Sonic Hedgehog–Modified Human CD34+ Cells Preserve Cardiac Function After Acute Myocardial Infarction


Rationale: Ischemic cardiovascular disease represents one of the largest epidemics currently facing the aging population. Current literature has illustrated the efficacy of autologous, stem cell therapies as novel strategies for treating these disorders. The CD34+ hematopoietic stem cell has shown significant promise in addressing myocardial ischemia by promoting angiogenesis that helps preserve the functionality of ischemic myocardium. Unfortunately, both viability and angiogenic quality of autologous CD34+ cells decline with advanced age and diminished cardiovascular health.

Objective: To offset age- and health-related angiogenic declines in CD34+ cells, we explored whether the therapeutic efficacy of human CD34+ cells could be enhanced by augmenting their secretion of the known angiogenic factor, sonic hedgehog (Shh).

Methods and Results: When injected into the border zone of mice after acute myocardial infarction, Shh-modified CD34+ cells (CD34Shh) protected against ventricular dilation and cardiac functional declines associated with acute myocardial infarction. Treatment with CD34Shh also reduced infarct size and increased border zone capillary density compared with unmodified CD34 cells or cells transfected with the empty vector. CD34Shh primarily store and secrete Shh protein in exosomes and this storage process appears to be cell-type specific. In vitro analysis of exosomes derived from CD34Shh revealed that (1) exosomes transfer Shh protein to other cell types, and (2) exosomal transfer of functional Shh elicits induction of the canonical Shh signaling pathway in recipient cells.

Conclusions: Exosome-mediated delivery of Shh to ischemic myocardium represents a major mechanism explaining the observed preservation of cardiac function in mice treated with CD34Shh cells. (Circ Res. 2012; 111:312-321.)

Key Words: angiogenesis ■ myocardial infarction ■ gene therapy ■ cell therapy ■ CD34 cells

Cell-based therapies are rapidly emerging as a predominant new strategy for the treatment of various cardiovascular diseases that often involve acute or chronic ischemia. Preclinical and clinical studies have illustrated the beneficial effects of using human bone marrow–derived CD34+ stem cells to treat conditions involving myocardial ischemia including refractory angina1-2 and acute myocardial infarction (AMI).3 A major benefit of using CD34+ stem cells in human patients is that the cell source is autologous, a unique characteristic that eliminates many of the inflammatory and toxicity concerns associated with non-autologous cells and/or pharmacological therapies. Although the exact nature of their therapeutic mechanism(s) is not entirely understood, it is believed that CD34+ stem cells promote the expansion of preexisting microvasculature (angiogenesis) and/or stimulate the de novo development of vascular structures (vasculogenesis) in ischemic regions of the cardiac muscle.4 Recent evidence has identified a therapeutic paracrine mechanism of CD34+ cells as mediated in part by the secretion of extracellular, membrane-bound nanovesicles known as exosomes5 that often carry proteins, RNAs,
and/or microRNAs. Nonetheless, the use of CD34+ cells as a strategy to enhance perfusion is known to preserve and/or improve cardiac function, which may extend and improve the quality of life for the patient.

Although CD34+ cell-based therapies exhibit strong efficacy and safety profiles, the general cardiovascular health of a patient predicts both the relative availability and the therapeutic activity of the isolated cells. Health factors including smoking and alcohol abuse negatively impact circulating CD34+ cell levels. Additionally, circulating levels of CD34+ cells serve as indicators of cardiovascular outcome since densities of CD34+ cells in the circulation are commonly inversely proportional to both age and the severity of disease, indicating a natural time-dependent decrease in the angiogenic potential of CD34+ mobilized cells. These findings suggest that as cardiovascular disease worsens, autologous CD34+ cells become less capable of providing the intended therapeutic benefit. To counteract this cellular functional decline, several attempts have been made to boost the potency of autologous cells by using combinatorial therapies that codeliver known angiogenic genes and/or proteins along with stem cells. Despite these efforts, gene therapy remains a largely inefficient procedure requiring large doses of DNA in order to establish measurable target gene translation that has resulted in some phase II and phase III gene therapy trials failing to show gene-mediated therapeutic benefit.

To improve delivery of the target gene while limiting the potential for adverse effects associated with off-target responses/poor gene transfer efficiency, we attempted to enhance the angiogenic quality of CD34+ cells by genetically modifying them to express the sonic hedgehog (Shh) protein. Shh is a well-established angiogenic morphogen and is known to play important roles in cardiac development and postnatal ischemic injury recovery. Specifically, AMI is a known stimulus for the induction of Shh and its signaling components including the g-protein–coupled receptor patched1 and the downstream Gli transcription factors. Importantly, postnatal induction of the Shh pathway has been shown to be protective against the tissue damage and cell death associated with ischemia because blockade of this pathway worsens outcome after experimental ischemia in rodents.

Accordingly, this study tested the hypothesis that Shh-modified human CD34+ cells provide enhanced functional benefit in the setting of AMI and that direct cellular modification provides a means to counteract age and disease related declines in the therapeutic potency of autologous cell therapy. Furthermore, these experiments also evaluated whether exosomes derived from Shh-modified CD34+ cells take part in this process.

**Methods**

Detailed Methods are provided in the Online Data Supplement.

**Animal Models**

Mice used in this study were obtained from The Jackson Laboratories (Bar Harbor, ME), and surgical procedures and animal care protocols were approved by the Northwestern University Animal Care and Use Committee. AMI injuries were induced (described previously) in 8-week-old male nude or NOD-SCID mice. Human CD34+ cells were delivered as 2 to 10 μL injections (on either side of the ligature) and contained either (1) a subtherapeutic dose of 2.5×10⁴ (25K) cells/mouse or (2) a therapeutic dose of 5.0×10⁴ (50K) cells/mouse. Treatment groups included (1) saline (n=16); (2) 25K unmodified CD34 cells (CD34NM) (n=8); (3) 25K CD34 cells transfected with an empty vector (CD34EV) (n=7); (4) 25K CD34 cells transfected with an Shh-coding vector (CD34Shh) (n=13); (5) 25K CD34NM and 200 ng Shh protein (n=7); or (6) 50K CD34NM (n=9).

**Results**

**Expression of Shh in Modified CD34+ Cells**

After gene modification, both Shh mRNA and protein expression levels were assessed using quantitative polymerase chain reaction and ELISA, respectively. As compared with both CD34NM and CD34EV cells, CD34Shh cells exhibited substantially higher levels of Shh mRNA (Figure 1A). Importantly, mRNA levels of Shh (Figure 1A) compared between CD34EV and CD34NM cells were not different indicating that modification alone failed to influence Shh expression. In terms of Shh protein (Figure 1B), both intracellular and extracellular (secreted) compartments of CD34Shh cells expressed significantly higher levels of Shh when compared with CD34EV cells. These results indicate that gene modification of CD34+ cells with the AMAXA system is effective and resulted in enhanced cellular Shh protein translation and secretion.

Additionally, fluorescence-activated cell sorting analysis with a panel of hematopoietic stem and non–stem cell surface antigens was used to evaluate whether gene modification or the presence of excess Shh acutely affected the CD34+ stem cell antigen profile. Analysis of the antigens CD34, CD45, CD38, CD41, Lin, CD133, and CD117 failed to reveal differences in antigen expression between CD34NM, CD34EV, and CD34Shh at 24 hours after modification, indicating that neither modification nor excess Shh acutely affects the antigenic identity of the cells (Online Figure I).

**CD34Shh Significantly Enhances Preservation of Cardiac Function After AMI**

After gene modification, cells were injected into the ischemic border zone of immune-compromised mice to assess whether modification with Shh could provide superior functional preservation and/or improvement. To provide the largest possible window for detection of potential functional differ-
ences between the various treatment groups, a subthreshold, nontherapeutic dose ($2.5 \times 10^4$ cells/mouse) of CD34NM was used. This subthreshold dose failed to provide any functional benefit as compared with mice receiving only saline after AMI (Online Figure II, A). Additionally, ejection fraction (EF), fractional shortening (FS), and left ventricular end-systolic and diastolic volumes were not different when compared between saline-treated, CD34NM-treated, and CD34EV-treated mice at 4 weeks after MI (Online Figure II, A). Conversely, doubling the cell dose to $5.0 \times 10^4$ CD34NM cells did provide functional benefit over saline, indicating (1) the CD34+ cells used did possess therapeutic activity (Online Figure II, A), and (2) the functional response was dose-dependent.

Interestingly, mice treated with $2.5 \times 10^4$ CD34Shh exhibited robust improvements in both EF and FS and also displayed decreased systolic and diastolic ventricular dilation as compared with all control mice (Figure 2A through 2E). Significantly improved cardiac function induced by CD34Shh was detected as early as 1 week after AMI and persisted to post-AMI weeks 2 and 4. These findings suggest that modification with Shh improves the therapeutic potency of CD34+ cells over CD34NM.

**CD34Shh Reduce Infarct Size and Increase Border Zone Capillary Density**

After euthanasia and tissue harvest at the 4-week time point, hearts from CD34Shh-treated mice exhibited significantly reduced infarct dimensions as compared with all other treatments (Figure 3A and 3B). Additionally, using immunofluorescent detection of BS-1 lectin staining, capillary density was assessed within the infarct border zone of all hearts. Density of capillaries within the infarct border zone was significantly increased in hearts of mice treated with CD34Shh as compared with control groups (Figure 3C and 3D). Additionally, infarct size and capillary density (compared between CD34NM and CD34EV-treated mice) were not different (Online Figure II, B and C), further validating the nontherapeutic nature of the subthreshold dose of CD34NM.

**CD34Shh Exhibit Enhanced Postinjection Myocardial Retention**

To address how the delivery of Shh via CD34Shh influences cardiac function, we sought to determine if modification affects the intramyocardial retention of cells after direct cardiac injection. To evaluate this parameter, a subthreshold dose of either CD34Shh or CD34EV was injected into the ischemic border zone of hearts of NOD-SCID mice after AMI. At 1 and 5 days after cell administration, hearts were harvested, enzymatically digested, and then stained with antibodies to HLA-ABC to quantify the viable human cells remaining in the mouse heart at each time point. As seen in Figure 4, modification of CD34+ cells with Shh significantly improved the retention of live human cells in the myocardium at 24 hours after injection. In contrast, no difference between cell types was observed at 5 days after injection, perhaps reflecting a time-dependent loss of HLA antigenicity or continued clearance of cells.

**Cotherapy of CD34NM With Shh Protein Does Not Improve Cardiac Function**

To understand the mechanisms behind the functional benefits observed with CD34Shh, we explored whether canonical Shh pathway genes are induced in CD34+ cells after treatment with Shh protein or genetic modification. As seen in Figure 5A, treatment of CD34NM with recombinant Shh (100 ng/mL) for 24 hours failed to stimulate the upregulation of mRNAs of Shh pathway components. Additionally, modification also failed to induce expression changes in Shh pathway components (Figure 5B). Last, several growth factors implicated in the paracrine, angiogenic activity of stem cells were also not regulated after Shh modification (data not shown), suggesting that activation of the canonical Shh signaling pathway in CD34+ cells is unlikely to be involved in the therapeutic benefit observed with CD34Shh.

To further explore the mechanism behind CD34Shh mediated functional preservation, we assessed whether cotreatment with the subtherapeutic dose of CD34NM along with recombinant Shh protein (200 ng/mouse) could replicate the functional benefits observed with CD34Shh. As seen in Figure 5, Shh protein coadministration along with CD34NM produced no improvements in FS or EF (Figure 5C and 5D) and also failed to improve infarct size (Figure 5E) or border zone capillary density (Figure 5F). These findings may indicate that the functional preservation seen with CD34Shh reflects the therapeutic necessity of a short-term, sustained secretion of Shh or the existence of a cellular Shh delivery process that...
is insufficiently replicated by the administration of a single injection of Shh protein.

**CD34<sup>Shh</sup> Specifically Package Overexpressed Shh in Secreted Exosomes**

Exosomes derived from CD34<sup>NM</sup> were recently characterized and have been shown to mediate a significant component of the angiogenic capacity of these cells. To assess the nature by which Shh was being secreted by CD34<sup>Shh</sup>, established exosomal purification methodologies were used to determine whether the exosomal fraction of CD34<sup>Shh</sup> conditioned media was involved. After purification (Figure 6A), it was determined that a significant fraction of the Shh protein secreted from CD34<sup>Shh</sup> was found to be associated with small, membrane-enclosed vesicles known as exosomes (Figure 6B). Modification of equal numbers of other cell types with Shh, such as human umbilical vein endothelial cells (HUVECs) or HeLa cells, failed to show substantial exosomal Shh deposition, indicating that this storage process appears to be largely specific to CD34<sup>+</sup> cells (Figure 6B). Although CD34<sup>Shh</sup> secrete slightly less absolute total Shh protein (Figure 6Bi) than other modified cells types, a far greater proportion of the total Shh secreted by CD34<sup>Shh</sup> exists in the form of exosomal Shh (Figure 6Bii). Supplementation of CD34<sup>NM</sup> with recombinant Shh (50 ng/mL) failed to result in exosomal deposition of the protein, indicating that genetic modification of CD34<sup>+</sup> cells is required to exploit any potential therapeutic benefit of exosome-mediated Shh delivery.

To determine whether Shh-containing exosomes derived from CD34<sup>Shh</sup> could be mediating the observed functional benefits seen in the in vivo AMI model, we assessed whether exosomes derived from modified CD34<sup>+</sup> cells could interact with another cell type. HUVECs were chosen as the recipient cell in this experiment for 2 specific reasons, including (1) the well-established importance of endothelial cell signaling and function in Shh-mediated angiogenesis and (2) the need for a recipient cell that fails...
to express measurable levels of Shh protein. After treatment of starved HUVECs with CD34\(^{Shh}\) derived exosomes for 16 hours, Shh ELISA was used to confirm that exosomes effectively transferred their Shh protein cargo to the recipient HUVECs (Online Figure III, A).

Last, we assessed whether exosome-mediated transfer of Shh could functionally influence recipient cells. To do this, luciferase activity in mouse embryonic (NIH3T3) fibroblasts cotransfected with both Gli-luciferase and β-galactosidase vectors was assessed after treatment with CD34\(^{Shh}\)-derived exosomes. Gli-reporter and β-gal transfected fibroblasts were treated with exosomes isolated from CD34\(^{Shh}\) for 16 hours. Fibroblasts treated with exosomes derived from CD34\(^{Shh}\) generated a modest increase in luciferase activity (Online Figure III, B), indicating that Shh protein transfer via exosomes produced measurable signaling events in recipient cells.

**Discussion**

Although stem cells continue to emerge as another tool within the therapeutic armamentarium available for addressing various cardiovascular disorders involving ischemia, their use comes with unique challenges not generally observed with...
standard pharmacological therapies. Perhaps the principal challenge to their adoption as standard therapy relates to the current inability to normalize their inherent reparative abilities across all patients, since individuals and their comorbidities vary immensely. Patient diversity in disease burden, age, and environmental factors alters the availability and therapeutic efficacy of isolated CD34+ cells.10–15 Given this serious and complex problem, we determined whether the therapeutic efficacy of CD34+ cells could be improved on by modifying them to express an established angiogenic protein, sonic hedgehog, to circumvent age and health related declines in CD34+ cell function. The use of the AMI model for these studies was directed by our previous success using autologous CD34+ cells for the treatment of refractory angina in human patients.1

As presented in this report, CD34Shh were observed to improve functional preservation of cardiac tissue as compared with control cells. Modification with Shh enhanced the therapeutic potency beyond that observed with a subtherapeutic dose (ie, 2.5×10^4 cells) of CD34NM, indicating that CD34Shh could theoretically be used at lower doses than would be possible in their conventional form. This finding is significant given the problems associated with utilizing cell based therapies in poorly mobilizing patient populations such as those with ischemic12,20 or diabetic30,31 conditions. The finding that treatment with CD34Shh induces robust increases in capillary development within the infarct border zone, accompanied by reduced infarct sizes, indicates that Shh-mediated angiogenesis is the likely mechanism for the observed functional improvements, although Shh-mediated transdifferentiation of resident fibroblasts to endothelial cells may also play a role. Given the abundance of information regarding the involvement of Shh-mediated repair of ischemic tissues,24,25,32,33 it is not surprising that cardiac function was preserved in mice treated with CD34Shh. However, these findings do provide a number of novel insights, including (1) short-term secretion of Shh appears to be required for functional benefits to occur, (2) modification with Shh improves the short-term retention of CD34+ cells, (3) CD34Shh deposit a proportionately greater amount of Shh in exosomes as compared with other Shh-modified cell types, (4) Shh-containing exosomes derived from CD34Shh are capable of transferring Shh to other cell types, and (5) exosomes containing Shh activate Shh signaling pathways in other cell types.

The observation that functional improvement requires sustained Shh secretion is derived from the finding that coadministration of a single dose of Shh protein along with CD34NM failed to replicate the in vivo functional benefits seen with CD34Shh. These results suggest that although the injected cells appear to be of low abundance in the ischemic myocardium, their presence for at least 5 days after injection allows for the sustained secretion of Shh, thus greatly enhancing the propensity for Shh-mediated angiogenic signaling. Recombinant Shh has a serum half-life of approximately 1 hour although chemical modification of these proteins can improve the half-life upwards to 12 hours with equal or greater potency.34 It remains to be determined whether (1) direct injection of modified forms of Shh or (2) modification of CD34+ cells with vectors expressing the
improved Shh variants would provide even greater functional benefit than seen here. Nonetheless, the evidence presented here for improved cardiac function with CD34Shh (and not for CD34NM plus Shh protein) indicates that Shh-mediated angiogenic signaling events that occur between days 1 to 5 after cell injection are critical for the detection of improved functional outcomes.

This argument is strengthened when considering that CD34Shh were retained within the heart after local injection to a greater extent than control cells. Although improved retention of CD34Shh cells was not resolved at day 5, a number of factors may be influencing the detection of retained cells at this time point, including (1) a loss of HLA-ABC antigenicity, (2) the general clearance of the cells over the 5-day time period, or (3) cell death. Given the difficulties of using immunohistochemical methods to quantify viable exogenous cells within cardiac tissue, we thought that enzymatic digestion of the whole heart followed by FACS analysis was a comprehensive method to quantify retention. Unfortunately, enzymatic digestion of the heart would be independently expected to cause cell death as well as potential antigen loss, suggesting that our results probably reflect an underestimate of the actual number of CD34+ cells retained in both conditions and at both time points. Further supporting the claim that sustained secretion of paracrine factors is required for the observed functional benefits of CD34Shh cells, is an elegant report that utilized programmed apoptosis of endothelial progenitor cells (EPCs) after cardiac injection to show that the presence of EPCs weeks after injection continues to contribute to the sustained improvements in cardiac function attributable to EPC function.35

One could also speculate that Shh-modification of CD34+ cells may affect adherence of cells within cardiac tissue by regulating expression of adhesion proteins and could be addressed in future experiments via cellular protein profiling. Another potential avenue of examination includes determining whether local Shh signaling processes alter the cell surface landscape of resident cardiac cells to assist in retaining therapeutic exogenous cells within that specific location. Along these lines, attempts to determine whether Shh treatment (ie, protein treatment or Shh-modification) acutely influenced CD34+ cells revealed no induction of endogenous Shh pathway components (Figure 5A and 5B) or the differentiation capacity of CD34+ cells (Online Figure I). Analysis at later time points for both assays may reveal more obvious long term effects not seen at the time points assessed in this study. With the recent characterization of a noncanonical Shh signaling pathway in endothelial cells,36,37 it is conceivable that the direct action of Shh on CD34+ cells may also utilize this pathway. Nonetheless, delivery of CD34Shh within 24 hours of modification was clearly sufficient to provide therapeutic benefit in vivo.

Together, these results indicate that CD34Shh acted primarily as a secretory vehicle to deliver Shh to the ischemic tissue...
they were injected into. Although an autocrine effect of Shh on CD34<sup>Shh</sup> was not observed (as has been seen in Shh-modified mesenchymal stem cells<sup>38</sup>), both modified stem cell types provide significant protection against functional deficits associated with AMI. Given our laboratory’s recent discovery that CD34+ cells actively secrete exosomes as a major component of their paracrine-mediated reparative abilities,<sup>5</sup> we explored whether the overexpression of Shh in modified CD34<sup>Shh</sup> would allow us to determine (1) whether CD34<sup>Shh</sup> selectively package Shh into exosomes and (2) whether this mechanism had any functional implications for Shh signaling in other cell types.

Fortunately, the gene modification approach allowed for the elucidation of exosomal mechanisms that, to this point, have been difficult to observe owing to their extremely small cargo load. We determined that CD34<sup>Shh</sup> divert approximately ∼25% of their secreted Shh into exosomes and that this process appears to be cell type–selective, given that other Shh-modified cell types such as HUVECs and HeLa cells secrete Shh almost exclusively as free protein. The observed exosomal deposition of Shh in CD34<sup>Shh</sup> does not appear to result from excess protein load, given that the absolute amount of protein secreted by the other cell types was generally higher. It should also be mentioned that exposing CD34<sup>NM</sup> to Shh protein did not result in its exosomal deposition, which reinforces the notion that CD34+ cells must be genetically modified in order to exploit therapeutic exosomal mechanisms.

Despite our knowledge that stem cell and cancer cell–derived exosomes possess angiogenic abilities independent of the cell type they are derived from,<sup>5,39–42</sup> the mechanistic basis of their action is still under investigation. Our finding that exosomes derived from CD34<sup>Shh</sup> physically transfer Shh to other cell types adds to this knowledge. Although emerging evidence is suggesting that exosomes also harbor mRNA and/or miRNAs that can be transferred to other cell types where they mediate expression changes in target cells,<sup>43,44</sup> to our knowledge, no report currently exists that depicts the ability of CD34+ cell-derived exosomes to transfer functional protein. Presumably, failure to observe exosomal–mediated protein transfer is derived from the difficulty associated with detecting low abundance proteins; however, the genetic modification procedure utilized here has allowed for the observation of this process. Although the possibility exists that Shh-containing exosomes were simply adhering to HUVECs, our methodology, which included multiple vigorous cell washes followed by centrifugation, indicates that any outer cell membrane adherence that did occur was apparently a physiologically relevant association.

Our observation that exosomal Shh also activated the Shh signaling pathway in fibroblasts, evidenced by upregulation of Gli-luciferase activity in NIH3T3 fibroblasts, supports the belief that exosomes promote meaningful communication between cells. Although the luciferase response induced by CD34<sup>Shh</sup> exosomes was modest as compared with free recombinant protein (see Online Figure III, B), the absolute level of the response was actually very robust when compared with the amount of Shh protein actually found in CD34<sup>Shh</sup> exosomes (see Online Figure III, A). This finding suggests that exosome-mediated activation of cell signaling may actually be more efficient than that caused by free protein. Because exosomes are membrane bound vesicles, they would be expected to protect their cargo from destructive extracellular proteases and thereby enhance cell signaling events. From a clinical standpoint, the delivery of exosomes may offer several advantages over cell delivery, a few of which include (1) the ability to deliver a treatment that may possess a higher therapeutic potency than modified cells, (2) the avoidance of triggering potentially damaging cell clearance mechanisms after injection, and (3) potential “expansion” of therapeutic exosomes via short-term culturing of isolated CD34+ cells. Current work in the laboratory is focused on further investigation of the mechanistic basis by which CD34+ cell–derived exosomes generate in vivo angiogenic responses in hind limb and myocardial ischemia models. As our knowledge regarding these interesting cellular components expands, it is likely that the true nature of their activity will resemble a combination of multiple mechanisms that will be regulated both contextually and temporally.

Acknowledgments

We thank K. Krueger for administrative assistance and D. Motlagh at Baxter Healthcare for providing the CD34+ cells used in these studies.

Sources of Funding

This study was supported in part by grants from the National Institutes of Health (HL-53354, HL-77428, HL-63414, HL-80137, HL95874, HLPO1-108795, HL-57516, HL-91983, and HL-105597).

Disclosures

D.L. is an employee of Baxter Healthcare.

References


Skog J, Wurdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, Curry WT Jr, Carter BS, Krichevsky AM, Breakefield XO. Glio-

320 Circulation Research July 20, 2012


### Novelty and Significance

**What Is Known?**

- CD34+ cells are known to stimulate therapeutic neovascularization in preclinical studies as well as in phase I and II human clinical trials, and the potency of CD34+ cells is greater than for unselected mononuclear cells.
- Sonic hedgehog (Shh) is a well-established angiogenic morphogen capable of stimulating postnatal angiogenesis after an acute ischemic insult.
- Both the number of CD34+ cells and their inherent angiogenic functionality are diminished in patients of increasing age and cardiovascular disease burden.
- Exosomes are small, membrane-bound vesicles secreted from many cell types that harbor protein and nucleic acids and have been implicated in cell-to-cell signaling mechanisms.

**What New Information Does This Article Contribute?**

- Genetic modification of CD34+ cells with Shh imparts therapeutic efficacy to a previously determined subtherapeutic dose of nonmodified CD34+ cells after myocardial infarction.
- When administered directly to ischemic myocardium, Shh-modified CD34+ cells significantly reduce both the negative anatomic and functional impact of an acute myocardial infarction in comparison with nonmodified and control modified cells.
- The CD34+ cells selectively deposit functional Shh protein into exosomes that can (1) be taken up by other cell types and (2) induce Shh signaling in other cells.

The therapeutic functionality and the yield of autologous CD34+ cells are reduced in the aged and those with significant cardiovascular disease. Therefore, improvement of current cell-based therapies is an important area of future study. We found that the in vivo therapeutic efficacy of CD34+ cells could be improved by genetic modification with the angiogenic morphogen Shh. In comparison with other exosome-secreting cell types such as HUVECs or HeLa cells, CD34+ cells appear to preferentially store Shh in exosomes. Additionally, Shh-containing exosomes, isolated from the culture media of genetically modified CD34+ cells, physically transfer exosomal Shh to endothelial cells and can also induce Shh-specific signaling events in cultured fibroblasts. These results indicate that Shh gene modification may prove beneficial in the further development of CD34+ cell/exosomal–based therapies for the treatment of ischemic disorders.
Sonic Hedgehog–Modified Human CD34+ Cells Preserve Cardiac Function After Acute Myocardial Infarction

Circ Res. 2012;111:312-321; originally published online May 10, 2012;
doi: 10.1161/CIRCRESAHA.112.266015
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/111/3/312

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2012/05/10/CIRCRESAHA.112.266015.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Supplemental Material

Supplemental Methods

Cell Isolation: CD34+ cells were isolated from healthy volunteers in collaboration with Baxter Healthcare Inc., (Deerfield, IL). Briefly, volunteers underwent mobilization of bone-marrow derived CD34+ cells with a short course of once daily injections of granulocyte-colony stimulating factor (5µg/kg per day) for five days. Leukoapheresis was then performed on the fifth day for the collection of mononuclear cells. The CD34+ fraction was then purified using a commercially available device (Isolex 300i, Baxter Healthcare, Deerfield IL) according to the manufacturer’s instructions.

CD34+ Cell Culture: Upon receiving purified CD34+ cells, the cells were cultured overnight in X-Vivo 10 culture media (Lonza, Walkersville, MD) supplemented with 50 ng/ml vascular endothelial growth factor, 50 ng/ml Flt-3 ligand, 20 ng/ml stem cell factor and 10 ng/ml thrombopoetin (PeproTech, Rocky Hill, NJ) and 0.25% human serum albumin (Baxter Healthcare, Deerfield, IL) at a density of one million cells/ml of media. All experiments involving CD34+ cells including exosome production and mRNA analysis were conducted in the complete media described above.

CD34+ Cell Nuclefection: Following the overnight recovery culture, CD34+ cells were nucleofected with the AMAXA nucleofector II device using a specific CD34+ cell AMAXA kit (Lonza, Walkersville, MD) as per the manufacturers recommendation protocol. The plasmid encoding human Shh was prepared as described previously. The coding sequence for the biologically active, 600-bp, amino-terminal domain of human Shh was
inserted into a mammalian expression vector pCMV-Script PCR (Stratagene, Santa Clara, CA); with the final Shh construct containing 4,878 bp. The empty expression vector was used as a control (denoted as empty vector). Cells were modified in batches of 3 million cells per reaction (per cuvette) and received 1µg of either the empty vector control plasmid (pCMV-Script) or pCMV-Script-Shh (Agilent Technologies, Santa Clara, CA) per million cells. Nucleofection efficiency was determined to be approximately 50-60% as determined using the pMAX-GFP vector (supplied by Lonza) and the subsequent detection of GFP+ cells via FACS analysis at 24 hours post-nucleofection (data not shown) which is in agreement with a previous report.\(^2\) Immediately following nucleofection, CD34+ cells were cultured in growth factor-supplemented CD34+ cell media (see above) overnight and were used for experiments the following day. For the purposes of data presentation, the following designations will be used when discussing the various modified CD34+ cell types. CD34\(^{Shh}\) will designate cells modified with the Shh plasmid listed above, CD34\(^{EV}\) will designate control cells modified with the only the empty vector plasmid listed above, CD34\(^{NM}\) will designate non-transfected cells while CD34\(^{NM}\) + Shh will indicate treatments that consisted of both CD34\(^{NM}\) cells and recombinant Shh protein.

**Animal Models:** All mice used in this study were obtained from The Jackson Laboratories (Bar Harbor, ME). All surgical procedures and animal care protocols were approved by the Northwestern University Animal Care and Use Committee. Acute myocardial infarction (AMI) injuries were induced in 8-week old male nude/J (cardiac
functional analysis study) or 8-week old male NOD-SCID mice (intra-myocardial CD34+ cell retention analysis).

Induction of Acute Myocardial Infarction and Myocardial CD34+ Cell Injections: AMI was induced as described previously. Briefly, mice were anesthetized, orally intubated and placed in a supine position. Respiration was controlled by mechanical ventilation using a rodent ventilator (Nemi Scientific, Inc., Framingham, MA) with tidal volume set to 0.4 ml at a rate of 110 strokes/min. The chest was then shaved, cleaned free of hair and sterilized. Under a dissecting microscope, a left thoracotomy was performed in the fourth intercostal space. After displacing the pericardium, an 8-0 monofilament nylon suture on a curved tapered needle was passed under the left anterior descending coronary artery (LAD) 4 mm below the left atrium and permanently tied to eliminate blood flow distal to the suture. Following verification of induced ischemia via epicardial blanching, CD34+ cells were re-suspended in sterile PBS and injected into the infarct border zone. Cells were delivered as two separate 10 µl injections (one on either side of the ligation) and contained either; 1) a previously determined sub-therapeutic dose of 2.5x10^4 cells/mouse, or 2) a therapeutic dose of 5.0x10^4 cells/mouse. Following injection of cells, the pericardium was re-draped over the heart, and the chest was then closed. A 22 gauge syringe was used to re-establish negative pressure within the chest cavity prior to extubation. Animals received post-surgical pain management with buprenorphine and surgical inflammation control with meloxicam. Animals were recovered until freely mobile on a heating pad at which point they were then placed into a clean cage and housed for the duration of the experiment.
Physiological Assessments of Left-Ventricular Function: Trans-thoracic 2-dimensional echocardiographic measurements were performed with a commercially available high-resolution echocardiographic system (VEVO 770™, VisualSonics Inc., Toronto, Canada) equipped with a 30-MHz transducer. Echocardiographic analysis was performed before AMI (baseline) and at 7, 14 and 28 days post-AMI on mice anesthetized with a mixture of 1.5% isoflurane and oxygen (1 L/min). M-mode tracings were used to measure LV wall thickness, end-systolic diameter (LVESD), and end-diastolic diameter (LVEDD). Systolic and diastolic left-ventricular areas were determined by M-mode in long-axis configuration and fractional shortening (FS) was measured at the mid-ventricular level. The left-ventricular chamber volumes in diastole and systole were derived from their respective measured 2D areas using a LV volume algorithm within the Vevo770 echo software. Cardiac ejection fraction was determined offline by the equation: $EF = (\text{Diastolic Volume} - \text{Systolic Volume} / \text{Diastolic Volume}) \times 100$.

Harvest of Cardiac Tissue, Histology and Immunofluorescent Assesments: All hearts were harvested after the 28 day echocardiographic analysis as described previously. To prepare paraffin-embedded sections, the abdominal aorta was cannulated with a polyethylene catheter containing phosphate buffered saline (PBS, 0.2 M, pH 7.4). Immediately after the cannulation, 1 mL of PBS/heparin solution was injected, and the heart was arrested in the diastolic phase by injecting 0.15 mL of 100 mM cadmium chloride through the catheter. The thorax was then opened and the heart was perfused at mean arterial pressure with methanol, and the right atrium was cut to allow drainage.
A 25G ¾" Vacutainer needle (Becton Dickinson, Franklin Lakes, NJ) was inserted into the left ventricle, and fixative was delivered at a pressure equal to the left-ventricular end-diastolic pressure. At the end of the procedure, whole heart was dissected from the body at the ascending aorta, and then embedded in paraffin and cut into sections.

**Infarct Size Determination:** All hearts were sectioned starting from the height of the suture and then sequentially at 250µm distances below the suture as far as effective sectioning would permit. Infarct size was evaluated on Masson-trichrome stained heart sections cut 1000µm below the ligation point with a computerized digital image analysis system (ImageJ: NIH image) and the transmural infarct perimeter was then assessed as a percentage of the entire LV chamber perimeter.

**Capillary Density Analysis:** Capillaries were identified by injecting mice with BS-1 lectin (Vector Laboratories, Burlingame, CA) ten minutes prior to sacrifice. Subsequent staining of sections included a goat anti-lectin primary antibody (Vector Laboratories) and Alexa 555-conjugated donkey anti-goat IgG secondary antibody (Invitrogen, Carlsbad, CA, USA). Sections were counter-stained with DAPI to allow quantification of the cell number per high power field. Slides were imaged using fluorescent microscopy (Zeiss) and capillary density was evaluated by counting positively stained tubular structures within the infarct border zone in sections 1000µm below the ligation point in all hearts. Three high power fields (20X) were analyzed in each section from three independent sections (nine images per animal).

**Standard Cell Culture:** HUVECs were cultured in endothelial complete medium-
2 (EGM™-2 MV) (Cambrex Corporation, Charles City, IA) and were used for experiments at passages 2-4. HUVEC starvation media was composed of EBM-2 media (Lonza, Walkersville, MD) supplemented with 0.25% BSA and 100 U/mL streptomycin/penicillin. NIH 3T3 embryonic fibroblasts (ATCC, Manassas, VA) were maintained in 4.5 g/L glucose containing Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% calf serum, 100 U/mL streptomycin/penicillin, 2 mmol/L-glutamine. NIH 3T3 fibroblast starvation media was composed of DMEM with only 100 U/mL streptomycin/penicillin and 2 mmol/L-glutamine.

Quantitative Real Time PCR (qRT-PCR): RNA was isolated from modified and Shh treated CD34+ cells cultured under complete media conditions with RNA STAT-60 (TELTEST Electronics Labs Inc, Austin, TX, USA) according to the manufacturer’s instructions. Total RNA was reverse transcribed with a Taqman cDNA Synthesis Kit (Applied Biosystems, Foster City, CA, USA) and amplification was performed with a Taqman 7500 (Applied Biosystems). All real time RT-PCR primer and probe sequences can be found in Online Table I. The relative expression of each mRNA was calculated by the comparative threshold cycle (C\text{\textsubscript{T}}) method and normalized to 18S expression.

ELISA: Detection of Shh protein in all cells types, exosomes and conditioned media was performed using a human Shh-N ELISA Kit (Raybiotech, Norcross, GA) as per the manufacturers’ recommended protocol.

Modified CD34+ Cell Myocardial Retention Study: 2.5\times10^4 CD34\textsuperscript{Shh} or CD34\textsuperscript{EV} were intramyocardially injected into NOD-SCID mice post-AMI as described above. At post-
AMI days 1 and 5, hearts were harvested from mice and washed with ice cold saline to remove all blood. Rinsed hearts were then minced to 1 mm³ pieces and placed in 5 ml of collagenase (2 mg/ml) for 30 minutes at 37°C. Using a 30 cc syringe attached to a sterile 14 gauge cannula, the suspension was then fully triturated. The triturated suspension was then passed through a 70 µm disposable cell strainer and then spun down at 400 g for 8 minutes at 4°C to pellet all cells. Cells were then subjected to staining for FACS analysis. An aliquot of cells resulting from the heart tissue digest, as well as 25,000 CD34+ cells, were pre-treated with FcR-blocking reagent (Miltenyi Biotec) and stained with anti-human anti-HLA-ABC, R-PE (Clone G46-2.6, BD Biosciences). Isotype-matched IgG antibody (BioLegend) was used as negative control. Data were acquired on the CyAn ADP (Beckman Coulter) flow cytometer. DAPI staining was used to gate dead cells. A defined number of flow cytometric counting beads (AbD Serotec) were added to each sample to allow for the determination of the absolute total number of HLA-ABC positive cells in each digested heart sample. The number of CD34+ cells retained in the heart as assessed by FACS was then converted to a percentage based on the initial number of cells injected into the heart (i.e. 2.5x10⁴ CD34Shh or CD34EV). All cell counts were derived from the FACS analysis of n=3 hearts for each condition (CD34Shh and CD34EV) and at each time point (post-AMI days 1 and 5).

Flow Cytometry: CD34+ cells were stained with Lineage-3, CD34-FITC (both BD Pharmingen Inc.), CD133-APC (Miltenyi Biotec), CD117-PC7, CD45-ECD, CD34-PC7 or -ECD, CD38-APC (IOTest). Isotype-matched IgG antibodies (BioLegend) were used as negative controls. Data were acquired on a CyAn ADP (Beckman Coulter) flow
cytometer. 50,000 events were counted per sample. DAPI staining was used to gate dead cells. Data were analyzed using Flow-Jo Software (Tree Star, Inc.).

**Exosome Purification:** Exosomes were isolated from CD34+, HUVEC and HeLa cells using a previously published ultracentrifugation protocol. Briefly, cells were pelleted by centrifugation at 400g for 15 minutes at which point the supernatant containing the exosomes was transferred to an ultracentrifuge tube. Samples were then centrifuged at 14,000g (~14000rpm) for 30 min at 4°C using a Beckman ultracentrifuge equipped with a Type 70 Ti rotor. The supernatant was then extracted and layered on top of 4 ml of 30% sucrose/D$_2$O (pH = 7.4) and centrifuged at 100,000g (37,000 rpm) for 60 min at 4°C. The top layer, including the blurry interface, was then aspirated. The remaining supernatant (approximately 3.5 ml) was then transferred to a new tube and re-suspended in 20 ml of PBS and centrifuged once more at 100,000g for 60 min at 4°C. The remaining supernatant was then aspirated leaving only the exosomal pellet. The pellet was then re-suspended in media according to which experiment was to be performed.

**Electron Microscopy:** Exosomes from CD34$^{\text{Shh}}$ cells were stained for electron microscopy following published methods. Briefly, the isolated exosomes were fixed with paraformaldehyde and deposited onto Formvar-carbon coated grids. The grids were washed with PBS, post-fixed with glutaraldehyde, then washed with water, and contrasted with uranyl oxalate. The samples were then embedded in a mixture of
methyl cellulose and uranyl acetate. Exosomes were examined with an FEI Tecnai Spirit G2 120 kV Transmission Electron Microscope.

**Transfer of Exosomal Shh to HUVECs:** HUVECs were plated at 50% confluency in 1.9 cm² wells one day before the experiment. The HUVECs were then starved in EBM-2 media supplemented with only 0.25% BSA and 100 U/mL streptomycin/penicillin for 8 hours prior to exosome treatment. Exosomes were prepared from CD34+ cells as described above and re-suspended in HUVEC starvation medium. The exosome samples were then added to wells of HUVECs (70-80% confluent in a 24 well plate) for 16 hours under normal cell culture conditions. After the incubation period, each well of cells was washed 3 times with 1 ml of PBS to remove any remaining exosomes. Each well was then scraped and cells were collected in ice cold PBS and centrifuged at 500g for 10 minutes at 4°C. The pellets were then solubilized in 40µl of lysis buffer (Pierce, Rockford, IL) supplemented with 1:200 protease inhibitor cocktail (Calbiochem, Gibbstown, NJ) and frozen at -80°C until detection of Shh protein via ELISA. Purified Shh protein (R&D Systems, Minneapolis, MN) added directly to the cells was used as the positive control whereas starvation media containing no exosomes served as the negative control.

**Induction of Gli transcriptional activity in 3T3 Fibroblasts:** NIH3T3 fibroblasts were seeded in 1.9 cm² wells (3.0x10⁴ cells/well) and one day later, transfected using Lipofectamine™ transfection reagent (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer’s instructions. Cells were co-transfected with 1.0 µg Gli-BS luciferase plasmid and 1.0 µg RSV-βgal plasmid. One day after transfection, cells were
starved in 0% serum media for 16 hours prior to treatment with purified exosomes. Exosomes were prepared from CD34Shh, CD34Ev and non-modified CD34+ cells as described above and were then re-suspended in 3T3 fibroblast starvation medium. The exosome samples were then added to wells of transfected 3T3 cells (70-80% confluent in a 24 well plate) for 16 hours under standard cell culture conditions. Purified Shh protein (R&D Systems, Minneapolis, MN) added directly to the cells was used as the positive control whereas starvation media served as the negative control. After the incubation period, each well of cells was washed 3 times with 1 ml of PBS. Each well was then scraped and cells were collected in ice cold PBS and centrifuged at 500g for 10 minutes at 4°C. The pellets were then solubilized in 50µl of 1X RLB lysis buffer (Promega, Madison, WI) supplemented with protease inhibitor cocktail (Calbiochem) and frozen at -80°C. Luciferase activity was assayed with a Luciferase assay system (Promega) and β-galactosidase activity was assayed as previously described. For each sample, luciferase activity was normalized to β-galactosidase activity to compensate for differences in transfection efficiency. Each condition was assayed in triplicate, and each experiment was performed at least two times. The Gli-BS luciferase plasmid was kindly provided by Dr H. Sasaki.

**Statistical Analysis:** All values are expressed as mean ± SEM, and a P value less than 0.05 was considered statistically significant. For comparisons between two groups, statistical significance was evaluated with an unpaired t-test. Comparisons among 3 or 4 groups were assessed by a one-factor analysis of variance (ANOVA) followed by the Holm-Sidak test when ANOVA P<0.05. Comparisons for measurements taken at
multiple time points were assessed by a two-way repeated measures ANOVA in order to assess differences related to both treatments (mice receiving saline, CD34^{EV}, CD34^{Shh} etc..) and time periods (baseline, day 7, day 14, day 28) followed by the Holm-Sidak post-hoc test.
References


Online Supplement Figure Legends

Online Figure I: FACS Analysis of Cellular Markers in Non-Modified (CD34\textsuperscript{NM}), or Modified CD34+ Cells. Modified (CD34\textsuperscript{Shh} and CD34\textsuperscript{EV}) and naïve cells (CD34\textsuperscript{NM}) were subjected to cell antigen analysis via FACS to determine whether the modification procedure was altering the short-term capacity of CD34+ cells to maintain expression of their stem cell antigens. Shown are representative examples of cultured CD34\textsuperscript{NM}, CD34\textsuperscript{EV} and CD34\textsuperscript{Shh} showing no change in the populational proportions of cells that express various stem cell and lineage markers at 24 hours post-modification. These images represent one of three independent experiments, all of which revealed similar findings.

Online Figure II: Validation of the sub-therapeutic CD34+ cell dose threshold. A. When intra-myocardially injected with 2.5x10\textsuperscript{4} CD34\textsuperscript{NM}, mice fail to display improvements in ejection fraction and fractional shortening as compared to saline treated mice. Conversely, mice injected with 5.0x10\textsuperscript{4} CD34\textsuperscript{NM} cells do show protection against losses in function at 4 weeks post-AMI. Both infarct size (depicted in B) and capillary density (depicted in C) are also not influenced by the sub-threshold 2.5x10\textsuperscript{4} CD34\textsuperscript{NM} dose. Bars on all graphs represent the group means ± SE. * represents p<0.05 assessed with a one-way ANOVA and the post-hoc Holm-Sidak test.

Online Figure III: CD34\textsuperscript{Shh} Produce Shh-containing Exosomes that then Physically Transfer Shh to Other Cell Types and Promote Shh Signaling. A. Treatment of
HUVECs with exosomes derived from CD34^{Shh} (CD34^{Shh} Ex) for 16 hours results in Shh protein transfer into HUVECS as assessed by Shh ELISA. B. Treatment of NIH3T3 cells (previously transfected with Gli-luciferase and β-galactosidase vectors) with exosomes derived from CD34^{Shh} (CD34^{Shh} Ex) for 16 hours results in enhanced induction of luciferase activity as compared to cells treated with exosomes from CD34^{EV}. Bars for both A and B depict replicate means ± SEM and are representative examples of at least 2 independent experiments.
### Online Table I

Human Primer and Probe Sequences for Real Time Quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shh</td>
<td>CGGCTTCGACTGGGTGTACT</td>
<td>GCAGCCTCCCCGATTTGG</td>
<td>CTCAGAGGTGTAAGGAC</td>
</tr>
<tr>
<td>Smo</td>
<td>CCTTTTGGCCATGTTGGAA</td>
<td>CCAGTACGCTCCAGATGA</td>
<td>TGGCATCGCCCATGAGCACCTG</td>
</tr>
<tr>
<td>Ptc1</td>
<td>CTGCCCAACAAAGTGTCACT</td>
<td>GATTCGGGATGGACCACAGT</td>
<td>AAGCCACAGAAACCCCCGTCCTTCCG</td>
</tr>
<tr>
<td>Gli1</td>
<td>TCGGACCATCCATTTCTA</td>
<td>TCAGTCTGCTTCTCCCTTGAT</td>
<td>CCTTCCGCTTCTGTTGGGCT</td>
</tr>
<tr>
<td>18S</td>
<td>ACGAGACTCTGGCATGCTAACTAGT</td>
<td>CCACCTTGTCCCTAAGAA</td>
<td>ACGCGACCCCGACGCGT</td>
</tr>
</tbody>
</table>
Online Figure I

A  B  C

CD34  CD117  CD45

D  E  F

Lineage  CD133  CD41

G

CD38

<table>
<thead>
<tr>
<th>Red</th>
<th>Blue</th>
<th>Green</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34\text{NM}</td>
<td>CD34\text{EV}</td>
<td>CD34\text{Shh}</td>
</tr>
</tbody>
</table>
Online Figure II

A

**Fractional Shortening %**

- **Saline (N=16)**
- **25K CD34NM (N=8)**
- **25K CD34LV (N=7)**
- **50K CD34NM (N=9)**

**Ejection Fraction %**

B

**Infarct Size (% of LV Circumference)**

- **Saline**
- **CD34NM**

C

**Counts/HPF**

- **Capillaries**
- **Nuclei**

- **Saline (N=16)**
- **25K CD34NM (N=8)**
Online Figure III

A

Shh (pg of protein)

CD34/SH Ex  Control  1  10  100

B

Lucifere Activity (Normalized)

CD34/SH Ex  Control  1  10  100

Shh (ng/ml)