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REVIEW

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Direct fibroblast reprogramming: an emerging strategy for treating organic fibrosis

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Abstract

Direct reprogramming has garnered considerable attention due to its capacity to directly convert differentiated cells into desired cells. Fibroblasts are frequently employed in reprogramming studies due to their abundance and accessibility. However, they are also the key drivers in the progression of fibrosis, a pathological condition characterized by excessive extracellular matrix deposition and tissue scarring. Furthermore, the initial stage of reprogramming typically involves deactivating fibrotic pathways. Hence, direct reprogramming offers a valuable method to regenerate target cells for tissue repair while simultaneously reducing fibrotic tendencies. Understanding the link between reprogramming and fibrosis could help develop effective strategies to treat damaged tissue with a potential risk of fibrosis. This review summarizes the advances in direct reprogramming and reveals their anti-fibrosis effects in various organs such as the heart, liver, and skin. Furthermore, we dissect the mechanisms of reprogramming influenced by fibrotic molecules including TGF-β signaling, mechanical signaling, inflammation signaling, epigenetic modifiers, and metabolic regulators. Innovative methods for fibroblast reprogramming like small molecules, CRISPRa, modified mRNA, and the challenges of cellular heterogeneity and senescence faced by in vivo direct reprogramming, are also discussed.

Keywords Fibrosis, Fibroblast, Myofibroblast, Direct reprogramming, In vivo direct reprogramming, In situ regeneration

Introduction

Replacing pathogenic cells and supplementing deficient ones are important strategies for treating various diseases [1]. To this end, a groundbreaking approach is cellular reprogramming [1]. In 2006, by manipulating four transcription factors (TFs; OCT4, SOX2, KLF4, and c-MYC, OSKM), Yamanaka et al. [2] discovered that somatic cells could be reprogrammed into induced pluripotent stem cells (iPSCs). The emergence of iPSCs overcomes the limitations of insufficient autologous stem cells and provides almost immeasurable stem cell sources, which propels the development of iPSC transplantation therapy [3]. However, concerns remain regarding this approach, such as abnormal differentiation, tumorigenicity, and potential trauma from transplantation [3, 4]. Yet another

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type of cellular reprogramming may provide a more viable approach, which is dubbed direct reprogramming or transdifferentiation [5]. During direct reprogramming, a differentiated cell changes its fate into another desired differentiated cell without going through the acquisition of pluripotency [5]. This method offers immense potential for in vivo application, able to simultaneously obtain the required cell types and replace cells related to pathogenic consequences, thereby achieving in situ direct repair and regeneration.

Fibrosis is a common pathological alteration that contributes significantly to organ dysfunction, often triggered by chronic inflammatory diseases and severe trauma [6]. Characterized by excessive deposition of extracellular matrix (ECM), fibrosis is primarily driven by fibroblasts [7, 8]. Fibroblasts are defined as spindle-like connective tissue cells that synthesize collagen (an essential component of the ECM), serving to maintain the ECM network [9]. In tissue repair, fibroblasts are indispensable. They support other cells for proliferation and differentiation and restore basic tissue structure through rebuilding the ECM network [10]. Nevertheless, when this repair is overly strong or incontrollable, fibroblasts constantly proliferate and secrete ECM, leading to the progression of fibrosis [11]. Moreover, activated fibroblasts can further differentiate into myofibroblasts, fibroblast-like cells that express α-smooth muscle actin (α-SMA) and are capable of contracting wounds and secreting massive amounts of collagen [12]. Hence, fibroblasts are active targets for improving fibrosis outcomes (Fig. 1). In this regard, direct reprogramming presents a promising new approach for reducing excessive fibroblasts and promoting tissue regeneration [13].

Successful direct reprogramming of fibroblasts requires the erasure of their fibrotic identity and the reconstruction of a gene expression pattern of the target cell type [14]. In the fibrotic microenvironment, however, profibrotic factors such as transforming growth factor-β (TGF-β), ECM stimuli, and inflammation sustain and augment the fibrotic identity, leading to the inhibition of reprogramming [14, 15]. Therefore, understanding the fibrosis program and mechanism is critical and may provide potential targets to facilitate in vivo reprogramming and further optimize the anti-fibrosis effect.

Based on the close relationship between direct reprogramming and fibrosis. This review first summarizes the current studies of direct reprogramming in anti-fibrosis and tissue regeneration, analyzing the influence of fibrotic programs in regulating the reprogramming of fibroblasts. Moreover, we focus on reprogramming methodology, proposing promising novel mediators for promoting the reprogramming application in treating fibrosis. Lastly, some challenges associated with the direct reprogramming of fibroblasts in vivo are briefly mentioned.

Direct reprogramming of fibroblasts in different organs and their anti-fibrosis effects

Numerous reprogramming strategies have been developed for various tissue cells. Among these, strategies with anti-fibrotic potential primarily focus on the heart, liver, and skin.

Direct reprogramming in the heart

The regenerative capacity of adult mammalian cardiomyocytes is highly limited. Cardiac fibroblasts (CFs) are widely present in the cardiac interstitium, providing structural support to the cardiomyocytes. Myocardial damage and inflammation resulting from ischemic heart diseases and other events extensively activate resident CFs, resulting in the development of cardiac fibrosis [16]. Therefore, resident CFs are crucial targets for reversing cardiac fibrosis, while in vivo reprogramming of CFs into induced cardiomyocytes (iCMs) together achieves the aims of anti-fibrosis and cardiac regeneration [17, 18].

In 2010, Ieda et al. [19] first identified that a TF cocktail GATA4, MEF2c, and TBX5 (GMT) enabled the reprogramming of mouse CFs into cardiomyocyte-like cells in vitro. A subset of these reprogrammed cells exhibited functional characteristics similar to normal cardiomyocytes, including action potentials, spontaneous beating, and calcium transients. Subsequently, this group demonstrated that the delivery of retroviral GMT to an injured heart in acute myocardial infarction (MI) mouse models can convert resident CFs into iCMs [20]. After eight weeks of GMT treatment, significant improvement in cardiac function and a reduction in scar area were observed. Strikingly, isolated iCMs from the GMT-treated heart also exhibited action potentials and held spontaneous contraction ability. These works exhibit the significance of direct reprogramming in organ regeneration and have inspired similar research. To address the problem of a virus vector carrying only one transcription factor. Inagawa et al. [21] designed a retroviral GMT-polycistronic construct with identical "self-cleaving" 2A sequences in a single messenger RNA (mRNA), further improving the in vivo reprogramming efficiency [21]. Interestingly, the transcriptional order of GMT in polycistronic construct can affect reprogramming efficiency, with the M-G-T order achieving the highest in vivo conversion rates [22, 23]. Moreover, the inclusion of HAND2 in the GMT cocktail (GMTH) facilitates the generation of more iCMs in vivo than using GMT and accelerates the reduction in the fibrotic area [24]. Recently, Zhou et al. [25] developed a novel compact TF cocktail containing MYΔ3A (an internal deletion of MYOCD without

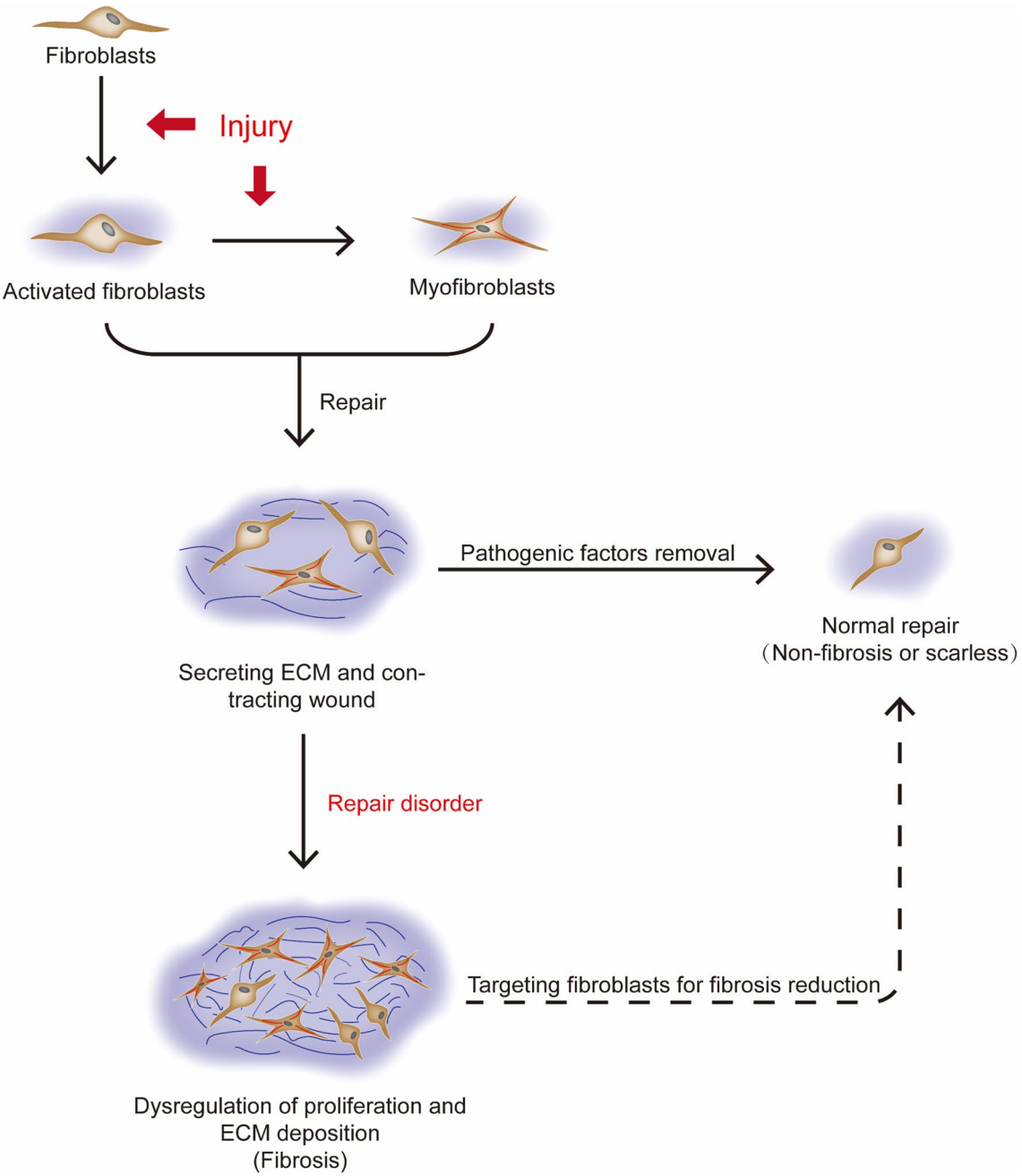

Fig. 1 The general process of fibrosis development. Injury triggers the activation of fibroblasts and induces activated fibroblasts to further differentiate into myofibroblasts. These activated fibroblasts and myofibroblasts secrete ECM and contract wounds, playing a crucial role in tissue repair. Once pathogenic factors are removed, the secretion of ECM by activated fibroblasts and myofibroblasts ceases, resulting in non-fibrosis or scarless repair. However, in cases of repair disorders, fibroblasts and myofibroblasts can proliferate uncontrollably and continuously secrete ECM, which leads to excessive ECM deposition and the formation of fibrosis. Targeting fibroblasts is another essential aspect of reducing fibrosis

functional reduction) and ASCL1, which reprogrammed CFs into iCMs in vivo in both acute and chronic MI models and alleviated cardiac fibrosis. Furthermore, delivering GTH and MEF2C fusion with MYOD transactivation domain was reported to generate twofold more iCMs in vivo than using GMTH. This approach also significantly improved cardiac function and reduced fibrosis [26]. Lin et al. determined that the conversion of fibroblasts to iCMs can be driven by regulating post-translational modifications. The activation of phosphorylation

code in 14–3-3 binding motifs (PC14-3–3) induces iCMs formation in vitro with the presence of only Tbx5, as PC14-3–3 promotes the release and phosphorylation of multiple key cardiac TFs [27].

MicroRNAs (miRNAs, miRs) are small non-coding RNAs that regulate gene expression and mediate reprogramming by degrading specific mRNAs [28]. Lentivirus-mediated delivery of miRNA combo (miR-1, miR-133, miR-208, and miR-499) could reprogram CFs into iCMs with calcium transients and beating capacity in vivo and significantly reduce cardiac fibrosis [29]. Using branched polyethyleneimine-coated nitrogen-enriched carbon dots can replace viral vectors to initiate the miRNA combo-mediated in vivo reprogramming of CFs into iCMs [30]. Wang et al. [31] developed a novel nanoparticle-coating FH peptide-modified neutrophil-mimicking membrane termed FNLM. The FH peptide can specifically bind to tenascin-C, an ECM glycoprotein that is abundant in activated fibroblasts and myofibroblasts [32]. Moreover, leveraging the properties of neutrophil membrane proteins, the FNLM can automatically migrate to the site of injury [31]. Therefore, this targeted delivery system successfully delivers the miRNA combo to CFs to the acute MI area, enhancing in vivo conversion efficiency and anti-fibrotic effects [31].

Small molecules, normally weighing less than 900 Daltons, mediate reprogramming by regulating signal transduction and epigenetic modifications [33]. A small molecule combination 9C (CHIR99021, A83-01, BIX01294, AS8351, SC1, Y27632, OAC2, SU16F, and JNJ10198409) converts human fibroblasts into iCMs in vitro [34]. These chemically induced iCMs demonstrate uniform contraction and electrophysiological properties akin to human cardiomyocytes. Furthermore, Huang et al. [35] exploited a combination CRFVPTM (C: CHIR99021; R: RepSox; F: Forskolin; V: VPA; P: Parnate; T: TTNPB; M: Rolipram), which successfully generated iCMs from CFs in vivo with action potentials and significantly reduced the scar area. Further optimization of these small molecule induction strategies will facilitate their clinical translation. Small molecules can also enhance TF-mediated reprogramming to improve conversion efficiency. Recently, small molecules SB431542 and Baricitinib were identified to improve in vitro reprogramming of fibroblasts to iCMs induced by GMT. Notably, SB431542 and Baricitinib plus MT (without GATA4) can selectively reprogram CFs rather than the fibroblasts from other organs [36].

However, since CFs are crucial supporters of cardiac structure, a concern of CFs reprogramming into iCMs is whether the non-fibrotic fibroblasts undergo conversion in vivo. Recently, Tani et al. [37] solved this problem by establishing a transgenic MI mouse model that expresses GMTH. They demonstrated that iCMs appeared only at the edge of the infarction area rather than at the infarction center or non-infraction area, suggesting that non-fibrotic fibroblasts do not initiate the reprogramming program [37].

The transformation of CFs into myofibroblasts plays a crucial role in the development of cardiac fibrosis. Cardiac myofibroblasts are enriched around the injured area in the early period and release massive ECM [16]. Therefore, reprogramming cardiac myofibroblasts may be more significant than reprogramming CFs in treating cardiac fibrosis. However, Zhao et al. [38] demonstrated that the GMT only induced 0.1% cardiac myofibroblasts to reprogram into iCMs in vitro, compared to 18% conversion in adult CFs. This disparity is likely due to heightened fibrotic gene activity in myofibroblasts [38]. Notably, the in vitro reprogramming efficiency of cardiac myofibroblasts into iCMs increased to 5.8% when the GMT cocktail was supplemented with the TFs SALL4 and MYOCD. This enhanced reprogramming cocktail was subsequently tested in vivo, successfully generating iCMs from myofibroblasts and significantly relieving cardiac fibrosis [39]. Moreover, the in vivo conversion efficiency was further augmented by treatment with the small molecules SB431542 and Ruxolitinib [39].

Overall, many reprogramming strategies have been established to induce in vivo iCM generation, showing considerable promise for cardiac fibrosis therapy and bringing clinical application closer to realization (Table 1). Further comparison of the anti-cardiac fibrosis effects between reprogramming CFs and cardiac myofibroblasts will be of significance. Moreover, it is essential to note that fibroblasts and myofibroblasts are fundamental in maintaining cardiac integrity via ECM secretion during the MI period [40]. Immoderate reduction in these cell populations may lead to serious complications, such as ventricular aneurysm and myocardial rupture [41]. Therefore, despite the absence of any reported adverse reactions, the safety of direct cardiac reprogramming should be thoroughly assessed.

Direct reprogramming in the liver

Hepatocytes hold remarkable regenerative capacity following acute injuries. However, constant hepatocyte impairment caused by chronic inflammatory diseases, such as viral hepatitis, parasitic infections, and alcoholic liver disease, can eventually give rise to liver fibrosis [42]. Myofibroblasts are the key mediators of liver fibrosis, mainly derived from portal fibroblasts or hepatic stellate cells [42]. In liver fibrosis and cirrhosis, the myofibroblasts dramatically increase in number along with the substantial reduction of hepatocytes. Accumulated myofibroblasts not only propel the progression of liver

Table 1 Representative direct reprogramming studies related to anti-fibrosis

	Reprogramming cocktails	Delivery methods	Anti-fibrosis effect	Ref
Reprogramming CFs into iCMs in vivo	GATA4, MEF2c, and TBX5 (GMT)	Retrovirus	Reduce merely 25% scar area after 8 weeks of treatment	[20]
	GATA4, MEF2c, and TBX5 + vascular endothelial growth factor	Lentivirus	Reduce merely 12% of fibrosis extent after 4 weeks of treatment	[165]
	GATA4, MEF2c, TBX5, and HAND2	Retrovirus	Reduce merely 23% fibrotic area after 4 weeks of treatment	[24]
	GATA4, MEF2c, and TBX5 (polycistronic construction)	Retrovirus	Not applicable	[21]
	Polycistronic construct MEF2c-GATA4-TBX5	Retrovirus	Further reduce fibrosis size than single G/M/T virus	[23]
	GATA4, MEF2c, and TBX5 + SB431542 and XAV939	Retrovirus	Significantly reduce scar size than GMT treatment alone	[64]
	GATA4, MEF2c, and TBX5	Sendai virus	reduce more fibrosis and collagen area than retroviral GMT with polycistronic construction	[166]
	GATA4, MEF2c, and TBX5	Polyethylimine conjugated Cationic gold nanoparticles	Reduce merely 30% scar size after 2 weeks of treatment	[167]
	MYOCD, ASCL1 and miR-133	AAV	Significantly inhibit fibrotic scar formation	[25]
	GTH and MEF2C fusion with MYOD transactivation domain	AAV	Significantly reduced fibrosis	[26]
	miR-1, 133, 208, and 499	Lentivirus	Reduce merely 15% fibrosis after 4 weeks of treatment	[29, 168]
	miR-1, 133, 208, and 499	Polyethyleneimine coated nitrogen-enriched carbon dots	Reduce merely 32% fibrosis area after 4 weeks of treatment	[30]
	miR-1, 133, 208, and 499	mesoporous silicon nanoparticles coating FH peptide-modified neutrophil-mimicking membranes	Reduce merely 40% fibrosis area after 4 weeks of treatment	[31]
	CHIR99021, RepSox, Forskolin, VPA, Parnate, TTNPB, Rolipram (CRFVPTM)	Oral administration + intraperitoneal injection	Reduce merely 15% fibrotic area after 6 weeks of treatment	[35]
Reprogramming cardiac myofibroblasts into iCMs in vivo	GATA4, MEF2c, TBX5, MYOCD, and SALL4 + SB431542 and Ruxolitinib	Lentivirus	Significantly alleviate fibrosis	[39]
Reprogramming myofibroblasts into iHeps in vivo	FOXA1, FOXA2, FOXA3, FOXA4, HNF1a, and HNF4a	AAV	Reduce liver fibrosis of 8 weeks CCL_4-induction	[43]
	FOXA3, GATA4, HNF1a, and HNF4a	AAV	Ameliorate liver fibrosis of 8 weeks CCL_4-induction	[44]
	CID755673, GSK429286A, ETC-1002, phenylpropanoid glycoside from Rhodiola Rosea L., and forskolin	Intraperitoneal injection	Reduce 2.5-fold fibrotic area	[46]
	Endogenously activate Gata4 and Foxa3 via CRISPRa	AAV	Reduce liver fibrosis of 4 weeks CCL_4-induction	[131]
Reprogramming fibroblasts into keratocytes in vivo	DNP63a, GRHL2, TFAP2a, and C-MYC	AAV	Ameliorate skin scar by accelerating wound healing	[51]
Reprogramming fibroblasts into DPCs in vivo	Tideglusib and Tamibarotene	Hydrogel microspheres	DPCs inhibit myofibroblasts and promote hair follicle regeneration to reduce fibrosis	[52]
Reprogramming myofibroblasts into adipocytes in vivo	BMP4 from hair follicles	Not applicable	Promote scarless wound healing	[56]

fibrosis but also hinder the regeneration of hepatocytes. Therefore, in vivo reprogramming of myofibroblasts into induced hepatocytes (iHeps) is attractive for liver fibrosis therapy.

Rezvani et al. [43] utilized adeno-associated virus (AAV) to deliver six TFs FOXA1, FOXA2, FOXA3, FOXA4, HNF1a, and HNF4a to convert approximately 1% of myofibroblasts into iHeps in vivo. The isolated iHeps possessed functional properties of normal hepatocytes, including storing glycogen, drug induction, and self-renewal. Similarly, Song et al. [44] reported the generation of iHeps in vivo through the delivery of FOXA3, GATA4, HNF1a, and HNF4a to myofibroblasts. These direct myofibroblast reprogramming approaches are effective in treating liver fibrosis following four or eight weeks of carbon tetrachloride (CCl_4) induction and improving liver function, yet they fall short in ameliorating liver cirrhosis (after 12 weeks of CCl_4 induction) [44]. Therefore, further optimizations are required to augment the efficiency of reprogramming myofibroblasts into iHeps. A small molecule combination (SB431542, CHIR99021, BIX01294, LDN193189, and DAPT) demonstrates a high in vitro conversion efficiency (80%) for fibroblast reprogramming into iHeps [45]. However, the efficiency of myofibroblast conversion and the in vivo anti-fibrosis effect of this approach require further evaluation. Lin et al. [46] newly identified a small molecule combination for hepatic reprogramming, including CID755673, GSK429286A, ETC-1002, phenylpropanoid glycoside from *Rhodiola Rosea L.*, and forskolin. In the treated mice, they observed the in vivo conversion of myofibroblasts to iHeps, accompanied by a reduction in the fibrotic area and improvements in liver injury markers.

Direct reprogramming in skin

Various types of skin damage, such as burns, trauma, and acne, can activate fibrosis repair, leading to scar formation. Many studies have focused on fibroblasts and myofibroblasts, attempting to suppress scars by inhibiting their activation or altering their fibrotic identity [47]. Moreover, some cells within the skin, including keratinocytes, dermal papilla cells (DPCs), and adipocytes have been shown to be beneficial in scar treatment [48–50]. Therefore, direct reprogramming of fibroblasts or myofibroblasts into these cells is a prospective approach for scarless healing.

Keratinocytes form the epidermal barrier [49]. Extensive loss or dysfunction of keratinocytes can result in the formation of scars due to prolonged wound healing [49]. Therefore, promoting the regeneration of keratinocytes may reduce scar by facilitating skin repair. Keratinocytes can be converted from skin fibroblasts in vivo by delivering the TFs DNP63a, GRHL2, TFAP2a, and c-MYC [51]. Epidermis reconstructed by the induced keratinocytes demonstrated a keratin pattern that was close to the natural skin epidermis, capable of resisting the toluidine blue dye penetration.

DPCs are mesenchymal cells in hair follicles [48]. They are able to inhibit myofibroblast formation and induce hair follicle regeneration [52]. Meanwhile, the presence of hair follicles also suppresses scar formation. Therefore, DPCs are a prospective target for scar treatment. The chemical drugs Tideglusib and Tamibarotene can reprogram fibroblasts into DPCs in vivo. The generated DPCs promoted keratinocyte migration and hair growth as well as alleviated skin scar formation [52]. However, further evaluation of anti-fibrotic efficiency is required to determine the therapeutic potential of this strategy.

The generation of adipocytes plays a crucial role in reducing scars. An increase in fat level diminishes the collagen content of ECM and contributes to arranging the collagen fiber networks orderly, thereby facilitating the restoration of the normal texture and elasticity of skin [50, 53]. Overexpressing the master adipogenic TF PPARγ2 or induction with the small molecule STK287794 is sufficient to generate mature adipocytes from fibroblasts in vitro [54, 55]. Interestingly, Plikus et al. [56] discovered that myofibroblasts situated in the proximity of hair follicles can be in vivo reprogrammed into adipocytes by BMP2/4 signaling derived from hair follicle cells. Moreover, sweat glands and sebaceous glands are conducive to scar reduction by regulating inflammation and ECM metabolism. Therefore, it will be valuable to further explore reprogramming approaches for directly regenerating skin appendages [57]. However, due to the complex structure of the skin, additional investigations are required to validate the anti-fibrotic effects of various skin cells derived from different reprogramming strategies.

Direct reprogramming in skeletal muscle

Injury and muscular dystrophies are the leading causes of skeletal muscle fibrosis [58]. Fibroblasts in the interstitial tissue of muscle and bone marrow-derived fibroblast precursors are the primary pro-fibrotic cells that replace injured muscle tissue and initiate fibrosis. Consequently, reprogramming fibroblasts into muscle cells represents a feasible strategy for both mitigating skeletal muscle fibrosis and promoting muscle regeneration.

Fibroblasts can be converted into induced muscle cells with non-proliferative capability in vitro by overexpressing the muscle lineage TF MYOD [59]. Proliferative muscle progenitor cells are the source of acquiring muscle cells. By forcing the expression of the TF cocktails PAX7 and MYF5, induced muscle progenitor cells (iMPCs) can

be induced from fibroblasts in vitro [60]. Treatment with a combination of small molecules (Forskolin, RepSox, and CHIR99210) along with the transduction of MYOD-mRNA can also generate iMPCs in vitro [61]. These iMPCs were capable of differentiating into multinucleated, contractile myotubes. Transplantation of these cells into the limb muscles of Duchenne muscular dystrophy mice led to a significant increase in myofiber numbers [60, 61]. Advancing research into the in vivo reprogramming of fibroblasts into iMPCs could unlock innovative therapeutic approaches for effectively addressing skeletal muscle fibrosis.

In summary, both fibroblasts and myofibroblasts are significant targets due to their critical roles in fibrosis. Fibroblasts are abundant in various tissues and easy to obtain, making them widely used in reprogramming research, particularly in studies involving the heart and skin. Myofibroblasts typically emerge in response to injury and accumulate at the injury site, suggesting that their reprogramming may provide more direct therapeutic benefits. Moreover, myofibroblasts play a pivotal role in fibrosis within specific organs, such as the liver. Therefore, selecting appropriate targets based on the pathophysiological mechanisms of fibrosis in different organs is critical.

Response of fibrotic mechanisms to reprogramming

TGF-β signaling pathway

TGF-β signaling is the dominant signaling pathway driving fibrosis initiation [15]. In the process of fibrosis, different kinds of inflammatory cells, particularly macrophages, release TGF-β [15]. Once released into the ECM, TGF-β binds to its receptor complex, triggering the activation of SMAD2/3, the central downstream mediators in TGF-β-driven fibrosis. [62]. SMAD2/3 further cooperated with SMAD4, starting a series of fibrotic programs, including fibroblast activation, myofibroblast transformation, ECM synthesis, and epithelial-mesenchymal transition (EMT) [62].

Inhibiting TGF-β signaling pathways facilitates direct fibroblast reprogramming. Fibrotic genes are highly enriched in the early stages of reprogramming events to protect fibroblast identity in cardiac reprogramming [63]. Early or preliminary inhibition of TGF-β effectively promoted fibroblast reprogramming into iCMs by downregulating the expression of fibrotic genes [39, 63–65]. Treatment with the TGF-β receptor inhibitors A83-01 or SB431542 also enhanced the quantity and contractile force of iCMs, which may be associated with the thorough erasure of fibrotic markers [64, 66]. In addition, Riching et al. [67] demonstrated that SMAD2/3 inhibited the histone demethylase Jumonji domain-containing protein-3 (JMJD3), leading to repressive modification H3K27me3 increasing in cardiac genes loci. SMAD2/3 also impedes the interaction between GATA4 and chromatin remodeling modifiers JMJD3 and Brahma-related gene 1, limiting chromatin accessibility and hindering cardiac reprogramming [67].

The mesenchymal-epithelial transition (MET) is necessary for fibroblast reprogramming, whereas TGF-β-induced EMT counteracts this process [68]. Inhibiting TGF-β can substitute KLF4 and c-MYC to induce MET, expediting fibroblast reprogramming into iHeps [69]. SNAI1, a key transcriptional regulator of EMT under TGF-β control, can be suppressed by reprogramming factors like miR-133 or GATA4, which facilitates the transformation of fibroblasts into iCMs [70, 71].

The novel TF MEOX1, regulated by the TGF-β pathway, has emerged as a crucial mediator of myofibroblast transformation [72]. MEOX1 overexpression markedly impairs the reprogramming of CFs, whereas MEOX1 is downregulated as CFs are gradually converted into iCMs [37].

Overall, the TGF-β pathway is a positive target for promoting direct fibroblast reprogramming (Fig. 2). Further studying the mechanism of the TGF signaling pathway in fibrosis may uncover novel targets to promote the direct reprogramming of fibroblasts.

ECM-related mechanical signaling pathways

The stiffness of fibrotic ECM is significantly elevated compared to normal ECM. Mechanical cues from the stiff ECM can be sensed by integrin receptors and activate two key mechanotransduction pathways: the Hippo pathway and the Rho/Rho-associated coiled-coil kinases (ROCKs) pathway. The activation of these pathways impedes cardiac and neuronal reprogramming [73–75] (Fig. 2).

The Hippo pathway has been demonstrated to be closely related to fibrosis. Its mechanosensitive transcription factors, yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding domains (TAZ), function as central downstream effectors [76]. Activated YAP/TAZ cooperates with the TEA domain (TEAD) and promotes the transcription of fibrotic genes, resulting in fibroblast proliferation, migration, maturation, and ECM deposition [76].

The Rho/ROCKs pathway further amplifies pro-fibrotic signaling, which is considered a key mechanism in the progression of pulmonary fibrosis [77]. ROCKs are the effectors of the Rho/ROCKs pathway, which is activated by RhoA [78]. The inhibition of ROCKs reduces the transcriptional activity of YAP/TAZ, thereby mitigating fibrosis [77]. Moreover, ROCKs regulate TGF-β by activating SMAD2/3 [79].

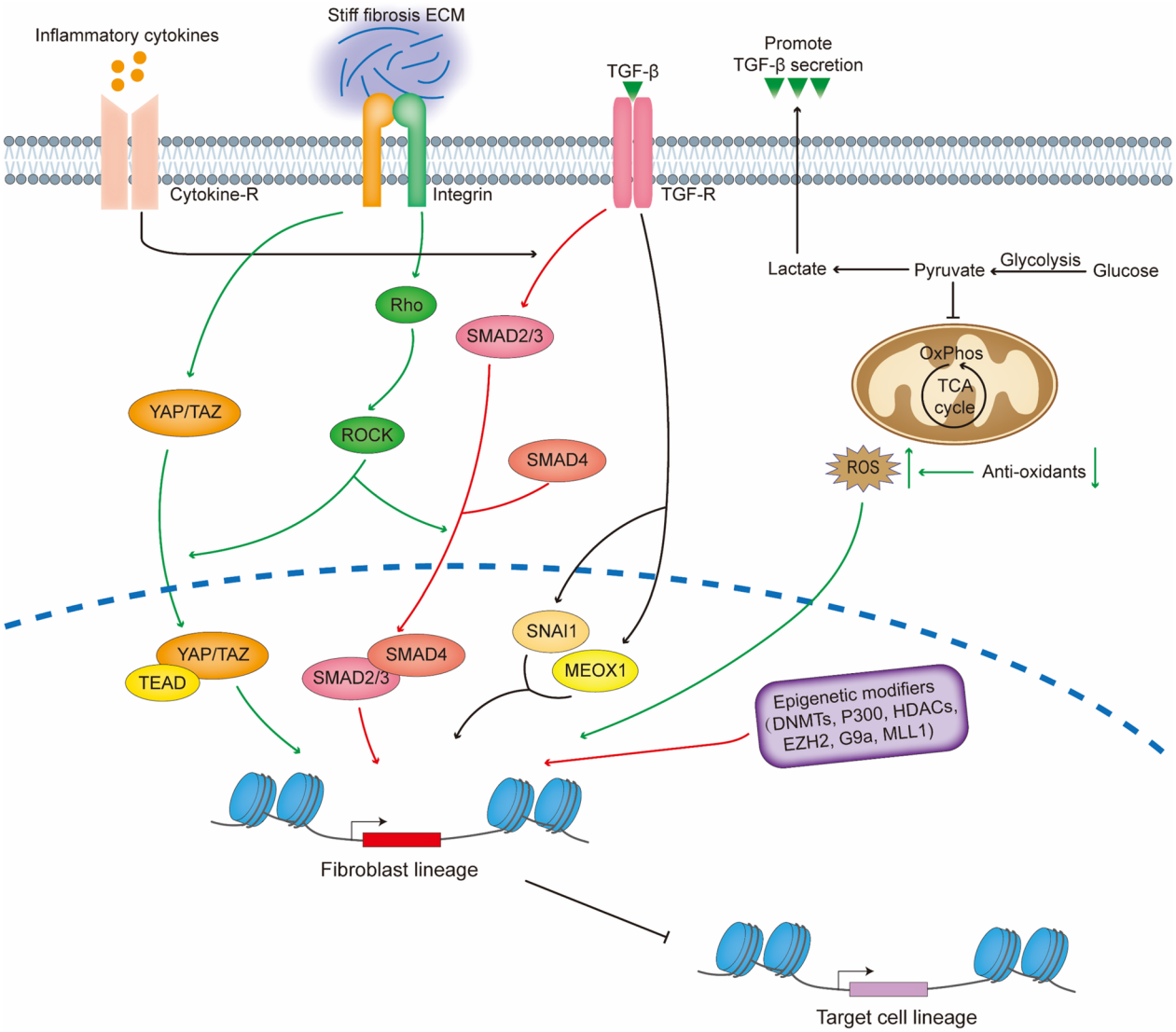

Fig. 2 Fibrosis programs potentially inhibit fibroblast reprogramming. Direct fibroblast reprogramming requires the inhibition of the fibrotic identity. However, fibrotic processes protect fibroblasts, which may impede the reprogramming of fibroblasts. The crucial pro-fibrotic pathway TGF-β/SMAD and the TGF-β pathway-regulated TFs SNAI1 and MEOX1 are potential barriers to fibroblast reprogramming. Stiff fibrosis ECM activates the Hippo and Rho/ROCKs pathways. The downstream TF of Hippo, YAP/TAZ, is activated and cooperates with TEAD to initiate the expression of fibrotic genes. Additionally, Rho/ROCK can enhance the Hippo and TGF-β pathways to facilitate fibrosis. The inhibition of YAP/TAZ and Rho/ROCKs is capable of promoting fibroblast reprogramming. Anti-inflammation also contributes to fibroblast reprogramming. Inflammatory factors may inhibit direct reprogramming by enhancing pro-fibrotic pathways like the TGF-β pathway. Several epigenetic modifiers, including DNMTs, P300, HDACs, EZH2, G9a, and MLL1, are upregulated in the fibrosis process and promote the activation of fibrotic genes, which may also hinder the reprogramming of myofibroblasts. Moreover, fibroblasts exhibit significant metabolic alterations during the fibrosis process. The enhancement of glycolysis and downregulation of OxPhos leads to an increase in lactate, which promotes extracellular TGF-β secretion. The reduction in antioxidants results in ROS accumulation, while too much ROS strongly inhibits fibroblast reprogramming. Regulating these fibrotic factors is a critical approach for promoting fibroblast reprogramming. (→ Only reported in cardiac reprogramming; → Reported in cardiac and hepatic reprogramming; → Reported in cardiac and neuronal reprogramming)

Nevertheless, the soft matrix has been shown to enhance the efficiency and quality of reprogramming fibroblasts into iCMs or induced neurons by directly inhibiting the Hippo and Rho/ROCKs pathways [74, 75]. Moreover, directly modulating these mechanotransduction pathways can also facilitate fibroblast reprogramming. The depletion of YAP/TAZ accelerates fibroblast reprogramming into iCMs [75]. Zhao et al. [63] reported that the ROCKs inhibitor Y-27632 downregulated pro-fibrotic events, inhibited the expression of the fibrotic markers Fn-EDA and α-SMA, and promoted the conversion of fibroblasts into iCMs.

The further advancement of methods for softening the fibrotic ECM may provide new approaches to facilitating fibroblast reprogramming. The application of injectable hydrogels may directly provide soft ECM [80]. Moreover, fibrolytic treatment is a prospective way to mitigate stiff fibrotic tissues [75]. However, this approach requires the development of clinically applicable effective drugs.

Inflammation signaling

Inflammatory cytokines, including interleukin (IL), tumor necrosis factor, and chemokines, play a certain role in promoting fibrosis [81]. These cytokines contribute to fibrotic processes by amplifying pro-fibrotic signaling pathways such as TGF-β, extracellular regulated protein kinases, and p38/mitogen-activated protein kinase [82–85]. Thus, anti-inflammation may enhance the conversion of the fibrotic lineage by inhibiting the pro-fibrotic pathways (Fig. 2).

Muraoka et al. [86] discovered that the anti-inflammatory drug diclofenac inhibited the COX2-PGE2 pathway to reduce IL-1β/IL-1 receptor signal transduction, which downregulated fibrotic genes and promoted cardiac reprogramming. Interestingly, diclofenac selectively promotes postnatal and adult fibroblast reprogramming into iCMs rather than embryonic fibroblast reprogramming, suggesting that the expression of fibrotic genes increases with cellular development [86]. Similarly, activating the C–C motif chemokine ligand-3, −6, and −17 hinders fibroblast reprogramming into iCMs [87]. Moreover, the TF Znf281 and the chromatin remodeling complex NuRD cooperate to inhibit inflammatory signaling, facilitating mouse fibroblasts to convert into iCMs [88].

However, Zhou et al. [89] reported that the depletion of inflammation-associated genes severely impaired the capacity of human fibroblasts to be reprogrammed into iCMs. These contradictory results may be attributed to species differences. Additional studies are required to elucidate the mechanism of inflammation signaling pathways in human fibroblast reprogramming, which may contribute to the translation of reprogramming treatments.

Epigenetic modification

Cell fate conversion entails a series of dynamic epigenetic modifications, including DNA methylation, histone modification, and chromatin remodeling [90, 91]. During the establishment of a fibrotic identity, some upregulated epigenetic modifiers significantly promote the expression of fibrotic genes [90, 91]. Consequently, regulating these modifiers may augment the reprogramming of fibroblasts (Fig. 2).

DNA methyltransferases (DNMTs) mediate DNA methylation to silence gene expression [92]. In the activated CFs, elevated DNMT activity facilitates the activation of fibrotic genes by repressing anti-fibrotic genes [93, 94]. Liu et al. [95] reported that excessive expression of DNMTs significantly impaired target gene expression during the later stages of cell fate conversion, preventing adult acinar cells from being reprogrammed into pancreatic β cells. Conversely, Muniyandi et al. demonstrated that hypomethylation induced by the DNMT inhibitor 5-azacytidine enhanced the conversion of fibroblasts into iCMs [96]. However, a delicate balance is required, as complete DNMT depletion disrupts fibrotic gene locus methylation and hampers cardiac reprogramming [95]. Therefore, the regulation of DNMTs should be precisely controlled.

The transcription coactivator P300 and CREB binding protein (P300/CBP) complex is a key histone acetyltransferase that plays a role in promoting fibrotic gene transcription and EMT in liver fibrosis [97]. Lim et al. [98] found that the P300/CBP inhibitor I-CBP112 effectively suppressed fibrotic gene expression, facilitating the conversion of fibroblasts into iCMs.

Another histone acetylation modifier, histone deacetylases (HDACs), is highly linked to the progression of fibrosis. Several types of HDACs drive fibrotic gene expression and myofibroblast transformation, which suggests that HDACs may be presented as a barrier to direct reprogramming [99–101]. Indeed, studies have shown that an HDAC inhibitor valproic acid promoted the conversion of fibroblasts into iCMs and iHeps [102, 103].

Histone methyltransferases (HMTs) such as enhancer of zeste homolog 2 (EZH2) and G9a contribute to pulmonary fibrosis by catalyzing the methylation of H3K27 and H3K9, respectively [104]. Elevated levels of EZH2 and G9a in activated fibroblasts promote histone hypermethylation, silencing anti-fibrotic genes [104]. However, transient inhibition of these enzymes promotes fibroblast reprogramming into iCMs [105].

Mixed lineage leukemia 1 (MLL1), another HMT that targets H3K4, is upregulated in fibroblasts differentiating into myofibroblasts, and promotes fibrotic gene expression by increasing H3K4 methylation in renal fibrosis [106]. Intriguingly, upregulated MLL1

suppresses direct cardiac reprogramming by enhancing the expression of genes related to the adipogenic lineage [107].

Other epigenetic modifiers, such as KDM3A, LSD1, and SET7/9, are also implicated in fibrosis [108–110], but their precise roles in direct reprogramming remain poorly understood. Further investigations are essential to uncover their functions, which will facilitate the identification of more targets to enhance the conversion of fibrotic identities.

Metabolic regulation

Profound metabolic alterations, including elevation of glycolysis, suppression of fatty acid oxidation, and glutaminolysis upregulation, are the hallmarks of fibrosis progression [6].

The shift from oxidative phosphorylation (OxPhos) to glycolysis represents the most salient metabolic change. Glycolysis rapidly supplies the energy to support fibroblast proliferation and collagen synthesis [111]. Additionally, glycolytic byproducts, such as lactic acid, lower the extracellular pH, promoting latent TGF-β activation [112]. In contrast, direct reprogramming of iCMs involves a metabolic transition from glycolysis back to OxPhos [89] (Fig. 2). Deletion of HIF-1α, a key glycolysis-related gene, significantly improved the efficiency of reprogramming fibroblasts into iCMs [113]. Moreover, fatty acid oxidation was enhanced in fibroblast-derived iCMs, aligning with the metabolic signature of mature cardiomyocytes [114]. However, the specific alteration and functional significance of glutamine metabolism in direct reprogramming remains unexplored.

Activated fibroblasts in the damaged heart maintain a high level of reactive oxygen species (ROS), which is a consequence of reduced antioxidant enzyme activity [115]. During the transition from glycolysis to OxPhos in direct reprogramming, ROS levels inevitably rise. The application of antioxidants such as ascorbic acid (vitamin C), tocopherol (vitamin E), and selenium greatly improves cardiac and neuronal reprogramming efficiency [116–118] (Fig. 2). These findings suggest that mitigating oxidative stress through ROS scavenging is a critical factor in facilitating successful fibroblast reprogramming.

Novel methodologies for mediating direct reprogramming in fibrosis

Current reprogramming factors such as TFs and miRNAs are limited by problems such as low efficiency, uncontrollability, and potential genetic integration [119]. Herein, we propose novel methods that may be beneficial for overcoming these obstacles.

Small molecules

As a non-viral strategy, small molecules circumvent concerns associated with genetic manipulation inherent in TF or miRNA-based approaches [120]. Moreover, small molecules mainly consist of chemical compounds that are cost-effective and readily accessible [33]. These advantages make them particularly appealing for clinical applications as reprogramming mediators. Currently, a considerable number of small molecule combinations have been developed to reprogram fibroblasts into various cell types [33].

Small molecules face challenges related to low efficacy, insufficient target specificity, and a lack of controllability. Innovative delivery systems are a key strategy to overcome these issues.[49, 121]. Scaffold-based drug delivery systems, such as hydrogels, nanoparticles, and liposomes, offer a dual strategy for enhancing small molecule-mediated reprogramming. These scaffolds provide a structure that mimics the normal ECM, creating a microenvironment conducive to reprogramming. Additionally, they act as reservoirs for small molecules, ensuring localized delivery and controlled release [122]. Ji et al. [52] recently utilized hydrogel microspheres as carriers to produce a wound adhesive and controlled release system for the administration of small molecules, significantly improving the reprogramming efficiency of skin fibroblasts.

Notably, the rational redesign of small protein molecules based on the structure of the receptor subtype further improves their specificity, affinity, and stability [123, 124]. With the flourishing development of artificial intelligence, small molecule modification technology may have a huge promotion on reprogramming.

CRISPRa

Using conventional exogenous TFs or miRNAs to open chromatin and promote gene expression is regarded as an inefficient and ponderous process [125]. An emerging alternative is CRISPR-based gene activation (CRISPRa), which has been developed to facilitate more efficient reprogramming. CRISPRa contains two main components: guide RNA and dead Cas9 (dCas9). dCas9 is fused with multiple transcriptional activators, such as VP64, p300, and p65, to form synthetic transcription activators. CRISPRa enables precise targeting of endogenous gene loci and robust activation of gene expression [126, 127]. In addition, the invention of chemical Cas9 inhibitors and attenuated guide RNA enhances the controllability of CRISPRa [128, 129]. Wang et al. [130] employed CRISPRa to activate the cardiac genes GATA4, NKX2.5, and TBX5, thereby reprogramming human foreskin fibroblasts into induced cardiac progenitor cells in vitro. Furthermore,

endogenous activation of GATA4 and FOXA3 via CRISPRa can effectively reprogram myofibroblasts into functional iHeps in vivo, resulting in a notable attenuation of liver fibrosis [131].

Despite the promise of CRISPRa, its translation is constrained by challenges in delivery. Currently, AAV vectors are the most commonly used for delivering CRISPRa [132]. However, the large size of CRISPRa requires multiple AAV vectors to carry its modules, potentially compromising delivery efficiency [133]. Novel bioengineering delivery systems offer a promising solution to this limitation while simultaneously addressing concerns about the immunogenicity of viral vectors [134, 135]. Nevertheless, additional research is imperative to clarify the safety of CRISPRa, given its involvement in gene manipulation and the potential for off-target effects.

Modified mRNA

Compared to TF or miRNA-based reprogramming, mRNA reprogramming eliminates the risk of gene integration, while bypassing the transcriptional process enhances reprogramming efficiency [136]. Transducing specific mRNA cocktails to fibroblasts has been shown to successfully generate iCMs and iMPCs [61, 137].

However, natural mRNA is inherently unstable and immunogenic, which limits its applicability in vivo [136]. Modified mRNA (modRNA) has emerged as a promising solution to bypass these obstacles. Modifications such as removing the miRNA binding sites in the untranslated region, introducing pseudoUridine, and optimizing 5'-cap structure significantly improve the stability and safety of mRNA [136]. Furthermore, integrating modRNA with hydrogel-based delivery systems facilitates controlled and localized release, further improving its therapeutic potential [138]. To date, modRNA-mediated reprogramming has been successfully used to generate iPSCs and iCMs [139, 140]. However, modRNA still needs to overcome its limitations, including high costs, manufacturing challenges, and short lifespans.

The three aforementioned approaches also exhibit substantial potential in advancing fibrosis treatment research [141–143]. Given the safety concerns associated with genetic manipulation, small molecules represent a more appropriate option for clinical applications. Moreover, several small-molecule strategies have been developed to alleviate fibrosis in multiple organs, including the lungs, liver, kidneys, and skin [144]. Although CRISPRa and modRNA show more powerful abilities in genetic regulation, small molecules continue to possess greater promise.

The challenges of direct reprogramming of fibroblasts in vivo

The population of fibroblasts is highly heterogeneous

Fibroblasts within fibrotic tissues are heterogeneous, comprising multiple subpopulations with different transcriptomic profiles [145–147]. Certain subpopulations can be sources of noise during reprogramming, leading to a reduction of overall efficiency (Fig. 3a). Consequently, determining the subpopulations within fibroblasts is crucial for advancing their direct reprogramming.

Single-cell RNA sequencing (scRNA-seq) can reveal the transcriptomic profiles of different fibroblast subpopulations, providing valuable insights into inefficient reprogramming caused by heterogeneity. For instance, a study using scRNA-seq identified four distinct subpopulations of fibroblasts in skin keloids, papillary fibroblasts, reticular fibroblasts, mesenchymal fibroblasts, and pro-inflammatory fibroblasts [147]. Mesenchymal fibroblasts, the largest subpopulation, exhibit enriched expression of genes associated with skeletal development, which may limit their efficiency in reprogramming into adipocytes within keloid tissue [147]. Meanwhile, reticular fibroblasts express elevated levels of adipose lineage markers, making them prone to converting into adipocytes [147, 148]. Other single-cell multi-omics technologies, including genomics, proteomics, and epigenomics, provide complementary information from different perspectives to precisely identify suitable cell subpopulations [149].

Single-cell lineage tracing techniques like CellTagging allow for the monitoring of reprogramming processes across different cell subpopulations. By introducing specific cell markers, such as genetic barcodes and fluorescent markers, and performing longitudinal tracking for these markers, the developmental trajectories of individual cells can be mapped. Subsequent single-cell sequencing analysis of these trajectories can further sort out subpopulations with high reprogramming efficiency and uncover the pivotal regulatory factors that drive the reprogramming process [150, 151].

Accumulation of senescent fibroblasts impacts the quality of direct reprogramming

A large number of senescent fibroblasts accumulate in chronic fibrotic areas [152–154]. One of the critical features of senescent cells is secreting pro-fibrotic factors, such as chemokines, IL, and TGF-β, which potentially perpetuate fibrosis and inhibit reprogramming [155].

Moreover, directly reprogrammed cells still preserve the aging transcriptional signature of their starting cells [156] (Fig. 3b). It has been demonstrated that neurons derived from cellular reprogramming almost completely inherit the mitochondrial aging defect of

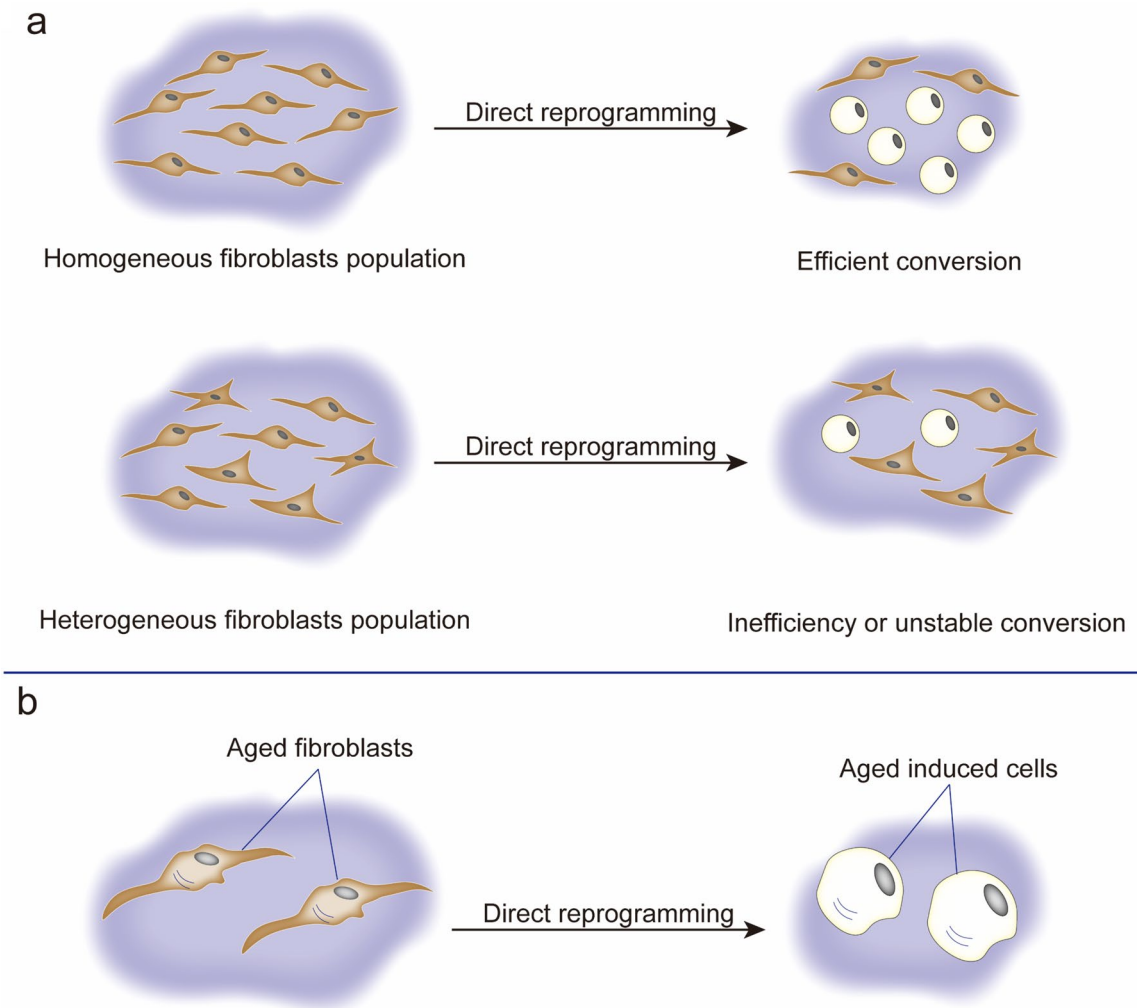

Fig. 3 Heterogeneity and senescence challenge the in vivo application of fibroblast reprogramming. **a**. A homogeneous population has stable reprogramming efficiency. However, the fibroblast population in fibrotic tissue is heterogeneous. Each subpopulation of fibroblasts has a different reprogramming sensitivity due to the variation in transcriptomic profiles, which may lead to inefficient reprogramming or unstable conversion. **b**. Cells induced by direct reprogramming still retain the aging features and deficiencies of the starting cells. However, senescent fibroblasts accumulate in fibrotic areas, which potentially impacts the quality of reprogramming and impedes the application of reprogramming in antifibrosis treatment

starting fibroblasts, exhibiting parallel metabolic disorder [157]. These findings suggest that anti-senescence treatments may have a positive effect on the quality of direct reprogramming. Several measures for counteracting cellular senescence have been developed, such as telomerase activation, mitochondrial protection, autophagy enhancement, and DNA repair promotion [158]. Notably, a breakthrough therapy known as Senolytics has shown great promise in reducing the population of senescent cells [159]. Senolytics therapy involves the employment of specific drugs or antibodies that selectively target and disrupt pathways associated with senescence, thereby inducing apoptosis of senescent cells [160]. The combination of Senolytics agents, dasatinib and quercetin, has been demonstrated to alleviate pulmonary fibrosis [161]. In addition to Senolytics, partial reprogramming has also gained tremendous attention. By temporarily expressing Yamanaka factors (OSKM), this approach eliminates senescence-associated epigenetic modifications, rejuvenating aged cells without altering their identity [162, 163]. A recent finding indicated that a combination of reprogramming small molecules can induce partial reprogramming, which paves the way for its translational application [164]. Thus, combining direct reprogramming with anti-senescence approaches holds the

potential to overcome cellular senescence barriers and achieve high-quality regeneration.

Conclusions

The increasing requirement for tissue repair and regeneration has spurred the rapid advancement of direct reprogramming. From in vitro to in vivo settings, reprogramming forms a designed pathway that includes starting cells, desired cells, reprogramming-mediated factors, and delivery systems. Currently, the heart, skin, and liver are the organs where direct reprogramming-mediated regeneration has been extensively studied in vivo. It is believed that these previous studies will act as valuable references to provide a feasible regeneration approach for other organs.

Fibroblasts are important contributors to fibrosis development and are regarded as promising targets for fibrosis inhibition. Leveraging fibroblast reprogramming for fibrosis treatment shows potential clinical implications: (1) Transplanting the reprogrammed cell can restore the function of fibrotic organs; (2) In situ fibroblast reprogramming reduces pro-fibrotic cells and simultaneously regenerates functional cells at the fibrotic lesion sites; (3) Various reprogramming induction strategies can provide targets for the development of tissue-specific anti-fibrotic drugs.

Despite the exciting preclinical advancements, several challenges remain, including fibroblast heterogeneity and senescence. Emerging single-cell technology and anti-senescence therapies provide new solutions to these issues. Additionally, safety concerns—such as gene integration, mutations, and inflammatory responses—along with the lack of standardized quality control metrics pose regulatory hurdles. Innovative bioengineering delivery systems and non-integrative small molecule induction are expected to improve reprogramming safety and efficiency. Further investigation into tissue-specific fibrosis mechanisms will not only advance fibrosis treatment but also identify additional targets to improve fibroblast reprogramming. Establishing unified technical standards in the future will be critical to accelerating the clinical application of direct reprogramming.

Abbreviations

TFs	Transcription factors
OSKM	OCT4, SOX2, KLF4, and c-MYC
iPSCs	Induced Pluripotent stem cells
ECM	Extracellular matrix
α-SMA	α-Smooth muscle actin
TGF-β	Transforming growth factor-β
CFs	Cardiac fibroblasts
iCMs	Induced cardiomyocytes
GMT	GATA4, MEF2c, and TBX5
MI	Myocardial infarction
GMTH	GATA4, MEF2c, TBX5, and HAND2
PC14-3–3	Phosphorylation code in 14–3-3 binding motifs
miRNAs	MicroRNAs
mRNA	Messenger RNA
AAV	Adeno-associated virus
iHeps	Induced hepatocytes
CCl_4	Carbon tetrachloride
DPCs	Dermal papilla cells
iMPCs	Induced Muscle progenitor cells
EMT	Epithelial–mesenchymal transition
MET	Mesenchymal-epithelial transition
JMJD3	Jumonji domain-containing protein-3
Rho/ROCKs	Rho/Rho-associated coiled-coil kinases
YAP/TAZ	Yes-associated protein and transcriptional coactivator with PDZ-binding domains
TEAD	TEA domain
IL	Interleukin
DNMTs	DNA methyltransferases
P300/CBP	P300 and CREB binding protein
HDACs	Histone deacetylases
EZH2	Enhancer of zeste homolog 2
HMTs	Histone methyltransferases
MLL1	Mixed lineage leukemia 1
OxPhos	Oxidative phosphorylation
ROS	Reactive oxygen species
CRISPRa	CRISPR-based gene activation
dCas9	Dead Cas9
modRNA	Modified mRNA
scRNA-seq	Single-cell RNA sequencing

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The authors report there are no competing interests to declare.

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