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Petite miracles: Insight to nano-management of scarless wound healing

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Teaser: Based on the foundation of many's painstaking investigation into scarring pathology, scientists work relentlessly via nanotechnology such as nanoparticles and nanofibers towards scarless wound healing through early detection, prevention and treatment of potential scars.

Abstract

Scars affect millions of patients worldwide, yet their treatment efficacy and options clinically remain very limited. In recent years, understanding of scar formation pathways and upsurgence of nanotechnology open up many opportunities for scar detection, prevention and treatment due to their nanoscale features and therapeutic delivery capabilities. Led by nanoparticles and nanofibers, these novel strategies can aid in reducing scar contracture, improving wound healing efficacy and greatly advance progress towards the much desired scarless wound healing.

1. Introduction

Scars are deficiencies or abnormalities originated from imperfect healing of damaged tissues induced by injuries and diseases. They come in many distinctions from hypertrophic scar and keloids being the most prominent caused by external wounds, to

internal scarring such as tendon adhesion, myocardial infarction and ocular occlusion [1,2]. Scars are formed due to the unusual deposition of collagen, proteoglycans and fibronectin by fibroblasts during the remodeling phase of wound healing, and are particularly prone to occur when inflammation is prolonged. They will lead to hypercellularity, vascularization and lower the function and appearance of the healed tissues [3].

Much effort has now been put into research of scar prevention due to its wide occurrence among all groups of the human population [4,5]. While the mechanisms of different scarring are slightly different, they follow the general pathway of hemostasis, inflammatory, proliferative and remodeling phase, and are treated traditionally through direct injection of therapeutics aiming at interfering with fibroblast activities. More advanced therapy options include surgical excision, laser and cryotherapy, and injection of steroids [6]. While initially effective, these strategies are only one-dimensional treatment strategy of scar tissue removal, whilst not being able to provide appropriate cues to support the subsequent healing after therapy, leading to extremely high recurrence rates [7]. Moreover, they are highly invasive: they disturb the pathophysiology of wound healing environment and often require multiple treatments, causing inconvenience to patients and hindering proper healing of tissues.

Recently, scar treatments have been attempted to combine wound healing with the aid of nanotechnology. Nanotechnology is particularly attractive in scar prevention due to its intrinsic nature to interact with cells on a molecular level, support wound healing and deliver therapeutics: nanoparticles can promote intracellular delivery, lowering drug dosage use while nanofibers allow for improved adhesion and proliferation of body cells, which improve wound healing efficacy [8,9]. For instance, silver nanoparticles (NPs) can penetrate the bacteria membrane and inhibit their replication by binding to the DNA [10], and were shown to elevate growth factors (GFs) and suppress both local and systemic inflammation, which are beneficial to suppressing scar formation [11]. For another example, Nanoskin® is a nano sized membrane made of cellulose, and clinical trials showed promising results for treatment of burn wounds due to its structural similarities to collagen, close adhesion to wound site and reduced infection rate [12]. Nanotechnology also allows for encapsulation and delivery of various therapeutics for

scar management: common nonsteroidal anti-inflammatory drugs (NSAIDs) such as celecoxib, diclofenac and ibuprofen have been encapsulated in NPs or nanofibers to prevent scar formation by knockdown of cyclo-oxygenase-2 (COX-2) activity without the record of impeded wound healing [13,14]; additionally, as GFs regulate cell behaviors, GFs including vascular endothelial growth factors (VEGFs), platelet-derived growth factors (PDGFs), hepatocyte growth factors (HGFs) and basic fibroblast growth factors (bFGFs) [15] as well as their interference molecules such as siRNAs and interference drug like Mitomycin C have been integrated with nanotechnology to mediate the actual healing and scar formation process to achieve scarless wound healing.

Nanotechnology such as NPs and nanofibers has showed great promise at improved healing and lowered scar formation for wound treatment by effective intracellular delivery, localized and programmed release and providing biochemical and biophysical cues to further supplement the effect of therapeutics (**Figure 1**). In addition to prevention of scar formation during wound healing, novel strategies in detection and treatment of scars have also emerged due to the wide variety of materials and functionalization methods derived from years of research. Early detection of scars allow for effective estimation of treatment options, and biosynthetic materials are on the rise to solve problems that autografts and allografts may possess, including hidden diseases and cellular remnants from a previous organism. Here we hope to summarize nanotechnology-based strategies used to manage scars including treatment, prevention and detection to provide insights to fellow researchers working towards scarless wound healing, which can be characterized by an absence of granulation tissue, fully reorganized extracellular matrix (ECM) and a complete rebuilding of hair follicles and sweat pores [16].

2. Nanostructures

Many nanostructures were studied and invented to be used in tissue engineering, including but not limited to NPs, nanofibers, graphene-based materials and nanogrooves. Among different structures, NPs and nanofibers are the most researched in scar prevention, detection and treatment due to their structural stability and ability to interact with wound tissues at a molecular level, allowing them to (1) support healing

and prevent scar formation with their nano-scaled features and/or (2) act as a cargo to bypass body barriers and limit dosage use through therapeutic preservation [17].

2.1. Nanoparticles (NPs)

NPs are materials at nanoscale throughout all dimensions, and are highly researched as drug carriers for scar management. Their relatively small size allows for activation of phagocytosis in mammalian cells, which engulfs the NPs alongside its encapsulated content including drug molecules and siRNAs. The molecules then freely diffuse within the cytoplasm into the nucleus and influence on transcription of genes. Additionally, NP materials are flexible in that both organic and inorganic options are available, and specific materials such as natural polymer like chitosan and hydroxyapatite can be chosen for specific applications such as programmed release in acidic environments in the context of wound healing. Synthetic polymers like polylactic acid (PLA), polyglycolic acid (PGA) and polylactide-co-glycolide (PLGA) have also been widely used as NPs for carrying drugs due to their high drug loading capacities, modifiability and biocompatibility [4]. Polymeric NPs possess lower degradability, effectively protecting the encapsulated drugs from degradation *in vivo*. Degradation rate of these NPs can be further manipulated by adjusting sizes and protective layers to achieve programmed drug release [5]. Utilization of NPs as carriers significantly reduces side effects of potentially poisonous drugs because of the lowered dosage due to the higher delivery efficiency, and reduced drug circulation time thanks to the localized delivery [6]. One notable inorganic carrier example is mesoporous silica nanoparticles with their iconic mesoporous structure, bolstering surface area and encapsulation efficiency [18-22]. Proven safe by the Food and Drug Administration and traditionally synthesized through templated Stöber process and sintering, they have wide applications in tissue engineering due to their structural niche allowing for versatile multifunctional drug delivery and their surface “OH” groups allowing for ready modification of various functional groups.

2.2 Nanofibers

While NPs have great properties as carriers for wound healing and scar management, direct usage proves difficult due to their fast dispersion in solutions. Unlike NPs, nanofibers show good tensile strength even in aqueous solutions [11]. This is important for exterior scar management since nanofibers are applied onto the surface of wound sites. Nanofibers are mimetic to native ECM fibrous structures, allowing for heightened cell proliferation and adhesion to these materials. Purely topographical nanofibers alone were able to promote wound healing and reduce scar formation [24]. Nanofibers are most commonly synthesized through electrospinning, where therapeutics can be incorporated in fibers by direct mixing of molecules in polymeric fiber materials or by encapsulation into NPs prior to electrospinning. They can also be absorbed or covalently bonded to the fiber surfaces. This will result in various drug release profile to meet the requirement of wound healing and scar formation [25,26].

3. Applications

3.1. Detection

The critical first step in determining scar prevention measures and treatment usage is to assess the severity and healing potential of a said wound [27]. Traditionally, this was performed visually by trained clinicians [28]. However, this poses limitations such as repeated disturbances, inability to predict scar formation and confinement to surface morphologies. With assistance from nanotechnology, other methods of wound monitoring have been researched and realized to provide more insights to healing and scar formation in real time.

Instead of direct visual assessment of scar tissues, targeted conjugation of area of interest with nano-molecules allows for optical imaging technology to be applied successfully on deep or internal wounds [29]. Clinical iodinated agents allow for short-term imaging (5 - 10 min) of internal organs, but were limited in their structure and composition for further refinement. By utilizing gold NPs functionalized with adhesion proteins, Kee et al. successfully performed computed tomography (CT) imaging on myocardial scars in the heart [30]. At 6 hours after injection, freely circulating gold NP clearance was complete, and clear enhancement of CT signals could be seen from scar

tissues with CNA35-conjugated gold NPs while control group with naked gold NPs saw no enhancement signals. In addition, reactive oxygen and nitrogen species (RONS) were recently researched as biomarkers for fluorescence probes. Keloids are characterized by their abnormally high RONS level, and Cheng et al. capitalized their nature to perform optical imaging using RONS-sensitive fluorescence probes [31]. To validate the probes' scar detection ability, keloid-derived fibroblasts along with normal dermal fibroblasts were seeded into mice models. After 3.5 h, fluorescence level of keloid-derived fibroblasts rose to over 3-folds of normal dermal fibroblasts. However, this visualization remains helpless at detecting abnormalities before scar tissue growth. In order to achieve scarless wound healing, the ability to track and predict growth of scar tissues would be extremely beneficial. Combining the concept of live-cell monitoring, Fang et al. performed real-time optical detection of abnormal skin scarring using RNA as a biomarker [32]. By introduction of nanoflares, which only respond to fluorescence under presence of connective tissue growth factor (CTGF) mRNAs, scarring can be visualized and quantified with fluorescence from CTGF overexpression, as demonstrated by applications in mice and rabbit scar models **(Figure 2)**. Nanoflares also reacted instantaneously to chemical changes in the environment, as proven by decrease in fluorescence signals after siRNA treatment, which could provide invaluable insight to testing scar prevention strategies in skin models.

3.2. Prevention

Scar preventive measures can be implemented as early as right after first aid treatment. Importance of early wound healing is stressed to preserve patients' lives, while later stages focuses more on preventing scar tissue formation or reducing undesirable tissue size. There are currently three primary principles to prevent and reduce scarring: reduced inflammation, which were shown by many studies to be directly related to scar formation due to promotion of pro-inflammatory cytokines, ECM modification and increased granulation tissue [33,34]; inhibition of adverse growth, which utilizes various remedies to lower activity of problematic cells responsible for scar tissue formation; and finally, improved healing. It is widely known that faster healing from trauma lowers the

probability and seriousness of scar formation. Recovery promotion thus focuses on providing the body with the appropriate cues to enhance healing efficacy.

3.2.1. Reduction of inflammation and/or infection

Immediately after injury, wounds are exposed to bacteria invasion due to the loss of skin as the protective layer. With bacteria colonization on the wound site, it prolongs the inflammatory phase and increases uptake of macrophages in the wound site, drastically reducing healing efficacy [33,34]. While tendon injuries are internal wounds that are usually free from infection, they often form scar tissues binding to surrounding tissue, which greatly lowers their functionality. Inflammatory reaction in tendon adhesion had been highly researched to alleviate the formation of adhesion tissues. While intrinsic mechanics of tendon adhesion had not been clearly revealed, scientists were able to derive strategies in reducing inflammation and isolation of wound site from exogenous cells. Celecoxib, an anti-inflammatory therapeutic widely used for prevention of joint and intra-abdominal adhesion, was shown to be effective at suppressing collagen expression through inhibition of the ERK1/2 and SMAD2/3 pathways [35,36]. However, its rapid clearance and potential side effects caused by oral administration limited its usage on scar prevention. Jiang et al. utilized poly(l-lactic acid)-polyethylene glycol (PELA) nanofibrous scaffolds for loading of Celecoxib due to their high surface-to-area ratio and anti-adhesion ability as a physical barrier [14]. The lowered proliferation and adhesion of tenocytes and fibroblasts, alongside the downregulation of collagen I, collagen III, ERK1/2, p-ERK1/2, SMAD2/3, p-SMAD2/3 and COX-2 expression evidenced by Western blot assay, were able to reduce the inflammatory response of peritendinous adhesions and attachment of tendon to surrounding tissues **(Figure 3)**. In thermal wounds, inflammation in response to infection is a major cause of morbidity in patients due to the easy colonization of bacteria at defenseless burn sites. Thakur et al. designed gel encapsulated chitosan-lipid NPs for carriers of antibiotic molecule fusidic acid to target *Staphylococcus aureus* (SA) and methicillin-resistant *Staphylococcus aureus* (MRSA) [37]. Compared to naked fusidic acid molecules, the gel NP system was more effective at inhibiting growth of bacteria colonies and had over 3-fold increased drug retention on skin. These improved therapeutic effects were further evidenced by *in*

vivo study. Pure fusidic acid treated wounds in mice thermal injury models still elicited serious infections, while fusidic acid loaded chitosan-lipid NPs were able to suppress inflammatory responses by elimination of bacteria and reduce the wound size by day 15. The better ability to inhibit infectious bacteria, provide hydration and carry drugs make this gel NP system a far more attractive strategy at inhibiting inflammation in burn wounds. On the other hand, maintaining moisture at wound site is also crucial for controlling inflammation. Chen et al. devised a sulphide-sulphide-silver bonded hydrogel with self-healing and anti-bacterial properties [38]. Through the unique disulphide bonds and their interactions with nanosilver, the formed self-healing hydrogel matrix can fuse, act as a physical barrier and resist twisting, pulling and cutting. By slow degradation leading to release of nanosilver, it protects wounds from further trauma, maintains wound site moisture and accelerates wound healing. Moreover, this hydrogel allows for encapsulation of small-molecule drugs within its matrix and as a proof-of-concept, this study loaded angiogenic drug desferrioxamine and greatly improved healing efficacy in diabetic rat models.

3.2.2. Inhibition of adverse growth

Scarring is the result of abnormal overexpression of collagen and connective tissues by traumatic signaling from body cells in the injury site. Successful knockdown of CTGF expression through siRNA interference would be effective at impeding scarring process, limited only by its low stability and transfection rate [39]. siRNAs were also attractive in their inhibition specificity, allowing for niche bio applications. Glaucoma is the leading cause in the world for irreversible blindness. While surgery could cure this condition, some patients still develop scarring despite antimetabolite therapy and fail the surgery. Compared to drug molecules such as mitomycin-C (MMC), siRNA possesses superior cell specificity, potency and duration to target particular genes such as Myocardin-related transcription factor B. For example, Fernando et al. utilized lipid-peptide-siRNA (LPR) NPs for treatment of glaucoma [40]. Targeting peptides Y, ME27, KG31 and KG32 were able to produce higher transfection efficiencies in human conjunctival fibroblasts. Their efficacy at preventing scar formation was assessed using rabbit models of glaucoma filtration surgery, whose failure was marked by significant scarring

and bleb failure. LPR NPs was able to nearly double bleb survival time from 11.0 ± 0.6 to 22.0 ± 2.1 days, preventing occurrence of conjunctival fibrosis and posed no local or systemic side effects. The high scar prevention efficacy of LPR NPs plus the absence of ocular or systemic poisoning suggest that they could become the next novel platform for glaucoma post-surgery treatment.

3.2.3. Promotion of wound healing

Nano-scaled features are highly beneficial to promoting wound healing. Nanogrooves and nanofibers were shown to be effective at aligning cells along the direction of bundles, which reduces the random accumulation of collagen in recovering wound sites. In addition, they direct cell migration towards wound gaps, which could provide novel strategies for wound closure other than sutures. For instance, Xie et al. demonstrated the efficacy of radially aligned polycaprolactone (PCL) electrospun fibers on dural wound closure [41]. Radial alignment was greatly beneficial to cell migration from edges to wound center to shorten healing time, and improved healing was observed in *ex vivo* models of dura mater injury. Dural fibroblasts migrated along aligned fibers and completely covered the scaffold over 4 days whereas a void was observed in the control randomized fibers. Nanotechnology also enables therapeutics to be encased in nanocarriers, which allows for longer circulation time and access to body areas originally unreachable via external injection with opportunities for smart delivery and programmed releases [42]. Traditionally, therapeutics were only able to enter the blood circulation through ingestion or injection into the scar site. This requires large quantity of drugs to be used, and therapeutic molecules often fail to bypass body innate barriers or be broken down by metabolism before reaching the target areas. Wang et al. modified liposomes with scar-homing tetrapeptides to simultaneously encapsulate docetaxel and brain-derived neurotrophic factor in the lipid and aqueous phase respectively [43]. It was shown in mice spinal cord injury (SCI) models that the loaded liposome group had enhanced recovery of spinal cord alongside improved locomotion following treatment, compared to control. Neural cell imaging also showed that therapeutics rearranged mitochondria in more elongated locations, which provided the energy needed for neurite growth as opposed to the normal accumulation of mitochondria near nuclei following

spinal injury. On another hand, certain scar prevention such as hypertrophic scarring requires long-term release of different therapeutics to effectively provide body cells with the suitable cues. To combine both concepts of nanotopography and drug loading, Cheng et al. fabricated ECM mimetic, electrospun PLGA nanofibrous scaffolds functionalized with various molecules (polyethylene glycol, RGD peptide and bFGFs) and loaded with 20(R)-ginsenoside (Rg3) for prevention of hypertrophic scarring [44]. *In vitro* drug release profile of scaffolds showed that the loaded Rg3 were steadily released over 40 days. Results showed that therapeutics combined with physical cues from the functionalized scaffolds were able to support wound healing in rabbit hypertrophic scar models with 10 mm diameter injury. While, many scar prevention strategies have been devised by researchers, most only are able to alleviate scar formation and speed up recovery while not being able to completely ward off scar tissues. Hence equal efforts have been put into continuing scar management through treatment therapies that focus on minimizing scar size during the last phases of wound healing.

3.3. Treatment

Treatment of scars currently relies on direct injection into scar site or intravenous injection of drug molecules after formation of scar tissues. The critical downfall of such strategy is the lack of specificity due to the dispersion of drug molecules into normal tissues, and the pain and risk of infection correlated to needle injections. As common anti-scarring drugs are usually chemotherapy drugs, they possess high toxicity to all types of cells and halt their proliferation indiscriminately. In one study, Zhang et al. studied the possibility of using gel with ethosomes to deliver traditional anti-scarring agent 5-fluorouracil (5-fu) transdermal with CO_2 fractional laser for treatment of hypertrophic scarring [45]. The laser opened up micro-channels in skin tissue, allowing for permeation of nano-scaled ethosomes (87.72 ± 9.27 nm) to permeate inside dermal layer. 7 days after intervention of *in vivo* treatment of rabbit hypertrophic scar models, experimental groups all showed reduced thickness of scar tissues and Haemotoxylin and Eosin (H&E) staining showed aligned collagen fiber remodeling with the aid of CO_2 fractional laser. These results showed that laser therapy with ethosomes were able to

achieve treatment of hypertrophic scarring without reliance on injections. In another study, Cho et al. developed a degradable poly(sorbitol-*co*-polyethylenimine) (PSPEI) nanocarrier to improve targeted delivery and reduce lysosomal degradation of siRNAs due to PEI's high efficiency of gene transfection in cells [46]. This greatly lowers cytotoxicity due to siRNA's specificity to CTGFs. To determine gene silencing efficacy, cells were transfected with PEI and PSPEI loaded with siRNAs, which showed lowered luciferase activity by up to 85%, decreased scar area and contracture significantly compared to negligible effects by direct injection of siRNAs in mice scar models **(Figure 4)**. Groups treated with small interfering connective tissue growth factors (siCTGFs) also showed noticeable difference in papillary structures in dermal layer of healed tissues, which were absent in control and scrambled siRNA group. The reduced skin contracture and low cytotoxicity of therapeutics suggested that siRNA-loaded PSPEI nanocarriers were a promising strategy for direct injection based treatment.

4. Conclusion and future outlook

A large gap has been bridged in scarless wound healing since the days of direct injection or ingestion of drugs, due to our linkage of exogenous effects of therapy with cellular signaling moieties. We are slowly moving from causal-effect treatments to therapies that tackle the root of scar formation made only possible by nanotechnology. Through study of molecular pathways involved in scarring, we have successfully identified Smad, Ras/ERK, JAK and PI3K signaling pathways and derived appropriate therapeutics corresponding to each mechanism, as well as the effect of macrophage phenotypes on debris clearance and collagen deposit on scarring [47,48]. However, there remain issues that require addressing in future studies.

While much improvement was demonstrated by many studies using animal models, the scar tissues remain highly visible compared to undamaged skin. This is hypothesized by the lack of inhibiting signals to halt fascia progression on skin surface. In studies with close adhesion of matrix to wound surface, the scar tissues were less prominent than usage of clinical dressings such as gauze. While there is no concrete evidence, this suggests that an interacting surface for cell adhesion is beneficial to signaling wound provisional matrix to halt its overgrowth. While physically this can be

tackled though nanofibrous membranes as proven by many studies, we still lack a biological and chemical understanding to provide appropriate cues to stop matrix growth, as movement of deep fascia matrix is unaffected by proliferation and accounts for over 80% of scar tissues [49], thus explaining the lack of effectiveness of interference drugs such as 5-fu, which primarily inhibits fibroblast proliferation but not movement.

Another thing of note is how behind research is at detecting scar formation. While novel methods have been invented to visualize scar formation via fluorescence and mRNAs, they remain impractical in a clinical setting as much time is required to perform staining and analyze tissue condition. Timing of application of therapeutics is of high importance as inappropriate control of dosage and schedule of drugs can hinder wound healing or aggravate scar formation. While we move towards an age of personalized medicine, individual assessment of scar formation will need to be developed to apply correct treatment in a timely fashion. Finally, it is a widely known fact that fetal wounds and certain organisms such as axolotls are able to completely heal tissue damage without any scar formation [50]. It would be the future trend to harness the ability of stem cells via nanotechnology since it provides the much needed niche for both culture and post-implant integration [51]. With their greater capacity for self-renewal and ability to differentiate to most types of cells, stem cells respond to injuries by heighted proliferation and reduce differentiation until cellular layer of tissue has been restored. Recently, the role of stem cells during wound healing has also been clarified through cell tracing, which revealed a loss in spatial confinement of stem cells during post-trauma homeostasis [52]. The position of stem cell proliferation also showed that they proliferated away from wound edge, which gives much insight to stem cell treatment strategies. For example, with preceding knowledge of cell phenotypes and their roles in wound healing, stem cells can be applied around wound edge while keratinocytes can be applied directly onto the trauma site as a cell sheet, resembling the intrinsic healing process of the native body. In addition, matrix-based therapies are slowly on the rise and by combination of nanotechnology with stem cell therapy [53-55], it is envisioned to be able to recapitulate the miraculous healing prowess of fetal tissues, and achieve the much-anticipated scarless wound healing.

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Figure 1. Schematic illustration of scar management strategies with nanotechnology to achieve scar detection, prevention and treatment. Therapeutics encapsulated in nanoparticles or nanofibers aid in scar prevention through inhibition of fibroblasts, anti-inflammatory actions and promotion of wound recovery. Created with Biorender.

Figure 2. Non-invasive detection of scar formation using Nanoflares. (A) Schematic of nanoflare matrigel for hypertrophic scar detection and (B) Schematic of nanoflare-laden matrigel and its imaging of human skin fibroblasts *in vitro*. (C)(i) Schematic and *in vivo* imaging system (IVIS) image of injected normal dermal fibroblasts (NDFS), peri-hypertrophic scar fibroblasts (PHSFS), human skin fibroblasts (HSFS) and control (blank gel). (ii) Dot plot of IVIS signals from injected gels. (iii) Schematic and IVIS image of Cy3 labeled glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and scrambled (SCRM) nanoflares topographically applied on mice and (iv) their respective normalized IVIS signals represented in Cy5 fluorescence images (v). Adapted from [32] with permission.

Figure 3. (A) Illustration of celecoxib-loaded PELA fibrous membrane for drug delivery and tendon adhesion prevention, and their respective morphologies. (B) Young's modulus of respective membranes under tensile stress and (C) cumulative release profile of celecoxib from fibrous membranes. (D) *In vitro* imaging of dermal fibroblasts on PELA membranes with control. (E) Evaluation of tendon adhesion using rabbit flexor digitorum profundus (FDP) model, with Masson staining (B) of control, PELA and celecoxib-loaded PELA groups. White arrows indicate interface without peritendinous adhesions while black arrows indicate adhesion between materials and tendon. Adapted from [14] with permission.

Figure 4. (A) Wound healing experiment schedule on mice. (B) Image of wounds before and after siCTGF (small interfering connective tissue growth factor), PSPEI/siScr (scrambled siRNA), PEI 25kD/siCTGF and PSPEI/siCTGF treatment. (C) Histological images of scar tissues stained with H&E, where papillary structures were indicated with white arrows. Adapted from [46] with permission.