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In situ Reprogramming as a Pro-Angiogenic Inducer to Rescue Ischemic Tissues

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Keywords
In situ reprogramming · Angiogenesis · Ischemic tissues

Abstract

Background: Enhanced regenerative therapeutic strategies are required to treat intractable ischemic heart disease. ***Summary:*** Since the discovery of putative endothelial progenitor cells (EPCs) in 1997, many studies have focused on their extraction, ex vivo processing, and autotransplantation under ischemic conditions. Nonetheless, numerous randomized clinical trials involving thousands of patients have yielded only marginal treatment effects, highlighting the need for advances regarding insufficient dosage and complex ex vivo processing. The prevailing paradigm of cellular differentiation highlights the potential of direct cellular reprogramming, which paves the way for in situ reprogramming. In situ reprogramming holds the promise of significantly enhancing current therapeutic strategies, yet its success hinges on the precise targeting of candidate cells for reprogramming. In this context, the spleen emerges as a pivotal "in situ reprogramming hub," owing to its dual function as both a principal site for nanoparticle distribution and a significant reservoir of putative EPCs. The in situ reprogramming of splenic EPCs offers a potential solution to overcome critical challenges, including the aforementioned insufficient dosage and complex ex vivo processing. ***Key Messages:*** This review explores the latest advancements in EPC therapy and in situ reprogramming, spotlighting a pioneering study that integrates those two strategies with a specific focus on the spleen. Such an innovative approach will potentially herald a new era of regenerative therapy for ischemic heart disease.

Introduction

Humanity's average lifespan has been progressively extending; however, it is noteworthy that the pace of this extension is decelerating [1]. Some argue that this deceleration is due to a lack of effective treatments for circulatory system diseases [2]. Indeed, recent world health statistics indicate that 28% of all human deaths are due to circulatory system diseases, suggesting that one in four individuals dies from vascular issues [1]. The statistics also indicate that circulatory system diseases accounted for 16% of preventable causes of mortality and 37% of treatable causes of mortality [1], underscoring the urgent need for more groundbreaking treatments for these conditions.

Ischemic heart disease comprises the majority of circulatory system diseases [1], primarily caused by the formation of atherosclerotic plaques in the coronary arteries [3–6]. These plaques form when endothelial cells

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are damaged followed by a combination with monocytes and lipids, eventually leading to thrombosis [3–7]. Consequently, current drug treatments for ischemic heart disease focus on lipid-lowering and antithrombosis effects [8, 9]. Lipid-lowering treatments range from classic statins to ezetimibe and proprotein convertase subtilisin/kexin type 9 inhibitors, with the prevailing view that "the lower the cholesterol, the better" in the context of ischemic heart disease [9]. In terms of antithrombosis therapy, classical aspirin and recently emerged superior agents such as clopidogrel, prasugrel, and ticagrelor are widely used, and in some cases, a combination with new oral anticoagulants shows excellent efficacy [8]. However, these treatments cannot reverse the development of pathological vessels once an irreversible cascade has begun [3–6]. Furthermore, revascularization methods, such as stent insertion and bypass surgery, delay progression rather than serve as fundamental cures [8].

Therefore, neovascularization has long been regarded as a revolutionary treatment for ischemic heart disease, with significant research focusing on endothelial progenitor cells (EPCs) capable of promoting angiogenesis [10–15]. Monocytes present at sites of atherosclerotic plaque formation, as mentioned above, are considered a promising source of EPCs [10–15]. Research involving the isolation of these cells from the body and their autotransplantation into sites of ischemic heart disease has shown excellent therapeutic effects in preclinical studies [10–15]. However, numerous clinical trials involving thousands of patients have failed to demonstrate significant treatment effects [16–19]. While some research in this area continues, many believe that the insufficient dose and complex ex vivo process are major challenges [20–23].

Achieving in situ reprogramming would constitute a substantial breakthrough in overcoming these limitations. In vivo cellular reprogramming, once deemed a futuristic concept, is now a plausible notion [24, 25]. Certain differentiated cells can be directly reprogrammed into other cell types without passing through induced pluripotent stem cell (iPSC) stage [26–30]. The actualization of direct reprogramming has prompted efforts toward in vivo reprogramming. While numerous enhancements are still necessary, this approach has demonstrated significant potential [25].

In this review, we examine the research on neovascularization conducted over the past decades and discuss in situ reprogramming as an improvement strategy. We also review recent research strategies that apply in situ reprogramming in neovascularization [31].

From the Discovery of "Putative" EPCs to Their Autotransplantations

In 1997, Asahara et al. [10] demonstrated that when CD34 (+) mononuclear cells were isolated from the blood and cultured, they showed increased expression of CD31 and vascular endothelial growth factor receptor 2, indicating their differentiation into endothelial cells. Furthermore, upon injecting these cells into an animal model of vascular injury, they differentiated into endothelial cells at the injury site, leading to the introduction of the concept of "putative" EPCs.

This study marked the beginning of the neovascularization narrative and changed the existing paradigm, which previously implied that adult neovascularization was only possible through the proliferation of existing endothelial cells. However, Asahara et al. [10] revealed that cells capable of becoming EPCs circulate in the blood and could contribute to vascular regeneration at injury sites.

Asahara et al. [11] established that circulating EPCs originate from bone marrow (BM). They also demonstrated therapeutic strategies using ex vivo-expanded EPCs and cytokines that increased the mobilization of EPCs from the BM [12, 13]. Other researchers joined the fray using treatment methods such as autotransplantation of BM cells to vascular injury sites in rabbits [14] and examining various cytokines and ligands to maximize the therapeutic effect of EPCs [15]. While the focus has been primarily on vascular diseases, such as coronary artery disease and peripheral artery disease, the therapeutic effects of EPCs have also been evaluated in damaged liver [32, 33], lung [34], and neuron [35, 36] models as vascular regeneration is important for other tissues.

Progression to clinical trials is the natural next step (Table 1). One of the initial randomized clinical trials, the 2004 BOOST trial, involved 60 patients who had undergone stent insertion following acute myocardial infarction and were divided into control and experimental groups [16]. In the experimental group, an average of 5.7 days after the procedure, the BM of patients was harvested and injected into the damaged coronary artery using a catheter after 6–8 h. The patients were followed up for up to 6 months, and the experimental group showed 6.0% increase in left ventricular ejection fraction (LVEF) compared to the control group.

Subsequent clinical studies followed a similar route, with BM cells extracted and injected into the coronary artery within a week of stent insertion following acute

Table 1. Principal clinical trials on EPC treatment

Year	Study design	*n*	f/u	Outcome
2004 [16]	RCT	60	6 M	- Improved LVEF
2006 [17]	RCT, placebo-controlled	204	12 M	- Improved LVEF, no difference in mortality
2009 [18]	RCT	200	6 M	- No difference in LVEF, mortality
2011 [40]	RCT	31	6 M	- Improved LVEF only in 2 higher dose groups
2017 [41]	RCT, placebo-controlled	153	6 M	- No difference in LVEF by dose escalation or γ-irradiation
2020 [37]	RCT	375	24 M	- No definite conclusions due to unexpectedly low recruitment

RCT, randomized clinical trial; M, months; LVEF, left ventricular ejection fraction.

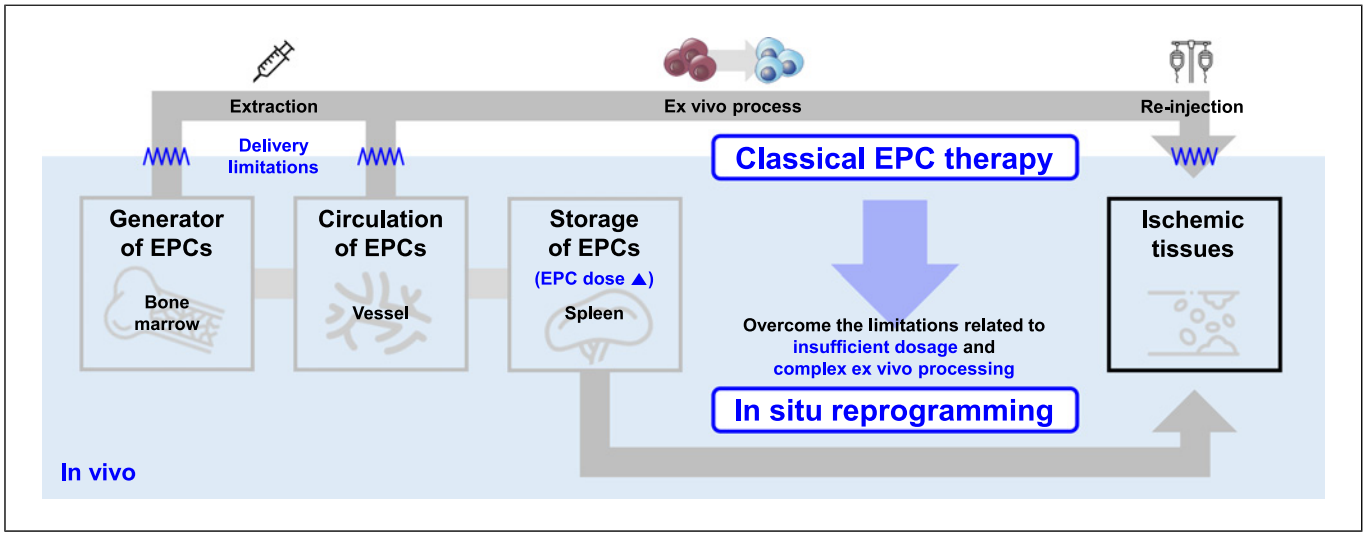

Fig. 1. Classical EPC therapy and overcoming insufficient dose and complex ex vivo processing issues through in situ reprogramming. In classical endothelial progenitor cell (EPC) therapy, EPCs are collected from the BM or blood vessels due to their ease of extraction. These extracted EPCs are then processed ex vivo and reinjected into the body using invasive delivery methods. Limitations associated with this approach can be mitigated through in situ reprogramming. This approach utilizes the abundant but otherwise unextractable EPC pool in the spleen. Moreover, these cells inherently target ischemic tissues, effectively addressing both insufficient dose and complex ex vivo processing limitations. Reproduced with permission.

myocardial infarction (Fig. 1). The 2006 REPAIR-AMI trial included 204 patients with a follow-up period of up to 1 year [17]. Although the treatment group showed therapeutic effects with respect to revascularization and some combined endpoints, there were no significant differences in the mortality or rehospitalization rates. The 2009 REGENT trial, conducted similarly with 160 patients over a 6-month follow-up period, also showed some effects in the experimental group, such as a significant increase in LVEF [18]. However, they conclude that there were no significant therapeutic effects in terms of mortality or major cardiovascular events.

A study published in 2015 conducted a meta-analysis regarding these studies including 41 clinical trials involving 2,732 participants [19]. The analysis found no therapeutic effect of EPCs on most parameters such as all-cause mortality, cardiovascular mortality, reinfarction, rehospitalization, morbidity, quality of life, or LVEF. To address these inconsistent outcomes, the 2020 BAMI trial, being the first phase 3 trial, recruited the largest cohort of 375 patients [37]. However, due to unexpectedly low recruitment for this trial, it failed to derive any meaningful conclusions.

The failure of EPC therapy in clinical trials has been attributed to various factors. A significant challenge is the lack of consensus on how to define EPCs and determine their specific markers [38]. Progenitor cells with the capability to differentiate into endothelial cells indeed

exist, but they can be categorized into at least two distinct types: circulating angiogenic cells (early EPCs) and endothelial colony-forming cells (late EPCs). This observation emphasizes the necessity for additional comprehensive research in this field [39].

Apart from the need for additional studies, there are numerous aspects in current EPC treatment strategies that require improvement. The first is the issue of insufficient dosage, with many studies highlighting the need for a further study on therapeutic threshold dose [20–22]. A small-scale clinical study conducted in 2011 showed that the therapeutic effect recorded with high doses of EPCs was not observed at low doses [40]. Furthermore, following the initial BOOST trial, the 2017 BOOST-2 trial divided the groups according to the treatment dose and found better effects in the high-dose treatment group [41]. These facts suggest that failure of prior clinical trials may be due to the insufficient dosage of EPCs.

The second issue is complex ex vivo processing [20, 21, 23]. Most clinical trials involve extracting EPCs from the BM and injecting them at the site of vascular disease. The process of extracting EPCs from the BM can be quite painful and risky for patients, requiring sedatives or anesthesia, and sometimes necessitates the cessation of antithrombotic agents to prevent bleeding. Furthermore, delivering these extracted EPCs to the damaged vascular site using a catheter often requires invasive interventions, such as coronary angioplasty, which can be a limitation of the current treatment approach. The complexity of these ex vivo processes may downgrade the therapeutic effect in each step.

Despite these issues, solving the problems of insufficient dose and complex ex vivo processing within the current treatment framework remains challenging. The number of cells that can be extracted from a patient's BM is limited, and improving the extraction and reinjection processes remarkably is challenging. This poses the question of whether the entire autotransplantation process could be conducted in vivo.

In situ Reprogramming: What Are the Benefits and Current Obstacles?

The Waddington model proposed in 1957 offered a classic perspective on cellular differentiation, likening it to a marble rolling down into grooves, suggesting that fully differentiated cells find it difficult to change into other types [42]. The model has been widely accepted for several decades. However, the premise of this model is now under scrutiny, particularly with the discovery of iPSCs. In 2006, Takahashi and Yamanaka [43] demonstrated that fully differentiated fibroblasts can be reprogrammed into iPSCs. This groundbreaking work, which earned them the 2012 Nobel Prize in Physiology or Medicine, proved that cells, once at the bottom of their differentiation "valley," could indeed climb back up. Further advancements include the concept of direct reprogramming, which allows specific cells to be converted into other cell types without passing through iPSC stage [24–30]. This is significant because it opens the door to in situ reprogramming, which has a vast therapeutic potential in vivo [25].

Early studies in in situ reprogramming include one where a team reprogrammed exocrine cells of the pancreas into β cells in vivo using an adenoviral vector [26]. These reprogrammed β cells were shown to function properly via secretion of insulin and synthesizing growth factors. Another study explored the common lineages of the pancreas and liver during embryonic development and successfully converted liver cells into insulin-secreting cells [27].

Similar approaches have been used to treat cardiovascular diseases. For example, one study reprogrammed cardiac fibroblasts into cardiomyocytes in vivo in mice [28]. After inducing myocardial infarction, the team delivered genes into cardiac fibroblasts using retroviruses, resulting in cells resembling cardiomyocytes. Some findings suggest that direct reprogramming may be more effective in vivo than in vitro as reprogrammed cells can integrate and function in harmony with surrounding tissues, thereby improving heart function [29, 30].

EPCs and chimeric antigen receptor (CAR)-T cell therapies share a similar framework. Similar to the extraction of BM cells for autotransplantation in EPC therapy, CAR-T cell therapy involves extracting T cells from blood, processing them, and reintroducing them to patients [44]. Despite its proven efficacy, CAR-T cell therapy poses significant challenges, including time and cost inefficiencies. It requires more than 3 weeks and costs up to USD 500,000 per patient [44]. Additionally, unnecessary preconditioning chemotherapy for T-cell extraction and ex vivo culture is linked to the cytokine release syndrome, which is a major side effect of CAR-T cell therapy [44]. To overcome these limitations, extensive research into the in situ reprogramming of CAR-T cells is underway, using methods such as viral vectors or lipid nanoparticles to deliver genes to T cells [44–46].

Nonetheless, in situ reprogramming encounters a significant obstacle in the form of targeting issues [25]. Successfully targeting specific cells for direct reprogramming with genes or drugs, without affecting

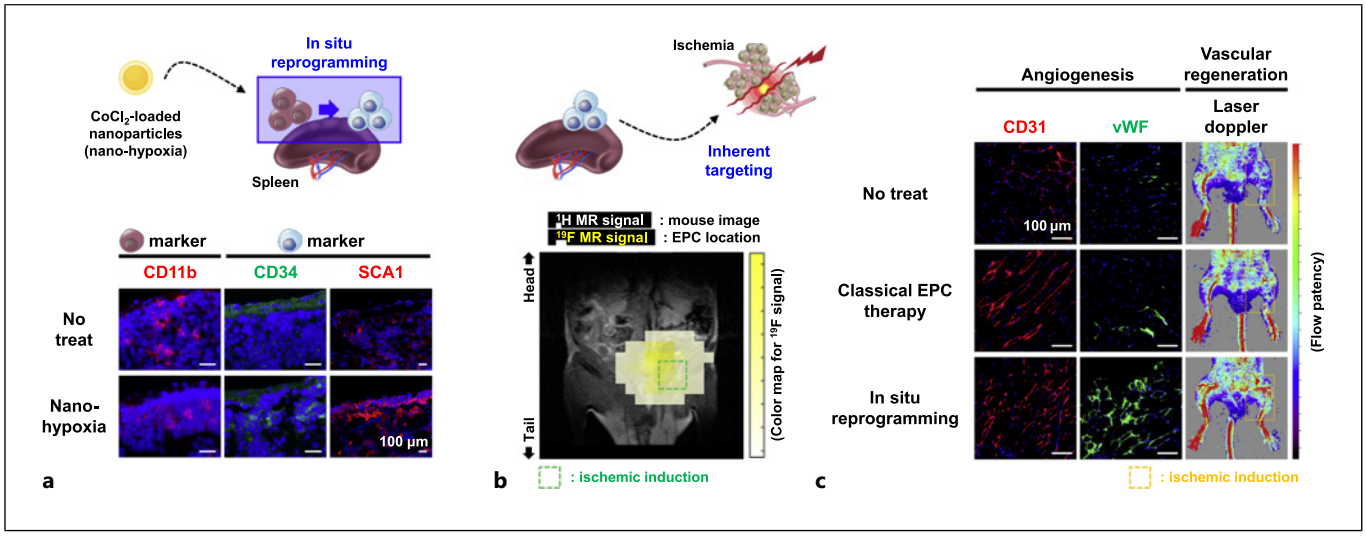

Fig. 2. Validation of in situ reprogramming as an angiogenesis induction method in a mouse hindlimb ischemia model. **a** The spleen was selected as a reprogramming site in vivo due to its dual role in nanoparticle clearance and as a monocyte reservoir. Inducing nano-hypoxia in splenic monocytes elevated the expression of EPC markers such as CD34 and stem cell antigen-1 (SCA-1) compared to that in the no-treat group. **b** The reprogrammed cells retained their inherent ability to target ischemic tissues, as confirmed by magnetic resonance (MR) imaging with ^{19}F-tagged EPCs (visualized as a yellow color map). **c** Consequently, in situ reprogramming facilitated angiogenesis and improved blood flow recovery in ischemic tissues, surpassing the outcomes observed in the no treat and classical EPC therapy groups. Reproduced with permission.

other cells or delivering sufficient quantities remains a significant challenge. Current drug delivery systems cannot prevent off-target effects, making targeting resolution a critical prerequisite for the advancement of in situ reprogramming.

In situ Reprogramming as a Pro-Angiogenic Inducer

One research team identified a convergence point by focusing on the spleen to overcome the insufficient dose and complex ex vivo processing issues of EPC therapy while simultaneously addressing the targeting issue of in situ reprogramming [31]. The spleen is an ideal site for in situ reprogramming because it stores a substantial number of monocytes that migrate to areas, such as sites of myocardial infarction, during inflammation [47, 48]. Additionally, the spleen is a major organ for nanoparticle distributions [7, 49]. Acting as a blood filter, the spleen not only filters out old red blood cells and bacteria but also captures many nanoparticles. Although the filtration function of the spleen has been a major limitation for most targeted nanoparticle applications [7], it implies that a significant number of nanoparticles can be naturally directed to the spleen without specific interventions.

Hence, if nanoparticles could be delivered to the spleen to reprogram splenic monocytes, they could present a new therapeutic strategy for EPC therapy. The research team used hypoxia, which is known as a priming agent for EPC therapy, as the reprogramming agent [50]. Monocytes isolated from the spleen were primed with hypoxia and the hypoxic-mimetic agent $CoCl_2$. Furthermore, in situ reprogramming was attempted by delivering $CoCl_2$-loaded nanoparticles to the spleen (nano-hypoxia).

In mice, nano-hypoxia induction on the spleen led to an augmentation of EPC characteristics, such as increased expression of CD34 and stem cell antigen (SCA) 1 (Fig. 2a), which was compared with actual hypoxia induced by splenic artery ligation. In situ reprogramming of splenic monocytes revealed an approximately 11.9-fold increase in EPC quantity compared to that observed with cells harvested from femur BM, demonstrating a clear advantage in terms of dose.

Additionally, it was confirmed both in vitro and in vivo whether the ability to target myocardial infarction sites remained in the reprogrammed cells. The reprogrammed cells targeted the inflammation sites surrounding the inflammation areas in a severity-dependent manner (Fig. 2b).

Finally, the research team evaluated the effects of in situ reprogramming on vascular regeneration in mouse ischemic hindlimb and partial hepatectomy models. In the ischemic hindlimb model, in situ reprogramming enhanced angiogenesis and blood flow recovery, proving to be more effective than the classical BM-derived cell autotransplantation (Fig. 2c). In the partial hepatectomy model, in situ reprogramming promoted angiogenesis and hepatic regeneration, which was not observed when the spleen was removed.

This study presents a new approach to overcome the insufficient dose and complex ex vivo processing issues associated with classical EPC therapy through in situ reprogramming of the spleen. While leveraging the distribution of nanoparticles, potential off-target effects in the liver or other organs remain a limitation, necessitating further research in larger animals. Nevertheless, this variant of in situ reprogramming signifies a potentially novel treatment strategy, presenting a prospective solution that has not been realized in more than 40 clinical trials encompassing thousands of patients [19].

Conclusion

Ischemic heart disease remains the leading cause of death worldwide, necessitating more effective regenerative treatments for ischemic tissues. Although traditional EPC-based therapies have shown significant potential, they are limited due to insufficient dose and complex ex vivo processing.

Recent advances in direct reprogramming, which can lead to in vivo reprogramming, have opened the door to novel therapeutic strategies. Improvements in EPC therapy can be achieved via in situ reprogramming. A study that utilized the spleen, a natural gathering site for nanoparticles, as an in situ reprogramming site demonstrated the potential for developing such innovative strategies.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Seyong Chung drafted the manuscript under organization and guidance of Hak-Joon Sung.

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