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CD34 positive cells isolated from traumatized human skeletal muscle require the CD34 protein for multi-potential differentiation

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Abstract

The CD34 protein is regarded as a marker of stem cells from multiple origins. Recently a mesenchymal progenitor CD34 positive cell identified from traumatized human skeletal muscle demonstrates differentiation capability into vascular endothelial cells, osteoblasts and adipocytes. Here they were treated with a small inhibitory RNA for CD34, which significantly reduced the cellular level of the CD34 protein. These treated cells had a reduced capacity to proliferate, and migrate. They were both unable to differentiate down multiple pathways and to undergo vascular endothelial differentiation as reflected by a lack of expression of VE cadherin, Tie 2 and CD31. Additionally the cells were unable to form tube-like structures in an endothelial tube assay. These treated cells were also unable to undergo osteogenesis, as revealed by lack of alizarin red and alkaline phosphatase staining and were unable to undergo adipogenesis as revealed by lack of oil red O staining. Finally, when CD34 was expressed in cells lacking this protein, the cells were able to undergo vascular endothelial differentiation as revealed by expression of Tie2, VE-cadherin and CD31. These data indicate that in cells derived from traumatized muscle the CD34 protein is required for enhanced proliferation, migration and differentiation down multiple pathways.

Introduction

Following musculoskeletal trauma, a wound healing/tissue regeneration response is rapidly set in motion^{1, 2}. Depending on the type of trauma, a number of outcomes ensue^{1, 2}. In war veterans that have been exposed to a blast injury to an extremity, for example, an

Author Contributions

Conceived and designed the experiments: GEW KMW. Performed the experiments: GEW KMW. Analyzed the data: GEW KMW. Contributed reagents/materials/analysis tools: GEW KMW. Wrote the paper: GEW KMW.

Credit Author Statement

Karen M. Wolcott: Methodology, Software, Validation, Formal Analysis, Investigation, Resources.

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Competing financial interests

The author(s) declare no competing financial interests.

inappropriate wound healing response arises that very often leads to bone formation in soft tissues³. This heterotopic ossification (HO) is a poorly understood complication that is evident in a majority of current wounded veterans³. HO also occurs to a much lesser extent following joint replacement therapy⁴ and following spinal cord and traumatic brain injuries⁵. In order to better understand the underlying pathology of HO, it is critical that we identify the cell types involved in bone formation⁶. A variety of distinct cell populations reside within the soft tissue component of these wounds (i.e. traumatized muscle), which could play a role in bone formation. It seems likely that endothelial cells⁷, fibrocytes^{8, 9}, vascular smooth muscle¹⁰, mesenchymal stem cells^{11, 12}, and fibroblasts^{13–15} have the greatest potential for forming bone due to their capacities to differentiate. In fact evidence shows that vascular endothelial cells (VECs) are able to differentiate into osteoblasts in vivo in an animal model of HO^{16, 17}, which provides compelling reasons that VECs could be the source of bone forming cells in this disease.

Recent additional evidence has shown that a population of CD34 positive cells reside in traumatized muscle¹⁸, comprising approximately 15 % of the single cell suspension derived from this tissue. These cells have multi-potentiality as shown by their ability to differentiate down endothelial, bone and fat pathways. Further, these cells are not of hematopoietic lineage or a muscle lineage and appear to be more of a vascular endothelial progenitor cell, with the additional capacity to differentiate down the osteogenic and adipogenic paths¹⁸. These results coupled with that of others^{16, 17, 19, 20} indicate the important role the vascular compartment plays in both the normal regeneration of tissues and the possible pathological differentiation of cells down an inappropriate pathway such as that of bone, which is observed in HO. For these reasons the CD34 positive cells within traumatized muscle need to be explored in greater detail if we are to better understand both the normal regeneration of muscle and the pathological occurrence of HO following severe muscle trauma.

While the gene and protein structure of CD34 has been known for some time, its functional role in cells which express it has been forthcoming in some areas but elusive in others²¹. For example, the one known ligand (L selectin) is present only on T cells²¹. Yet the receptor is likely to be active, or activated, in a variety of circumstances where there is a lack of immune response²¹. CD34 plays a role in both proliferation and cell migration, in systems where there appears to be minimal involvement of T cells^{22–25}. Additionally, the involvement of CD34 in differentiation²⁶ has also been elucidated in a cell culture system lacking any immune cells. Thus it seems likely that activation of CD34 must be due to an interaction with an as yet unidentified factor or factors such as the extracellular matrix, receptors on adjacent cells or some as yet unidentified ligand in serum. Further, it is not clear what signal transduction cascade is activated by CD34. The only downstream target of the CD34 protein is CRKL, an adaptor protein present in a human myeloid leukemia cell line that appears to bind to SH3 domains on the cytoplasmic face of CD34²⁷. Additional downstream targets have yet to be identified.

Here we have hypothesized that the CD34 protein is not only a marker for multi-potential vascular progenitor cells but plays a functional role in the initiation of multiple differentiation pathways. We have attempted to elucidate the role of CD34 protein in the differentiation response of a population of CD34 positive cells derived from traumatized

human skeletal muscle. We find that the protein is required for differentiation down multiple pathways and is also required for optimal cell migration and proliferation.

Materials and Methods

Cell Isolation

Soft tissue samples were collected from traumatic extremity wound debridements comprised mostly of injured human muscle from lower extremity wounds sustained as a result of high-energy trauma from Operation Enduring Freedom and Operation Iraqi Freedom. All experimental protocols and samples were collected with Institutional Review Board approval at Walter Reed Army Medical Center or Walter Reed National Military Medical Center (G1 90QY). The Walter Reed National Military Medical Center Institutional Review Board waived the need for consent as tissue was classified as biological waste. All methods were carried out in accordance with the approved guidelines of the Institutional Review Board. Cells were extracted from traumatized muscle tissue using a methodology that is a modification of prior work¹¹. Connective tissue, fat and necrotic tissue were dissected away from the healthy margin of the debrided muscle sample. The remaining tissue (0.5 ml) was washed three times in Hanks' Balanced Salt Solution (Gibco, Carlsbad, California) and then minced in a Dulbecco's Modified Eagle Medium (Gibco) with penicillin, streptomycin and Fungizone(3X) (Gibco). The minced tissue was digested with 0.5 mg/mL collagenase type 2 (Worthington Biochemical, Lakewood, New Jersey) at 37°C for two hours, then filtered through a 40-mm cell strainer (Falcon) and centrifuged. The pelleted cells were resuspended in PBS plus 0.1 % FBS with the antibodies indicated below. Human dermal fibroblasts (HDFs) were derived from human newborn foreskin.

Transfections.

Transfections were performed in HDFs, CD34+/CD45– and CD91+/CD45– cells using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). In all experiments, a second transfection was performed 2 days after the primary transfection. The transfected cells were analyzed 2 days after the second transfection or 10 days if grown in Endothelial Differentiation Media. The CD34 small interfering RNA (siRNA) knockdowns were performed in cells by following a previously described transfection protocol²⁸(Krumins & Gilman, 2006). CD34 siRNAs and control siRNAs were purchased from Dharmacon. For CD34+ siRNA silencing, an ON-TARGET plus SMARTpool L-019503-00-0005 was used (sequences 5' - UAACCUCAGUUUAUGGAAA -3', 5' - GCACUAGCCUUGCAACAUC -3', 5' - GCGCUUUGCUUGCUGAGUU -3', and 5' - CCACUAAACCCUAUACAUC -3' based on GenBank accession number [NM_001773](#)). The siCONTROL Non-Targeting siRNA Pool #1 was used for control siRNA transfections (Dharmacon).

FACS analysis, Cell Sorting, Immunophenotyping Fluorescence Microscopy

For flow cytometry the crude cell suspension derived from muscle ($>2 \times 10^5$ cells) were washed and resuspended in phosphate– buffered saline (PBS) + 0.1% FBS along with IgG1, κ anti– human monoclonal antibodies (BD Biosciences, San Jose, CA): CD34– PE, CD91– PE and CD45– FITC for 1 h at 4°C. The cell suspension was also simultaneously negatively

sorted for either CD45-FITC or CD117-PE to exclude hematopoietic stem cells with the CD34-PE clone being specific for the variant of CD34 in MSCs and not HSCs. Cell suspensions were washed twice and resuspended in either a FACS buffer for analysis on a Cell Sorter (FACS Aria, BD Biosciences) or in 1% paraformaldehyde for analysis on a flow cytometer (Fortessa, BD Biosciences). Subsequent analyses were performed using the FACS DIVA software (BD Biosciences, San Jose, CA) or FlowJo (Tree Star, Ashland, OR).

Fluorescence microscopy utilizing antibodies against the endothelial makers Tie2, VE-Cadherin and CD31, was used to identify vascular endothelial cells.

Proliferation and Migration Assays

Cells were seeded at 1,000 cells per well (6 well plate) in 200 μ l final volume 96 well plates. Six replicate samples were prepared for, along with control (no cells) for media background fluorescence measurement. The cells were incubated at 37°C in 5% CO₂. Every 2 days, the growth medium was replaced with fresh growth medium. Microplates of sub-population cells were harvested on days 0 through day 15 when 200 μ l of the CyQUANT GR dye/lysis buffer was added to each sample well. The samples were incubated in darkness for 2 to 5 min. Sample fluorescence was measured with a BioTek Synergy H1 Hybrid Multi-Mode Microplate Reader, and growth curves were plotted as cell number per well versus time.

Cell migration was measured using a Cell Biolabs (San Diego, CA, USA) 2D gap-closure assay in a 96-well plate, according to manufacturer's instructions. Migration was evaluated at 18 and 36 hrs in replicates. Cells were stained with a Calcein AM non-toxic green fluorescent metabolic dye, for 10 min. at 37°C at each time interval (Biotium, Hayward, CA). Images were taken and gap-closure was measured.

Vascular endothelial differentiation, osteogenesis and adipogenesis

For vascular endothelial differentiation, the CD34 positive/CD45 negative cells were seeded at a density of 5,000 cells/cm² into 24-well plates (Corning). The cells were treated for 2 weeks with endothelial progenitor cell medium (Promocell), consisting of 10% FBS, and supplemented with 50 ng/ml VEGF (Promocell) or were treated with growth media as a control.

For endothelial tube formation, primary cultures of human umbilical vein endothelial cells (HUVECs) were cultured in EGM-2 medium (Lonza) and compared with CD34+ cells. The 96-well trays were coated with 10 μ L of Matrigel (Cell Biolabs). To analyze the effect of CD34+ siRNA on endothelial tube formation in vitro, 3×10^3 and 6×10^3 cells per well were transfected with CD34+ siRNA and compared to those transfected with siRNA control.

Cells were induced to undergo adipogenic and osteogenic differentiation, as described previously¹¹. For adipogenic differentiation the cells were seeded into six-well tissue-culture plates at a density of 20,000 cells/cm² and treated for 3 weeks with adipogenic medium (DMEM with 10% FBS, and supplemented with 0.5 mmol/L 3-isobutyl-1-methylxanthine (IBMX), 1 μ g/ml insulin, and 1 μ mol/L dexamethasone (Sigma)). The cells were then fixed in 2 % paraformaldehyde and stained with Oil Red O for lipid accumulation. For osteogenic differentiation, cells were seeded into six-well plates (Corning) at a density of 20,000

cells/cm², and treated for 3 weeks with osteogenic medium, (DMEM with 10% FBS, and supplemented with 10 mmol/L β -glycerolphosphate, 10 nmol/L dexamethasone, 50 μ g/ml ascorbic acid-2-phosphate, and 10 nmol/L 1,25 dihydroxyvitamin D₃ (Biomol International L.P., Plymouth Meeting, PA)). The cells were then fixed in 2 % paraformaldehyde and stained with Alizarin Red for mineralization

Statistical Analysis

The Students T-test or the Mann-Whitney U test was used to determine statistical significance. P values less than 0.05 were considered significant. The data are expressed as a mean plus or minus the standard error of the mean (SEM) with sample sizes where indicated (n).

Results

CD34 positive cells from traumatized muscle.

A population of CD34 positive and CD45 negative cells (referred to as CD34+/CD45-) can be isolated from traumatized muscle as shown in the flow cytometry analysis of Figure 1A. Selection against CD45 aids in the elimination of hematopoietic stem cells. The CD34+/CD45-population comprises 15.4 % of the total initial single cell population derived from debrided tissue. Because the CD34 negative/CD45 positive (2.49%) and CD34positive/CD45 positive (0.79 %) populations are much less abundant they will not be analyzed in this study. When the CD34+/CD45- population is expanded in culture (Figure 1B) they appear to have an elongated cell shape typical of progenitor cells^{11, 29-31}. These cells also display expression of the CD34 protein as shown in the immunoblot in Figure 1C. As a control CD34 is not expressed in human dermal fibroblasts (HDF, Figure 1C).

The CD34+/CD45-cells were assessed for differentiation capability. These cells were able to undergo vascular endothelial differentiation as shown Figure 2A where the cells express Tie2, VE cadherin and CD31, all markers of vascular endothelial cells. Additionally these cells were able to form endothelial tube-like structures (Figure 2B) under endothelial differentiation conditions. When assessed in osteogenic and adipogenic differentiation conditions (Figures 2C & D) it was clear that the CD34+/CD45-cells had the ability to both mineralize and synthesize fat droplets shown by Alizarin Red and Oil Red O staining respectively. In total these data show that a subpopulation of cells can be derived from traumatized human muscle that express CD34 and that are capable of undergoing osteogenesis, adipogenesis and vascular endothelial differentiation.

Suppression of CD34 by Inhibitory RNA

To begin to assess the function of CD34 we attempted to lower its levels in the CD34 positive cells by using an inhibitory RNA. A set of small inhibitory RNAs targeting the CD34 mRNA were transiently transfected into the CD34+ cells. At seven days post transfection, the cells were analyzed by flow cytometry for the number of CD34 positive cells. As shown in Figure 3A, the number of CD34 positive cells dropped significantly. Additionally, when extracts of the cells were analyzed for the presence of the CD34 protein (Figure 3B), it was found to be significantly reduced compared to the control transfection.

To ensure that the inhibitory RNAs were not toxic to the cells, the phase contrast image in Figure 3C shows that the cells transfected with the CD34 inhibitory RNA appear nearly identical to the control RNA transfected cells. These data show that the inhibitory RNA is very efficient in reducing the cellular levels of the CD34 protein, yet is not overtly toxic to the cells.

Down regulation of CD34 inhibits optimal cell migration and proliferation

The well described functions of the CD34 protein are that it supports both cell migration and proliferation (reviewed in 21). It was therefore expected that reducing the cellular levels of CD34 would correspondingly reduce the ability of the cells to migrate and proliferate. As shown in the migration assay in Figure 4A, transfection with the inhibitory CD34 small RNA reduced the ability of the cells to migrate across the gap. In the quantitation of the assay, shown in the graph in Figure 4B, reduction in CD34 significantly reduced the ability of the cells to migrate across the gap while it had no effect on the migration of human dermal fibroblasts (HDF) which do not express CD34.

A similar effect of the CD34 inhibitory RNA was observed in a proliferation assay. As shown in Figure 5, reducing CD34 levels significantly reduced the ability of the cells to proliferate, while it had no effect on the proliferation of HDFs, which do not express CD34.

Down regulation of CD34 blocks vascular endothelial differentiation, osteogenesis and adipogenesis

The CD34⁺ cells were then assessed for vascular endothelial differentiation following down regulation of the CD34 protein by inhibitory RNA transfection. As shown in immunofluorescence images in Figure 6, the cells were able to undergo efficient differentiation as indicated by expression of Tie2, CD31 and VE cadherin in the presence of a control RNA. However in the presence of a CD34 inhibitory RNA, expression of these three vascular endothelial marker proteins was completely inhibited, indicating that CD34 is required for efficient differentiation. As controls, the DAPI stain shows abundant cells in both the control and CD34 inhibitory RNA transfection conditions, indicating roughly equal numbers of cells following both treatments.

As an additional test for endothelial differentiation a tube assay was performed following transfection and differentiation with the inhibitory and control RNA (Figure 7). As evident in the figure, tube-like structures form in the control RNA transfection but not in the CD34 inhibitory RNA transfection condition. In total these data indicate that CD34 is required for vascular endothelial differentiation.

Experiments were then performed to determine if CD34 was required for osteogenesis. As shown in Figures 8A & B, following transfection of the control and CD34 inhibitory RNA, CD34 appeared to be required for efficient mineralization (Figure 8A) and alkaline phosphatase expression (Figure 8B), indicating that CD34 is necessary for osteogenesis along with other osteogenic markers.³²

Additional experiments were then performed to determine if CD34 was required for adipogenesis. As shown in Figure 8C, following transfection of the control and CD34

inhibitory RNA, CD34 appeared to be required for efficient lipid accumulation in the cells following differentiation, indicating that CD34 is also necessary for adipogenesis along with other adipogenic markers.³²

Overexpression of CD34 in CD34 negative cells enhances endothelial differentiation.

From the data above it seems clear that CD34 is required for efficient vascular endothelial differentiation in CD34+ cells. This would suggest that expression of CD34 in a CD34 negative cell may provide the signals necessary for vascular endothelial differentiation. To test this idea we utilized two distinct CD34 negative cell populations; CD91 positive/CD45 negative cells (CD91+/CD45-) derived from human traumatized muscle and human dermal fibroblasts (HDF). We transiently expressed CD34 in these cells as shown in the western blot in Figure 9. The data show that the CD34 protein is expressed in these cells.

The CD91+/CD45- cells and HDFs that were transiently expressed with CD34 were then induced to differentiate into vascular endothelial cells. As shown in Figure 10A & B, both the CD91+ and HDF cells displayed expression of Tie2, VE-cadherin and CD31. The expression levels were low but detectable indicating that CD34 provides the necessary signals for differentiation into vascular endothelial cells. However the differentiation level may not as robust as that of the CD34+ cells, likely due to a requirement of additional factors not present in the CD91+ and HDF cells. The control transfection with an empty vector led to no detectable visual fluorescence of vascular endothelial markers (Figure 10C & D).

Discussion

Here we have addressed the functional role of the CD34 protein in a population of CD34 positive cells derived from traumatized human muscle. We have recently found that these cells have multi-potentiality and that they comprise about 15% of the total cell number within traumatized muscle expressing high levels of TGF- β 1 in conditioned media¹⁸. A large number of studies have been performed utilizing CD34 as a selection agent to isolate cells with differing characteristics, such as hematopoietic stem cells³³, satellite cells³⁴, vascular endothelial cells³⁵, and vascular endothelial progenitor cells^{16, 33, 36}. However there are much fewer studies addressing the function of CD34 in these cells, with one such study looking at the muscle regenerative response after NTX, a snake peptide venom toxin, muscle injury in CD34-/- mice²⁴. To arrive at a better understanding regarding the function of the CD34 protein in the CD34 positive multipotential cells isolated from traumatized human muscle, we have both down regulated and up regulated its normal levels in these human cells and assessed its function with regard to differentiation, proliferation and cellular migration.

When the levels of CD34 were down regulated by transfection of the cells with a small inhibitory RNA, we find that differentiation is blocked. The cells were unable to undergo vascular endothelial differentiation, osteogenesis and adipogenesis. Thus CD34 appears to be required for three very distinct differentiation pathways. This novel finding is in contrast to previously published data showing that CD34 actually inhibited differentiation of a transformed hematopoietic cell line²⁶. It is possible that the difference in these previous

results with that of our study is due to the fact that we are using primary human mesenchymal cells and not a transformed hematopoietic cell line.

The primary question that arises from our results is how does CD34 function to aid in three distinct differentiation pathways. It is possible that CD34 aids in signaling required for multi-potential differentiation. This is difficult to test since little is known of the downstream signaling of CD34. One known adaptor protein, CRKL, that binds to the intracellular portion of CD34²⁷, is present in our CD34 positive cells and in fact associates with CD34 (data not shown). However, to date it is not known how CRKL aids in signaling of CD34. A further complexity is that the one known ligand for CD34, L-selectin (reviewed in 21), is expressed only in T-cells which are not present in our cell culture. Other as yet unknown ligands for CD34 could be present in our culture system, such as extracellular matrix proteins (e.g. fibronectin or collagen). While there is no RGD sequence on CD34 there is an RGE. A ligand could also be present in serum, growth factors or inflammatory factors could be possible candidates for binding CD34. Additionally a ligand could even be a receptor on adjacent CD34+ cells. Because the downstream signaling of CD34 remains obscure, at this time it is not known how CD34 would aid in the differentiation down multiple pathways. Podocalyxin and endoglycan, part of the CD34 transmembrane receptor family, were not detected in the cells used in this study. Podocalyxin and endoglycan, despite having a similar transmembrane domain and intracellular domain, their extracellular domains are very different, and their proteins are expressed with very novel, distinct and different functions in other cell types²¹

An additional way CD34 would be necessary for multi-potentiality is that it maintains the stemness of progenitor cells. It is possible that CD34 keeps the cells in a primed stem-like state. Loss of CD34 may direct the cells down a more myofibroblast lineage, resistant to differentiation. In support of this possibility we have examined the levels of alpha smooth muscle actin, a known marker for myofibroblasts³⁷. Alpha smooth muscle actin levels increase dramatically when CD34 levels are down regulated in CD34+ cells (data not shown). Thus it may be that CD34 keeps the cells in a state necessary for differentiation. However, CD34 would still need to maintain signaling within the cells to keep them in this state and it is unclear how it accomplishes this. While it is likely that it does so through CRKL, the downstream factors affected by this protein appear to be as yet unknown.

A surprising find in these studies is that expression of CD34 in CD34-null cells leads to enhanced vascular endothelial differentiation. Both HDFs and CD91+/CD45- cells showed enhanced expression of vascular endothelial markers Tie2, VE-cadherin and CD31 following ectopic expression of CD34 and culture in endothelial differentiation medium. Since both HDFs and CD91+/CD45- cells do not express CRKL, the data suggest that CRKL is not required for vascular endothelial differentiation. However it must be noted that the level of expression of the endothelial markers appears lower in the HDFs and CD91+ cells than in the CD34+ cells. Thus it may be that CRKL necessary for optimal VEC differentiation.

Finally it must be noted that while the number of CD34+ cells appears to be roughly 15% in traumatized muscle, in preliminary experiments the levels in normal muscle are much lower (< 1%). This would suggest that these cells are recruited and/or stimulated to proliferate

following trauma. The source of these CD34 cells remains unclear although the most likely source would appear to be the peripheral vascular system which would contain VEC precursors.

In conclusion the data here show that the CD34 protein is critical for multi-potential differentiation, proliferation and migration in progenitor cells derived from traumatized human muscle.

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Highlights

- CD34+ confers progenitor function in FACS sorted traumatized human muscle cells in culture
- CD34+/CD29+ cells are the majority progenitor cell type found in traumatized human muscle cells
- FACS sorted CD34+ cells from traumatized human muscle will progress down an osteogenic lineage in osteogenic differentiation media in culture

Figure 1. CD34 positive/CD45 negative cells derived from traumatized human muscle.

A. Cells isolated by a single cell suspension were analyzed directly by flow cytometry using fluorescent conjugated antibodies against CD34 and CD45. The dot plot shows the resulting subpopulations CD34 positive/CD45 negative (15.4 %), CD34 positive/CD45 positive (0.79 %) and CD34negative/CD45positive (2.49%). (N=12) B. The CD34positive/CD45 negative cells from (A) were cultured in monolayer to confluence and imaged at 10X). The cells as in (B) were lysed and the protein extracts were analyzed by western blot for CD34 protein using a CD34 antibody. Human dermal fibroblast extracts (HDF) were used as a negative control. GAPDH was used as a control for equal loading of lanes.

Figure 2. CD34 positive/CD45 negative cells display multipotentiality

A & B. CD34 positive/CD45 negative cells were cultured in either growth medium (GM) or in endothelial differentiation medium (EM). The cells were either processed for immunofluorescence for Tie2, VE-cadherin and CD31 (A) or for endothelial tube formation (B). CD34 positive/CD45 negative cells were cultured either in growth medium (GM), in osteogenic medium (OM) (C) or in adipogenic medium (AM) (D) then stained for mineralization via Alizarin Red (C) or for fat via Oil Red O (D). (N=5)

Figure 3. Inhibitory small RNA efficiently reduces the CD34 protein levels in the CD34 positive/CD45 negative cells.

A set of small inhibitory RNAs targeting the CD34 mRNA were transfected into the CD34 positive cells. A group of random generated small RNAs served as a transfection control. At seven days post-transfection the cells were processed for flow cytometry (A), immunoblotting (B) or phase contrast microscopy (C). (N=5)

Figure 4. Down regulation of CD34 inhibits cell migration

A set of small inhibitory RNAs targeting the CD34 mRNA were transfected into the CD34 positive cells. A group of random generated small RNAs served as a transfection control. The cells were seeded into wells of a Gap-Closure Assay plate. When the wells were confluent the circular plug was removed to allow the cells to migrate into the space (A). The graph (B) indicates the % closure of the circular space at the indicated times (N=8)

Figure 5. Down regulation of CD34 inhibits cell proliferation.

A set of small inhibitory RNAs targeting the CD34 mRNA were transfected into the CD34 positive cells. A group of random generated small RNAs served as a transfection control. The cells were seeded at 1×10^5 cells per well in 6-well plates on day 0 and cultured for an additional two weeks. The graph indicates the cell number on the indicated days. Significant differences were noted between the CD34 positive cells transfected with the CD34 inhibitory RNA compared to the control RNA transfection at days 5 through 15. (N=5)

Figure 6. Down regulation of CD34 inhibits endothelial differentiation: Tie2, VECadherin, CD31 expression

A set of small inhibitory RNAs targeting the CD34 mRNA were transfected into the CD34 positive cells. A group of random generated small RNAs served as a transfection control. The cells were cultured in endothelial differentiation medium and processed for immunofluorescence for Tie2, VE-cadherin and CD31 as shown. The nuclei (DNA) of the cells were also stained with DAPI (blue fluorescence) as a control for cell number. (N=5).

Figure 7. Down regulation of CD34 inhibits endothelial differentiation: tube assay.

A set of small inhibitory RNAs targeting the CD34 mRNA were transfected into the CD34 positive cells. A group of random generated small RNAs served as a transfection control. The cells were cultured in either growth medium (GM) or in endothelial differentiation medium (EM) and endothelial tube formation. The cells were imaged under phase contrast microscopy (4X) as shown. (N=5).

Figure 8. Down regulation of CD34 inhibits osteogenesis and adipogenesis.

A set of small inhibitory RNAs targeting the CD34 mRNA were transfected into the CD34 positive cells. A group of random generated small RNAs served as a transfection control. The cells were cultured either in osteogenic medium (OM) (A & B) or in adipogenic medium (AM) (C) then stained for mineralization via Alizarin Red (A), alkaline phosphatase (B) or for lipid accumulation via Oil Red O (C). (N=5)

Figure 9. Expression of CD34 in human dermal fibroblasts and CD91 positive/CD45-cells. Human dermal fibroblasts (HDFs) or CD91 positive/CD45 negative cells derived from human traumatized muscle were grown in culture and transfected with a CD34 expression plasmid (+) or control plasmid (-). At 7 days post-transfection, extracts were generated and processed form immunoblotting using a CD34 specific antibody or an alpha tubulin antibody to demonstrate equal levels of protein loading per lane. The developed blots are shown.

Figure 10. Expression of CD34 in human dermal fibroblasts and CD91 positive/CD45-cells enhances endothelial differentiation

Human dermal fibroblasts (HDFs) or CD91 positive/CD45 negative cells derived from human traumatized muscle were grown in culture and transfected with a CD34 expression plasmid (A,B) or control plasmid (C,D). At 7 days post-transfection, the cells were cultured either in growth medium (GM) or in endothelial differentiation (EM) medium and processed for immunofluorescence for Tie2, VE-cadherin and CD31 as shown.