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REVIEW ARTICLE:

Sonic Hedgehog Signaling Pathway in Endothelial Progenitor Cell Biology for Vascular Medicine

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Abstract: The Hedgehog (Hh) signaling pathway plays an essential role in embryonic and postnatal vasculature development and homeostasis of organs. Under physiological condition, Hh family's member Sonic Hedgehog (SHh) regulates endothelial cell growth, promotes cell migration, and induces the construction of blood vessels. In this review, we highlight recent topics in EPC biology, regarding current advance in SHh canonical and non-canonical signaling pathway in EPCs and EC biology in terms of homeostasis, extracellular SHh signal transmission by parental cell-derived extracellular vesicle (exosomes containing single-strand non-codding miRNA), and SHh signal impairment in cardiovascular diseases. Also, we discuss SHh signaling pathway activation of EPCs as a promising therapeutic tool against cardiovascular disease patients.

Keywords: sonic hedgehog; endothelial cells; endothelial progenitor cells; canonical signals; non-canonical signals; extracellular vesicles

INTRODUCTION:

Endothelial progenitor cells (EPCs) were first isolated from adult peripheral blood (PB) in 1997[1], were shown to derive from bone marrow (BM) and to incorporate into foci of physiological or pathological neovascularization[2]. The finding that EPCs can home to sites of neovascularization and differentiate into endothelial cells (ECs) *in situ* is consistent with "vasculogenesis", a critical paradigm well described for embryonic neovascularization, but recently also proposed for the adult organism in which a reservoir of progenitor cells contributes to post-natal neovascular formation[3]. The discovery of EPCs has therefore expanded vascular biology field, formerly considered only by EC biology, in organ regeneration and vascular diseases. Therefore, a lot of EPC biology researches have been investigated to elucidate the differentiation cascade for vascular development and regenerative medicine. While many signal cascade and expressional profiles, as expected, are common between EPC and differentiated ECs, the uniqueness of signals during differentiation and bioactivity will elucidate the originality in EPC vascular development and pathology in postnatal life.

The Hedgehog (Hh) signaling pathway has an essential biological function that orchestrates the development of embryonic, postnatal angiogenesis, and organogenesis. In an early embryological development of vertebrates Hh morphogen plays the crucial role on the development of organs such as limb[4,5], neuron (axon elongation, and astrocyte)[6-8], and cardiac and vascular (septal cardiogenesis, angiogenesis, and vasculogenesis) development[9-13].

In mammals, there are three Hh family members - Sonic, Indian, and Desert hedgehogs (SHh, IHh, and DHh, accordingly) have been known. Among them, SHh signaling pathway has been attracted researchers' attention due to involvement in an induction of postnatal vasculogenesis in homeostasis and pathological condition[14-18]

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In this review, we shall highlight three recent topics in EPC biology; i) current advance in SHh canonical and non-canonical signaling pathway in EPCs and EC biology in terms of homeostasis ii) extracellular SHh signal transmission by parental cell-derived extracellular vesicle (exosomes containing single-strand non-codding miRNA), iii) SHh signal impairment in cardiovascular diseases. Also, iv) we discuss SHh signaling pathway activation of EPCs as a promising therapeutic tool against cardiovascular disease patients.

SHh Signaling Pathway in Vascular Development

Classical canonical signaling is initiated by secreting sonic hedgehogs, that recognizes a cell surface receptor protein, PTCH1 on target cells[19]. De-repressed smoothened (SMO) protein is a central signal transducer of SHh pathway, activates downstream glioma-associated oncogene homolog (Gli) transcription factors (**Figure 1**). At the nucleolus level Gli transcription factor accumulation activates target genes including, proliferation (e.g., *Cyclin-D1*, *MYC*), apoptosis (e.g., *Bcl-2*), angiogenesis (e.g., *ANG1/2, PDGF-BB, VEGF*), epithelial-to-mesenchymal transition (e.g., *SNAIL*), and stem cell self-renewal (e.g., *NANOG*, *SOX2*)[19-21].

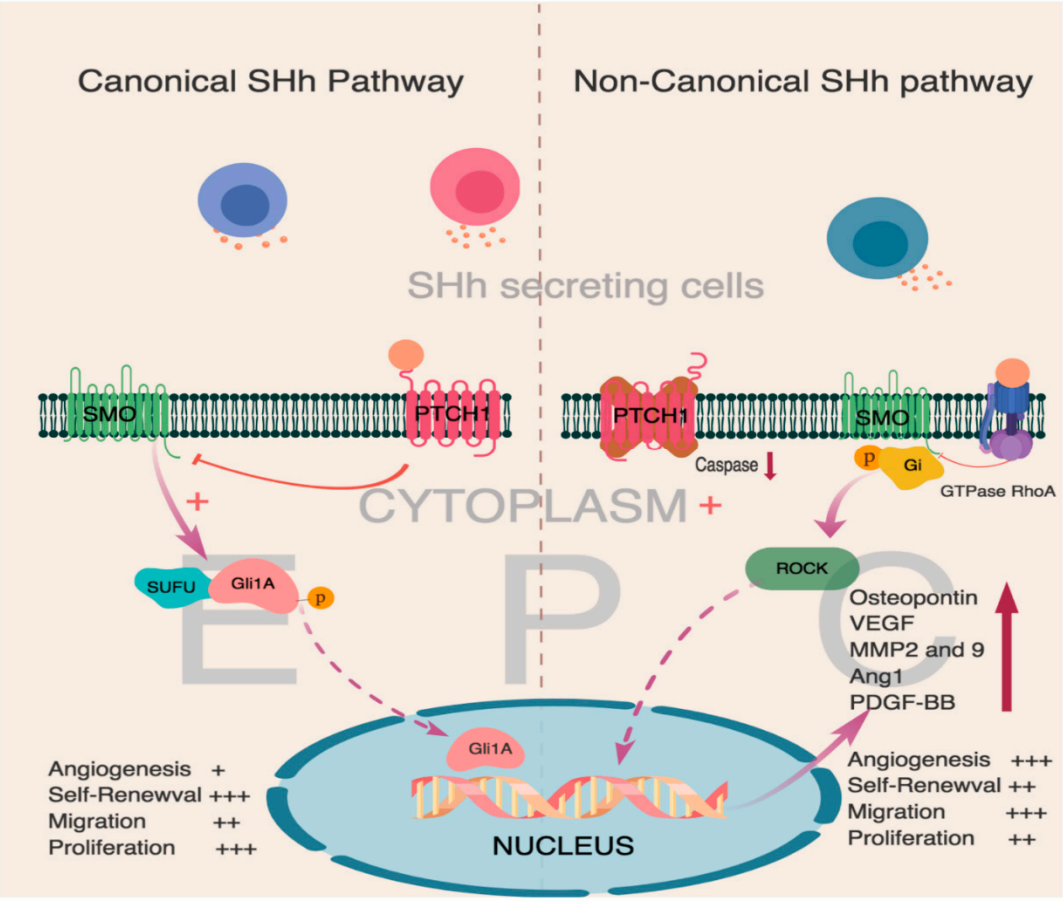

Figure 1. Canonical and Non-Canonical SHh signaling pathway of EPCs and ECs. As shown here, SHh molecules activate membrane surface PTCH1, which inhibits SMO receptors to activate binding of SUFU/Gli1A complex by autophosphorylation. Emerging evidence showed that mostly EPCs are activated by non-canonical SHh signaling pathway for angiogenesis.

Recent researches in vascular biology, however, have shown that non-canonical SHh signaling significantly contributed to vascular development rather than canonical signals in either pathological or homeostasis conditions[11,15,22]. While all of Hh family members; SHh, IHh, DHh can trigger endothelial cells via non-canonical pathway depending on strength of signal[23,24], the role of SHh morphogen is essential on endothelial cell lineage vascular differentiation, maturation, and function[12,15-17]. An application of recombinant SHh molecules, but not IHh and DHh morphogens, to a confluent endothelial cell monolayer resulted in a change of the overall

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morphology from the typical EC "cobblestone" shape to a swirling pattern of elongated cells oriented in bundles, a pattern characteristic of the activated endothelial cells engaged in angiogenesis [22,25].

There are several non-canonical SHh signaling has been disclosed in the literature. SHh protein activation of membrane RhoA GTPase, by SMO/Gi protein-dependent manner is initially investigated [22]. The membrane RhoA family of small GTPases have been shown to play an essential role in cell migration and invasion by collectively guanine nucleotide exchange factors and GTPase-activating proteins complex orchestrating [26]. Depending on cell lines, traditional RhoA GTPase signaling during cell migration is different. There are more than 20 members of the Rho family can be divided into classical and non-typical types, and till now which RhoA GTPase family member responsible for signal regulation to activate Gi/SMO complex of EC are less studied [27]. *In vitro*, in tube formation assay showed that Hh proteins induced a rapid activation of RhoA levels in human umbilical-vein EC cells to stimulate tubulogenesis via SMO, and Gi proteins whereas prevention of RhoA activation disturbed tube formation [22,28]. These data also showed that RhoA GTPase concentration increased 3-fold after binding with Hh molecule, perhaps, that can deliver a strong angiogenic signal to activate non-canonical SMO- dependent SHh pathway for further EPCs maturation toward EC (**Figure 1**)[22,24,28].

SMO independent manner through decreasing caspase-3 activity for further inhibition of PTCH1 pro-apoptotic function to keep EC survival[22,24]. Lee et al., using a combined transcriptomic and proteomic approach, identified a 101-gene endothelial signature that could be further used to characterize endothelial commitment. Among these genes, Hedgehog-interacting protein is a strong negative regulator of late EPCs (LEPCs) through regulation of Gli-dependent canonical Hh signaling. On the one hand, HIP knockdown in LEPCs improves angiogenic activity and enhances LEPC survival under oxidative stress.[29] This may indicate that exogenous SHh treatment can beneficially preserve EPC survival after onset ischemic events.

Also, SHh morphogen stimulates through Rho/ROCK pathway to increase downstream MMP-9, osteopontin (OPN), and PGF-BB expression (**Figure 1**), which are essential for SHh-induced angiogenesis *in vitro*[12,22,24]. The potential involvement of Smo, the Rho/ROCK pathway, MMP-9, OPN, and the Gli transcription factors in SHh-induced angiogenesis was investigated *in vivo* with the mouse corneal angiogenesis model by implanting pellets containing phosphate-buffered saline (PBS) or cyclopamine (SMO protein inhibitor) alone, and in combination with SHh to evaluate angiogenesis via *in vivo* fluorescein-BS-1 lectin perfusion. The PBS+SHh combination sharply increased angiogenesis whereas the presence of cyclopamine abolished it. Then, whether the Rho/ROCK pathway essential to a contribution of the SHh signaling, pellets containing PBS, SHh, Y27632, or SHh and Y27632 (ROCK inhibitor) were implanted. Pellets containing SHh alone substantially increased the ROCK downstream targets MMP-9 and OPN dependent angiogenesis, but no enhancement was observed after transplantation of pellets containing both SHh and Y27632[24]. Another mouse corneal angiogenesis model demonstrated that SHh contributed to PDGF-BB–induced pericyte cell recruitment, which is essential for the maturation of newly formed vessels[12].

To sum up, among Hh family members- SHh, IHh, DHh, SHh morphogen play a vital role in terms EC and EPC: migration, angiogeneic bioactivity, survival or anti-apoptotic, and maturation effects mostly via non-canonical rather than canonical signaling pathway.

Another non-canonical SHh pathway is an indirect effect of SHh molecule for EC and EPCs mediated angiogenesis. Recently Gupta et al. demonstrated that effects of SHh on EC proliferation and migration *in vitro* are limited by direct incubation of SHh in culture, but are

significantly enhanced by conditioned media from SHh-treated fibroblasts or stromal cells. Moreover, SHh treatment of fibroblasts sharply stimulated angiogenic growth factor expression profiles, including PDGF-B, VEGF-A, HGF and IGF. PDGF-B was the most upregulated and may contribute to the large neo-vessels associated with SHh-induced indirect angiogenesis [23]. In the corneal angiogenesis model, response to exogenous Shh was assessed and there was no significant difference in corneal angiogenesis induced by administration of SHh pellets between eSmoWT and eSmoNull mice. *In vivo*, hindlimb ischemia (HLI) model was applied to $eSmo^{Null}$ mouse and wild-type littermates to define the importance of SMO protein for downstream signaling, and

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demonstrated that equal recovery findings between both animals in terms of perfusion ratio, limb motor function, limb necrosis, and blood vessel formation[23] [14]. This may suggest that the fibroblast-derived pro-angiogenic genes play an important role on EC and EPCs, that can be indirectly activated angiogenesis without SMO protein.

Extracellular Vesicle for SHh signals

Extracellular vesicles (EVs) secrete from parental cell 30-150nm in size lipid bilayer enclosed cargo, containing mRNA, miRNA, growth factors and proteins for transfer recipient cells[30,31]. All of EVs share common three main stages of EVs from biogenesis to release; i) the first stage takes place on plasma membrane by outward budding, fission and (ii) formed early endosomes packaging and sorting occurs endoplasmic reticulum to form (iii) exosomes or extracellular vesicles to release intracellular space which has been described in review article in detail[30,32]. The recent EVs research highlighted that mammals SHh secretes distinct EVs/exosomes to directly or indirectly downstream target gene activation [33]. Vyas et al[33]. isolated two distinct exosome fractions, P150 and P450 using differential ultracentrifugation techniques from full-length SHh transfected HEK293T cells culture media, and both extracellular vesicular pools derived from an endocytic origin, due to endocytosis protein expression of Rab was higher in two fractions.

SHh signaling pathway regulation by EV-derived miRNA is divided in to three level of activation; in the first level, miRNA binds with membrane surface receptors or proteins (PTCH1 or activates Rho GTPase) and second level, with cytoplasmic proteins Gli1,2,3 and final, nuclear level of activating estimates as the strongest activation of SHh among three level[34-36] (**Figure 2**). The clinical study showed that osteoarthritis patient's chondrocytes were highly expressed on SHh, PTCH1, Gli1, and MMP-13, and positively correlated with overexpression of miRNA-602 and miRNA-608 which may activate all three level[37]. In addition, tumor studies revealed that cancerous cell-derived EVs abundantly enhanced with SHh and Gli gene. expression, and these genes positively correlated with the microvascular density of tumor tissue [38].

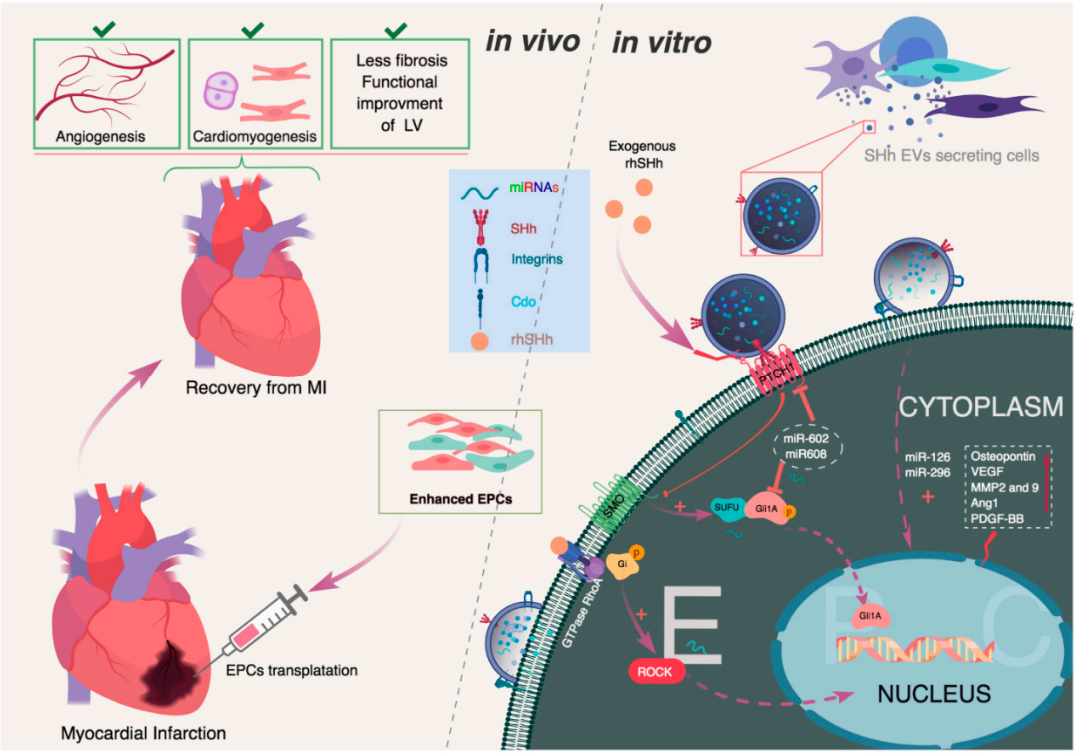

Figure 2. SHh molecule or SHh EVs secreting cells mediated functional improvement of EPCs. Exogenous SHh molecules implementation increased vasculogenic EPCs, and transplantation of enhanced EPCs beneficially recovered from MI in *in vivo* studies.

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In vitro study disclosed that SHh secreted EVs activates EPCs by canonical PTCH1-Gli1 or non-canonical by pro-angiogenic miRNA, integrin-linked kinase, and ROCK pathway-dependent manner for downstream signal transduction [24,28,39,40](**Figure 2**). *In vivo* study also documented that SHh-codding vector transfected CD34+ cells or EPCs exosomes were obtained strong vasculogenic potential to improve myocardial infarcted tissues recovery by enhancing angiogenesis and reducing left ventricle fibrosis [15]. Transcriptional profile analysis also depicted that EPCs derived exosomes overexpression to miRNA-126a and miRNA-296 were high and *in vivo* study, self-renewal and vasculogenic functions improved EPCs enhanced angiogenesis in the murine LHI model **Table 1** [41]. These may imply that that EVs derived SHh molecules or miRNAs play an important role in postnatal angiogenesis and tumor metastasis. Activation of SHh signaling by miRNA in aforementioned levels have been attracting researchers focus to extensively investigate SHh EVs derived miRNA functions to develop new drugs against cardiovascular ischemic disease patients (see **Table 1**).

Table 1. Summary of pro-angiogenic and anti-senescence miRNAs EPC and EC biology.

Name of miRNA	Expression	Target cells	Outcome	Target genes	Ref.
miR-126-3p	Up	EPC and EC	*In vitro,* presence of miR126-3p enhanced tube formation length. *In vivo*, increased HLI inducted animals MVD.	VEGF, Ang-1, Ang-2, and MMP-9	[41]
miR-106b-25	Up	EC and EPC, Sca-1 and BMMSC	Increased tube formation capacity. Overexpression of individual members of the miR-106b-25 cluster increases viability, proliferation, and migration of endothelial cells.	VEGF, Sca-1, and Flk-1	[42]
miR-126	Down	EPC, Sca-1, and Lin-	Silencing of miR-126 in HLI inducted animals increased EPC, Sca-1, and Lin- cells mobilization from bone marrow to the site of injury, consequently improved angiogenesis	SDF-1	[43]
miR-10A and miR-21	Down	EPC	miR-10A and miR-21 regulate EPC senescence via suppressing Hmga2 expression	Hmga2	[44]
miR-361	Down	EPC	KO of miR-361-5p not only restored VEGF levels and angiogenic activities of diseased EPCs *in vitro*, an *in vivo* further promoted blood flow recovery in ischemic limbs of mice.	VEGF	[45]
miR-34a	Down	EPC	miR-34a overexpression led to a significantly increased EPC senescence and impairment, paralleled with an approximately 40% Sirt1 reduction. KO of Sirt1 by its siRNA resulted in diminished EPC angiogenesis and increased senescence	Sirt1 and FoxO1	[46]

Abbreviations: EPC-endothelial progenitor cells; EC- endothelial cells; HLI- hindlimb ischemia; BMMSC-bone marrow mesenchymal stromal cells; MVD- microvascular density; Lin- lineage negative cells; KO- knock-out.

SHh Signal Impairment in Cardiovascular Diseases

Cardiovascular diseases share almost 32% all of the death in the world. Among them, ischemic diseases are a leading cause of mortality and morbidity [47,48]. EPCs in patients with comorbidities such as atherosclerosis, DM, hypertension, and obesity, as well as risk-associated factors, such as smoking and western diet style, may cause impairment of functional quality and quantity in peripheral bloodstream [19,49,50]. Preclinical experiments modeling of DM, AMI, wound healing and chronic vascular inflammatory diseases have concluded that endogenous SHh pathway was aggravated in a non-treated group whereas therapeutic exogenous SHh implemented group represented the functional recovery of EPCs resulting in enhanced angiogenesis, cardiomyogenesis and wound healing [23,51,52]. Other studies demonstrated that SHh molecules contributed to the neovascularization process in LHI and AMI modeled animal ischemic tissues through a biological effect on EPC enriched cell population, CD34+ cell[15,17,53] (**Figure 2**). Kanaya et al have tried SHh conditioning to human CD34+ cells, isolated from the healthy volunteers or Burger's disease patients after granulocyte colony-stimulating factor (G-CSF) administration, and realized that SHh protein superiorly increased pro-angiogenic gene expressions in a dose-dependent manner, particularly from patient-derived CD34+ cells in comparison with or without G-CSF mobilized healthy controls [17]. Qin et al disclosed EPC migration, tube formation ability, and mobilization of EPCs in streptozotocin-induced DM type 1 animals were impaired when compared to the healthy control group. Also, they

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found cross-talk of Shh and PI3K/AKT pathways in EPCs in DM type 1 animals which decreased AKT activity, led to an increased GSK-3β activity and degradation of the Shh pathway transcription factor Gli1/Gli2[54]. This may suggest that endogenous SHh PTCH1-Gli1 protein complex in cardiovascular disease patient EPCs was "exhausted" due to chronic inflammation, risk factors etc., and exogenous administration of SHh molecules may beneficially improve EPC functional profiles by PTCH1-Gli1 molecule recovery[54-56]. In fact animal experiments depicted that SHh, PTCH1 and Gli1 proteins in myocardial infarction models in DM type 1 mice significantly decreased in myocardial tissues compared to control littermates, which resulted in extended left ventricle infarct size and reduced capillary density, accompanied by cardiac dysfunction[56]. The Gli1 protein function is essential for regulating cell-cycle, survival, apoptosis, and migration[21]. EPCs transplantation studies on ischemic diseases such as AMI, ischemic cardiomyopathy, heart failure, PAD, and in stroke have documented that aged and DM patients EPCs did not recover from ischemia in comparison with control group. This may indicate that PTCH1-Gli1 molecule concentrations or SHh signaling pathway receptors sensitivity in EPCs decreased depending on severity, age, type, and timing of diseases [57-59]. Aging animal studies by Renault et al confirmed it by decreased SMO expression in skeletal muscle in aged mice [60].

Therapeutic Application of SHh Signals for Cardiovascular Diseases

In no-option patients or the terminal stages of the ischemic diseases, the effectiveness of interventional reperfusion therapy is hampered [61-63]. Following consideration, EPCs therapy is a promising therapeutic option against ischemic cardiovascular diseases in terms of angiogenesis, vasculogenesis, and contemporary organ preservation. G-CSF mobilized EPCs transplantation is safe and feasible for patients with advanced coronary artery disease or PAD who are not amenable to surgical or percutaneous revascularization [64-66]. However, EPC mobilization efficacy with G-CSF is very low in DM or previously EPC mobilized patients due to quality and quantity impairment of EPCs [67]. In this regard, we showed that SHh-mediated activation of EPC is one of the best options for functional recovery of EPC for cardiovascular patients who previously underwent cell mobilization [17]. The preclinical studies revealed that therapeutic implementation of SHh proteins or SHh pathway activation notably improved several ischemic disease models such as AMI [15,43], myocardial ischemic- reperfusion[48, LHI [17,23,45,[68], , stroke [69], diabetic wound healing[44], skeletal myogenesis[70], and osteogenesis and bone tissue formation[71] **Table 2**. Below we discuss the pros and cons of SHh molecule therapeutic application in terms of cardiovascular related diseases.

Table 2. Endogenous and Exogenous SHh Signaling Activation in Cardiovascular Diseases.

Disease Model	SHh pathway and Cell Tx.	Results	Ref.
AMI	Endogenous and exogenous SHh signaling activation by SHh–modified human CD34+ cells and its exosomes	Treatment with SHh–modified human CD34+ cells reduced infarct size, and increased border zone capillary density, and cardiac function was improved; EF, FS, compared with unmodified CD34 cells or cells transfected with the empty vector.	[15]
AMI and Chronic MI	Exogenous recombinant Shh administration and gene transfer of naked DNA encoding human SHh	MI fibrosis size and apoptotic cardiomyocytes were reduced. MVD increased. Shh gene transfer also enhanced the contribution of bone marrow–derived endothelial progenitor cells to myocardial neovascularization.	[53]
Myocardial Ischemia-Reperfusion–Induced Injury	Endogenous Hh signaling activation and exogenous recombinant Shh administration	Reduced apoptosis, fibrosis, and increased vascularization. Exogenous SHh administration reduced apoptosis, increased vascularization, and reduced	[55]
Post-myocardial ischemic-reperfusion injury	Endogenous Hh signaling activation and exogenous recombinant Shh administration	Exogenous SHh administration significantly increased vasculogenesis-related factors including VEGF, FGF and Ang as well as the SHH signal proteins including Patch-1, Gli1, Gli2 and SMO.	[79]
HLI	Shh-treated human G-CSF mobilized EPCs locally injected into the HLI muscles	Exogenous SHh molecule incubation of CD34+ cells significantly increased vasculogenesis-related factors including VEGFA, VEGFB, HGF, and Pecam 1 as well as the SHH signal proteins including Patch-1, Gli1, Gli2, and SMO at dose 1μg/mL. *In vivo* experiment;	[17]

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		significantly increased angiogenesis and vasculogenesis and blood perfusion recovery following HLI.	
HLI	SHh conditioned fibroblast media or exosomes	PDGF-B, VEGF-A, HGF, and IGF. PDGF-B was the most upregulate to contribute MVD. Improved blood flow perfusion after HLI.	[23]
HLI	Combinational treatment SHh and EPC	Increased incorporation of EPC with host vessels, reduced apoptotic EPC, and initiated to generate a new myocyte.	[52]
Diabetic wound healing	Exogenous nanoscale polymer encapsulated SHh administration	Accelerated diabetic-induced wound closure.	[44]
DM type 1mouse was inducted AMI	SHh + EPCs Tx	EPC migration, tube formation ability, and mobilization were impaired in diabetic mice vs. control, and all were improved by in vivo administration of the Shh pathway receptor agonist. SHh molecule significantly increased capillary density and blood perfusion in the ischemic hindlimbs of diabetic mice	[54]
Ischemic Stroke	Exogenous SHh administration	SHh treatment results in enhanced functional recovery both in locomotor function and in cognitive function at 1 month after stroke. Increased the cerebral blood flow map by arterial spin labeling, and immunohistochemistry α-smooth muscle actin and CD31 immunostaining.	[78]

Abbreviation: *Tx- transplantation; AMI- acute myocardial infarction; EF-ejection fraction; FS- fractional shortening; SV- stroke volume; MVD- microvascular density; HLI- hindlimb ischemia; DM- diabetic mellites.Ischemic Heart Diseases*

As shown in earlier publications, a young human heart is thought to be composed of cardiomyocytes (approximately 18%), endothelial cells (24%), and mesenchymal cells or fibroblasts 58% [72]. However, Pinto et al, using sophisticated cardiac single cell preparation and immunohistochemistry analysis methods, has recently shown that endothelial cells constitute over ~51-54%, hematopoietic-derived cells ~3%, and fibroblasts under equating to ~11% of the total cells of the heart when assuming ~30-33% of the cells are cardiomyocytes[73]. Considering the main cardiac cellular composition is endothelial cells, it opens therapeutic an avenue for beneficially treat with SHh molecules (**Figure 2)**. The therapeutic application of EPCs +SHh molecules has demonstrated that SHh signaling can preserve cardiac function and improve cardiac recovery in the context of myocardial ischemia. To this end, combination therapy of intramyocardial Shh gene transfer and AMD3100-induced progenitor-cell mobilization significantly improved cardiac functional recovery after the onset of MI in a mouse. In histology, increased MVD and reduced left ventricle fibrosis area, and in transcriptome analysis, SDF-1α mRNA expression was significantly elevated after MI, in mice administered combination therapy[74]. One of the limitations of broad use of SHh molecules is its short half-life in the body. In this regard, study-controlled delivery or a coacervate delivery system of SHh morphogens to the ischemic myocardium is one of the solutions to prolong the therapeutic efficacy of SHh molecules. They developed a coacervate delivery system which incorporates with SHh and protects from degradation and releases at least for 3 weeks[75]. EPCs transplantation and controlled delivery of SHh morphogens to the ischemic myocardium will be one of the promising therapeutic tools against cardiovascular diseases. Various therapeutic application to AMI and Myocardial Ischemia-Reperfusion–Induced Injury models were given in **Table 2**.

Peripheral Arterial Diseases (PAD)

The most of SHh signaling and therapeutic effects were investigated n PAD model in mouse and rat species. One of the importance of SHh therapeutic application is that its regulatory function of limb development during embryogenesis as well as postnatal; skeletal myogenesis, vasculogenesis, and neurogenesis etc. [4,5,37]. An endogenous SHh increase macrophage infiltration in mice deficient for SHh signaling in myocytes was associated with increased VEGFA expression and a transiently increased angiogenesis but not in healthy control, demonstrating that Shh limits inflammation and angiogenesis indirectly by signaling to myocytes, whereas exogenous administration of SHh molecules has previously been shown to promote ischemia-induced angiogenesis and skeletal myogenesis **Table 2** [17,68,70]. Studies on aged mouse HLI provided that combination of Shh gene

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transfer and BM-derived EPCs transplantation more effectively promotes angiogenesis and muscle regeneration than BM-EPCs along. Moreover, incorporation into host blood vessels was increased in SHh + EPC treatment, thus suggesting that SHh therapy increases transplanted EPCs migratory effect into the site of ischemia to enhance angiogenesis and vasculogenesis. On the other hand, the combination of SHh + EPC significantly reduced apoptotic EPC cells and increased of myoblast proliferation *in vivo* after HLI induction Table 2 [52]. This may suggest that SHh and EPC combinational treatment accelerated quiescent myogenic stem cells (satellite cells) proliferation and to fuse with each other to form myotubes, which eventually become mature myofibers supplied with a new blood vessel.

Post Diabetic Mellitus Complication

Patients with diabetes are at increased risk of cardiovascular diseases, and associated clinical complications has been becoming an increasingly common disease, estimated to affect 552 million people worldwide by 2030[76]. The recent a cohort study in 1.9 million people with type 2 diabetes and incidence of cardiovascular diseases results showed a strong positive associations between type 2 diabetes and peripheral arterial disease, ischemic stroke, stable angina, heart failure, and non-fatal myocardial infarction[77]. From this valuable data, we know that post diabetic clinical complications are a global burden which requires new therapeutic strategies to overcome. To this end, preclinical experiments in the animal with DM type one was inducted AMI and HLI models and showed that Hh signaling downstream proteins such as PTCH1, SMO, and Gli1,2,3 functionally were impaired, one the other hand, exogenous combination of SHh molecule and EPCs significantly improved histological and functional parameters. Moreover, after treatment expression of PTCH1, SMO, and Gli1,2,3 genes were strikingly upregulated in transcriptome analysis, thus may again prove the "exhaustion" of SHh signaling pathway in cardiovascular diseases[54,56]. Furthermore, nanoscale polymer SHh conjugates via activation of SHh pathway, accelerate wound closure in a diabetic animal model. Authors also found the beneficial effect of the SHh treatments directly on wound revascularization using immunohisto-chemistry to quantify endothelial cells, CD31, and to assess the formation of neovascular structures in the wound tissues[51].

Post-stroke immediately treatment with the SHh pathway agonist remarkably increased expression of vasculogenesis-related factors including VEGF, FGF and Ang, as well as the SHh signal proteins including PTCH1, Gli1, Gli2 and SMO. SHh also improved neurological scores, reduced infarct volume, microvascular density, and promote angiogenesis, and neuron survival in the ischemic boundary zone in histology[69]. Strikingly, delayed treatment (post 3–8 days) of the ischemic stroke with SHh pathway agonist enhanced the locomotor behavioral recovery and cognitive function at 1 month after stroke, suggesting that a prolonged treatment window for potential treatment strategy to modulate sonic hedgehog pathway after the stroke, **Table 2** [78].

Conclusion:

The SHh signaling pathway is one of the key regulators of postnatal vasculogenesis. According to its valuable regulatory effect, an impairment of endogenous SHh pathway may have aggravated cardiovascular patients' disease severity such as AMI, stroke, and PAD due to the less angiogenic potential of EPCs. Almost all of the aforementioned preclinical experimental studies have concluded that combinational treatment of SHh molecule with EPCs has a strong angiogenesis, vasculogenesis, cardiomyogenesis, skeletal myogenesis, and neurogenesis effect even in delayed treatment. Certainly, to maintain EPC functional activity, the role of interactions between SHh pathway and other pathways or key regulators such as *Wnt* or *Notch* also important. Further investigations need to be addressed to elucidate these issues.

Conflict of interest: The authors have no potential conflicts of interests.

Author's contributions: Conception and design, Acquisition of data, and manuscript writing: A.A.S, A.K.S. Final approval: R.P & T.A

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