Development 139, 2857-2865 (2012) doi:10.1242/dev.079665

Hypoxia promotes satellite cell self-renewal and enhances the efficiency of myoblast transplantation

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SUMMARY

Microenvironmental oxygen (O_2) regulates stem cell activity, and a hypoxic niche with low oxygen levels has been reported in multiple stem cell types. Satellite cells are muscle-resident stem cells that maintain the homeostasis and mediate the regeneration of skeletal muscles. We demonstrate here that hypoxic culture conditions favor the quiescence of satellite cell-derived primary myoblasts by upregulating Pax7, a key regulator of satellite cell self-renewal, and downregulating MyoD and myogenin. During myoblast division, hypoxia promotes asymmetric self-renewal divisions and inhibits asymmetric differentiation divisions without affecting the overall rate of proliferation. Mechanistic studies reveal that hypoxia activates the Notch signaling pathway, which subsequently represses the expression of miR-1 and miR-206 through canonical Hes/Hey proteins, leading to increased levels of Pax7. More importantly, hypoxia conditioning enhances the efficiency of myoblast transplantation and the self-renewal of implanted cells. Given the robust effects of hypoxia on maintaining the quiescence and promoting the self-renewal of cultured myoblasts, we predict that oxygen levels in the satellite cell niche play a central role in precisely balancing quiescence versus activation, and self-renewal versus differentiation, in muscle stem cells in vivo.

KEY WORDS: Hypoxia, Satellite cells, Self-renewal, Quiescence, microRNA, Mouse

INTRODUCTION

The term 'stem cell niche' refers to the local microenvironment, including cellular and acellular components, that houses, nourishes and regulates stem cells. Within the niche of many adult stem cells, oxygen levels are usually much lower than those in the ambient air or even in the host tissue (Simon and Keith, 2008; Simsek et al., 2010). For instance, oxygen levels in the niches of mesenchymal, hematopoietic and neural stem cells are 2-8%, 1-6% and 1-8%, respectively (Clarke and van der Kooy, 2009; Eliasson and Jonsson, 2010; Krinner et al., 2009; Mohyeldin et al., 2010). Low oxygen tension (hypoxia) is critical for multiple stem cells to maintain their stemness and the hypoxia niche plays a crucial role in the self-renewal and proliferation of the resident stem cells. Recent studies have demonstrated that cancer stem cells, the tumor-initiating cells, are located in a hypoxic microenvironment, and the extent of hypoxia is strongly correlated with the undifferentiated states of tumors (Das et al., 2008; Pear and Simon, 2005; Sahlgren et al., 2008). Notably, mild hypoxic conditions even enhanced the generation of induced pluripotent stem cells (Yoshida et al., 2009), suggesting a previously unappreciated role of hypoxia in stem cell biology.

Satellite cells (muscle stem cells) reside in a defined anatomical niche, beneath the basal lamina and adhered to muscle fibers (Mauro, 1961). Upon muscle injury, quiescent satellite cells are activated to proliferate, differentiate and fuse into multinuclear myofibers in order to regenerate muscles (Kuang and Rudnicki, 2008). Recent studies demonstrated that these different cell cycle states can be readily distinguished based on Pax7 and MyoD expression: $Pax7^+MyoD^-$ (quiescence/self-renewal), $Pax7^+MyoD^+$ (proliferation) and $Pax7^-MyoD^+$ (differentiation) (Halevy et al., 2004; Olguin and Olwin, 2004; Zammit et al., 2004). The systemic environment of satellite cells that precisely regulates quiescence or activation, and self-renewal or differentiation, is largely unknown. Prior studies demonstrated that hypoxia maintains cultured myoblasts in an undifferentiated state by inhibiting myogenic differentiation through activating the Notch or PI3K signaling pathways (Gustafsson et al., 2005; Majmundar et al., 2012). However, it remains unknown whether hypoxia actively promotes the self-renewal of proliferating cells to maintain them at a more quiescent state. Also, the relevance of a hypoxic niche in vivo is unclear.

In this study, we demonstrate that a hypoxic culture conditions favors the quiescent state and diminishes the differentiated state of satellite cell-derived primary myoblasts. Hypoxia also promotes self-renewal divisions without affecting the overall proliferation of primary myoblasts. Mechanistic studies reveal that hypoxia exerts its effects by upregulating Pax7, mediated by downregulation of miR-1 and miR-206. Gain- and loss-of-function studies show that the Notch signaling pathway, activated by hypoxia, dictates expression of both miR-1 and miR-206. Furthermore, we demonstrate that hypoxia-conditioned myoblasts exhibit enhanced transplantation and self-renewal efficiency in vivo. These results establish O_2 level as a physiological regulator of satellite cell activity and suggest that manipulating O_2 exposure could be an effective approach for improving muscle regeneration.

MATERIALS AND METHODS

Animals

All procedures involving the use of animals were performed in accordance with the guidelines presented by Purdue University's Animal Care and Use Committee. Mice were housed in the animal facility with free access to water and standard rodent chow. Mice were from the Jackson Laboratory (*mTmG*: stock #007576; Rosa26-N1ICD: stock#006850; $Notch1^{fl/fl}$: stock#007181; MDX: stock#001801; $MyoD^{iCre}$: stock#014140) and PCR genotyping was carried out using protocols described by the supplier.

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Accepted 28 May 2012

Primary myoblast isolation and culture

Primary myoblasts were isolated from hind limb skeletal muscle. Muscles were minced and digested in type I collagenase and dispase B mixture (Roche Applied Science). Cells were then filtered from debris, centrifuged and cultured in growth media (F-10 Ham's medium supplemented with 15% fetal bovine serum, 4 ng/ml basic fibroblast growth factor and 1% penicillin-streptomycin) on collagen-coated dishes.

Induction of hypoxia

Normal humidified tissue culture incubators with 5% CO_2 were used for the normoxic cultures. For hypoxic (~1% O_2) cultures, cells were put into gas-tight modular incubator chambers (Billups-Rothenberg, Del Mar, CA, USA), which were flushed with a custom gas mixture containing 5% air, 5% CO_2 and 90% N_2, 25 psi per minute, for 3 minutes each day.

Locked nucleic acid (LNA) oligonucleotide transfection

Control LNA and miR-1/206 LNA were purchased from Exiqon. To perform the transfection experiments, primary myoblasts were seeded at 70-80% confluency in 6-cm plates. On the second day, the medium was changed and cells were transfected with LNAs (50 nM) using Lipofectamine 2000 reagent. After 12 hours the transfection complex was replaced with fresh Ham's complete medium and after an additional 48 hours of culture under normoxia and hypoxia, the cells were collected for protein and RNA analysis.

Viral infection of myoblasts

Adenoviruses were gifts from X. Y. Wang (University of Massachusetts Medical School, MA, USA). Adenovirus-Cre and adenovirus-GFP were infected to 10-cm plates of Rosa-NICD and Notch1$^{fl/fl}$ myoblasts. After 6 hours, fresh Ham's complete medium was added to replace the virus solution and samples were collected after an additional 48 hours.

Cell cycle arrest

Myoblasts were seeded at 5-10% confluence in 4-well chambers and cultured in 21% O_2 or 1% O_2 for 24 hours. LNA-transfected myoblasts were recovered with fresh Ham's complete medium for 12 hours before 24 hours culture under normoxic or hypoxic conditions. To synchronize myoblasts at the late telophase stage, cells were then treated with 2.5 mM thymidine for 16 hours, released for 8 hours, treated with 2.5 mM thymidine again for 16 hours, released for 4 hours, and then treated with 68 μM monastrol for 8 hours, released and treated with 10 μM MG132 for 2 hours, followed by 1 hour release and fixation in 2% paraformaldehyde (PFA).

Quantitative real-time polymerase chain reaction (qPCR)

RNA was extracted and purified from muscle tissue or cell cultures using Trizol and contaminating DNA was removed with DNase I. Random hexamer primers were used to convert RNA into cDNA. For miRNA qPCR, multiple adenosine nucleotides were added to the 3′ end of RNAs by *Escherichia coli* DNA polymerase and cDNAs were synthesized with a specific RT primer (supplementary material Table S1). Genomic DNA of cell transplantation samples was extracted and purified with a phenol:chloroform mixture. qPCR was performed using a Light Cycler 480 (Roche) machine for 40 cycles and the fold change for all the samples was calculated by $2^{-\Delta\Delta ct}$ methods. *18s* was used as housekeeping gene for mRNA qPCR. 18s and u6 were used as housekeeping genes for miRNA qPCR.

Western blots

Cultured cells were washed with PBS and homogenized in lysis buffer [50 mM Tris-Cl (pH 8.0), 1% SDS, 200 mM NaCl, 50 mM NaF, 1 mM dithiothreitol (DTT), and protease inhibitors]. Proteins were resolved on each lane on 12% SDS-PAGE, electrotransferred onto PVDF membrane, and probed with specific antibodies (Pax7 mouse IgG1 from Developmental Studies Hybridoma Bank, MyoD rabbit IgG from Santa Cruz, α-tubulin mouse IgG from Sigma, GAPDH mouse IgG from Santa Cruz, NICD rabbit IgG from Calbiochem) and detected by chemiluminescence. The bands were quantified using Carestream molecular imaging software.

Myoblast transplantation

Muscle regeneration in MDX mice was induced by injecting cardiotoxin (CTX, 50 μl of 10 μM solution, Sigma) into the mid-belly of tibialis anterior (TA) muscles one day before cell transplantation. About 1×10^5 *m*T*m*G myoblast cells that were cultured for 48 hours under 21% O_2 or 1% O_2 were transplanted into the mid-belly of left or right TA muscle, respectively. Muscles were harvested 1-28 days after injection and fixed in 4% PFA.

Cryosectioning

TA muscle samples (fresh or fixed) were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek) and quickly frozen in dry ice-cooled isopentane. Muscles were cut at 10 μm thickness by a Leica CM 1850 cryostat and the sections were placed on Superfrost Plus glass slides (Electron Microscopy Sciences).

Immunostaining and image capture

Fixed cells or tissue sections were blocked for 30 minutes with blocking buffer containing PBS, 5% horse serum, 2% bovine serum albumin, 0.2% Triton X-100 and 0.1% sodium azide. Tissues and cells were then incubated with primary antibodies diluted in blocking buffer for 1 hour at room temperature, then incubated with secondary antibodies and Hoechst (diluted in PBS) for 30 minutes at room temperature, and mounted with Dako fluorescent mounting media (Glostrup, Denmark). Fluorescent images were captured using a Coolsnap HQ CCD camera (Photometrics, USA) driven by IP Lab software (Scanalytics, USA) using a Leica DMI 6000B fluorescent microscope (Mannheim, Germany) with a 20× objective (NA=0.70). The relative intensity of Pax7 (red) and MyoD (green) immunofluorescence was used to classify the cell cycle status of myoblasts. If the merged color appeared to be red, yellow or green, then the cell was classified as $Pax7^+/MyD^-$ (self-renewal), $Pax7^+/MyD^+$ (proliferation) or $Pax7^-/MyD^+$ (differentiation), respectively. The absolute intensity (for example, bright versus dim yellow) was not discriminated in the analysis. During the analysis, gray scale images of the green and red channels were also assessed. If the signal intensity was below a certain threshold, then the protein was considered not to be expressed. As the assessment was rather qualitative, identical image handling and fluorescence scoring criteria were used in all experiments.

Statistical analysis

The data are presented with mean ± s.e.m. *P*-values were calculated using two-tailed Student's *t*-test. *P*-values >0.05 were considered to be statistically significant.

RESULTS

Hypoxia promotes quiescence in cultured myoblasts

To examine how O_2 levels affect satellite cell behavior, skeletal muscle satellite cell-derived primary myoblasts were cultured under ambient air (21% O_2) or 1% O_2 for 48 hours. Expression of Pax7, the satellite cell self-renewal marker, and MyoD, a marker for satellite cell activation and myogenic differentiation, was then examined by immunofluorescence (Fig. 1A). Previous studies have established that $Pax7^+MyoD^-$, $Pax7^+MyoD^+$ and $Pax7^-MyoD^+$ expression profiles mark subpopulations of self-renewed (quiescent), proliferating and differentiating myoblasts, respectively (Halevy et al., 2004; Olguin and Olwin, 2004; Zammit et al., 2004). We confirmed by cell proliferation marker Ki67 (Mki67 – Mouse Genome Informatics) and metaphase cell marker phospho-histone H3 (pHH3) labeling that 90-97% $Pax7^+MyoD^-$ cells are negative for these markers (supplementary material Figs S1, S2), indicating $Pax7^+MyoD^-$ cells are predominantly in the quiescent state.

The relative expression of Pax7 and MyoD was assessed as detailed in Materials and methods. Overall, the percentage of myoblasts expressing Pax7 was increased (from 83.8% to 88.9%), but the percentage of myoblasts expressing MyoD was decreased (from 89.2% to 70.9%), under hypoxic conditions (Fig. 1B).

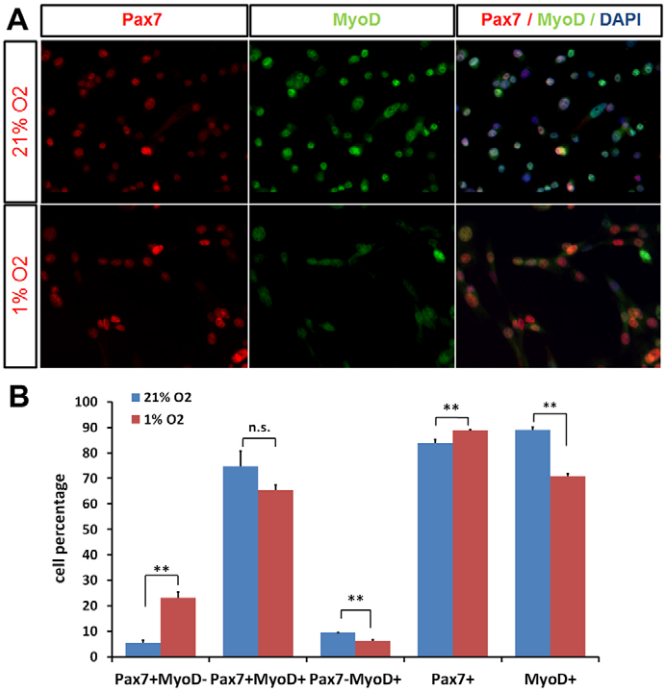

Fig. 1. Hypoxia favors the quiescent state in cultured myoblasts. (**A**) Representative images of primary skeletal myoblasts cultured under 21% O_2 (normoxia) or 1% O_2 (hypoxia) for 48 hours and labeled with Pax7 (red) and MyoD (green). Nuclei were counterstained with Hoechst33342 (blue). (**B**) Percentage of quiescent ($Pax7^+MyoD^-$), proliferating ($Pax7^+MyoD^+$) and differentiating ($Pax7^-MyoD^+$) cells. *n*=3 independent experiments, with five different areas (at least 150 cells/area) analyzed in each experiment. Error bars represent s.e.m. $^{**}P<0.01$. n.s., not significant.

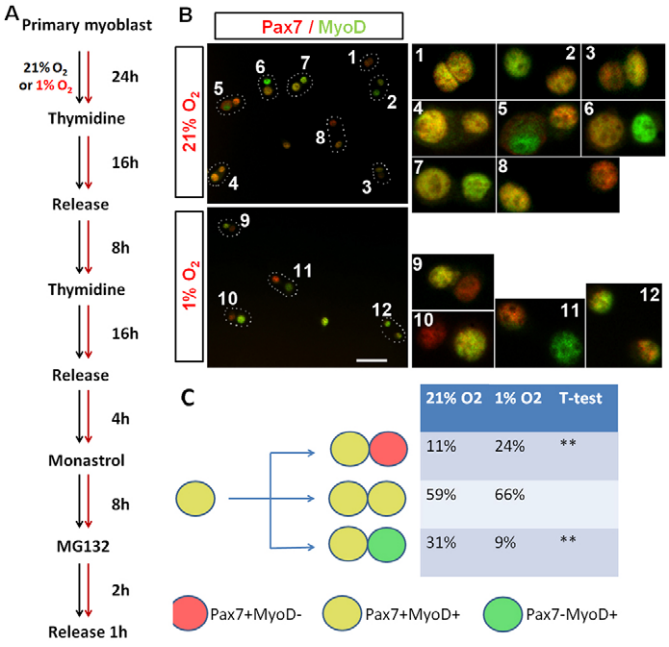

Fig. 2. Hypoxia promotes asymmetric self-renewal division in cultured myoblasts. (**A**) The strategy for synchronizing cultured myoblasts in late telophase stage. (**B**) Primary myoblasts were synchronized at the telophase stage and stained for Pax7 (red) and MyoD (green). Doublets of $Pax7^+MyoD^+$:$Pax7^+MyoD^-$ sister cells (8-10) represent asymmetric self-renewal division; $Pax7^+MyoD^+$:$Pax7^+MyoD^+$ (1-4, 7, 12) represent symmetric proliferative division; and $Pax7^+MyoD^+$:$Pax7^-MyoD^+$ (5, 6, 11) represent asymmetric differentiation division. Scale bar: 25 μm. (**C**) Percentages of asymmetric self-renewal, symmetric proliferative and asymmetric differentiation divisions under exposure of 21% O_2 or 1% O_2. *n*=3 independent experiments, with at least 60 doublets analyzed in each experiment. $^{**}P<0.01$.

Notably, the percentage of $Pax7^+MyoD^-$ quiescent (self-renewed) cells was dramatically increased by hypoxia (23.1% in hypoxia versus 5.4% in normoxia; Fig. 1B). By contrast, the percentage of $Pax7^-MyoD^+$ differentiating cells was decreased by hypoxia (6.2% under hypoxia versus 9.5% under normoxia; Fig. 1B), and the percentage of $Pax7^+MyoD^+$ proliferating myoblasts was marginally decreased by hypoxia (65.3% under hypoxia versus 74.7% under normoxia, *P*=0.067; Fig. 1B). These data demonstrate that hypoxia exposure promotes the quiescence of activated myoblasts and inhibits myogenic differentiation. Consistent with this notion, myogenin expression was also inhibited by hypoxia (supplementary material Fig. S3). Interestingly, myoblasts adopted endothelial cell morphology and formed vasculature-like arrangement in culture (Fig. 1A). Furthermore, when cells were cultured with less nutrient (5% fetal bovine serum, compared with 20% in normal cultures), hypoxia exposure resulted in 40% of myoblasts being in the quiescent state, compared with only 5% of myoblasts in the quiescent state under normoxia culture (supplementary material Fig. S4), indicating that hypoxia plays a more crucial role in myoblasts when nutrient supply is limited.

Hypoxia facilitates asymmetric self-renewal division of myoblasts

Hypoxia increases the proportion of myoblasts in the quiescent state, raising the possibility that hypoxia might influence daughter cell fate determination (i.e. self-renewal, proliferation or differentiation) during cell division. To address this, we followed a cell cycle synchronization strategy (Li et al., 2010), combined with Pax7 and MyoD labeling to examine the fate of newly divided daughter cells. Primary myoblasts were cultured at very low density under 21% or 1% O_2, and their cell cycle was synchronized as shown in Fig. 2A, which resulted in 20-40% of cells arrested at telophase stage. This strategy allowed us to document precisely the process of cell fate decision in newly divided daughter cells based on Pax7 and MyoD expression (Fig. 2B).

To demonstrate that these doublets are newly divided sister cells rather than random cell aggregates, mixtures of labeled and non-labeled myoblasts were subjected to the same culture procedure shown in Fig. 2A. The labeled primary myoblasts were derived from the *m*T*m*G mouse (Muzumdar et al., 2007), which ubiquitously expresses membrane-targeted tandem dimer Tomato (mT, a red fluorescent protein), and the non-labeled cells were from wild-type littermates. As we mixed equal numbers of mT^+ (50%) and mT^- (50%) cells, if after cell division the doublets are random aggregates of two separate cells, then ~50% of doublets should be mT^+/mT^-, the other 50% should be mT^+/mT^+ or mT^-/mT^-. We observed that only 6% doublets (χ^2 test, $P<0.01$; $n=303$) were mT^+/mT^- (supplementary material Fig. S5). These results provide strong evidence that the doublets in our experiments predominantly represent true sister cells.

As the $Pax7^+MyoD^-$ (quiescent) and $Pax7^-MyoD^+$ (differentiating) cells have both withdrawn from the cell cycle, all doublets of sister cells should be divided from $Pax7^+MyoD^+$ cells

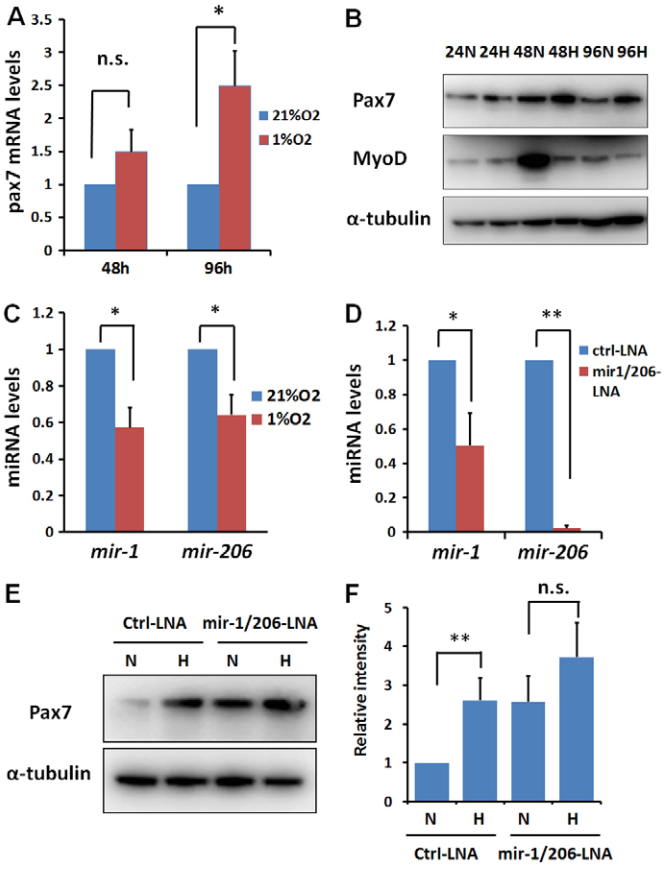

Fig. 3. Hypoxia upregulates Pax7 by downregulating miR-1/206. Primary myoblasts were cultured under normoxia (N; 21% O_2) or hypoxia (H; 1% O_2). (**A**) qPCR for relative expression of *Pax7* mRNA normalized to 18s. (**B**) Western blot analysis for Pax7, MyoD and α-tubulin at the indicated time points. (**C**) Hypoxia inhibits miR-1 and miR-206 expression. Myoblasts were cultured under normoxia or hypoxia for 48 hours and qRT-PCR results were normalized to 18s and U6. (**D**) Efficient knockdown of miR-1/206 by LNAs. Myoblasts were transfected with 50 nM control LNA or LNAs against miR-1/206 and cultured for 48 hours. (**E**,**F**) Pax7 protein (E) and *Pax7* mRNA (F) levels under hypoxia and normoxia, in the presence or absence of LNA against miR-1/206. Data are from five independent experiments. **P*<0.05, ***P*<0.01. n.s., not significant.

that are in the cell cycle and proliferate (Halevy et al., 2004; Olguin and Olwin, 2004; Zammit et al., 2004). We found that doublets of sister cells are predominantly $Pax7^+MyoD^+$:$Pax7^+MyoD^-$, $Pax7^+MyoD^+$:$Pax7^+MyoD^+$, or $Pax7^+MyoD^+$:$Pax7^-MyoD^+$ (Fig. 2B), presumably as a result of asymmetric self-renewal division, symmetric division and asymmetric differentiation division, respectively. Strikingly, hypoxia robustly increased the frequency of asymmetric self-renewal divisions (24.4% under hypoxia versus 10.5% under normoxia cultures; Fig. 2C). The frequency of symmetric cell divisions was not significantly affected by hypoxia exposure (Fig. 2C). However, the frequency of asymmetric differentiation divisions was inhibited (8.7% under hypoxia versus 30.9% under normoxia; Fig. 2C). Overall, our data suggest that hypoxia exposure affects myoblast fate decision by promoting self-renewal but inhibiting differentiation, without affecting cell proliferation.

Hypoxia upregulates Pax7 by downregulating miR-1 and miR-206 (miR-1/206)

To investigate the mechanism by which hypoxia maintains myoblast at the undifferentiated state, we first investigated how hypoxia regulates Pax7, a crucial determinant of self-renewal. Hypoxia did not affect *Pax7* mRNA expression at 48 hours (P=0.23), but significantly upregulated *Pax7* transcription at 96 hours (~2.5-fold increase, P<0.05; Fig. 3A). Interestingly, Pax7 protein levels were increased dramatically by hypoxia at both time points (Fig. 3B), suggesting that post-transcriptional regulation of Pax7 occurs under hypoxia exposure. Consistent with previous studies, we noted that MyoD and myogenin proteins were decreased under hypoxia culture (Fig. 3B; supplementary material Fig. S3E).

We next investigated what mediates the effect of hypoxia on Pax7. Recent studies demonstrated that some small regulatory RNAs, such as miR-1, miR-206 and miR-486, can recognize the 3′UTR of *Pax7* mRNA and downregulate Pax7 protein production (Chen et al., 2010; Dey et al., 2011). Under hypoxic cultures, we found that miR-1 and miR-206 were downregulated by 43% and 36%, respectively (Fig. 3C), whereas miR-486 was not detectable by qRT-PCR. These observations prompted us to hypothesize that hypoxia-induced downregulation of miR-1/206 accounts for the upregulation of Pax7 protein. To examine this, we used antisense LNA oligonucleotides to block miR-1/206 specifically, which gave us 50% and 98% knockdown of miR-1 and miR-206, respectively (Fig. 3D). When myoblasts were treated with a mixture of miR-1/206 LNA, the *Pax7* mRNA level was slightly increased (supplementary material Fig. S3F) and Pax7 protein was dramatically increased (Fig. 3E,F). We then examined whether depletion of miR-1/206 can abolish hypoxia-stimulated upregulation of Pax7 protein. As predicted, control LNA-transfected myoblasts showed dramatic upregulation of Pax7 upon hypoxia exposure (2.6-fold, P<0.01; Fig. 3E). By contrast, hypoxia only elicited a small and non-significant upregulation of Pax7 in miR-1/206 LNA-transfected myoblasts (1.2-fold, P=0.23; Fig. 3F). Thus, knockdown of miR-1/206 abolishes the effect of hypoxia on Pax7, suggesting that hypoxia upregulates Pax7 protein production through the downregulation of miR-1/206.

Inhibition of miR-1/206 abolishes hypoxia-promoted self-renewal in myoblasts

As hypoxia downregulates miR-1/206, we investigated whether these miRNAs are necessary to mediate the effect of hypoxia on myoblasts. We used a mixture of LNAs to knockdown miR-1/206 and examined the cell cycle state of primary myoblasts (supplementary material Fig. S6). Under normoxic conditions, mock LNA-transfected (control) myoblasts had 5.8% quiescent ($Pax7^+MyoD^-$) cells and knockdown of miR-1/206 by LNA led to ~2.5-fold increase in the proportion of quiescent cells (Fig. 4A). The percentage of differentiating ($Pax7^-MyoD^+$) myoblasts was reduced, whereas the proportion of proliferating ($Pax7^+MyoD^+$) myoblasts was not affected, by miR-1/206 knockdown (Fig. 4A). Thus, blockage of miR-1/206 mimicked the effect of hypoxia on myoblast quiescence and differentiation. Importantly, hypoxia exposure failed to induce further increases in the quiescent cells (21.8% versus 19.5%), or decreases of the differentiating cells (10.0% versus 8.2%), in cultures treated with miR-1/206 LNA compared with those treated with mock LNA (Fig. 4A). The non-additive effects of miR-1/206 blockage and hypoxia exposure suggest that the effect of hypoxia on myoblast quiescence and differentiation is mediated by miR-1/206.

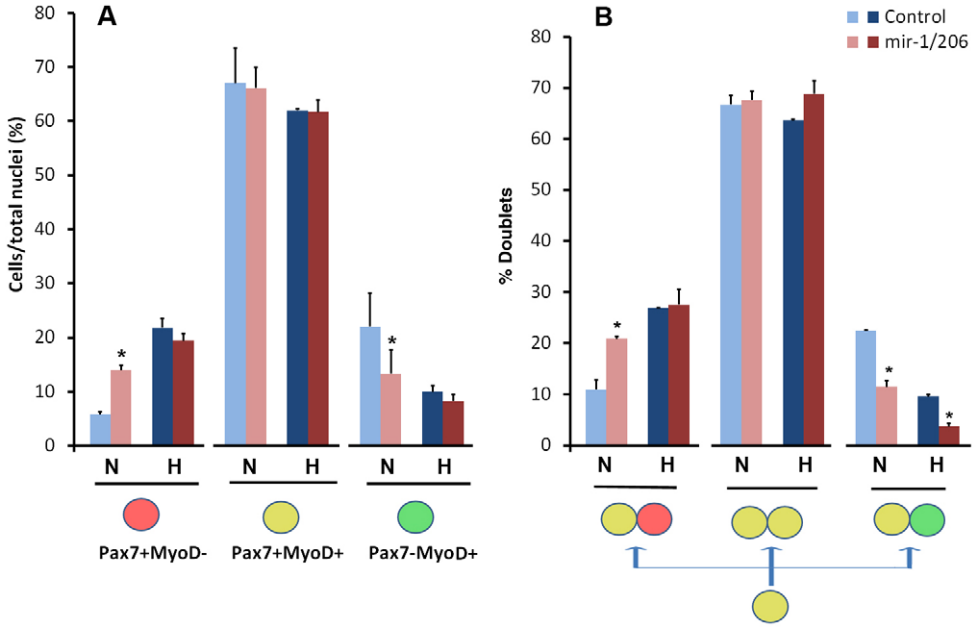

Fig. 4. Inhibition of miR-1/206 mimics the effect of hypoxia on the quiescence and self-renewal of myoblasts. Myoblasts were transfected with 50 nM control LNA or LNAs against miR-1/206, cultured under 21% O_2 (N, normoxia) or 1% O_2 (H, hypoxia) for 48 hours. (**A**) Relative abundance of quiescent ($Pax7^+MyoD^-$), proliferating ($Pax7^+MyoD^+$) and differentiating ($Pax7^-MyoD^+$) cells. *n*=3. (**B**) Sister cells were synchronized at telophase stage and their cell fates were analyzed based on Pax7 (red) and MyoD (green). The graph shows the frequency of asymmetric self-renewal, symmetric proliferative and asymmetric differentiation divisions. *n*=3. **P*<0.05, ***P*<0.01. n.s., not significant.

Next, we examined the effect of miR-1/206 knockdown on myoblast cell fate determination following cell division (supplementary material Fig. S7) using the cell cycle synchronization paradigm illustrated in Fig. 2A. Under normoxic conditions, LNA-mediated knockdown of miR-1/206 led to an approximate doubling of the incidence of asymmetric self-renewal divisions that give rise to a $Pax7^+MyoD^-$ and a $Pax7^+MyoD^+$ cell, echoed by a halving of the incidence of asymmetric differentiation divisions that give rise to a $Pax7^-MyoD^+$ and a $Pax7^+MyoD^+$ cell (Fig. 4B). Knockdown of miR-1/206 had no effect on symmetric cell divisions that give rise to two $Pax7^+MyoD^+$ sister cells (Fig. 4B). Notably, miR1/206 knockdown abolished further increases of asymmetric self-renewal divisions induced by hypoxia exposure (Fig. 4B), suggesting that the effects of miR-1/206 and hypoxia on myoblast self-renewal are not additive. Together, these results provide strong evidence that hypoxia regulates the self-renewal division and quiescence of primary myoblasts through inhibition of miR-1/206.

The Notch signaling pathway, activated under hypoxia, represses miR-1/206

To investigate how hypoxia inhibits miR-1/206, we examined the Notch signaling pathway, which has been shown to be activated by hypoxia, although a recent study indicated that hypoxia had no effect on Notch activation in C2C12 myoblasts (Gustafsson et al., 2005; Majmundar et al., 2012). In our study, we consistently found that activated Notch1 protein (cleaved Notch1 intracellular domain, N1ICD) was accumulated under hypoxia culture (Fig. 5A). The expression of two Notch target genes, *Hes1* and *Hey1*, was also increased under hypoxia (Fig. 5B), suggesting that hypoxia activates the Notch signaling pathway in primary myoblasts. To examine whether the activated Notch signal pathway accounts for the downregulation of miR-1/206 under hypoxia, we cultured myoblasts from Rosa26-N1ICD mice (Murtaugh et al., 2003), in which N1ICD overexpression, driven by the ubiquitously expressed endogenous Rosa26 promoter, can be induced by Cre. Indeed, adenovirus mediated *Cre* transduction into the Rosa-N1ICD myoblasts resulted in strong inhibition of miR-1 and miR-206 (~70% reduction; Fig. 5C). These results suggest that hypoxia activates the Notch signaling pathway, which represses miR-1/206 expression.

To examine whether Notch1 inactivation affects miR-1/206 expression, we isolated myoblasts from the $Notch1^{fl/fl}$ mice (Yang et al., 2004), in which the first exon of *Notch1* is flanked by LoxP sites and can be deleted upon adenovirus-Cre infection, leaving an immediate non-sense mutation. Adeno-Cre-mediated deletion of *Notch1* led to 69% and 48% upregulation of miR-1 and miR-206, respectively (Fig. 5D). It is worth mentioning that our adeno-Cre infection protocol resulted in ~70% reduction of *Notch1* expression (Fig. 5E,F), indicating that miR-1 and miR-206 would be more robustly upregulated if *Notch1* were completely ablated. Importantly, *Pax7* mRNA and protein levels both decreased dramatically (Fig. 5E,F). Consistent with the results from genetic depletion of *Notch1*, when Notch signaling was blocked by DAPT, a pharmacological inhibitor of γ-secretase that cleaves Notch receptor and releases functional NICD, we found that both miR-1 and miR-206 were significantly upregulated (Fig. 5G). Consequently, *Pax7* mRNA and protein were both downregulated, together with reduced N1ICD protein levels (Fig. 5H,I). Therefore, inactivation of Notch signaling leads to upregulation of miR-1 and miR-206, and downregulation of Pax7.

To determine whether Notch signaling activation is required for hypoxia-mediated repression of miR-1 and miR-206, and upregulation of Pax7, we cultured primary myoblasts under normoxia and hypoxia in the presence of DAPT. Strikingly, DAPT treatment abolished hypoxia-mediated repression of miR-1 and miR-206 (Fig. 5J). Consequently, hypoxia-stimulated Pax7 upregulation was also abolished when Notch signaling was blocked by DAPT (Fig. 5K). Thus, hypoxia acts through Notch to repress miR-1 and miR-206 expression.

We conducted additional experiments to investigate how Notch regulates the miRNAs. As Notch inhibits MyoD, a positive regulator of miR-1/206 (Rao et al., 2006), we first investigated whether Notch can inhibit miR-1/206 independently of MyoD using primary myoblasts derived from $MyoD^{iCre/iCre}$ mice, in which Cre is knocked into the MyoD locus to disrupt this gene (Kanisicak et al., 2009). We found that overexpression

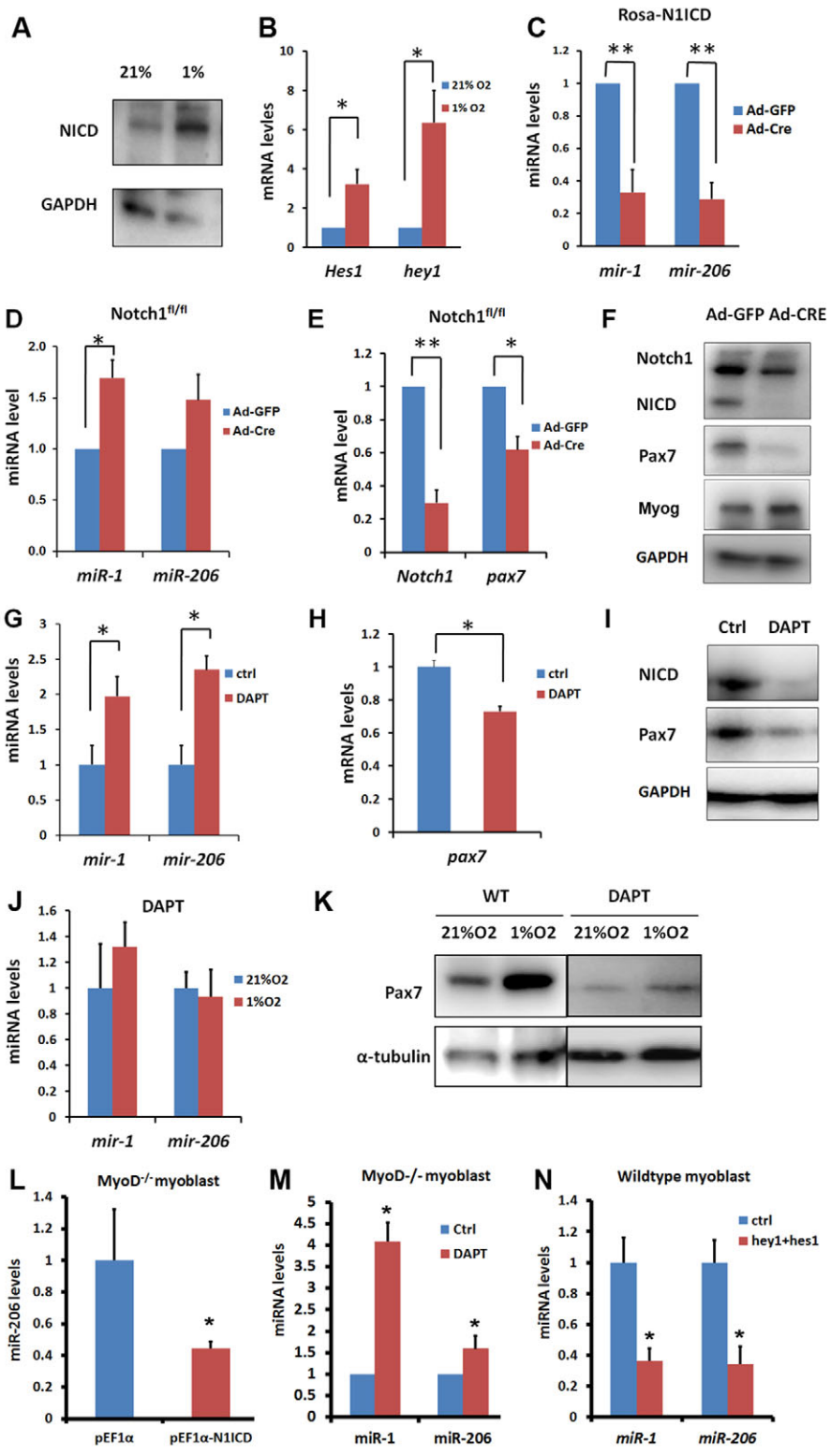

Fig. 5. Notch signaling represses miR-1/206. (**A**,**B**) Myoblasts were cultured under 21% O_2 and 1% O_2 for 48 hours and cells were collected for western blot analysis of N1ICD (A) and qPCR for Notch targets *Hes1* and *Hey1* (B) expression. (**C**) Myoblasts derived from Rosa26-N1ICD mice were infected with adenovirus-GFP or adenovirus-Cre. Cells were collected after 48 hours of infection for qPCR analysis of miR-1/206. (**D-F**) Notch1$^{fl/fl}$ knockout mice, in which the first exon of Notch1 is floxed and can be deleted by Cre, leading to mutation of Notch1. Myoblasts derived from Notch1$^{fl/fl}$ mice were infected with adenovirus-GFP and adenovirus-Cre. Cells were collected after 48 hours of infection for qPCR detection of miR-1/206 expression (D) and *Notch1* and *Pax7* expression (E), and western blot analysis for Notch1, Pax7, myogenin and GAPDH expression (F). (**G-I**) Wild-type myoblasts were treated with 10 μM DAPT, a pharmacological inhibitor of γ-secretase that cleaves Notch receptor and releases functional NICD. Cells were collected after 48 hours of DAPT treatment for qPCR detections of miR-1/206 (G) and *Pax7* mRNAs (H), and western blot analysis for N1ICD, Pax7 and GAPDH proteins (I). (**J**,**K**) Wild-type myoblasts were treated with 10 μM DAPT 12 hours before exposure to 21% O_2 or 1% O_2 for an additional 48 hours, and analyzed for miR-1/206 expression by qPCR (J) and Pax7 expression by western blot (K). (**L**) MyoD$^{iCre/iCre}$ (MyoD$^{-/-}$) myoblasts were electroporated with the pEF1a-N1ICD plasmid expressing N1ICD or with the empty vector pEF1a, cultured for 48 hours, and analyzed for miR-206 expression by qPCR. (**M**) MyoD$^{-/-}$ myoblasts were treated with 10 μM DAPT for 48 hours, and analyzed for miR-1/206 expression by qPCR. (**N**) Wild-type myoblasts were electroporated with the plasmids expressing *Hes1* and *Hey1* or with the empty vector, cultured for 48 hours, and analyzed for miR-1/206 expression by qPCR. Expression levels of mRNAs were normalized to 18s and miRNAs were normalized to 18s and U6. n=3-6. *P<0.05, **P<0.01.

of N1ICD repressed miR-206 expression (Fig. 5L), whereas inhibition of Notch signaling with DAPT significantly upregulated the expression of both miR-1 and miR-206 in the absence of *MyoD* (Fig. 5M). Next, we investigated whether canonical Notch targets can regulate miR-1/206 expression. We co-expressed the hypoxia-induced Notch targets *Hey1* and *Hes1* in primary myoblasts. *Hey1* and *Hes1* overexpression was sufficient to inhibit the expression of miR-1 and miR-206 (Fig. 5N). These results indicate that Notch signaling can repress miR-1/206 through Hes/Hey family targets and independently of MyoD. Collectively, we conclude that hypoxia represses miR-1 and miR-206 expression through activating Notch signaling, leading to the upregulation of Pax7 and enhanced self-renewal of myoblasts.

Enhanced transplantation efficiency of hypoxia-conditioned myoblasts

As hypoxia promotes myoblast self-renewal, a bottle-neck limiting the long-term success of myoblast transplantation, we examined whether hypoxia conditioning improves the efficiency of myoblast transplantation. We derived primary myoblasts from the *m*T*m*G mouse (Muzumdar et al., 2007), which ubiquitously expresses membrane-targeted red fluorescent protein (mT). Myoblasts were cultured under normoxia or hypoxia for 48 hours and injected into regenerating TA muscles of MDX mice, which lack the expression of dystrophin. Throughout the first four weeks following cell transplantation, more mT^+ (Fig. 6A,B) and $dystrophin^+$ (Fig. 6C,D) fibers were observed in the muscles that received hypoxia-conditioned myoblasts compared with muscles that received non-conditioned myoblasts. The mT^+ cells are bona fide myofibers that express the sarcomeric myosin heavy chain protein (supplementary material Fig. S8). Quantitative analysis indicates that there were approximately two times more mT^+ cells in muscles injected with hypoxia-conditioned myoblasts than in muscles injected with non-conditioned myoblasts within 4 days post injection (dpi) of myoblasts (Fig. 6E). The improved grafting efficiency of hypoxia-conditioned myoblasts appeared to be more obvious with time, as there were ~2.5-fold and ~5.5-fold as many mT^+ cells in the muscles injected with hypoxia-conditioned myoblasts compared with muscles injected with non-conditioned myoblasts, at 1-2 and 3-4 weeks post injection, respectively (Fig. 6E). Consistently, the number of $dystrophin^+$ myofibers and the *mTmG* genomic DNA levels increased 2.6- and 3.2-fold, respectively, in muscles injected with hypoxia-conditioned myoblasts compared with muscles injected with non-conditioned myoblasts at 2-4 weeks post transplantation (Fig. 6F,G). Our data demonstrate that hypoxia-conditioned myoblasts have better transplanting efficiency.

The time-dependent increases in the grafting efficiency between hypoxia-conditioned and non-conditioned myoblasts (Fig. 6E) indicate a superior self-renewal ability of hypoxia-conditioned myoblasts. We examined this possibility directly using M-cadherin as a marker to quantify the number of mT^+ satellite cells attached to host fibers (supplementary material Fig. S9). At three weeks post transplantation, significantly more satellite cells were enumerated per unit microscopic area in muscles transplanted with hypoxia-conditioned myoblasts compared with muscles injected with non-conditioned myoblasts (3.7±0.6 cells in hypoxia group versus 1.6±0.6 cells in normoxia group, $P<0.05$, $n=3$ mice; Fig. 6H). These results demonstrate that hypoxia-conditioned myoblasts exhibit enhanced self-renewal and occupy the satellite cell compartment more readily in vivo.

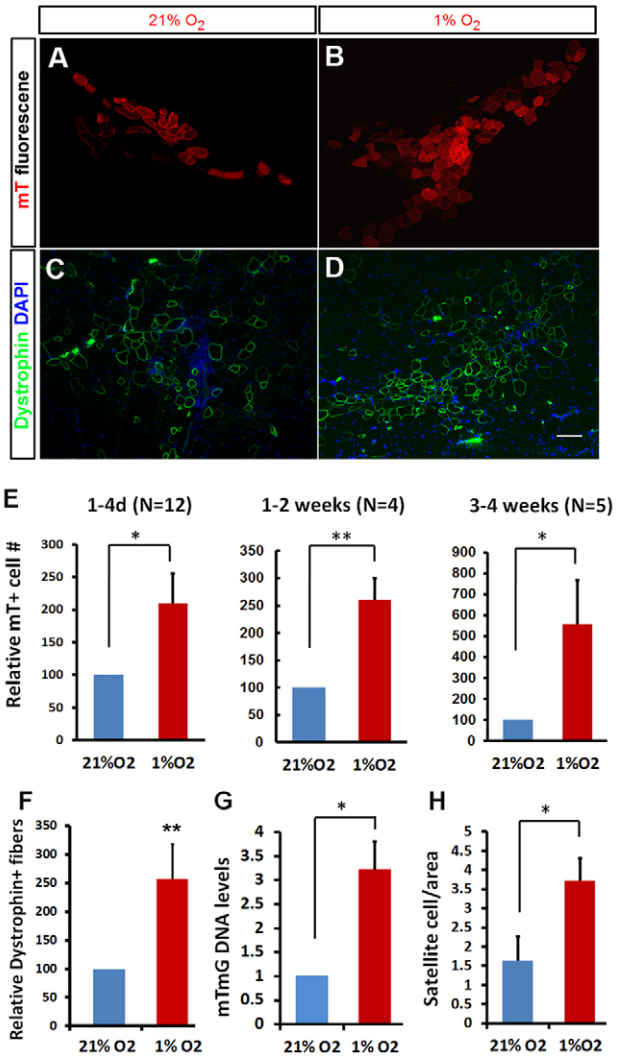

Fig. 6. Enhanced transplantation efficiency of hypoxia conditioned myoblasts. *m*T*m*G myoblasts expressing membrane-targeted tandem dimer Tomato (*m*T, a red fluorescent protein), cultured under normoxia or hypoxia for 48 hours, were transplanted into the left and right TA muscles of MDX mice, respectively. (**A-D**) Representative images of mT^+ (A,B) and $dystrophin^+$ fibers (C,D) at 3 weeks post injection (wpi). (**E**) The relative number of mT^+ fibers after 1-4 dpi, 1-2 wpi and 3-4 wpi. N represents the number of mouse analyzed, with five sections counted in each muscle. (**F**) The relative number of $dystrophin^+$ myofibers ($n=5$) after 2-3 wpi. (**G**) Relative *mTmG* genomic DNA ($n=4$) from samples of 3-4 wpi. (**H**) M-Cadherin$^+$ RFP^+ satellite cell numbers per microscopic region. Scale bar: 50 μm. $^*P<0.05$, $^{**}P<0.01$.

DISCUSSION

In this work, we identify a novel role of hypoxia in promoting asymmetric self-renewal division and facilitating the undifferentiated state of muscle progenitors. We illustrate the underlying molecular pathway through which hypoxia activates Notch signaling, which leads to suppression of miR-1/206 and upregulation of Pax7. Furthermore, our cell transplantation experiments demonstrate that hypoxia-conditioned myoblasts exhibited improved self-renewal in vivo and contributed more efficiently to the reconstitution of damaged host muscles. The results indicate that microenvironmental oxygen levels play a crucial role in balancing quiescence versus activation, and self-renewal versus differentiation of muscle stem cells in vivo.

Our finding that hypoxia upregulates Pax7 and promotes self-renewal complements previous observations that hypoxia inhibits myogenic differentiation through downregulation of MyoD and myogenin (Gustafsson et al., 2005; Majmundar et al., 2012). It is worth mentioning that increased self-renewal is not a necessary consequence of inhibition of differentiation. In theory, when proliferating cells are prevented from differentiation, they can either continue proliferating or undergo self-renewal. Our analysis of self-renewal, proliferation and differentiation based on Pax7 and MyoD expression supports the latter possibility. As the two transcription factors Pax7 and myogenin, which are crucial for self-

renewal and differentiation, respectively, can reciprocally inhibit each other (Olguin et al., 2007), it remains to be determined whether reduced differentiation is a secondary effect of increased self-renewal, or vice versa.

Hypoxia can potentially exert its function to maintain undifferentiated state via two modes of action: it might statically upregulate Pax7 and/or downregulate MyoD in myoblasts regardless of their cell cycle stages or, alternatively, it might promote asymmetric cell divisions that generate self-renewal progeny and inhibit asymmetric divisions that generate differentiating progeny. Our analysis of cell divisions demonstrates that although hypoxia has no effect on the overall frequency of symmetric versus asymmetric cell divisions, it robustly influences the daughter cell fate of asymmetrically divided cells. Specifically, the proportion of asymmetric division giving rise to a self-renewal and a proliferating progeny is increased, whereas that giving rise to a differentiating and a proliferating progeny is decreased by hypoxia. Under normoxia, ~10% of cell divisions are asymmetric self-renewal divisions that can theoretically result in 5% of cells being in the quiescent state, which is exactly what we observed. Asymmetric cell division has been proposed as a mechanism through which the balance between self-renewal and differentiation/commitment is maintained. In satellite cells, asymmetric segregation of immortal DNA strands, inheritance of the cell fate determinant Numb, and expression of the myogenic commitment marker Myf5 or the self-renewal maker Pax7 have been reported in previous studies (Conboy and Rando, 2002; Conboy et al., 2007; Kuang et al., 2007; Rocheteau et al., 2012; Shinin et al., 2006). However, the environmental cues that regulate asymmetric cell division have not been reported. Our study identifies environmental oxygen level as a regulator of stem cell fate by promoting asymmetric divisions. Interestingly, under hypoxia, ~24% of the cell divisions are asymmetric self-renewal divisions that will theoretically generate 12% cells in the quiescent state ($Pax7^{+}/MyoD^{-}$). As we actually observed that 23% of myoblasts are in the quiescent state under hypoxia, this result indicates that, in addition to regulating asymmetric divisions, hypoxia also regulates the relative levels of Pax7 and MyoD in the absence of cell division to maintain myoblasts in the undifferentiated state.

We illustrate a molecular pathway involving Notch signaling and microRNAs that mediates the effect of hypoxia on satellite cell self-renewal. Previous studies in myoblasts reported contradicting results as to whether hypoxia activates Notch signaling (Gustafsson et al., 2005; Majmundar et al., 2012). Our results not only support the notion that hypoxia activates Notch signaling, but further demonstrate that Notch signaling is required to mediate the effect of hypoxia on self-renewal of satellite cells. These results are in agreement with several recent studies. Specifically, genetic mutation of RBP-Jκ, a central mediator of Notch signaling, in quiescent satellite cells leads to their spontaneous loss of quiescence and precocious differentiation (Bjornson et al., 2012; Mourikis et al., 2012). Conversely, constitutive ligand-independent activation of Notch upregulates Pax7 and promotes the self-renewal of satellite cells (Wen et al., 2012).

Our current gain- and loss-of-function studies show that Notch signaling dictates the expression of miR-1/206, leading to Pax7 upregulation. As Notch pathway functions by binding to the RBP-Jκ nuclear adaptor and activating downstream targets such as *Hes* and *Hey*, it is possible that the expression of miR-1/206 is regulated directly by NICD through consensus RBP-Jκ binding sites, or by Hes/Hey proteins and their downstream targets. Intriguingly, Notch signaling is known to activate Hes1, which then inhibits MyoD expression (Kopan et al., 1994). Hirai et al. reported that MyoD regulates miR-1 expression through directly binding to the upstream enhancer (Hirai et al., 2010). Sun et al. reported that mTOR controls miR-1 expression by regulating MyoD stability (Sun et al., 2010). These previous studies point to the possibility that activated Notch represses miR-1/206 expression by downregulating MyoD. Our study demonstrates that Notch signaling, acting through activation of Hes1/Hey1, can inhibit miR-1/206 independently of MyoD. However, our study does not rule out the possibility that MyoD suppression is involved in the inhibition of miR-1/206 by hypoxia. In this scenario, a greater inhibition of miR-1/206 can be achieved through both MyoD-dependent and independent mechanisms. Importantly, both miR-1 and miR-206 have been shown to attenuate Pax7 expression in primary myoblasts (Chen et al., 2010). Therefore, downregulation of miR-1/206 under hypoxia leads to Pax7 upregulation and enhanced self-renewal. Meanwhile, hypoxia- and NICD-mediated downregulation of MyoD and myogenin might also contribute to maintaining myoblasts in the undifferentiated state. Together, our results suggest that hypoxia activates Notch signaling, which subsequently represses MyoD to inhibit differentiation, and represses miR-1/206 to upregulate Pax7 and promote self-renewal of myogenic progenitors.

Skeletal myoblasts have been applied in cell transplantation therapies to expand the existing satellite cell pool and to regenerate new functional muscles, in both mouse models and human patients. However, only few transplanted myoblasts survive and contribute to new satellite cells, leading to a limited application of the therapy (Kuang and Rudnicki, 2008). We found that hypoxia-conditioned myoblasts have improved homing and regenerative efficiency when grafted into MDX mice. Our finding is consistent with recent reports that hypoxia conditioning improves the efficiency of mesenchymal stem cell transplantation (Rosova et al., 2008). Intriguingly, $MyoD^{-/-}$ myoblasts survived better upon transplantation and resulted in higher efficiency of new muscle formation (Asakura et al., 2007). In our study, hypoxia-conditioned myoblasts have dramatically reduced MyoD expression, which might have contributed to their increased survival and self-renewal after transplantation. Consistent with this notion, we observed that hypoxia-conditioned myoblasts had delayed differentiation kinetics after being transplanted, evidenced by the smaller size of new myofibers derived from the transplanted cells (supplementary material Fig. S8). However, hypoxia-conditioned myoblasts are much more competent in self-renewal and homing to the satellite cell compartment. Ultimately, the self-renewed satellite cell population is crucial for the long-term contribution of transplanted cells to the formation of new myofibers in the host.

Acknowledgements
We thank Michael Rudnicki for comments on the manuscript.

Funding
The project is partially supported by funding from the Muscular Dystrophy Association (MDA), the National Institutes of Health (NIH) [R01AR060652] and the United States Department of Agriculture (USDA) [AFRI-2009-35206-05218 to S.K.]. Deposited in PMC for release after 12 months.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.079665/-/DC1

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