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Hypoxia preconditioned mesenchymal stem cells improve vascular and skeletal muscle fiber regeneration after ischemia through a Wnt4 dependent pathway

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Short title: role of hypoxia on MSC regenerative properties

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Abstract

Mesenchymal stem cells (MSC) are multipotent postnatal stem cells, involved in the treatment of ischemic vascular diseases. We investigate the ability of MSC, exposed to short term hypoxic conditions, to participate in vascular and tissue regeneration in an *in vivo* model of hindlimb ischemia. Transplantation of hypoxic preconditioned murine MSC (HypMSC) enhanced skeletal muscle regeneration at day 7, improved blood flow and vascular formation compared to injected non-preconditioned MSC (NormMSC). These observed effects were correlated with an increase in HypMSC engraftment and a putative role in necrotic skeletal muscle fiber clearance. Moreover, HypMSC transplantation resulted in a large increase in Wnt4 expression and we demonstrate its functional significance on MSC proliferation and migration, endothelial cell migration, as well as myoblast differentiation. Furthermore, suppression of Wnt4 expression in HypMSC, abrogated the hypoxia-induced vascular regenerative properties of these cells in the mouse hind limb ischemia model. Our data suggest that hypoxic preconditioning plays a critical role in the functional capabilities of MSC, shifting MSC location *in situ* to enhance ischemic tissue recovery, facilitating vascular cell mobilization and skeletal muscle fiber regeneration via a paracrine Wnt dependent mechanism.

INTRODUCTION

Bone marrow mesenchymal stem cells (MSC) can be isolated from bone marrow-derived mononuclear cells (MNC), expanded *in vitro* and have been shown to possess functional multi lineage differentiation capacity [1]. MSC have been shown to differentiate into pericytes [2], vascular endothelial cells [3, 4] and cardiomyocytes [5], and their transplantation has improved recovery from ischemia in rat hindlimb and swine dilated cardiomyopathy models [6]. Their precocious role in the formation and the maintenance of neo vessels has been studied extensively [2, 7]. Such reports have led to the hypothesis that MSC may be used in the treatment of ischemic cardiovascular pathologies. One major limitation demonstrated in both animal and human experiments has been the inability to maintain a large number of cells after grafting into hypoxic sites. In the current study we employed a strategy of low oxygen (O₂)- preconditioning of MSC before tissue implantation. We hypothesized that creating this hypoxic environment might mimic the stem cell niche microenvironment *in vivo* which others have hypothesized to likely create a hypoxic cavity [8]. We chose to study 1% O₂ concentration since it has been reported as the level found *in vivo* in bone marrow stem cell niche areas [9]. Moreover, data from animal studies have demonstrated a role of hypoxic gradients in stem cell trafficking in ischemic tissue [10]. On the molecular level, temporary oxygen deprivation is known to regulate gene transcription and differentiation of stem cells [11, 12].

Several reports suggest that the Wnt pathway plays a role in maintaining hematopoietic stem cell self-renewal and pluripotency [13, 14]. The Wnt family of genes encodes over twenty cysteine-rich secreted glycoproteins to regulate canonical and non canonical signaling pathways [15]. The canonical Wnt pathway regulates gene expression due to the stabilization and nuclear localization of β catenin. We have previously demonstrated that MSC have a structural role in forming a precocious network and facilitating neovessel recruitment in an angiogenic model and reported that overexpression of a soluble modulator of Wnt functions, sFRP-1, in MSC, enhanced their angiogenic capacities [2]. Experimental manipulation of a pluripotent mouse embryonal carcinoma

stem cell line via another secreted Wnt modulator, sFRP2, identified the Wnt pathway in cardiomyogenic differentiation [16]. Wnt3a activation modulated proliferation and differentiation of human MSC and murine embryonic mesenchymal cells [17, 18]. Recently, another study demonstrated a role of the Wnt signaling pathway in the acquisition of the invasion property of human MSC [19]. These data have been particularly important to help elucidate the molecular mechanism that enables MSC to contribute to tissue regeneration under ischemic conditions.

In the current study, we compare the ability, of hypoxic preconditioned murine MSC (HypMSC) and non-preconditioned MSC (NormMSC), to engraft into ischemic tissue and assessed the ability of these cells to participate in skeletal tissue and vascular regeneration. A sub acute murine limb ischemia model was used to mimic the clinical setting of critical limb ischemia in patients. Our results demonstrate that HypMSC have a greater capacity for engraftment in ischemic tissue compared to NormMSC. HypMSC also improved skeletal muscle regeneration and induced more neoangiogenesis, and elicited significant improvement in blood flow as compared to NormMSC. Localization of HypMSC compared to NormMSC suggests that HypMSC participate in the remodeling of the stromal environment via structural interactions, first in encircling necrotic myocytes and then shaping a tight network around endothelial cells along growing neovessels. This preferential localization led us to speculate that MSC could functionally interact via paracrine effectors with myocytes and endothelial cells (EC). Our data demonstrates that MSC express Wnt4 and *in vivo* Wnt4 transcripts were significantly increased in ischemic muscles engrafted with HypMSC while in muscle engrafted with NormMSC no significant increase was detected. We tested the biological activity of Wnt4 *in vitro* and found that it could stimulate EC migration, myogenic differentiation and MSC proliferation but had no effect on EC proliferation. Importantly, inhibition of Wnt4 expression in MSC abrogated HypMSC-induced vascular regeneration. Our data provides strong evidence that Wnt signaling could be an important factor in the mechanism by which MSC contribute to tissue regeneration, as well as vessel formation and stabilization. As such, hypoxia preconditioning of MSC could be an attractive therapeutic approach for the treatment of ischemic vascular diseases.

RESULTS

Vascular and muscle regeneration of the ischemic hindlimb was improved after transplantation of HypMSC vs NormMSC

We examined the comparative effectiveness of transplanted murine HypMSC vs NormMSC for vascular and ischemic muscle regeneration after femoral ligation in C57BL/6J mice. Three groups were set up: a control sham group receiving saline injection, a NormMSC group receiving by intramuscular injection 0.5×10^6 MSC cultured in normoxia (20% O₂) and a HypMSC group transplanted with 0.5×10^6 MSC pretreated during 36 hours in a reduced oxygen tension (1% O₂).

HypMSC enhanced vascular response

Blood flow recovery in the limb of HypMSC-treated animals was significantly improved when compared with sham-treated animals or NormMSC-treated animals (**Figs. 1a,b**).

The therapeutic action of HypMSC was also evident at the angiogenic level, as evidenced by quantification of only viable muscle, excluding angiogenesis involved in an inflammatory response to granulation tissue. At day 15, there was an increase in CD31-positive capillary number in HypMSC-treated muscles compared respectively with that in sham-treated control ($P < 10^{-5}$) and in NormMSC groups ($P < 10^{-3}$) at day 15 (**Figs 1c-f**). These results suggest a potent role of HypMSC in new-vessel formation. Consistent with improved limb perfusion and capillary density, micro scanner (mCT) analysis of the whole arterial network revealed the global impact of HypMSC on vessel formation in the hindlimb after ischemia. Images showed a greater number of vessels in HypMSC-treated hind limb compared to NormMSC or sham-treated injected hindlimb at day 8 after the insult (**Figs 1g-k**). We calculated a 2 fold increase in the vessel number (number of vessels/ μm^3 , $P < 0.01$) and a 1.7 fold increase in their connectivity and branching ($P < 0.01$) in the HypMSC-treated hindlimb compared with sham-treated control animals while NormMSC-treated animals were at an intermediate level ($P < 0.05$) (**Figs 1 j-k**).

HypMSC enhanced muscle regeneration

Histological evaluation of muscle tissue, performed to analyze the impact of HypMSC transplantation on the repair process, revealed that administration of either HypMSC or NormMSC accelerated the repair process. At day 7, almost no overt necrotic area was found in either of the MSC-treated groups, while necrotic muscle fibers were markedly observed in sham treated muscles (25% out of total muscle surface). However, HypMSC engraftment had a significant beneficial effect on muscle regeneration when compared with NormMSC-treated group. Less than 10% of the histological area in the ischemic muscle were still actively regenerating in HypMSC-treated tissue while in the NormMSC treated group, the process was delayed with over 21% of the total muscle surface was still actively regenerating (**Figs 1l-q**).

Hypoxic pretreatment modified MSC engraftment in ischemic hindlimb

MSC were infected with a lentivirus encoding for the GFP gene (GFP-MSC) and expression was followed as a marker of viable GFP-positive MSC [2]. HypMSC robustly engrafted the adductor and tibial muscles in large patches, as revealed by direct fluorescence at day 7 (**Figure S1a**) or antiGFP immunohistochemistry at day 14 (**Figure S1b**).

Hypoxic treatment improved MSC survival capacities in vivo

Real time RT-PCR was performed using total RNA obtained from HypMSC and NormMSC injected muscles at day 7 after injury to determine the percentage of remaining GFP expression, and demonstrated that hypoxic pre-treatment contributes to effective enhancement of MSC recruitment *in vivo*. In the HypMSC group, there was a significant increase in MSC engraftment at day 7 (150% of initially engrafted HypMSC) where as in NormMSC group, the total number of MSC was not maintained after transplantation (63% of initially engrafted NormMSC) (**Fig. 2a**). At day 7, caspase 3 labeling showed apoptotic muscle fibers surrounding by surviving Hyp and Norm GFP-MSC (**Fig. 2b**). Double BrdU/GFP labeling revealed that BrdU incorporation rate was significantly increased in HypMSC group compared to NormMSC group (16.3 ± 8.1 vs 13.1 ± 6.6 BrdU/GFP + cells/mm² P<0.05)

(Figs 2c-d).

HypMSC were widespread in hypoxic zones and may play a macrophage-like function

HypMSC appeared to have a different pattern of engraftment as compared to NormMSC. To better understand this, we evaluated the scattering of MSC in hypoxic zones using the 2-nitroimidazole hypoxia marker, pimonidazole. Hypoxic sections of muscle produced a mottled staining with a characteristic hypoxic brown center. At day 7, a large number of GFP-positive HypMSC were evenly distributed in deep hypoxic zones around necrotic myocytes whereas GFP-positive NormMSC were consistently detected at the periphery of ischemic zones (**Fig 3a and Figure S2**). The distribution of MSC around necrotic myocytes suggested a potential macrophage-like, phagocytic function for these cells. GFP immunofluorescent staining revealed that MSC harbor a phenotype with long filopodial protrusions that can encircle and penetrate into dead myocytes (**Figs 3b-d**). To evaluate the phagocytic capacity of MSC in regards to myocytes, *in vitro* experiments were conducted. Fluorescent microscopy examination revealed that GFP-MSC co-cultured with necrotic cell bodies of PKH-labeled murine myoblasts (red) were able to robustly engulf part of the myoblast cell membranes (**Figs 3f,g**). GFP-MSC co-cultured with non-necrotic PKH-labeled murine myoblasts (red) did not have this property (**Fig. 3e**). At day 15, HypMSC appeared to be preferentially associated with capillaries and microvessels, behaving as perivascular cells shaping along the whole length of the vessel, covering growing CD31- forming tube-like structures, as mural cells (**Figure S3**).

HypMSC paracrine Wnt dependent effect on angiogenesis and muscle regeneration

The effects of temporary hypoxia on Wnt factor expression were also investigated. Several studies have demonstrated a role of the Wnt pathway in vascular development [20] as well as stem cell proliferation and maintenance [13]. We hypothesized that MSC express Wnt and use the Wnt

pathway as a mechanism for activation of muscle regeneration processes. Screening by RT-PCR for the expression of different *Wnt* transcripts revealed that MSC express *Wnt4*, *5A*, *5B* and *11*. *In vivo*, *Wnt4* transcripts were significantly increased in HypMSC injected muscles (ratio *Wnt4/P0*: 15.6 ± 5.2 , $P=0.01$ vs control tissues) while NormMSC implantation did not significantly increase *Wnt4* transcripts (ratio *Wnt4/P0*: 3.8 ± 1.8 , $P=NS$ vs control tissues) (**Fig. 4**). *Wnt5A*, *5B* and *11* expressions were not significantly modified in either HypMSC and NormMSC injected muscles compared with control muscle at day 7 after ischemia. Corroborating evidence was found *in vitro* where a specific strong up-regulation of *Wnt4* was detected after exposing MSC to hypoxia for 36 hours compared to normoxia-treated MSC (*Wnt4/P0*: 4.78 ± 1.8 , $P=0.01$ in comparison to control) (**Figure S4**). In parallel, we report preconditioning effects on angiogenic growth factor expressions by HypMSC (**Figure S4 a-c**).

Functional validation of HypMSC secreted factors on cell functions

To further elucidate the functional paracrine and autocrine role of HypMSC secreted factors in vascular response and muscle regeneration enhancement, we studied the effect of *ex vivo* incubation of the different cellular components namely EC, muscle fibers and MSC with HypMSC and *Wnt4* conditioned medium (HypCM and *Wnt4* CM, respectively).

HypMSC secreted factors and Wnt4 played a role on EC migration but not on EC proliferation

Transwell filters revealed that HypCM and *Wnt4* CM induced significantly more migration of EC than did respectively CM from NormMSC (NormCM) and control CM ($P<0.05$). Moreover, addition of a secreted Wnt antagonist, rb sFRP-1, blocked the HypCM-induced migration suggesting that Wnt dependent factors are the major active factors present in HypCM. (**Fig 5a and Figure S5**). It is worth noting that rb sFRP-1 enhanced migration of both NormCM-treated EC and control EC, which is in agreement with previous reports [21]. Interestingly, HypCM had no effect on EAhy926 proliferation compared with CM from NormMSC (NorCM) (data non shown). Taken together, these results support a

role of HypCM and Wnt4 on EC migration.

Wnt4 induced skeletal myoblast differentiation

To explore the potential role of Wnt4 on skeletal muscle differentiation, intact primary murine skeletal myoblasts were cultured in the presence of Wnt4 CM for 16 hours and compared with myoblasts treated with control CM. The results revealed that the number of myogenin-expressing cells was significantly increased in Wnt4-treated myoblast culture compared to control condition ($P < 0.01$) (**Fig. 5b**).

Autocrine role of Wnt4 promoted MSC proliferation, migration and Wnt canonical β -catenin pathway activation

We then tested the hypothesis that enhancement of HypMSC engraftment could be due in part to *Wnt4* upregulation. The role of Wnt4 as a potent inducer of MSC migration was demonstrated (**Fig. 5c**). In addition, data revealed an effect of Wnt4 on the number of MSC but only under hypoxic conditions (**Fig. 5d**). Moreover, Wnt4 was able to activate the canonical Wnt pathway as shown by T-Cell factor (TCF) -dependent transcriptional activity measured under hypoxic conditions; transfection of MSC with a plasmid coding for Wnt4 provoked a 15-fold increase in luciferase activity (**Fig. 5e**). No activation was seen by using the corresponding control reporter with mutated TCF binding sites. Inhibition of Wnt signaling using *rb sFRP1* also effectively suppressed TopFlash luciferase reporter activities in response to Wnt4. Our data are consistent with the interpretation that Wnt4 signals are transduced via the canonical branch of the Wnt pathway and reveal that MSC respond to Wnt4 stimuli in a hypoxic environment.

Suppression of Wnt4 expression in HypMSC prevented the vascular regenerative response induced by hypoxia pre treatment

An *in vivo* model of *Wnt4* suppression was employed to demonstrate the importance of Wnt4 as a key paracrine factor mediating pro-angiogenic effects of HypMSCs. As such, we treated HypMSC with two siRNA against *Wnt4* (**Fig. 6a**) and transplanted them into the mouse ischemic hindlimb model. We

showed that siControl (siCtl) treated HypMSC compared to siCtl treated NormMSC enhanced significantly the blood flow ($P<0.05$) (**Fig. 6b**), and vascular density ($P<0.05$) and connectivity ($P<0.05$) (**Fig. 6 c-d**). Treatment of HypMSC with siRNA against *Wnt4* abrogated the difference between HypMSC and NormMSC groups; either blood flow or vascular density and connectivity were not significantly different between siWnt4 treated HypMSC and siCtl treated NormMSC groups.

DISCUSSION

The influence of temporary hypoxic preconditioning of MSC on the stimulation of angiogenesis and muscle regeneration was investigated and the data revealed that *ex vivo* hypoxic preconditioning of MSC plays a unique role in the enhancement of vessel formation and repair of the necrotic zone after a hindlimb ischemia via a Wnt dependent pathway.

We hypothesized that the 1% oxygen atmosphere in which MSC were maintained could condition them to survive in a hypoxic environment *in vivo* as reported *in vitro* [22, 23]. Oxygen tension in bone marrow compartments has been reported to be very low as in injured tissue, suggesting that tissue hypoxia may be a fundamental mechanism governing stem cell recruitment and retention [10]. As a result, we demonstrated that hypoxic pre-treatment can more effectively overcome the hurdle of cell death after implantation than normoxic conditioning, promoting MSC survival and proliferation *in situ*. HypMSC engrafted robustly in ischemic tissue and their proliferation index in ischemic sites was superior to that of NormMSC. The decline MSC number that has been previously described could be due to cytokine secretion and tissue remodeling [24] or immune rejection by the host as they expressed GFP a well-known immunogen [25].

MSC have been successively administered as a treatment in both animal studies [6, 24, 26] and human clinical trials [27, 28]. Studies showed their possible contribution to vascular structures and skeletal muscle *in vivo*. For example, injection of MSC in a rat model of dilated cardiomyopathy revealed that some of the MSC were differentiated into cardiomyocytes [6]. The application of MSC improves

wound healing regeneration through differentiation [24]. Recent studies suggest that monocytes under activation could express EC markers and form tube-like structures [29]. The setting of hypoxic preconditioning in this study did not enhance cell differentiation into EC, as HypMSC kept their pericyte-like properties and behaved as perivascular cells as reported [2, 30]. Their tissue localization led us to hypothesize a potential macrophage-like role in which they participate in phagocytosis of necrotic muscle fibers. Our data indicates that MSC have the ability to participate in the phagocytic process of muscle fibers *in vitro* and to encircle and send extensions into necrotic muscle fibers *in vivo*, which could indicate that these cells participate in clearing skeletal muscle cellular debris. This process has similarities with previously published data which demonstrated that macrophages reorganize their actin cytoskeleton to form phagocytic lamellipodia structures to engulf apoptotic cells [31]. Moreover, at early timepoints, MSC appear to play a role in neovessel formation encircling endothelial cells in capillaries and microvessels and participating in their stabilization as previously described [32, 33]. Our results support the notion that MSC play a cooperative role in both myocyte regeneration and vascular formation by structural and functional interaction with both myocytes and EC.

As MSC have been shown to secrete a large quantity of angiogenic factors [34], we investigated the potential involvement of paracrine and/or endocrine factors produced by MSC. Previous reports have demonstrated that conditioned medium from MSC can promote EC migration, differentiation and survival [35]. Our data indicate that conditioned medium from HypMSC is more effective than conditioned medium from NormMSC in inducing EC migration as well as MSC proliferation and migration. As the Wnt signaling pathway has been shown to play a role in stem cell fate [13], muscle regeneration [36], vascular formation [37] and invasion capacity of MSC [19], we hypothesized that this pathway may be involved in the mechanism involved in this process. Our study identified that Wnt4 was specifically over expressed *in vitro* in conditioned media from HypMSC and that, *in vivo*, injection of HypMSC into the ischemic hindlimb strongly induced Wnt4 expression. We also demonstrate that Wnt4 exerts an effect on myoblast differentiation, *in vitro*. We have established a role of Wnt4 as a potent mediator in vascular regeneration as it affects EC migration. It is worth noting

that Wnt4 conditioned medium could affect MSC migration and proliferation but only under hypoxic conditions through a canonical β -catenin dependent Wnt signaling. Thus, it is likely that, *in situ*, residual tissue hypoxia confers responsiveness of MSC to environmental signals. Moreover, Wnt4 signaling was shown to stimulate myogenic proliferation and differentiation during chick development [38] and satellite cell proliferation in skeletal muscle in myostatin-knockout mice [39]. Finally we demonstrate here that, knockdown of Wnt4 expression resulted in inhibition of the beneficial impact of HypMSC on vascular regeneration compared with that induced by NormMSC. Collectively, these results strongly support the hypothesis that Wnt4 secreted by MSC is an important factor in mediating tissue regeneration in the setting of ischemia.

In conclusion, we have demonstrated that hypoxic preconditioning of MSC prior to transplantation could be a potent strategy in overcoming the current limitations in the field of regenerative medicine. This study suggests that many of the cellular functions associated with the ability of MSC to regenerate ischemic tissue are often not expressed constitutively by MSC but rather only transiently. MSC respond to contextual signals according to their microenvironment.

Materials and Methods

MSC [2, 26] were treated for 36 hours at 1% O₂ in a hypoxia chamber (HypMSC) (C21 Proox chamber, BioSpherix, New York) or maintained at 20% O₂ (NormMSC) before being injected into a mouse ischemic hind limb. Mice were randomly assigned to three treatment groups. One group underwent sham treatment (i.e., aperture and saline injection) (control group). The other groups received injection of either 0.5 x10⁶ NormMSC or HypMSC. Detailed description of materials, animal procedures, MSC preparation, hindlimb ischemia model, tissue collection and preparation for biochemical assays, Laser Doppler Perfusion Imaging (LPDI), microCT analysis, immunohistochemistry, macrophagic assays, and *in vitro* cell functional assays can be found in the Online Data Supplement.

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FIGURE LEGENDS

Figure 1: augmented vascular regeneration by intramuscular transplantation of MSC in a hindlimb ischemia model.

(a) Images of representative LPDI in sham mice (control W/O cell) and in HypMSC or NormMSC transplanted groups at day 7 (n=21) and day 15 (n=15) after ischemia. (b) Quantitative analysis of hindlimb blood perfusion by calculating the ischemic/normal limb perfusion ratios after the induction of hindlimb ischemia. Blood perfusion of the ischemic hindlimb markedly increased in the HypMSC and NormMSC group one week after transplantation. LPDI index in the HypMSC group was the highest among the three groups at day 7 and day 15. Data are means \pm S.E.M.; 'K, $P < 0.05$ and 'K'K, $P < 0.01$ vs control; \$ $P < 0.05$ vs. NormMSC.

Vessel numbers were calculated after CD31 immunolabelling (c-f) in the control, NormMSC and HypMSC groups. 'K, $P < 0.00001$ vs. control; \$, $P < 0.001$ vs NormMSC.

Reconstructed micro-CT images of control, NormMSC or HypMSC injected hindlimb 7 days after ischemia in age- and gender-matched mice (g-i) (μm^3 resolution). Note marked increase in vessel number (j) and in connectivity (k) in HypMSC treated mice versus NormMSC treated mice and control mice. Data are % \pm S.E.M.; 'K, $P < 0.05$ and 'K'K, $P < 0.001$ vs. control; \$, $P < 0.05$ vs. NormMSC. n=6 in each group.

Repaired surface measurement of control and MSC group (n=6 in each group) in hindlimb ischemic mice. Representative repaired surface and necrotic area were quantified as ratio of necrotic or in repair or repaired surface out of total muscle area (l-q).

Figure 2: Engraftment, survival and proliferation of MSC in ischemic hindlimb tissues:

(a) Engraftment of GFP-MSC in muscle. The levels of GFP expression was determined by performing real time RT-PCR. The percentage of engrafted MSC was assessed by the ratio of GFP level at day 7 with that of GFP level at D0 (initial MSC graft) (n=4 in NormMSC and HypMSC groups). The histogram showed a higher persistence of MSC in the muscle at day 7 after injury in HypMSC-treated groups compared to NormMSC-treated group. 'K, $P < 0.05$, n=10.

After 7 days of ischemia, (b) survival assay of MSC and (c-d) induction of MSC proliferation were assessed using respectively a double GFP (green) /caspase 3 (red) and a double GFP (green) /BrdU (red) immunofluorescent labelling performed on NormMSC and HypMSC transplanted tissue sections. (b) Representative photomicrographs at day 7 after ischemia showed MSC (arrowhead) surrounding caspase3 positive myocytes (white star). (c) The levels of double GFP/BrdU NormMSC and HypMSC (white arrowheads) were quantified and reported in histogram graphs. (d) Representative photomicrographs of ischemic hind limb sections at day 7 after ischemia revealed a higher level of proliferating GFP-MSC in HypMSC injected tissues compared to that in NormMSC injected tissues. *K, P<0.05, n=10.

Figure 3: HypMSC engraftment sites could assist MSC in their macrophagic-like role

(a) At day 4, hindlimb muscles transplanted with either NormMSC or HypMSC have large hypoxic zones as revealed using pimonidazole binding (Hypoxyprobe). Immuno staining of GFP-MSC with an antibody directed against GFP (AC anti GFP) showed localization of NormMSC at the peripheral front of hypoxia although HypMSC displayed a scattered distribution inside hypoxia zone.

MSC macrophagic activity. (b) immunofluorescent analysis for GFP expression (green) with CD31 (red) on hindlimb tissue section at day 4, revealed that during muscle regeneration, GFP-positive MSC (green) surrounded necrotic muscle fiber, harboring a spindle shape with long filopodial extensions. (c) Arrow heads point to MSC extensions, which enter through necrotic skeletal muscle fibers (d) close-up of the boxed region in the larger image. Stars indicate necrotic muscle fiber. Cell nuclei are stained with DAPI (blue).

In vitro, GFP-positive MSC were mixed with intact PKH-labeled skeletal muscle fibers (e) or in contact with PKH-labeled skeletal muscle fiber particulate debris (f-g). In presence of muscle fiber membrane debris, MSC have the capacity to phagocyte particles. The cartoons at right and bottom end side represent perpendicular views of the confocal acquired images (d-g). White lines

show myocyte red particulate debris in GFP-MSc cytoplasm (f-g).

Figure 4: effects of HypMSC transplantation on Wnt gene expressions. Wnt4, 5A, 5B and 11 expressions in hindlimb ischemic muscle were analyzed by performing real-time PCR. Hindlimb muscles transplanted with NormMSC (Grey bars) or HypMSC (Black bars) or without cells (sham group, white bars) were extracted at day 7 after ischemia. Values are means \pm SD; n=3; the assays were carried out in triplicate. * <0.05 , P, §, P <0.01 vs control.

Figure 5: effect of HypCM and Wnt4 CM on cell properties

(a) Endothelial cell chemotactic migration was quantified using transwell filters. Fluorescent-labelled EAHy926 migrated in the presence in the lower chamber of either NormCM, HypCM and Wnt4CM with or without rb sFRP-1 in the lower chamber. Migrated EC were then lysed and quantified on a fluorimeter. All the CM induced EAHy926 migration. *, P <0.05 , §, P <0.01 vs Control. rbsFRP-1 induced migration in NormCM assays and blocked HypCM and Wnt4CM induced EAHy926 migration (*, P <0.05).

(b) Muscle fibers were cultured either in control CM (Cont CM) or in Wnt4 CM during 48 hours. Increase expression of myogenin assessed an effect of Wnt4 on myoblast differentiation. The numbers of myogenin-positive nuclei were reported on the graph. §, P <0.01 .

(c) The effect of Wnt4 on MSC migration was quantified after in an *in vitro* wound assay. Confluent MSC were wounded and placed during 16 hours in presence of either control CM (cont CM) or Wnt4 CM. Analysis of Wnt4 effect on wound closure was measured as area of migration ($\square\text{m}^2$) on phase contrast microscopy images. §, P <0.01 .

(d) MSC proliferation of MSC was influenced by Wnt4 overexpression (pWnt4 vs pCDNA3, §, P <0.01) in hypoxia but not in normoxia. MSC were maintained during 24 hours either in normoxia or in hypoxia after transfection before counting. Values correspond to total cell numbers per well.

(e) Wnt4 activates the TopFlash reporter in MSC. Cotransfection of MSC with TopFlash or FopFlash reporter and Wnt4 resulted in more than a 15 fold increase in TopFlash over FopFlash activity compared with MSC transfected with an empty vector (pcDNA3) in hypoxia condition (\$, P<0.01). Adding of rb sFRP-1 blocked either Wnt4 overexpression induced TopFlash over FopFlash activity (*, P<0.05). The experiments were conducted in hypoxia. The error bars indicate on standard deviation from the mean of triplicate samples.

Figure 6: effect of Wnt4 knock down on NormMSC or HypMSC treated with siControl (siCont) or with siWnt4 (a) on Wnt4 expression *in vitro* followed by performing real-time PCR, (b-d) on vascular effect induced by MSC: quantitative analysis of hindlimb blood perfusion by calculating the ischemic/normal limb perfusion ratios after the induction of ischemia (b), and microCT quantification of (c) vessel number and (d) connectivity at D7 after injury in mice transplanted either with NormMSC or HypMSC treated with siControl (siCont) or with siWnt4 or not transplanted (Cont). Data are % \pm S.E.M.; *, P <0.05 and **, P<0.001 vs. control. \$, P<0.05 vs. NormMSC. N=9 in each group.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1: MSC detection: (a) cell patches at day 7 were found in whole muscle by direct fluorescent imaging. (b) Note the presence of green GFP-positive MSC intercalated between muscle fibers (white arrows).

Figure S2: double immunolabelling of GFP and CD31 in ischemic tissues at day 7 and day 15 after injury transplanted with either NormMSC or HypMSC. Confocal acquired images show that GFP-positive NormMSC were frequently found in highly in repair areas at day7 and day 15 although GFP-positive HypMSC were found around necrotic myocytes at day 7 and then at day 15 organized around neo vessels.

Figure S3: MSC harbored a pericyte like phenotype after implantation in ischemic muscle at day 7.(a) GFP in MSC (green) colocalize with NG2 marker (red) in ischemic tissues but (b) did not express CD31 marker (red) in extensive reparative areas. Higher magnifications show convolution of GFP-positive perivascular cells around CD31 positive- EC in neo-vessels but GFP labelling do not colocalize with the endothelial marker CD31 (red). Arrows point to GFP-MSC crawling around muscle fibers and CD31 positive neo vessels. Stars indicate necrotic muscle fibers. Dapi labeling reveal nucleus.

Figure S4:

(a) effects of HypMSC transplantation on growth factor (VEGF, TGF- β 1, bFGF, Ang1, 2 and PDGFB) gene expressions followed by performing real-time PCR.

(b) effects of temporary hypoxia on growth factor (VEGF, TGF- β 1, bFGF, Ang1, 2 and PDGFB) gene expressions in MSC followed by performing real-time PCR.

(b) Wnt expressions in MSC followed by performing real-time PCR.

(c) Western blot analysis of lysates of MSC (control) or MSC exposed during 8 or 24 hours at 1% O₂ atmosphere for VEGF expression. a-tubulin was used for equal loading.

(d) effects of temporary hypoxia on Wnt (Wnt4, 5A, 5B and 11) expressions in MSC followed by performing real-time PCR.

MSC were exposed to either normoxic (21% O₂, NormMSC, gray bars) or hypoxic (1% O₂, HypMSC, black bars) conditions for 48 hours. Values are means \pm SD; n=3; the assays were carried out in triplicate. *, P<0.05, \$, P<0.01 HypMSC vs NormMSC.

Figure S5: effect of HypCM or Wnt4 CM on EC wound migration. Wnt4 CM and HypCM stimulate EC migration in an *in vitro* scratch angiogenesis assay compared respectively to Cont CM and NormCM. Rb sFRP-1 and Dkk1 blocked the HypCM-induced migration. After the wound, EC were maintained either in Wnt4 CM vs cont CM or in HypCM vs NormCM. Analysis of CM effect on wound closure was measured as area of migration (μm^2) on phase contrast microscopy images. *, P<0.05.