathways, one of the most obvious classes is represented by ion channels. However, of the 215 ion channels that exist in the human genome, with 85 ion channels that have been linked to nociception, only a minor number has been successfully targeted for pain research (117).

TRPV1 is a Ca^{2+} permeant non-selective cation channel expressed in various subset of populations of primary afferent neurons and with a well-established role in nociception (118,119). To date, optical control of TRPV1 has been investigated and the result is the development of several azo-capsaicin derivatives (AzCAs). These molecules are photo-switchable agonists of TRPV1 channels, they are fairly inactive in the dark and are activated upon irradiation with UV-A light (120). Among these, *cis*-AzCA4 (121) has been shown to be one of the most effective in activating TRPV1 and to possess a reversible action. In addition, *in vivo* tests demonstrated a TRPV1-mediated hyperalgesia exerted after the application of this compound (**Figure 2C**) (16,120,122).

A photo-switchable compound (Optovin) that reversibly activates another member of the TRP channel family, Transient Receptor Potential Ankyrin 1 (TRPA1), has also been developed so far (123,124). This molecule has been used to modulate TRPA1b channels in zebrafish (**Figure 2C**) (125). Recently, photo-switchable diacylglyerols have also been used to optically-tune the activity of TRPC2, TRPC6 (126) and TRPC3 (127).

GABA-A receptors are chloride-selective pentameric ligand gated ion channels activated by Gamma Ammino-Butirric Acid (GABA). In post-synaptic neurons, GABA receptors trigger a decrease of action potential firing upon their activation. Given that, GABA-A receptors have been investigated as potential target for the development of anaesthetics (128,129). Photo-compounds that act on GABA-A receptors have been synthetized resembling the structure of Propofol, a lipophilic anaesthetic agent that acts through potentiation of GABA-induced currents (128). These compounds operated as allosteric modulators, potentiating GABA currents in the dark and being inactivated upon application of light. Additional Azo-benzene derivatives of propofol were produced (AP1-16) and among these, AP2 showed anaesthetic activity in an *in vivo* animal model in albino *Xenopus laevis* tadpoles (128). Also, the so-called LiGABAR, that is a genetically modified light-controlled GABA receptor, has been developed, so far, by using tethered photopharmacology (130). The resulting design of a transgenic line of mice constitutively expressing LiGABAR, facilitated the development of higher efficient new PTLs (PAG-1C) and finally allowed to control the activity of cortical neurons in mice by using the light (131).

Voltage-gated ion channels (VGICs) play an essential role in the generation of action potentials and in synaptic transmission and represent a privileged target of photopharmacology. They have also been fundamental for the development of the field (132). The photo-switchable azobenzene derivative QAQ is structurally composed of two azo-linked quaternary amines and, together with its derivative QX-314, has been developed on the basis of lidocaine, a local anaesthetic that blocks VGICs (133,134). These compounds are blockers of KV, NaV, and CaV channels and, importantly, are membrane-impermeable and thus they need to be transported into the cell via TRPV1 channels or P2X receptors, allowing the selective targeting of TRPV1 expressing cells for the optical control of nociception. These molecules have been used, in addition to capsaicin, to selectively block TRPV1-positive nociceptors (135,136). So far, a QAQ derivative has also been developed, namely QENAQ, that is controlled by using visible light. This compound allows to photo-control the pain signalling without issues deriving from invasiveness and with high specificity and fast kinetics (**Figure 2C**) (137). Another compound (fotocaine) based on azologisation of the local anaesthetic fomocaine has been also developed. Neurophysiological application of this compound has opened up the way to test its applicability as a potential analgesic (135,136).

μ-opioid receptors are GPCRs that activate inhibitory G-proteins. They assemble as homo- and hetero-dimeric complexes and scaffold a variety of proteins. GPCRs are potentially involved in all physiological processes in eukaryotic organisms, including acute and chronic pain (91). Indeed, most of the potent analgesics currently in use act through the μ-opioid receptor. Moreover, they belong to the class A (Rhodopsin-like family) of GPCRs and thus they have been an exclusively amenable class of proteins for the development of phototunable compounds. For these reasons, photo-switchable opioids have been under thorough investigation in the last few years. The usage of such compounds, as possible photo-analgesics, may enable the optical-control of μ-opioid receptors. The first compound that has been developed was an azobenzene derivative of the synthetic μ-opioid receptor agonist Fentanyl (photofentanyl-2 or PF2) (**Figure 2C**) (138). The development of this compound generated interest in a potential future use of photo-analgesics (16,139).

Photopharmacology is constantly growing and its usage to control nociception is an emerging but interesting field. New compounds are frequently synthetized in order to get accurate control of novel targets (ionotropic glutamate receptors (37,140), metabotropic glutamate receptors (141,142), adrenergic receptors, muscarinic acetylcholine

receptors, dopamine, histamine, serotonin receptors, calcium and potassium channels and a number of transporters and pumps (12,13,75)).

Advancements in light delivery methods

Optogenetics and photopharmacology have the great potential to dissect the somatosensory circuitry and the key molecular players involved in pain biology and pathobiology (143,144). However, one of the major limitations of these approaches, particularly in behavioural experiments, is the complexity to deliver light especially to neurons in the spinal cord and in the periphery in freely behaving mice (143). Brain imaging and optogenetics in awake rodents with chronic optic fibre implants is currently well established and can be used also in combination with electrophysiology to optically stimulate and record, at the same time, from different neuronal circuits *in vivo* (**Figure 3A, B**) (94,144). Imaging peripheral tissues however poses major technical difficulties in the absence of a solid structure like the skull, that can help to stabilise the implants. The first attempts involved peripheral light delivery to the hind paws by implementing optical fibres or Light-Emitting Diode (LED) arrays in cages to target opsin-expressing afferents for behavioural and place aversion tests (**Figure 3G**) (63,65,110). To overcome the limitations of this approach, and in the effort to target more central structures like the spinal cord, tethered optical fibres have been adapted for peripheral nervous system stimulation. Laser-driven optical fibres have been implanted chronically in the epidural space of the spinal cord, allowing for direct modulation of opsin-expressing peripheral sensory neurons innervating the dorsal horn of the spinal cord, as well as interneurons in the substantia gelatinosa (**Figure 3C**) (113,145). Another similar approach involves the use of a nerve cuff that surrounds the peripheral nerve: the light stimulation is provided by an optical fibre tethered to the skull and delivered subcutaneously to reach the implanted cuff (**Figure 3E**) (146). These new technologies have propelled the use of optogenetics to investigate peripheral nociception. However, these implants are still dependent on an apparatus that is partially fixed to the skull, hindering the free movement of the animals. Wireless implantable LED devices for the stimulation of superficial areas in the brain, spinal cord and peripheral tissue have seen a great popularity in the last years. Different laboratories have used similar approaches for the construction of miniaturised probes that utilise microscale LEDs to allow light stimulation in freely behaving rodents (107,109,110,147–149). These implants utilise inductive coupling to remotely power the μLEDs, eliminating the need for batteries and circuits and dramatically reducing the dimension of the implants themselves, that can be as small as 10 mm^3 and weight as less as 20 mg (**Figure 3D, F, H**) (110). The most recent versions of these devices use near-field power coupling and radio frequencies transmission to power and activate the LEDs, as well as softer and more durable encapsulation of the microcircuits, strategies that reduce both the fabrication cost and the technical expertise necessary to produce such devices (109,148,149). Further technological improvements of these wireless approaches will make the simultaneous stimulation and recording of responses possible, as it has been demonstrated in the central nervous system (150), and will help to render a more complete picture of the somatosensory coding of multi-modal stimuli in freely moving animals.

Therapeutic potential and challenges of light-based pharmacology

The possibility to achieve a high spatial and temporal resolution in controlling the signalling of defined neuronal populations throughout the nervous system opens the path towards the development of more effective therapies for disease and pain treatment. Pain management and chronic pain treatment, as stated before, are fundamental problems that are poorly addressed by current treatments and often burdened by unwanted side effects.

Optogenetic and photopharmacological tools employ a spatially defined beam of light as stimulus to elicit a response in the desired target. It is exactly this spatial definition that may be a very effective way to modulate chronic pain in suffering patients (151). The obvious targets to exploit are the numerous ion channels that are expressed centrally and peripherally and are involved in nociception: photo-controllable drugs have been designed to modulate TRPV1, TRPA1, μ-opioid, GABA-A and metabotropic glutamate receptors (118,120,123,128,137,139,152,153). Photochemical and optogenetic controllers of opioid signalling harbour the most promise in delivering peripheral analgesia without involving central circuits linked to addiction (53,139). Another interesting approach is the use of photo-reversible local anaesthetics that target TRPV1-positive nociceptors (QAQ and QENAQ) and have been effective in controlling pain signalling in behaving rodents (134,137). Moreover, well-established light-based techniques now exist for bidirectional control of primary afferents via transdermal stimulation: these techniques could potentially harbour a future of non-invasive, implant-free optogenetic control of chronic pain disorders (147). A fascinating, similarly non-invasive use of light-based therapy is the prolonged exposure of patients to specific light wavelengths to treat pain and anxiety; this kind of therapy has already been used to control depression in chronic pain and disease suffering patients (154,155), and has been recently associated with profound, opioid-dependent peripheral and central anti-nociception in naïve and neuropathic pain suffering rodents (156).

Despite harbouring great promise, several hurdles have still to be overcome in order to deliver a safe and effective therapy for pain management. The two principal issues in the implementation of light-based therapies are the genetic delivery of the opsins or photo-switches to their targets and the delivery of light to inaccessible organs like the brain and the spinal cord. As stated before, the development of wireless light delivery methods using μLEDs, that are miniaturised, injectable and programmable,

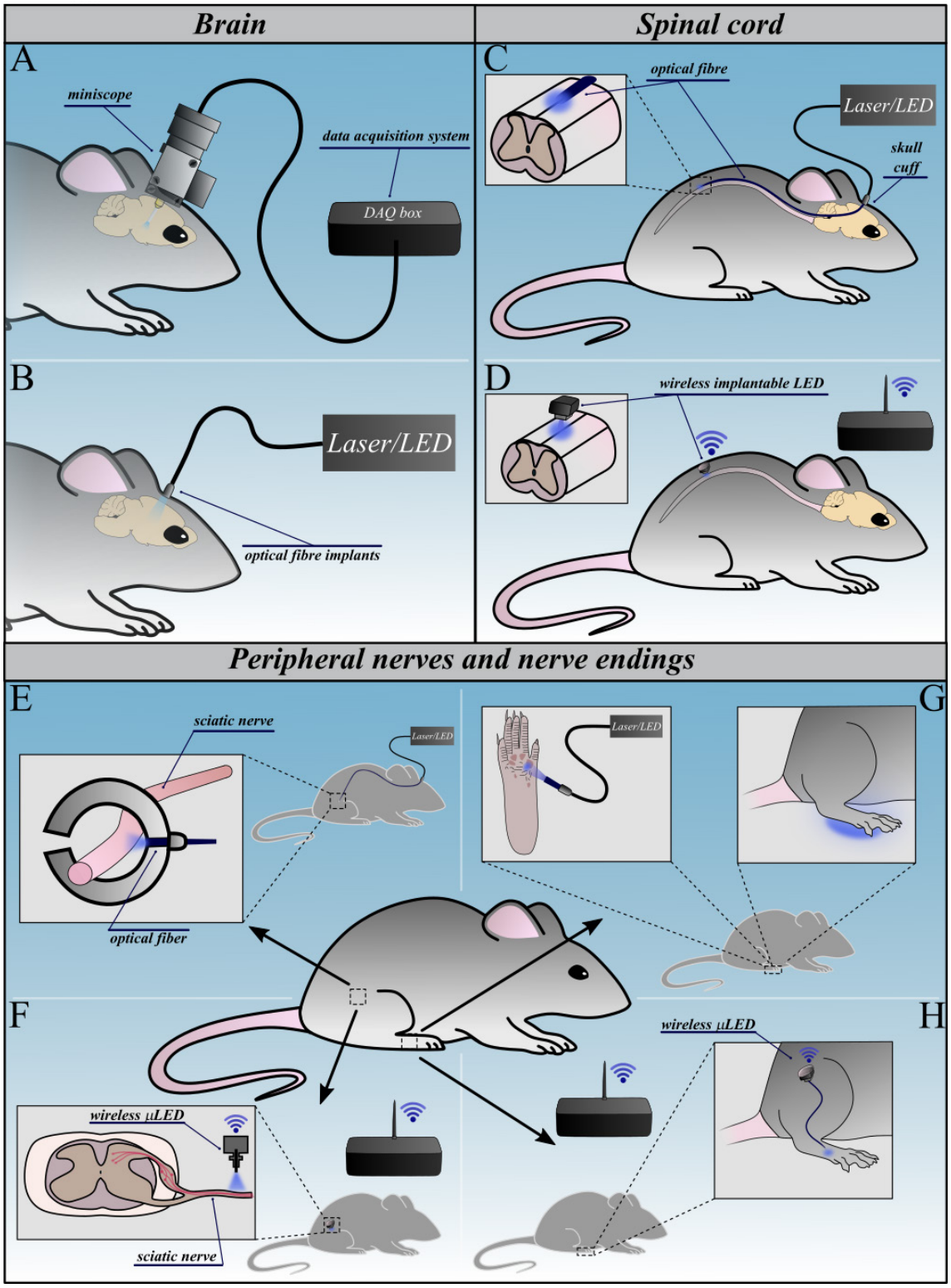

Figure 3. Past, present and future approaches for light delivery within the nervous system. Cartoon illustrating all the different approaches used to deliver light to different regions within the Central and Peripheral Nervous Systems, discussed in this review. **(A, B)** Light delivery approaches for brain imaging. **(A)** Head-mounted microscope system. **(B)** Skull-implanted cuff with an optical fibre cannula; **(C, D)** Light delivery techniques used in the spinal cord imaging. **(C)** Skull cuff with implanted epidural flexible light-emitting diode (LED). **(D)** Wirelessly powered µLED device for stimulation of spinal afferents or spinal cord neurons. **(E, F)** Light delivery approaches for Peripheral Nerves. **(E)** The sciatic nerve is represented, as an example. Fiber-optic coupled nerve cuffs are implanted subcutaneously and connected to the skull. **(F)** Small, wireless µLED devices can directly deliver light to the nerve. **(G, H)** Nerve endings light delivery techniques. **(G)** Transdermal illumination of sensory nerve endings through an external source of light. In the picture two alternative methods are represented (enlargements). **(H)** µLEDs implanted subcutaneously for wireless light-delivery to the area of interests.

is becoming more and more effective, and these devices allow efficient remote photocontrol with minimal tissue damage (107,109,147,157,158). Concomitant light and drug delivery is currently being explored via a combination between light-emitting and microfluidic devices (159).

Gene therapy is the principal tool to successfully and safely deliver photo-controllable molecules to patients. The use of viral vectors has already been effective in the peripheral delivery of transgenes to patients, albeit most studies addressing chronic pain involve direct production and release of analgesic molecules, like GABA or opioid agonists (160,161). AAV vectors are currently used to express ChR2 in retinal ganglion cells of patients, and Herpes Simplex Virus vectors have been used to successfully deliver gene products in humans through intradermal injections (162,163).

Other current limitations of light-based approaches for therapy are the safeness as well as the transient nature of the expression of opsins and photoswitches. Maximal expression of AAV-delivered proteins takes a few weeks, after which the level decreases: routine administration may solve this problem maintaining optimal expression levels. Delivery of the newly engineered bi-stable opsins may partially solve the problem by eliciting long-lasting changes in neuronal activity following low light stimulation (47). Moreover, continuous increase in clinical trials that employ virally mediated gene therapy will boost the improvement of safer vectors for therapeutic treatment, reducing, therefore, the potential occurrence of immune responses.

Thus, despite the critical issues stated before, light-based approaches already represent a powerful and fundamental tool in the study of pain physiology and pathology. Future technological, as well as biological improvements will help to surmount their current obstacles making them a promising candidate for the development of novel therapies in the challenging field of pain management.

Conclusion and future remarks

Light-based pharmacology and genetics have undergone great development in the past two decades. Researchers from various fields recognise the impact that the implementation of these techniques has on their research, as well as the great clinical potential of these approaches, and new interesting targets and applications are emerging at a swift rate. The further development of more and more specific photo-switchable molecules and optogenetic probes, coupled with the advancements in gene therapy and engineering of non-invasive tools to visualise and manipulate their functions *in-situ*, may enable selective and powerful therapeutic interventions and will continue to refine the research on complex neuronal circuits and functions. Finally, the high temporal resolution and cell specificity allowed by these techniques offer great potential for the development of phototherapy as a routinary, powerful and personalised approach to pain treatment that could overcome the limitation of conventional pharmacology.

Acknowlegements
The authors would like to thank Prof. John Wood for the insightful discussion and suggestions during the making of this article. We also thank the core funding from AstraZeneca (“Spinal circuitry of mechanical nociception”) and Wellcome Trust (200183/Z/15/Z).

Conflict of interest
The authors declare no conflicts of interest in the writing of this paper.

Funding body
AstraZeneca, Cambridge, UK.
Wellcome Trust – Charitable Health Foundation, London, UK.

References

1. Pain terms: a list with definitions and notes on usage. Recommended by the IASP Subcommittee on Taxonomy. Pain. 1979 Jun;6(3):249.
2. Nahin RL. Estimates of Pain Prevalence and Severity in Adults: United States, 2012. J Pain. 2015 Aug 1;16(8):769–80.
3. Breivik H, Collett B, Ventafridda V, Cohen R, Gallacher D. Survey of chronic pain in Europe: prevalence, impact on daily life, and treatment. Eur J Pain. 2006 May 20;10(4):287–287.
4. Todd AJ. Neuronal circuitry for pain processing in the dorsal horn. Nat Rev Neurosci. 2010 Dec;11(12):823–36.
5. Peirs C, Seal RP. Neural circuits for pain: recent advances and current views. Science. 2016 Nov 4;354(6312):578–84.
6. von Hehn CA, Baron R, Woolf CJ. Deconstructing the neuropathic pain phenotype to reveal neural mechanisms. Neuron. 2012 Feb 23;73(4):638–52.
7. Markenson JA. Mechanisms of chronic pain. Am J Med. 1996 Jul 31;101(1 A):S6–18.
8. Meacham K, Shepherd A, Mohapatra DP, Haroutounian S. Neuropathic pain: central vs. peripheral mechanisms. Curr Pain Headache Rep. 2017 Jun 1;21(6):28.
9. Saab CY. Chronic pain and brain abnormalities. Chronic Pain and Brain Abnormalities. 2013;1–148.
10. Usoskin D, Furlan A, Islam S, Abdo H, Lönnerberg P, Lou D, et al. Unbiased classification of sensory neuron types by large-scale single-cell RNA sequencing. Nat Neurosci. 2015 Jan 1;18(1):145–53.
11. Boyden ES, Zhang F, Bamberg E, Nagel G, Deisseroth K. Millisecond-timescale, genetically targeted optical control of neural activity. Nat Neurosci. 2005;8(9):1263–8.
12. Paoletti P, Ellis-Davies GCR, Mourot A. Optical control of neuronal ion channels and receptors. Nat Rev Neurosci. 2019 Sep 1;20(9):514–32.
13. Hüll K, Morstein J, Trauner D. In vivo photopharmacology. Chem Rev. 2018 Nov 14;118(21):10710–47.
14. Rost BR, Schneider-Warme F, Schmitz D, Hegemann P. Optogenetic tools for subcellular applications in neuroscience. Neuron. 2017 Nov 1;96(3):572–603.
15. Velema WA, Szymanski W, Feringa BL. Photopharmacology: beyond proof of principle. J Am Chem Soc. 2014 Feb 12;136(6):2178–91.
16. Lerch MM, Hansen MJ, van Dam GM, Szymanski W, Feringa BL. Emerging targets in photopharmacology. Angew Chem Int Ed. 2016 Sep 5;55(37):10978–99.
17. Nikolaev DM, Panov MS, Shtyrov AA, Boitsov VM, Vyazmin SY, Chakchir OB, et al. Perspective tools for optogenetics and photopharmacology: from design to implementation. Springer Ser Chem Phys. 2019;119:139–72.
18. Oesterhelt D, Stoeckenius W. Rhodopsin-like protein from the purple membrane of Halobacterium halobium. Nat New Biol. 1971 Sep 29;233(39):149–52.
19. Matsuno-Yagi A, Mukohata Y. Two possible roles of bacteriorhodopsin; a comparative study of strains of Halobacterium halobium differing in pigmentation. Biochem Biophys Res Commun. 1977 Sep 9;78(1):237–43.

20. Nagel G, Ollig D, Fuhrmann M, Kateriya S, Musti AM, Bamberg E, et al. Channelrhodopsin-1: a light-gated proton channel in green algae. Science. 2002 Jun 28;296(5577):2395–8.
21. Zemelman B V., Lee GA, Ng M, Miesenböck G. Selective photostimulation of genetically chARGed neurons. Neuron. 2002 Jan 3;33(1):15–22.
22. Lima SQ, Miesenböck G. Remote control of behavior through genetically targeted photostimulation of neurons. Cell. 2005 Apr 8;121(1):141–52.
23. Banghart M, Borges K, Isacoff E, Trauner D, Kramer RH. Light-activated ion channels for remote control of neuronal firing. Nat Neurosci. 2004 Dec;7(12):1381–6.
24. Aravanis A, Wang L, Zhang F, Meltzer L, Mogri M, Schneider M, et al. An optical neural interface: in vivo control of rodent motor cortex with integrated fiberoptic and optogenetic technology. J Neural Eng. 2007;4(3):S143–S156.
25. Tye KM, Deisseroth K. Optogenetic investigation of neural circuits underlying brain disease in animal models. Nat Rev Neurosci. 2012 Apr;13(4):251–66.
26. Wang H, Vilela M, Winkler A, Tarnawski M, Schlichting I, Yumerefendi H, et al. LOVTRAP: an optogenetic system for photoinduced protein dissociation. Nat Methods. 2016 Aug 30;13(9):755–8.
27. Berndt A, Lee SY, Ramakrishnan C, Deisseroth K. Structure-guided transformation of channelrhodopsin into a light-activated chloride channel. Science. 2014;344(6182):420–4.
28. Berndt A, Lee SY, Wietek J, Ramakrishnan C, Steinberg EE, Rashid AJ, et al. Structural foundations of optogenetics: determinants of channelrhodopsin ion selectivity. Proc Natl Acad Sci USA. 2016 Jan 26;113(4):822–9.
29. Iyer SM, Vesuna S, Ramakrishnan C, Huynh K, Young S, Berndt A, et al. Optogenetic and chemogenetic strategies for sustained inhibition of pain. Sci Rep. 2016 Aug 3;6:30570.
30. Airan RD, Thompson KR, Fenno LE, Bernstein H, Deisseroth K. Temporally precise in vivo control of intracellular signalling. Nature. 2009 Apr 23;458(7241):1025–9.
31. Schindler SE, McCall JG, Yan P, Hyrc KL, Li M, Tucker CL, et al. Photo-activatable Cre recombinase regulates gene expression in vivo. Sci Rep. 2015 Sep 9;5:13627.
32. Jarrin S, Finn DP. Optogenetics and its application in pain and anxiety research. Neurosci Biobehav Rev. 2019 Oct 1;105:200–11.
33. Bieth J, Vratsanos SM, Wassermann N, Erlanger BF. Photoregulation of biological activity by photocromic reagents. II. Inhibitors of acetylcholinesterase. Proc Natl Acad Sci USA. 1969;64(3):1103–6.
34. Deal WJ, Erlanger BF, Nachmansohn D. Photoregulation of biological activity by photochromic reagents. 3. Photoregulation of bioelectricity by acetylcholine receptor inhibitors. Proc Natl Acad Sci USA. 1969;64(4):1230–4.
35. Fehrentz T, Schönberger M, Trauner D. Optochemical genetics. Angew Chem Int Ed. 2011 Dec 16;50(51):12156–82.
36. Polosukhina A, Litt J, Tochitsky I, Nemargut J, Sychev Y, De Kouchkovsky I, et al. Photochemical restoration of visual responses in blind mice. Neuron. 2012 Jul 26;75(2):271–82.
37. Laprell L, Hüll K, Stawski P, Schön C, Michalakis S, Biel M, et al. Restoring light sensitivity in blind retinae using a photochromic AMPA receptor agonist. ACS Chem Neurosci. 2016 Jan 20;7(1):15–20.
38. Miesenböck G. Optogenetic control of cells and circuits. Annu Rev Cell Dev Biol. 2011 Nov 10;27(1):731–58.
39. Nagel G, Szellas T, Huhn W, Kateriya S, Adeishvili N, Berthold P, et al. Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. Proc Natl Acad Sci USA. 2003 Nov 25;100(SUPPL. 2):13940–5.
40. Gradinaru V, Mogri M, Thompson KR, Henderson JM, Deisseroth K. Optical deconstruction of parkinsonian neural circuitry. Science. 2009 Apr 17;324(5925):354–9.
41. Zhang F, Wang LP, Brauner M, Liewald JF, Kay K, Watzke N, et al. Multimodal fast optical interrogation of neural circuitry. Nature. 2007 Apr 5;446(7136):633–9.
42. Chow BY, Han X, Dobry AS, Qian X, Chuong AS, Li M, et al. High-performance genetically targetable optical neural silencing by light-driven proton pumps. Nature. 2010 Jan 7;463(7277):98–102.
43. Berndt A, Schoenenberger P, Mattis J, Tye KM, Deisseroth K, Hegemann P, et al. High-efficiency channelrhodopsins for fast neuronal stimulation at low light levels. Proc Natl Acad Sci USA. 2011 May 3;108(18):7595–600.
44. Gunaydin LA, Yizhar O, Berndt A, Sohal VS, Deisseroth K, Hegemann P. Ultrafast optogenetic control. Nat Neurosci. 2010 Mar;13(3):387–92.
45. Zhang F, Prigge M, Beyrière F, Tsunoda SP, Mattis J, Yizhar O, et al. Red-shifted optogenetic excitation: a tool for fast neural control derived from Volvox carteri. Nat Neurosci. 2008 Jun;11(6):631–3.
46. Chuong AS, Miri ML, Busskamp V, Matthews GAC, Acker LC, Sørensen AT, et al. Noninvasive optical inhibition with a red-shifted microbial rhodopsin. Nat Neurosci. 2014;17(8):1123–9.
47. Berndt A, Yizhar O, Gunaydin LA, Hegemann P, Deisseroth K. Bi-stable neural state switches. Nat Neurosci. 2009 Feb;12(2):229–34.
48. Oh E, Maejima T, Liu C, Deneris E, Herlitze S. Substitution of 5-HT1A receptor signaling by a light-activated G protein-coupled receptor. J Biol Chem. 2010 Oct 1;285(40):30825–36.
49. Gunaydin LA, Grosenick L, Finkelstein JC, Kauvar I V., Fenno LE, Adhikari A, et al. Natural neural projection dynamics underlying social behavior. Cell. 2014 Jun 19;157(7):1535–51.
50. Li P, Rial D, Canas PM, Yoo JH, Li W, Zhou X, et al. Optogenetic activation of intracellular adenosine A2A receptor signaling in the hippocampus is sufficient to trigger CREB phosphorylation and impair memory. Mol Psychiatry. 2015 Nov 1;20(11):1339–49.
51. van Wyk M, Pielecka-Fortuna J, Löwel S, Kleinlogel S. Restoring the ON switch in blind retinas: opto-mGluR6, a next-generation, cell-tailored optogenetic tool. PLoS Biol. 2015 May 7;13(5):e1002143.
52. Barish PA, Xu Y, Li J, Sun J, Jarajapu YPR, Ogle WO. Design and functional evaluation of an optically active μ-opioid receptor. Eur J Pharmacol. 2013 Apr 5;705(1–3):42–8.
53. Siuda ER, Copits BA, Schmidt MJ, Baird MA, Al-Hasani R, Planer WJ, et al. Spatiotemporal Control of Opioid Signaling and Behavior. Neuron. 2015 May 20;86(4):923–35.
54. Luo L, Callaway EM, Svoboda K. Genetic Dissection of Neural Circuits. Neuron. 2008 Mar 13;57(5):634–60.
55. Zhang F, Gradinaru V, Adamantidis AR, Durand R, Airan RD, De Lecea L, et al. Optogenetic interrogation of neural circuits: technology for probing mammalian brain structures. Nat Protoc. 2010 Mar;5(3):439–56.
56. Zeng H, Madisen L. Mouse transgenic approaches in optogenetics. Prog Brain Res. 2012;196:193–213.
57. Zhang F, Wang LP, Boyden ES, Deisseroth K. Channelrhodopsin-2 and optical control of excitable cells. Nat Methods. 2006 Oct;3(10):785–92.
58. Nagy A. Cre recombinase: the universal reagent for genome tailoring. Genesis. 2000 Feb 1;26(2):99–109.
59. Branda CS, Dymecki SM. Talking about a revolution: The impact of site-specific recombinases on genetic analyses in mice. Dev Cell. 2004 Jan;6(1):7–28.
60. Schnütgen F, Doerflinger N, Calléja C, Wendling O, Chambon P, Ghyselinck NB. A directional strategy for monitoring Cre-mediated recombination at the cellular level in the mouse. Nat Biotechnol. 2003 May 1;21(5):562–5.
61. Cardin JA, Carlén M, Meletis K, Knoblich U, Zhang F, Deisseroth K, et al. Driving fast-spiking cells induces gamma rhythm and controls sensory responses. Nature. 2009 Jun 4;459(7247):663–7.
62. Li B, Yang XY, Qian FP, Tang M, Ma C, Chiang LY. A novel analgesic approach to optogenetically and specifically inhibit

pain transmission using TRPV1 promoter. Brain Res. 2015;1609(1):12–20.
63. Daou I, Tuttle AH, Longo G, Wieskopf JS, Bonin RP, Ase AR, et al. Remote optogenetic activation and sensitization of pain pathways in freely moving mice. J Neurosci Res. 2013;33(47):18631–40.
64. Gong S, Doughty M, Harbaugh CR, Cummins A, Hatten ME, Heintz N, et al. Targeting Cre recombinase to specific neuron populations with bacterial artificial chromosome constructs. J Neurosci Res. 2007 Sep 12;27(37):9817–23.
65. Beaudry H, Daou I, Ase AR, Ribeiro-Da-Silva A, Séguela P. Distinct behavioral responses evoked by selective optogenetic stimulation of the major TRPV1+ and MrgD+ subsets of C-fibers. Pain. 2017;158(12):2329–39.
66. Madisen L, Mao T, Koch H, Zhuo JM, Berenyi A, Fujisawa S, et al. A toolbox of Cre-dependent optogenetic transgenic mice for light-induced activation and silencing. Nat Neurosci. 2012 May;15(5):793–802.
67. Baumbauer KM, Deberry JJ, Adelman PC, Miller RH, Hachisuka J, Lee KH, et al. Keratinocytes can modulate and directly initiate nociceptive responses. eLife. 2015 Sep 2;4:e09674.
68. Madisen L, Garner AR, Shimaoka D, Chuong AS, Klapoetke NC, Li L, et al. Transgenic mice for intersectional targeting of neural sensors and effectors with high specificity and performance. Neuron. 2015 Mar 4;85(5):942–58.
69. Bourane S, Duan B, Koch SC, Dalet A, Britz O, Garcia-Campmany L, et al. Gate control of mechanical itch by a subpopulation of spinal cord interneurons. Science. 2015 Oct 30;350(6260):550–4.
70. Robertson SD, Plummer NW, De Marchena J, Jensen P. Developmental origins of central norepinephrine neuron diversity. Nature Neuroscience. 2013 Aug;16(8):1016–23.
71. Fenno LE, Mattis J, Ramakrishnan C, Hyun M, Lee SY, He M, et al. Targeting cells with single vectors using multiple-feature Boolean logic. Nat Methods. 2014 Jun 8;11(7):763–72.
72. Brieke C, Rohrbach F, Gottschalk A, Mayer G, Heckel A. Light-controlled tools. Angew Chem Int Ed. 2012 Aug 20;51(34):8446–76.
73. Ellis-Davies GCR. Caged compounds: Photorelease technology for control of cellular chemistry and physiology. Nat Methods. 2007 Aug 30;4(8):619–28.
74. Klán P, Šolomek T, Bochet CG, Blanc A, Givens R, Rubina M, et al. Photoremovable protecting groups in chemistry and biology: reaction mechanisms and efficacy. Chem Rev. 2013 Jan 9;113(1):119–91.
75. Broichhagen J, Frank JA, Trauner D. A Roadmap to success in photopharmacology. Acc Chem Res. 2015 Jul 21;48(7):1947–60.
76. Mutter NL, Volarić J, Szymanski W, Feringa BL, Maglia G. Reversible photocontrolled nanopore assembly. J Am Chem Soc. 2019 Sep 11;141(36):14356–63.
77. Morstein J, Trauner D. New players in phototherapy: photopharmacology and bio-integrated optoelectronics. Curr Opin Chem Biol. 2019 Jun 1;50:145–51.
78. Mehta ZB, Johnston NR, Nguyen-Tu MS, Broichhagen J, Schultz P, Larner DP, et al. Remote control of glucose homeostasis in vivo using photopharmacology. Sci Rep. 2017;7(1):291.
79. Yeoh YQ, Yu J, Polyak SW, Horsley JR, Abell AD. Photopharmacological control of cyclic antimicrobial peptides. Chembiochem. 2018 Dec 18;19(24):2591–7.
80. Sansalone L, Bratsch-Prince J, Tang S, Captain B, Mott DD, Raymo FM. Photopotentiation of the GABAA receptor with caged diazepam. Proc Natl Acad Sci USA. 2019 Oct 15;116(42):21176–84.
81. Qazi R, Gomez AM, Castro DC, Zou Z, Sim JY, Xiong Y, et al. Wireless optofluidic brain probes for chronic neuropharmacology and photostimulation. Nat Biomed Eng. 2019 Aug 1;3(8):655–69.
82. Mayer G, Hechel A. Biologically active molecules with a “light switch.” Angew Chem Int Ed. 2006 Jul 24;45(30):4900–21.
83. Gautier A, Gauron C, Volovitch M, Bensimon D, Jullien L, Vriz S. How to control proteins with light in living systems. Nat Chem Biol. 2014;10(7):533–41.
84. Kamiya H. Photochemical inactivation analysis of temporal dynamics of postsynaptic native AMPA receptors in hippocampal slices. J Neurosci. 2012 May 9;32(19):6517–24.
85. Hansen MJ, Velema WA, Lerch MM, Szymanski W, Feringa BL. Wavelength-selective cleavage of photoprotecting groups: Strategies and applications in dynamic systems. Chem Soc Rev. 2015 Jun 7;44(11):3358–77.
86. Peterson JA, Wijesooriya C, Gehrmann EJ, Mahoney KM, Goswami PP, Albright TR, et al. Family of BODIPY Photocages Cleaved by Single Photons of Visible/Near-Infrared Light. J Am Chem Soc. 2018 Jun 13;140(23):7343–6.
87. Jedlitzke B, Yilmaz Z, Dörner W, Mootz HD. Photobodies: light-activatable single-domain antibody fragments. Angew Chem Int Ed. 2020 Jan 20;59(4):1506–10.
88. Hermanson GT. Bioconjugate techniques: 3rd edn. Elsevier Inc.; 2013. p. 229–58.
89. Krishnamurthy VM, Semetey V, Bracher PJ, Shen N, Whitesides GM. Dependence of effective molarity on linker length for an intramolecular protein-ligand system. J Am Chem Soc. 2007 Feb 7;129(5):1312–20.
90. Yasuike N, Lu H, Xia P, Woolley GA. Intramolecular cross-linking of proteins with azobenzene-based cross-linkers. Methods Enzymol. 2019 Jan 1;624:129–49.
91. Gazerani P. Shedding light on photo-switchable analgesics for pain. Pain management. 2017 Mar 1;7(2):71–4.
92. Carr FB, Zachariou V. Nociception and pain: Lessons from optogenetics. Front Behav Neurosci. 2014 Mar 25;8:69.
93. Tovote P, Fadok JP, Lüthi A. Neuronal circuits for fear and anxiety. Nat Rev Neurosci. 2015 Jun 26;16(6):317–31.
94. Corder G, Ahanonu B, Grewe BF, Wang D, Schnitzer MJ, Scherrer G. An amygdalar neural ensemble that encodes the unpleasantness of pain. Science. 2019 Jan 18;363(6424):276–81.
95. Kiritoshi T, Ji G, Neugebauer V. Rescue of impaired mGluR5-driven endocannabinoid signaling restores prefrontal cortical output to inhibit pain in arthritic rats. J Neurosci. 2016 Jan 20;36(3):837–50.
96. Zhang Z, Gadotti VM, Chen L, Souza IA, Stemkowski PL, Zamponi GW. Role of prelimbic GABAergic circuits in sensory and emotional aspects of neuropathic pain. Cell Rep. 2015 Aug 4;12(5):752–9.
97. Huang J, Gadotti VM, Chen L, Souza IA, Huang S, Wang D, et al. A neuronal circuit for activating descending modulation of neuropathic pain. Nat Neurosci. 2019 Oct 1;22(10):1659–68.
98. Mcdonald AJ. Cortical pathways to the mammalian amygdala. Prog Neurobiol. 1998 Jun 27;55(3):257–332.
99. Neugebauer V. Amygdala pain mechanisms. Handb Exp Pharmacol. 2015;227:261–84.
100. Bernard JF, Besson JM. The spino(trigemino) pontoamygdaloid pathway: electrophysiological evidence for an involvement in pain processes. J Neurophysiol. 1990;63(3):473–90.
101. Sugimura YK, Takahashi Y, Watabe AM, Kato F. Synaptic and network consequences of monosynaptic nociceptive inputs of parabrachial nucleus origin in the central amygdala. J Neurophysiol. 2016 Jun 1;115(6):2721–39.
102. Crock LW, Kolber BJ, Morgan CD, Sadler KE, Vogt SK, Bruchas MR, et al. Central amygdala metabotropic glutamate receptor 5 in the modulation of visceral pain. J Neurosci. 2012 Oct 10;32(41):14217–26.
103. Li JN, Sheets PL. Spared nerve injury differentially alters parabrachial monosynaptic excitatory inputs to molecularly specific neurons in distinct subregions of the central amygdala. Pain. 2020 Jan 1;161(1):166–76.
104. Abraira VE, Ginty DD. The sensory neurons of touch. Neuron. 2013 Aug 21;79(4):618–39.
105. Montgomery KL, Iyer SM, Christensen AJ, Deisseroth K, Delp SL. Beyond the brain: optogenetic control in the spinal

cord and peripheral nervous system. Sci Transl Med. 2016 May 4;8(337).
106. Wang H, Zylka MJ. Mrgprd-expressing polymodal nociceptive neurons innervate most known classes of substantia gelatinosa neurons. J Neurosci. 2009 Oct 21;29(42):13202–9.
107. Park S Il, Brenner DS, Shin G, Morgan CD, Copits BA, Chung HU, et al. Soft, stretchable, fully implantable miniaturized optoelectronic systems for wireless optogenetics. Nat Biotechnol. 2015 Dec 1;33(12):1280–6.
108. Mishra SK, Tisel SM, Orestes P, Bhangoo SK, Hoon MA. TRPV1-lineage neurons are required for thermal sensation. EMBO J. 2011 Feb 2;30(3):582–93.
109. Samineni VK, Yoon J, Crawford KE, Jeong YR, McKenzie KC, Shin G, et al. Fully implantable, battery-free wireless optoelectronic devices for spinal optogenetics. Pain. 2017 Nov 1;158(11):2108–16.
110. Iyer SM, Montgomery KL, Towne C, Lee SY, Ramakrishnan C, Deisseroth K, et al. Virally mediated optogenetic excitation and inhibition of pain in freely moving nontransgenic mice. Nat Biotechnol. 2014;32(3):274–8.
111. Daou I, Beaudry H, Ase AR, Wieskopf JS, Ribeiro-da-Silva A, Mogil JS, et al. Optogenetic silencing of Nav1.8-positive afferents alleviates inflammatory and neuropathic pain. eNeuro. 2016;3(1):702–5.
112. Draxler P, Honsek SD, Forsthuber L, Hadschieff V, Sandkühler J. VGluT3+ primary afferents play distinct roles in mechanical and cold hypersensitivity depending on pain etiology. J Neurosci. 2014 Sep 3;34(36):12015–28.
113. Bonin RP, Wang F, Desrochers-Couture M, Ga¸secka A, Boulanger ME, Côté DC, et al. Epidural optogenetics for controlled analgesia. Mol Pain. 2016 Feb 27;12.
114. Ghitani N, Barik A, Szczot M, Thompson JH, Li C, Le Pichon CE, et al. Specialized mechanosensory nociceptors mediating rapid responses to hair pull. Neuron. 2017 Aug 16;95(4):944-954.e4.
115. Maksimovic S, Nakatani M, Baba Y, Nelson AM, Marshall KL, Wellnitz SA, et al. Epidermal Merkel cells are mechanosensory cells that tune mammalian touch receptors. Nature. 2014;509(7502):617–21.
116. Moehring F, Cowie AM, Menzel AD, Weyer AD, Grzybowski M, Arzua T, et al. Keratinocytes mediate innocuous and noxious touch via ATP-P2X4 signaling. eLife. 2018 Jan 16;7.
117. Skerratt SE, West CW. Ion channel therapeutics for pain. Channels. 2015 Jan 1;9(6):344–51.
118. Zussy C, Gómez-Santacana X, Rovira X, De Bundel D, Ferrazzo S, Bosch D, et al. Dynamic modulation of inflammatory pain-related affective and sensory symptoms by optical control of amygdala metabotropic glutamate receptor 4. Mol Psychiatry. 2018 Mar 1;23(3):509–20.
119. Frias B, Merighi A. Capsaicin, nociception and pain. Molecules. 2016 Jun 1;21(6).
120. Frank JA, Moroni M, Moshourab R, Sumser M, Lewin GR, Trauner D. Photoswitchable fatty acids enable optical control of TRPV1. Nat Commun. 2015 May 22;6.
121. Konrad DB, Frank JA, Trauner D. Synthesis of redshifted azobenzene photoswitches by late-stage functionalization. Chem Eur J. 2016 Mar 18;22(13):4364–8.
122. Gilbert D, Funk K, Dekowski B, Lechler R, Keller S, Möhrlen F, et al. Caged capsaicins: new tools for the examination of TRPV1 channels in somatosensory neurons. Chembiochem. 2007 Jan;8(1):89–97.
123. Kokel D, Cheung CYJ, Mills R, Coutinho-Budd J, Huang L, Setola V, et al. Photochemical activation of TRPA1 channels in neurons and animals. Nat Chem Biol. 2013 Apr;9(4):257–63.
124. Fajardo O, Friedrich RW. Optopharmacology: A light switch for pain. Nat Chem Biol. 2013 Apr;9(4):219–20.
125. Lam PY, Mendu SK, Mills RW, Zheng B, Padilla H, Milan DJ, et al. A high-conductance chemo-optogenetic system based on the vertebrate channel Trpa1b. Sci Rep. 2017 Dec 1;7(1):11839.
126. Leinders-Zufall T, Storch U, Bleymehl K, Mederos y Schnitzler M, Frank JA, Konrad DB, et al. PhoDAGs enable optical control of diacylglycerol-sensitive transient receptor potential channels. Cell Chem Biol. 2018 Feb 15;25(2):215-223.e3.
127. Lichtenegger M, Tiapko O, Svobodova B, Stockner T, Glasnov TN, Schreibmayer W, et al. An optically controlled probe identifies lipid-gating fenestrations within the TRPC3 channel article. Nat Chem Biol. 2018 Apr 1;14(4):396–404.
128. Stein M, Middendorp SJ, Carta V, Pejo E, Raines DE, Forman SA, et al. Azo-propofols: photochromic potentiators of GABA a receptors. Angew Chem Int Ed. 2012 Oct 15;51(42):10500–4.
129. Lutz T, Wein T, Höfner G, Pabel J, Eder M, Dine J, et al. Development of new photoswitchable azobenzene based γ-aminobutyric acid (GABA) uptake inhibitors with distinctly enhanced potency upon photoactivation. J Med Chem. 2018 Jul 26;61(14):6211–35.
130. Lin WC, Davenport CM, Mourot A, Vytla D, Smith CM, Medeiros KA, et al. Engineering a light-regulated GABAA receptor for optical control of neural inhibition. ACS Chem Biol. 2014 Jul 18;9(7):1414–9.
131. Lin WC, Tsai MC, Davenport CM, Smith CM, Veit J, Wilson NM, et al. A comprehensive optogenetic pharmacology toolkit for in vivo control of GABAA receptors and synaptic inhibition. Neuron. 2015 Dec 2;88(5):879–91.
132. McKenzie CK, Sanchez-Romero I, Janovjak H. Flipping the photoswitch: ion channels under light control. Adv Exp Med Biol. 2015;869:101–17.
133. Mourot A, Tochitsky I, Kramer RH. Light at the end of the channel: optical manipulation of intrinsic neuronal excitability with chemical photoswitches. Front Mol Neurosci. 2013 Mar 6;6:5.
134. Mourot A, Fehrentz T, Le Feuvre Y, Smith CM, Herold C, Dalkara D, et al. Rapid optical control of nociception with an ion-channel photoswitch. Nat Methods. 2012 Apr;9(4):396–402.
135. Binshtok AM, Gerner P, Oh SB, Puopolo M, Suzuki S, Roberson DP, et al. Coapplication of lidocaine and the permanently charged sodium channel blocker QX-314 produces a long-lasting nociceptive blockade in rodents. Anesthesiology. 2009;111(1):127–37.
136. Schoenberger M, Damijonaitis A, Zhang Z, Nagel D, Trauner D. Development of a new photochromic ion channel blocker via azologization of fomocaine. ACS Chem Neurosci. 2014 Jul 16;5(7):514–8.
137. Mourot A, Herold C, Kienzler MA, Kramer RH. Understanding and improving photo-control of ion channels in nociceptors with azobenzene photo-switches. Br J Pharmacol. 2018 Jun 1;175(12):2296–311.
138. Römpler H, Stäubert C, Thor D, Schulz A, Hofreiter M, Schöneberg T. G protein-coupled time travel: evolutionary aspects of GPCR research. Mol Interv. 2007 Feb;7(1):17–25.
139. Schönberger M, Trauner D. A photochromic agonist for μ-opioid receptors. Angew Chem Int Ed. 2014 Mar 17;53(12):3264–7.
140. Levitz J, Popescu AT, Reiner A, Isacoff EY. A toolkit for orthogonal and in vivo optical manipulation of ionotropic glutamate receptors. Front Mol Neurosci. 2016 Feb 2;9:2.
141. Goudet C, Rovira X, Llebaria A. Shedding light on metabotropic glutamate receptors using optogenetics and photopharmacology. Curr Opin Pharmacol. 2018 Feb 1;38:8–15.
142. Levitz J, Broichhagen J, Leippe P, Konrad D, Trauner D, Isacoff EY. Dual optical control and mechanistic insights into photoswitchable group II and III metabotropic glutamate receptors. Proc Natl Acad Sci USA. 2017 Apr 25;114(17):E3546–54.
143. Mickle AD, Gereau RW. A bright future? Optogenetics in the periphery for pain research and therapy. Pain. 2018 Sep 1;159:S65–73.
144. Copits BA, Pullen MY, Gereau RW. Spotlight on pain: Optogenetic approaches for interrogating somatosensory circuits. Pain. 2016 Aug 19;157(11):2424–33.
145. Christensen AJ, Iyer SM, François A, Vyas S, Ramakrishnan C,

Vesuna S, et al. In Vivo Interrogation of Spinal Mechanosensory Circuits. Cell Rep. 2016 Nov 1;17(6):1699–710.
146. Towne C, Montgomery KL, Iyer SM, Deisseroth K, Delp SL. Optogenetic Control of Targeted Peripheral Axons in Freely Moving Animals. PLoS ONE. 2013 Aug 21;8(8).
147. Montgomery KL, Yeh AJ, Ho JS, Tsao V, Iyer SM, Grosenick L, et al. Wirelessly powered, fully internal optogenetics for brain, spinal and peripheral circuits in mice. Nat Methods. 2015 Sep 29;12(10):969–74.
148. Samineni VK, Mickle AD, Yoon J, Grajales-Reyes JG, Pullen MY, Crawford KE, et al. Optogenetic silencing of nociceptive primary afferents reduces evoked and ongoing bladder pain. Sci Rep. 2017 Dec 1;7(1).
149. Shin G, Gomez AM, Al-Hasani R, Jeong YR, Kim J, Xie Z, et al. Flexible Near-Field Wireless Optoelectronics as subdermal implants for broad applications in optogenetics. Neuron. 2017 Feb 8;93(3):509-521.e3.
150. Lu L, Gutruf P, Xia L, Bhatti DL, Wang X, Vazquez-Guardado A, et al. Wireless optoelectronic photometers for monitoring neuronal dynamics in the deep brain. Proc Natl Acad Sci USA. 2018 Feb 13;115(7):E1374–83.
151. Beaudry H, Daou I, Ribeiro-da-Silva A, Séguéla P. Will optogenetics be used to treat chronic pain patients? Pain Manag. 2017 Jul 1;7(4):269–78.
152. Stein M, Breit A, Fehrentz T, Gudermann T, Trauner D. Optical control of TRPV1 channels. Angew Chem Int Ed. 2013 Sep 9;52(37):9845–8.
153. Font J, López-Cano M, Notartomaso S, Scarselli P, Di Pietro P, Bresolí-Obach R, et al. Optical control of pain in vivo with a photoactive mGlu5 receptor negative allosteric modulator. eLife. 2017 Apr 11;6.
154. Golden RN, Gaynes BN, Ekstrom RD, Hamer RM, Jacobsen FM, Suppes T, et al. The efficacy of light therapy in the treatment of mood disorders: a review and meta-analysis of the evidence. Am J Psychiatry. 2005 Apr;162(4):656–62.
155. Figueiro MG, Plitnick BA, Lok A, Ejones GE, Higgins P, Rhornick TR, et al. Tailored lighting intervention improves measures of sleep, depression, and agitation in persons with Alzheimer's disease and related dementia living in long-term care facilities. Clin Interv Aging. 2014 Sep 12;9:1527–37.
156. Ibrahim MM, Patwardhan A, Gilbraith KB, Moutal A, Yang X, Chew LA, et al. Long-lasting antinociceptive effects of green light in acute and chronic pain in rats. Pain. 2017 Feb 1;158(2):347–60.
157. Park S Il, Shin G, McCall JG, Al-Hasani R, Norrisf A, Xia L, et al. Stretchable multichannel antennas in soft wireless optoelectronic implants for optogenetics. Proc Natl Acad Sci USA. 2016 Dec 13;113(50):E8169–77.
158. Kim T Il, McCall JG, Jung YH, Huang X, Siuda ER, Li Y, et al. Injectable, cellular-scale optoelectronics with applications for wireless optogenetics. Science. 2013 Apr 12;340(6129):211–6.
159. Jeong JW, McCall JG, Shin G, Zhang Y, Al-Hasani R, Kim M, et al. Wireless optofluidic systems for programmable in vivo pharmacology and optogenetics. Cell. 2015 Aug 1;162(3):662–74.
160. Chattopadhyay M, Mata M, Fink DJ. Vector-mediated release of GABA attenuates pain-related behaviors and reduces Na V1.7 in DRG neurons. Eur J Pain. 2011 Oct;15(9):913–20.
161. Braz J, Beaufour C, Coutaux A, Epstein AL, Cesselin F, Hamon M, et al. Therapeutic efficacy in experimental polyarthritis of viral-driven enkephalin overproduction in sensory neurons. J Neurosci. 2001 Oct 15;21(20):7881–8.
162. Fink DJ, Wechuck J, Mata M, Glorioso JC, Goss J, Krisky D, et al. Gene therapy for pain: results of a phase I clinical trial. Ann Neurol. 2011 Aug;70(2):207–12.
163. Wolfe D, Mata M, Fink DJ. A human trial of HSV-mediated gene transfer for the treatment of chronic pain. Gene Ther. 2009;16(4):455–60.

RESEARCH ARTICLE

Inflammatory events drive neural stem cell migration by elevating stromal-derived factor 1 alpha

Ziyun JIANG, Mingliang TANG*

Joint Research Institute of Southeast University and Monash University, Suzhou 215123, China; Melbourne 500301, Australia.

*Correspondence: mltang2010@126.com
https://doi.org/10.37175/stemedicine.v1i3.59

ABSTRACT

Background: Ischemic stroke is the most common cause of ischemia-related death globally. Brain injuries due to stroke and trauma are typically followed by inflammation reactions within the central nervous system (CNS). Neural stem cell (NSC)-based therapeutic strategies show great potential for treating stroke and ischemia-mediated brain injuries, and migration of NSCs is a critical step involved in NSC-based therapy.

Methods: In order to examine the effects of microglial activation upon ischemia and stroke on NSC behaviors, oxygen-glucose deprivation (OGD) *in vitro* model was established for mimicking *in vivo* stroke and ischemia pathological conditions in this study. By combining of enzyme-linked immunosorbent assay, migration assay, Western blot and immunostaining, we found that OGD insult induced microglial activation by releasing cytokines and chemokines.

Results: The conditioned media (CM) of OGD-treated groups impaired the proliferation and capability of neurosphere formation. Moreover, we found the stromal cell-derived factor 1α/CXC chemokine receptor 4 (CXCR4) pathway was an active player that facilitated the migration of NSCs, since a CXCR4 specific antagonist AMD3100 was able to impair NSC migration both *in vitro* and *in vivo*.

Conclusion: The current study presents a potential interaction between NSC behaviors and microglial activation underlying brain injuries, such as ischemia and stroke. More importantly, we reveal the underlying mechanisms of microglia-induced NSC migration under OGD conditions and it should be beneficial to stem cell-based therapies to treat acute brain injuries.

Keywords: Microglia · Oxygen-glucose deprivation · Neural stem cells · Migration

Introduction

Ischemic stroke is a dominant cause of ischemia-related deaths around the world. The immune system and triggered inflammation reactions are key elements involved following ischemia as they respond to brain injuries after stroke (1). Various evidences have suggested the appearance of inflammatory reactions after stroke and trauma in the central nervous system (CNS) (2). Infiltration of macrophages and neutrophils into the respective brain parenchyma, as well as the activation of resident microglia, is the main characteristic of brain injury-mediated inflammation. Microglia are a type of resident macrophages of the spinal cord and the brain, which account for 10-15% of total cells from the brain (3) and are responsible for the scavenge of damaged neurons, plaques and infectious agents within the CNS (4). Microglia usually maintain a ramified morphology at resting status to monitor the environment. In the case of brain injuries, the microglial cells are activated (5). It is unclear whether inflammation followed brain injury would benefit or damage post ischemia (6, 7). Some reports have revealed a strong

Received: Jun 2, 2020; Accepted: Jun 21, 2020.

correlation between microglial activation along with excess amount of secretion of cytotoxic interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6 (8), and neuronal degeneration after transient cerebral ischemia (9).

Neural stem cells (NSCs) are self-renewing ones with the capacity to differentiate into astrocytes, oligodendrocytes and neurons (10). They are capable to replace lost cells within the CNS in principle (11). Both NSCs and microglia are important components of the CNS. NSCs have shown the potential in cell transplantation therapy for stroke. The critical step involved in neural regeneration is the migration of NSCs. NSCs present a capacity to precisely migrate toward distant pathological targets such as tumors and various types of brain injuries, which might suggest an adaptive response to limit and/or repair damage (5). However, substantial death of grafted stem cells has been observed in patients receiving NSC transplantation, probably due to host inflammatory reaction (4). Thus, it would be beneficial to promote the migration of endogenous NSCs to the areas of pathology besides the grafted stem cells. In fact, several *in vitro* and *in vivo* investigations have confirmed characteristic migration of inflammatory-mediated NSCs (3, 12). Several animal models have evidenced the migration of endogenous and exogenous NSCs towards damaged regions mediated by released soluble factors from the microglial cells (11). Human NSCs have been seen migrating *in vivo* towards a pathological area, which is mediated through the stromal cell-derived factor 1α (SDF-1α)/CXC chemokine receptor 4 (CXCR4) pathway (3). Monocyte chemo attractant protein-1 (MCP-1), a glia secreted chemokine, is also involved in up-regulating the migration capacity of NSCs in rats (13). Therefore, it is of importance to study the interaction between microglial cells and NSCs, especially during certain pathologies and injuries.

In this study, both oxygen-glucose deprivation (OGD) *in vitro* model and ischemic spinal cord injury mouse model have been established to mimic pathological ischemia and stroke. Several studies have focused on microglia under OGD insult, however, there are very few reports investigating how NSC migration is influenced by microglia activation in the condition of ischemia and stroke. Herein, by combining immunofluorescence, enzyme-linked immunosorbent assay (ELISA) technique and Western blotting analysis, we attempted to interpret the enhanced migration of NSCs as well as the underlying mechanisms mediated by microglia activation upon OGD.

Materials and Methods

Cell culturing

BV2 cells, a murine microglial cell line, were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY, USA) containing 100 mg/ml streptomycin (Gibco), 100 mg/ml streptomycin (Gibco), and 10% fetal bovine serum (FBS, Gibco) at 37°C in a humidified 5% CO2 incubator.

NSCs were derived from hippocampal hemispheres obtained from day 1 postnatal (P1) ICR mice. Cells were digested in TryplE (Life Technologies, Pleasanton, CA, USA) at 37 °C for 15 min and then gently dissociated with pipettes. To determine cell viability, NSCs (5×10^4/ml) were plated on 96 well plates pre-coated with laminin and then maintained in proliferative medium consisted of DMEM-F12 with 2% B27 supplement (Life Technologies), fibroblast growth factor (FGF, 20 ng/ml, R&D Systems, Minneapolis, MN, USA) and epidermal growth factor (EGF, 20 ng/ml, R&D Systems). For the condition medium (CM) treatment, after 24 h culturing, supernatants of the NSC culture were replaced with CM collected earlier, with additional 20 ng/ml EGF, 20 ng/ml FGF, and 2% B27 supplement, followed by another 24 h of culture. This current study was designed in conformity with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The experimental protocols obtained approval from Joint Research Institute of Southeast University and Monash University. All surgical procedures were conducted under deep anesthesia using sodium pentobarbital, and all precautions were taken to avoid unnecessary suffering of the animals.

CM collection

Cells were initially incubated for 24 h in BV2 media discussed above. The supernatants of the cell culture were then replaced with DMEM-F12 FBS-free media to undergo OGD treatment for 2 h and 5 h respectively for the purpose of activation of microglia. CM was collected and free-floating cells were eliminated by centrifugation at 1000 g for 5 min, followed by sterile filtration of the samples, which were then frozen at -80°C prior to use. Collected CM was utilized for culturing NSCs for observing the consequences and behaviors of NSCs as described above.

Establishment of OGD model

OGD treatment was conducted for simulating and establishing microglial cell ischemia model following the method explained by Zhang et al. (14). Suspensions of microglial cells at a final density of 1×10^6 cells/ml were seeded into culture plates. Microglial cell culture medium was replaced with DMEM (glucose-free) followed by 2 h or 5 h of non-oxygen incubation in a sealed tank. This oxygen deprivation condition in sealed tank was established by constant low-flow (1.5 L/min) 5% CO2 and 95% N2 for 20 min to clear oxygen.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

The MTT assay was used to examine the viability of BV2 cells treated with 2 or 5 h of OGD respectively. BV2 cells exposed to 100 ng/ml glucose after 24 h culturing, along with control cells, were subjected to 4 h incubation at 37 °C in MTT solution (0.5 mg/ml). The formazan crystals formed within each well were extracted using dimethyl sulfoxide to measure the absorbance at 490 nm wavelength on a Multilabel Reader (VictorTM 4,

PerkinElmer, Singapore). The cell viability of each well was normalized to that of the control. All cytotoxicity assays were conducted in triplicates (six wells per sample for each time point). Viability of the NSCs after treatments of various CM was determined using the MTT assay as well as LIVE/DEAD viability/cytotoxicity kit (Invitrogen, USA).

LDH assay

Cells were kept on ice in cold Assay Buffer (0.5 ml), and then centrifuged at 4 °C for 15 min at 10,000 g to collect the supernatant for further use. Positive controls were diluted at 1:9 (v/v) in Assay Buffer. Samples (2-50 µl) were added to a 96-well plate and brought to a final volume of 50 µl using Assay Buffer. Various volumes (0, 2, 4, 6, 8 and 10 µl) of NADH standard solution (1.25 mM) were added to 96-well plate separately to generate NADH standard curve. Reaction mix, consisted of 2 µl of Substrate Mix Solution and 48 µl of Assay Buffer, was added to each sample, positive control as well as standard. Measurements of OD 450 nm at T1 and T2 were performed for final analysis.

Live/dead viability assay

The cells were treated with OGD for 2 h or 5 h, and then incubated with the combined LIVE/DEAD cell staining solution (200 ml, containing 4 mM EthD-1 in PBS and 2 mM calcein AM) at 37 °C for 20 min. Images were acquired with an inverse light fluorescence microscope (Eclipse Ti-E, Nikon, Japan).

ELISA

The levels of IL-1β, IL-8, IL-6, TNF-α, and SDF-1α in culture supernatants secreted by microglia were detected using commercially available ELISA Kits (Boster Biological Technology, Wuhan, China) following the provided protocols. The absorbance at 450 nm was measured on a microplate reader. The IL-1β, IL-8, IL-6, TNF-α, and IL-10 concentrations were normalized and standardized against standard samples.

Western blot

Cells were lysed in radioimmunoprecipitation assay buffer (Beyotime, Shanghai, China) that contained 2 mM phenylmethylsulfonyl fluoride as well as Complete Protease Inhibitor Cocktail. Total protein (~20 mg) was resolved through 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, which was then transferred onto 0.45 mm nitrocellulose membrane (Millipore, Billerica, MA, USA). BCA Protein Assay Kit (Beyotime) was utilized to assess protein abundance. The membranes were first hybridized with the primary antibody (anti-CXCR4, 1:1000, Abcam, Cambridge, MA, USA) overnight at 4 °C, followed by incubation with the HRP-conjugated secondary antibody (1:5000, Amersham Pharmacia Biotech) at ambient temperature for 2 h. Protein bands were detected by an ECL detection system.

Establishment of spinal cord ischemic injury mouse model

Spinal cord ischemic injury mouse model was established as previously reported (15). Briefly, mice were subjected to deep anesthetization and bilateral L3-5 lumbar arteries were bluntly isolated, and occluded with a vascular clamp for 25 min. After removal of the vascular clamp, the incision was sutured. Motor function was evaluated using the modified Tarlov scale after spinal cord injury. Mice scoring less than 3 were considered successful models.

Transplantation and tracking of NSCs

4 µg/ml of CM-DiI dye (Molecular Probes, USA) was added to a single-cell suspension from a primary culture of NSCs. CM-DiI-labeled NSCs were measured using flow cytometry. The CXCR4 antagonist, AMD3100 octahydrochloride, was purchased from Sigma-Aldrich and dissolved in distilled normal saline prior to use. 6 weeks old C57BL/6 mice with spinal cord ischemic injury were transplanted with either AMD3100 pretreated NSCs (AMD3100) or normal saline treated NSCs (Control). NSCs were delivered into the cerebrospinal fluid of cryoanesthetized mice following a previously established method (16). In brief, cells were injected along with a maker dye solution (lissamine green). The tracker appearing along the spinal cord as well as in the fontanelles, which was visible through the skin, was an indication of a successful transplantation of the NSCs into the spinal canal. In total, 2 µl of cell suspension containing about 20,000 cells was slowly delivered.

AMD3100 *in vivo* treatment

AMD3100 was intraperitoneally administered at a daily dose of 15 µg per gram of body weight to mice transplanted with AMD3100 pretreated NSCs continuously for 7 days, while normal saline administered to mice transplanted with normal saline treated NSCs was used as control.

NSC migration observed by laser scanning confocal microscope

Eight mice were selected randomly from each group at 2, 4 or 7 days after transplantation and sacrificed. L3–5 spinal cord tissue was obtained and freeze-sectioned. The sections were assessed using a laser scanning confocal microscope (Nikon, Tokyo, Japan) at 553 nm excitation and 570 nm emission wavelengths.

Immunohistological staining

Cells were processed as instructed in the manual of the Fast ImmunoFluorescence Staining Kit (BPIF30-1KT, Protein Biotechnologies, USA). Anti-nestin primary antibody was obtained from Abcam.

Statistical analysis

All experiments were carried out in triplicates and data from three separate repeats were expressed as mean ± S.E.M.. One-way ANOVA analysis followed by Tukey's test was conducted. A p value less than 0.05 was regarded as statistical significance.

Results and discussion

The purpose of this study was to demonstrate the effects of microglia activation upon ischemia and stroke on NSC migration. Here, we established an *in vitro* model of mimicking ischemia and stroke by introducing OGD treatment to the BV2 cell culture following previously established method (14), as it has been used widely for simulating and establishing microglial cell ischemia model. We first characterized the viability and activation of microglia at different time points after OGD treatment and found that OGD treatment less than 3 h caused microglial activation, while OGD treatment longer than 3 h induced even severe reaction (data not shown). We therefore selected two representative durations of OGD treatment (2 h and 5 h) in the following experiments.

The MTT assay and LDH assay were employed to assess the effects of OGD insult on microglial cell growth. We found that the BV2 cells in the experimental groups exhibited a diversity of morphological features under optical microscope, including spindle, amoeboid, spherical and ramified shapes, while no obvious morphological changes were caused by OGD treatment (**Figure 1A**). However, the cell numbers in either 2 h or 5 h OGD treated groups were significantly reduced compared to control group in a time-dependent manner, as indicated by the MTT assay (**Figure 1B**). This result was validated by LDH assay (**Figure 1C**), which assesses impaired integrality of the plasma membrane as a necrosis hallmark. Our findings here confirmed previous reports on the impairments of mobility and viability of microglia under hypoxia or OGD conditions, where 2 h of OGD treatment induced increased cell death as well as reduced cell motility (17).

Upon the activation by OGD which mimics ischemia and stroke conditions, microglial cells are triggered to become hypertrophic, amoeboid shaped, highly phagocytic and proliferating rapidly (18). They migrate to inflammatory sites along with the production of proinflammatory

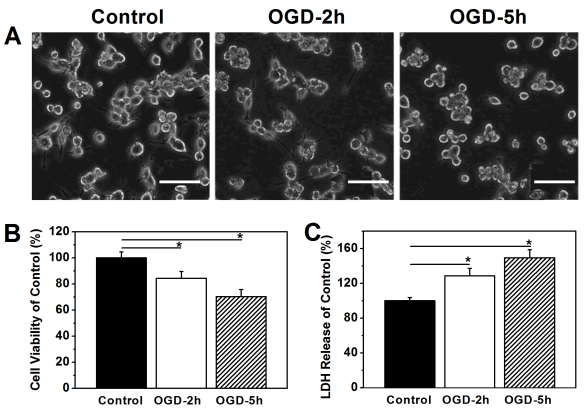

Figure 1. OGD treatment induced BV2 cell death. (A) Representative images of BV2 cells without or with OGD treatment. Scale bar = 200 μm. (B) Cell viabilities in control, OGD treated for 2 h or 5 h groups, examined by MTT assay. (C) OGD treatment increased LDH release in BV2 cells. Data were presented as mean ± S.E.M. *p < 0.05 vs control.

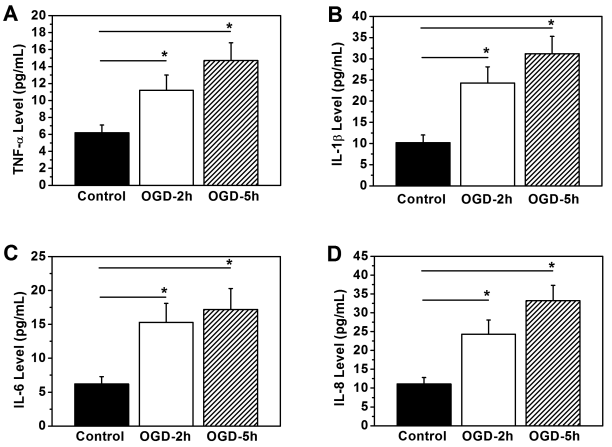

Figure 2. Secretion of proinflammatory factors from microglia cells under OGD treatment. Compared to the control group, levels of TNF-α (A), IL-1β (B), IL-6 (C) and IL-8 (D) were markedly increased by OGD-induced damage either for 2 h or 5 h. Data were presented as mean ± S.E.M. *p < 0.05 vs control.

cytokines and chemokines including IL-1β, IL-8, IL-6, TNF-α, MIP-1α and MIP-1β (19-23). Although these inflammatory factors could prevent further brain injury by producing tissue repair and neuronal survival associated factors (24), they also exert some cytotoxic effects on neurons and glial cells (25). Some studies suggest the degree and type of brain injuries would contribute to different outcomes through which these chemokines and cytokines perform neuro-destructive or neuro-protective effects (2). Therefore to elucidate the possible proinflammatory response following OGD insult, we examined the content of IL-1β, IL-8, IL-6, and TNF-α in the culture supernatants through ELISA assay. IL-1β, IL-8, IL-6, and TNF-α are all mediators of the inflammatory responses. In **Figure 2**, we found drastically elevated levels of all four cytokines in both OGD-2 h and OGD-5 h groups compared with the control group. In addition, OGD-5 h induced even higher levels of all of those cytokines than OGD-2 h, which illustrated a time-dependent inflammatory response. These results demonstrated OGD treatment triggered microglial activation and cytokine secretion, which may induce detrimental or beneficial effects to other cells in the neighboring area.

To exclude the interference of OGD treatment themselves to the behaviors of NSCs, we employed the microglia-treated CM in the current study, to evaluate the effects of OGD microglial activation on the NSCs, without actual co-culturing of microglia and NSCs. Here, mouse NSCs were purified and cultured. A single NSC can proliferate and form neurospheres after several days of culturing. As demonstrated in **Figure 3A** and **3B**, most of the cells either in adherent culture or suspension culture were immunostained positive for nestin, a marker for NSCs (26). We next assessed the effects of CM from OGD-treated microglia on the viability and proliferation of NSCs. The Calcein-AM and EthD-1 staining assays indicated that approximately 90% of the cells in both OGD-treated and control groups were viable (**Figure 3C-E**). The MTT

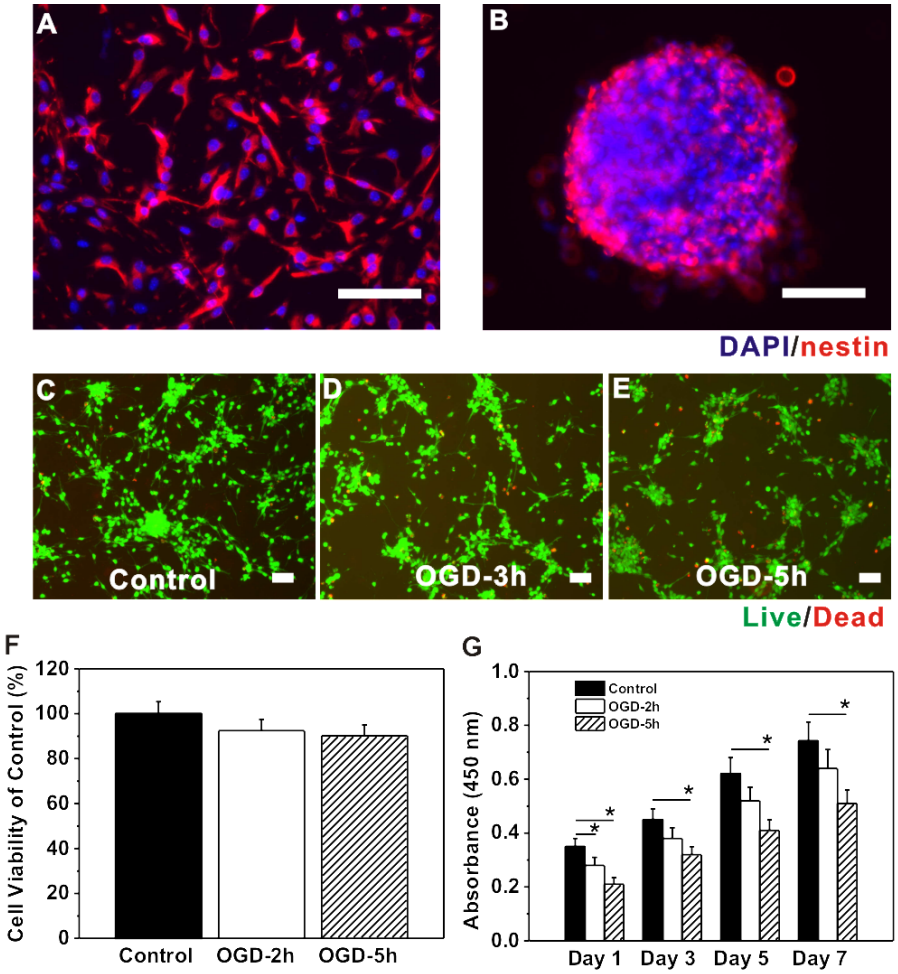

Figure 3. Cell viability and proliferation capacity of NSCs under OGD treatment. The fluorescent images of the adhered NSC (A) and the neurosphere (B). Nestin (red) and DAPI (blue) were used for immunostaining. (C-E) Cell viability assay of NSCs in control, OGD treated for 2 h and 5 h groups respectively as determined by live/dead assay. Live cells are stained green and dead cells are stained red. (F) MTT-measured viability of NSCs in these three groups. (G) Proliferation kinetics of NSCs were assessed at indicated time points in these three groups. Scale bar = 50 μm. Data were presented as mean ± S.E.M. *p < 0.05 vs control.

assay further confirmed that there was no obvious impairment on cell viability in OGD-treated CM (**Figure 3F**). Proliferation kinetics of NSCs was assessed at indicated time points in these three groups by WST-based cell proliferation assay (**Figure 3G**), which clearly showed significantly lower absorbance in both OGD-treated groups than control, indicating the inhibitory effect of OGD-treated CM on cell proliferation of NSCs.

Furthermore, we examined the formation of neurospheres from NSCs in these three kinds of CM at day 3 or day 5 (**Figure 4A-F**). Neurospheres are clonal structures generated from NSCs *in vitro* that exhibit neural cell-lineage intra-clonal diversity (27). It is an important indicator of NSC differentiation and proliferation status (28, 29). NSCs start to form neurospheres of different sizes after 3-5 days of culturing in the CM supplemented with 20 ng/ml FGF and 20 ng/ml EGF, and cells in the neurospheres were positive for NSC marker nestin immunostaining (**Figure 3B**). **Figure 4A-F** illustrated a declined capacity of neurosphere formation following 2 h and 5 h of OGD treatment compared to control-treated CM. We next assessed the neurosphere formation by measuring the average size and the total number of the neurospheres, in both OGD-treated and control groups (**Figure 4G** and **H**). After 3 days of OGD insult, OGD-5 h group exhibited the smallest sphere number as well as the lowest sphere diameter, while there was no statistically significant difference between control, OGD-2 h and OGD-5 h groups after 5 days of culturing. In **Figure 4H**, a significant reduction in sphere diameter was observed in OGD-2 h and OGD-5 h groups compared to control, following both 3 days and 5 days of culturing. The above results again confirmed the damage caused by OGD CM on NSCs.

It is interesting to point out the markedly larger sphere diameter after 5 days of culturing in all OGD and control-treated groups (**Figure 4H**), which might reflect the slow recovery of NSCs with time. Indeed, previous report has demonstrated that microglia may produce chemokines to enhance the proliferation of NSCs under certain circumstances (11). In fact, if properly activated, microglia are able to promote cell survival via the production of anti-inflammatory and trophic factors (30, 31). However, once they become over-activated, microglia do induce death to the surrounding cells, as indicated by the clinical observation of substantial death of grafted stem cells in

patients receiving NSC transplantation, which is probably due to host inflammatory reaction (4).

We further explored the effects of CM collected from activated microglial cells on NSC migration (**Figure 5**). It is well established that migration of NSCs to pathological areas is the initial key step in the NSC-based therapies for treating neural disorders. To start with, we chose individual neurospheres of comparable size from the three different experimental groups for further statistical analysis. In the control group, a part of cells migrated away from the neurospheres after 48 h of culturing, demonstrating a healthy state of those cells (**Figure 5A**). On the other hand, visibly higher amount of cells started to migrate from the neurospheres in OGD-2 h treatment group (**Figure 5B**), while in OGD-5 h group even more migrating NSCs could be seen (**Figure 5C**), and statistical results confirmed the observation (**Figure 5D**). Besides the number of migrating cells, the maximal distance migrated was another indicator to assess NSC migration. As shown in **Figure 5E**, an increasing maximal distance migrated have been observed from control, OGD-2 h and OGD-5 h treated groups at both (10 h and 48 h) time points. Our results here demonstrated the NSC migration triggered by microglia activation following OGD treatment, which suggested the important role microglial cells played in migration and proliferation of NSCs *in vitro*.

Activated microglia secrete anti-inflammatory factors that contain chemokines (32-34). Glial cells-localized SDF-1α is one of the best characterized chemokines involved in migration (3, 34, 35). CXCR4, a specific α-chemokine receptor for SDF-1α, also exerts significant function in neuronal guidance and dissemination mediation (36), as well as invasion and proliferation in various cancers

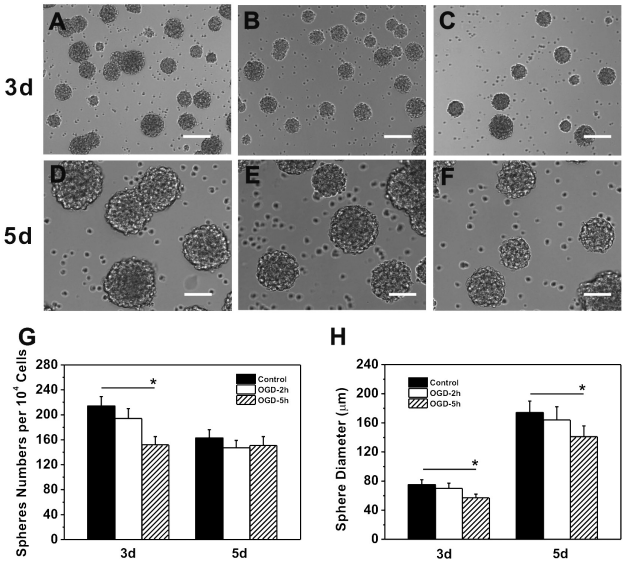

Figure 4. The formation of NSC neurosphere under OGD treatment. (A-F) The bright field photos of the neurospheres for three or five days of culture under OGD treatment for 2 h and 5 h. The number of neurospheres generated per 104 NSCs (G) and the average sphere diameter (H) in control, OGD-2 h and OGD-5 h groups for culturing three or five days. Scale bar = 100 μm. Data were presented as mean ± S.E.M. *p < 0.05 vs control.

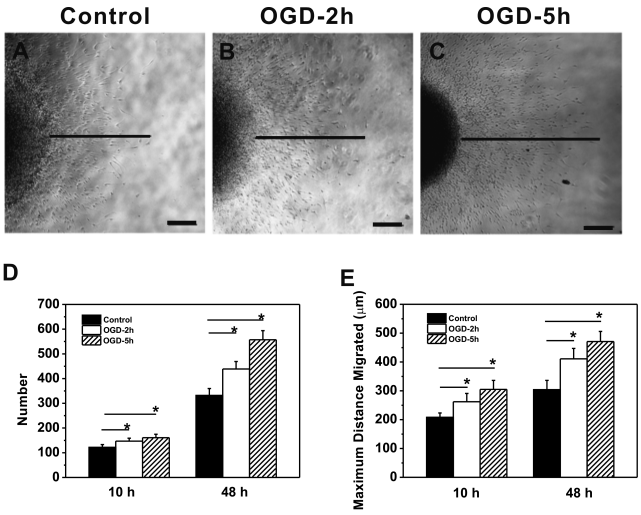

Figure 5. NSC migration under OGD treatment. (A-C) The representative images of the migrated cell from the neurospheres for 48 h in control, OGD-2 h and OGD-5 h groups, respectively. Scale bar = 100 μm. The inset lines indicate the maximum length of the cells migrated from the neurospheres in the three groups. (D) Numbers of the migrated cells from the neurospheres. (E) Mean maximal distance migrated from the neurospheres for 10 h and 48 h. Data were presented as mean ± S.E.M. *p < 0.05 vs control.

(3, 37). Studies have demonstrated elevated proliferation, promoted chain migration as well as transmigration after exposing quiescent NSCs to SDF-1α (3). Therefore we next determined the level of the SDF-1α in the three different CM by specific ELISA. Expectedly, SDF-1α in the CM of OGD-treated groups were significantly higher compared to the control group (**Figure 6A**). The migrating NSCs were also collected and Western blot analysis was employed to assess their cellular CXCR4 levels. Increased expression levels of CXCR4 were observed in OGD-treated groups in comparison with the control group (**Figure 6B** and **C**), which suggested the involvement of SDF-1α/CXCR4 in the migration of NSCs.

From the results above, we propose that SDF-1α/CXCR4 signaling pathway is an active player in the facilitation of NSC migration by OGD-induced microglial activation. To confirm this idea, we next assessed the effect of loss-of-function of CXCR4 on NSC migration *in vitro* as well as *in vivo*. However, because CXCR4 is an essential gene in mice and CXCR4 knockout results in embryonic fatality (38, 39), we then employed AMD3100, a selective CXCR4 antagonist (40). Neurospheres of comparable size in different experimental groups were chosen for further statistical analysis similarly as in **Figure 5**. As expected, 48 h after AMD3100 treatment, no statistical differences in the distance and amount of cells migrated away from the neurospheres were observed between OGD-2 h and OGD-5 h groups compared to the control (**Figure 7A** to **C**), indicating impaired NSC migration in cultures. Quantification results in **Figure 7D** and **E** further confirmed the observation, where both maximal distance migrated and number of migrated cells were almost the same among all three experimental OGD groups after 10 h and 48 h of culturing. As OGD is merely an *in vitro* model established to reproduce ischemia and stroke like

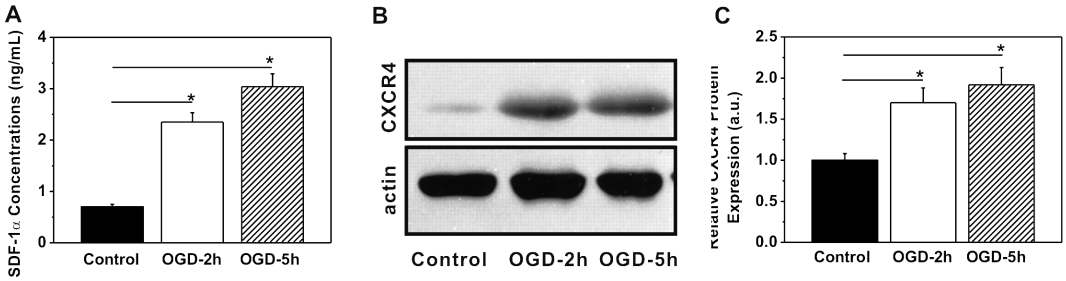

Figure 6. (A) The content of SDF-1α in the conditioned mediums of the three groups. (B) Western blot analysis of CXCR4 protein of NSCs expression when treated with OGD for 2 h or 5 h. (C) Relative optical densities of CXCR4 bands. Data were presented as mean ± S.E.M. *p < 0.05 vs control.

symptoms, in order to determine the involvement of SDF-1α/CXCR4 on NSC migration in more pathologically and physiologically relevant context, we next examined the *in vivo* effect of AMD3100 in a mouse ischemic spinal cord injury model. The mice with ischemic spinal cord injury were transplanted with either AMD3100 pretreated (**Figure 8A**, AMD3100) or normal saline treated (**Figure 8A**, Control) NSCs labelled with CM-DiI dye. The two groups of mice were also intraperitoneally administered with daily dose of AMD3100 or normal saline, respectively. We then tracked the migration of the transplanted NSCs *in vivo*, at various time points (**Figure 8A**). Few NSCs were detected in the injured site at 2 days following transplantation, but the number gradually increased at 4 and 7 days. AMD3100 treatment significantly delayed migration, where NSCs were only first detected in the injured site at 4 days after transplantation, increasing by 7 days. Quantification analysis revealed there were consistently much fewer NSCs in the AMD3100 group than control throughout the entire duration of the experiment (**Figure 8B**). Taken together, these results convincingly demonstrated that the SDF-1α/CXCR4 pathway actively contributed to NSC migration in vitro responding to OGD treatment, as well as in vivo responding to ischemic spinal cord injury.

Conclusion

In conclusion, we hereby have showed that the effects of the CM of microglia upon OGD insult on the NSC behaviors in vitro. The results show that: 1) OGD insult could induce microglia death and activation by secreting cytokines and chemokines; 2) the CM of OGD-treated groups did not affect NSC viability instead of impairing the proliferation and capability of neurosphere formation; 3) the CM of OGD-treated groups facilitated NSC migration via the SDF-1α/CXCR4 pathway *in vitro*;

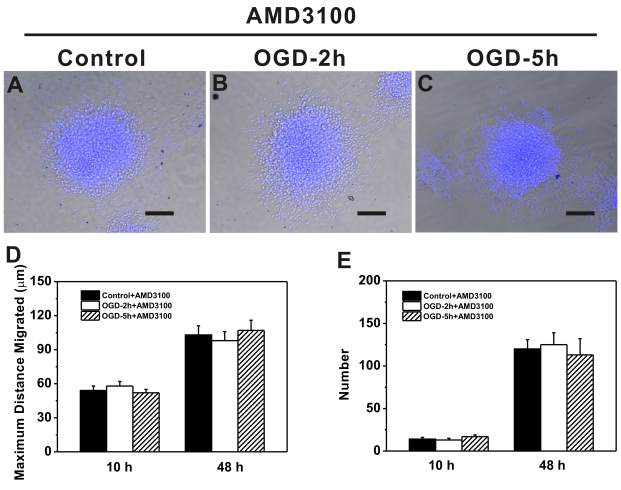

Figure 7. Inhibition of NSC migration under AMD3100 treatment. (A-C) The representative images of NSC migration from the neurospheres for 48 h in control, OGD-2 h and OGD-5 h groups under 20 μg/ml AMD3100 treatment, respectively. Scale bar = 100 μm. The cells were stained blue for the nucleus. (D) Mean maximal distance migrated from the neurospheres for 10 h and 48 h. (E) Numbers of the migrated cells from the neurospheres. Data were presented as mean ± S.E.M.

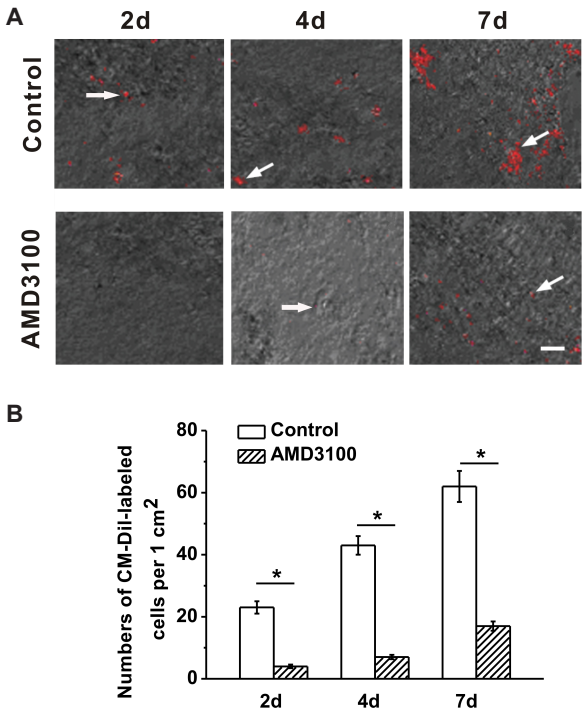

Figure 8. NSC migration in vivo during ischemic spinal cord injury. (A) Representative photos of NSC migration under ischemic spinal cord injury with or without AMD3100 treatment at day 2, 4 and 7 post-transplantation. Red: CM-DiI-labeled cells. Scale bar = 100 μm. (B) Histogram of the numbers of CM-DiI-labeled cells per 1 cm^2 in (A). Data were presented as mean ± S.E.M. *p < 0.05 vs control.

4) SDF-1α/CXCR4 also contributed to NSC migration in ischemic spinal cord injury mouse model. Our study presents the possible interactions between NSCs and microglia under OGD insult. More importantly, we reveal the underlying mechanisms of microglia induced NSC migration in the OGD conditions. These findings may help to understand the interactions between microglia and NSCs in the context of brain injuries, such as ischemia and stroke. Also, it should be beneficial to stem cell-based therapies to treat acute brain injuries.

Conflict of interest

The authors declare that they have no conflicts of interest to disclose.

Acknowledgement

This work was supported by Jiangsu Province Natural Science Foundation (BK20181435).

References

1. Iadecola C, Anrather J. The immunology of stroke: from mechanisms to translation. Nat Med. 2011;17(7):796-808.
2. Neumann J, Gunzer M, Gutzeit HO, Ullrich O, Reymann KG, Dinkel K. Microglia provide neuroprotection after ischemia. FASEB J. 2006;20(6):714-6.
3. Imitola J, Raddassi K, Park KI, Mueller FJ, Nieto M, Teng YD, et al. Directed migration of neural stem cells to sites of CNS injury by the stromal cell-derived factor 1alpha/CXC chemokine receptor 4 pathway. Proc Natl Acad Sci U S A. 2004;101(52):18117-22.
4. Gehrmann J, Matsumoto Y, Kreutzberg GW. Microglia: intrinsic immuneffector cell of the brain. Brain Res Brain Res Rev. 1995;20(3):269-87.
5. Block ML, Zecca L, Hong JS. Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nat Rev Neurosci. 2007;8(1):57-69.
6. Shichita T, Sugiyama Y, Ooboshi H, Sugimori H, Nakagawa R, Takada I, et al. Pivotal role of cerebral interleukin-17-producing gammadeltaT cells in the delayed phase of ischemic brain injury. Nat Med. 2009;15(8):946-50.
7. Gliem M, Mausberg AK, Lee JI, Simiantonakis I, van Rooijen N, Hartung HP, et al. Macrophages prevent hemorrhagic infarct transformation in murine stroke models. Ann Neurol. 2012;71(6):743-52.
8. Dheen ST, Kaur C, Ling EA. Microglial activation and its implications in the brain diseases. Curr Med Chem. 2007;14(11):1189-97.
9. Moon JB, Lee CH, Park CW, Cho JH, Hwang IK, Yoo KY, et al. Neuronal degeneration and microglial activation in the ischemic dentate gyrus of the gerbil. J Vet Med Sci. 2009;71(10):1381-6.
10. Clarke DL, Johansson CB, Wilbertz J, Veress B, Nilsson E, Karlstrom H, et al. Generalized potential of adult neural stem cells. Science. 2000;288(5471):1660-3.
11. Aarum J, Sandberg K, Haeberlein SL, Persson MA. Migration and differentiation of neural precursor cells can be directed by microglia. Proc Natl Acad Sci U S A. 2003;100(26):15983-8.
12. Bye N, Turnley AM, Morganti-Kossmann MC. Inflammatory regulators of redirected neural migration in the injured brain. Neurosignals. 2012;20(3):132-46.
13. Magge SN, Malik SZ, Royo NC, Chen HI, Yu L, Snyder EY, et al. Role of monocyte chemoattractant protein-1 (MCP-1/CCL2) in migration of neural progenitor cells toward glial tumors. J Neurosci Res. 2009;87(7):1547-55.
14. Zhang W, Smith C, Shapiro A, Monette R, Hutchison J, Stanimirovic D. Increased expression of bioactive chemokines in human cerebromicrovascular endothelial cells and astrocytes subjected to simulated ischemia in vitro. J Neuroimmunol. 1999;101(2):148-60.
15. Gaviria M, Haton H, Sandillon F, Privat A. A mouse model of acute ischemic spinal cord injury. J Neurotrauma. 2002;19(2):205-21.
16. Corti S, Locatelli F, Papadimitriou D, Donadoni C, Del Bo R, Crimi M, et al. Transplanted ALDHhiSSClo neural stem cells generate motor neurons and delay disease progression of nmd mice, an animal model of SMARD1. Hum Mol Genet. 2006;15(2):167-87.
17. Eyo U, Dailey ME. Effects of oxygen-glucose deprivation on microglial mobility and viability in developing mouse hippocampal tissues. Glia. 2012;60(11):1747-60.
18. Batchelor PE, Liberatore GT, Wong JY, Porritt MJ, Frerichs F, Donnan GA, et al. Activated macrophages and microglia induce dopaminergic sprouting in the injured striatum and express brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor. J Neurosci. 1999;19(5):1708-16.
19. Lee JC, Cho GS, Kim HJ, Lim JH, Oh YK, Nam W, et al. Accelerated cerebral ischemic injury by activated macrophages/microglia after lipopolysaccharide microinjection into rat corpus callosum. Glia. 2005;50(2):168-81.
20. Yrjanheikki J, Keinanen R, Pellikka M, Hokfelt T, Koistinaho J. Tetracyclines inhibit microglial activation and are neuroprotective in global brain ischemia. Proc Natl Acad Sci U S A. 1998;95(26):15769-74.
21. Yrjanheikki J, Tikka T, Keinanen R, Goldsteins G, Chan PH, Koistinaho J. A tetracycline derivative, minocycline, reduces inflammation and protects against focal cerebral ischemia with a wide therapeutic window. Proc Natl Acad Sci U S A. 1999;96(23):13496-500.
22. Giulian D, Corpuz M, Chapman S, Mansouri M, Robertson C. Reactive mononuclear phagocytes release neurotoxins after ischemic and traumatic injury to the central nervous system. J Neurosci Res. 1993;36(6):681-93.
23. Giulian D, Vaca K, Corpuz M. Brain glia release factors with opposing actions upon neuronal survival. J Neurosci. 1993;13(1):29-37.
24. Polazzi E, Contestabile A. Reciprocal interactions between microglia and neurons: from survival to neuropathology. Rev Neurosci. 2002;13(3):221-42.
25. Wang J, Li PT, Du H, Hou JC, Li WH, Pan YS, et al. Impact of paracrine signals from brain microvascular endothelial cells on microglial proliferation and migration. Brain Res Bull. 2011;86(1-2):53-9.
26. Jin F, Zhao L, Zhao HY, Guo SG, Feng J, Jiang XB, et al. Comparison between cells and cancer stem-like cells isolated from glioblastoma and astrocytoma on expression of anti-apoptotic and multidrug resistance-associated protein genes. Neuroscience. 2008;154(2):541-50.
27. Suslov ON, Kukekov VG, Ignatova TN, Steindler DA. Neural stem cell heterogeneity demonstrated by molecular phenotyping of clonal neurospheres. Proc Natl Acad Sci U S A. 2002;99(22):14506-11.
28. Lobo MVT, Alonso FJM, Redondo C, oacute, pez-Toledano MA, Caso E, et al. Cellular characterization of epidermal growth factor-expanded free-floating neurospheres.J Histochem Cytochem. 2003;51(1):89-103.
29. Laks DR, Masterman-Smith M, Visnyei K, Angenieux B, Orozco NM, Foran I, et al. Neurosphere formation is an independent predictor of clinical outcome in malignant glioma. Stem Cells. 2009;27(4):980-7.
30. Liao H, Bu WY, Wang TH, Ahmed S, Xiao ZC. Tenascin-R plays a role in neuroprotection via its distinct domains that coordinate to modulate the microglia function. J Biol Chem. 2005;280(9):8316-23.
31. Morgan SC, Taylor DL, Pocock JM. Microglia release activators of neuronal proliferation mediated by activation of mitogen-activated protein kinase, phosphatidylinositol-3

kinase/Akt and delta-Notch signalling cascades. J Neurochem. 2004;90(1):89-101.
32. Carbajal KS, Schaumburg C, Strieter R, Kane J, Lane TE. Migration of engrafted neural stem cells is mediated by CXCL12 signaling through CXCR4 in a viral model of multiple sclerosis. Proc Natl Acad Sci U S A. 2010;107(24):11068-73.
33. Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, et al. Cell migration: integrating signals from front to back. Science. 2003;302(5651):1704-9.
34. Lima e Silva R, Shen J, Hackett SF, Kachi S, Akiyama H, Kiuchi K, et al. The SDF-1/CXCR4 ligand/receptor pair is an important contributor to several types of ocular neovascularization. FASEB J. 2007;21(12):3219-30.
35. Won Y-W, Patel AN, Bull DA. Cell surface engineering to enhance mesenchymal stem cell migration toward an SDF-1 gradient. Biomaterials. 2014;35(21):5627-35.
36. Bagri A, Gurney T, He X, Zou Y-R, Littman DR, Tessier-Lavigne M, et al. The chemokine SDF1 regulates migration of dentate granule cells. Development. 2002;129(18):4249-60.
37. Ehtesham M, Mapara KY, Stevenson CB, Thompson RC. CXCR4 mediates the proliferation of glioblastoma progenitor cells. Cancer Lett. 2009;274(2):305-12.
38. Ma Q, Jones D, Borghesani PR, Segal RA, Nagasawa T, Kishimoto T, et al. Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. Proc Natl Acad Sci U S A. 1998;95(16):9448-53.
39. Ratajczak MZ, Zuba-Surma E, Kucia M, Reca R, Wojakowski W, Ratajczak J. The pleiotropic effects of the SDF-1-CXCR4 axis in organogenesis, regeneration and tumorigenesis. Leukemia. 2006;20(11):1915-24.
40. Hatse S, Princen K, Bridger G, De Clercq E, Schols D. Chemokine receptor inhibition by AMD3100 is strictly confined to CXCR4. FEBS lett. 2002;527(1-3):255-62.