Myf5 haploinsufficiency reveals distinct cell fate potentials for adult skeletal muscle stem cells

Barbara Gayraud-Morel[1], Fabrice Chrétien[1,2,*], Aurélie Jory[1,*,‡], Ramkumar Sambasivan[1,*], Elisa Negroni[6], Patricia Flamant, Guillaume Soubigou[3], Jean-Yves Coppée[3], James Di Santo[4], Ana Cumano[5], Vincent Mouly[6] and Shahragim Tajbakhsh[1,§]

[1]Stem Cells and Development, Department of Developmental Biology, Institut Pasteur, CNRS URA 2578; [2]Unité d'Histopathologie humaine et modèles animaux; [3]Unité Transcriptome et Epigénome; [4]Unité d'Immunité Innée; [5]Unité du Développement des Lymphocytes, Institut Pasteur, 25 rue du Dr Roux, 75105 Paris, France
[6]Université Pierre et Marie Curie (Paris 6) UM76, INSERM UMRS974, CNRS UMR7215, Institut de Myologie, Groupe Hospitalier Pitié-Salpétrière, 47-83 bd de l'Hopital, 75651 Paris Cedex 13, France

*These authors contributed equally to this work
‡Present address: Columbia University Medical Center, 701 W168th Street, HHSC 1104, New York, NY 10032, USA
§Author for correspondence (shaht@pasteur.fr)

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Summary

Skeletal muscle stem cell fate in adult mice is regulated by crucial transcription factors, including the determination genes *Myf5* and *Myod*. The precise role of Myf5 in regulating quiescent muscle stem cells has remained elusive. Here we show that most, but not all, quiescent satellite cells express Myf5 protein, but at varying levels, and that resident *Myf5* heterozygous muscle stem cells are more primed for myogenic commitment compared with wild-type satellite cells. Paradoxically however, heterotypic transplantation of *Myf5* heterozygous cells into regenerating muscles results in higher self-renewal capacity compared with wild-type stem cells, whereas myofibre regenerative capacity is not altered. By contrast, *Pax7* haploinsufficiency does not show major modifications by transcriptome analysis. These observations provide a mechanism linking Myf5 levels to muscle stem cell heterogeneity and fate by exposing two distinct and opposing phenotypes associated with *Myf5* haploinsufficiency. These findings have important implications for how stem cell fates can be modulated by crucial transcription factors while generating a pool of responsive heterogeneous cells.

Key words: Satellite cell, Myogenic regulatory factors, Myf5, Pax7, Regeneration, Stem cell, Heterogeneity, Transcriptional priming, Haploinsufficiency

Introduction

Vertebrate organogenesis, growth and regeneration rely on tissue-specific stem cell populations that generate committed precursors and differentiated cells while maintaining a reservoir of stem cells for long-term tissue maintenance in the adult. Distinguishing how these decisions are balanced is a major topic of interest in stem cell biology. Recent studies have suggested that some tissues might harbour multiple stem cell entities, or stem cells with distinct states of commitment and potential (Li and Clevers, 2010; Lugert et al., 2010; Mitchell et al., 2010; Tajbakhsh, 2009). It has been proposed also that in the blood, intestine and hair follicle lineages, a more engaged stem cell population could serve to function in routine repair, whereas a more primitive, infrequently dividing fraction might assure progeny, stem and committed cells for longer periods (Li and Clevers, 2010; Wilson et al., 2008).

Adult skeletal muscle stem (satellite) cells, reside between the myofibre plasmalemma and the surrounding basement membrane, and they are in a reversible quiescent state. They enter the cell cycle after muscle injury to produce myoblasts that fuse to the existing myofibres, or generate de novo myofibres to effect tissue repair. Muscle stem cell self-renewal and commitment are controlled by a genetic network of transcription factors including the paired-box homeodomain proteins Pax3 and Pax7, and basic helix-loop-helix muscle regulatory factors (MRFs), Myf5, Mrf4, Myod and myogenin (Kassar-Duchossoy et al., 2004; Relaix et al., 2005; Rudnicki et al., 1993; Tajbakhsh et al., 1997). Myf5, Mrf4 and Myod act as the obligate determinants for the acquisition of myogenic cell fate (Kassar-Duchossoy et al., 2004; Rudnicki et al., 1993), whereas myogenin functions as a differentiation factor (Hasty et al., 1993; Nabeshima et al., 1993). Unlike *Pax3*, which is downregulated in most muscles from fetal stages, *Pax7* expression marks stem and progenitor cells from the early embryo to adult satellite cells; hence it is currently the most reliable marker for satellite cells (Bosnakovski et al., 2008; Relaix et al., 2006; Seale et al., 2000). In addition, mice null for *Pax7* have no overt deficits in myogenesis prenatally, but they are severely deficient in muscle satellite cells after birth (Kuang et al., 2006; Oustanina et al., 2004; Relaix et al., 2006; Seale et al., 2000). Notably however, *Pax3:Pax7* conditional double mutants do not compromise adult satellite cell self-renewal and differentiation, therefore, it remains unclear which genes regulate adult muscle satellite cell self-renewal and fate (Lepper et al., 2009).

Other markers of satellite cells include the $Myf5^{nlacZ}$ mouse, and the cell surface markers M-cadherin, syndecan-3 and syndecan-4, CD34, α7 integrin, CXCR4 and SM/C2.6 antigen (Vcam) (Beauchamp et al., 2000; Cerletti et al., 2008; Cornelison et al., 2004; Fukada et al., 2007; Gnocchi et al., 2009; Kuang and Rudnicki, 2008). Although Myod protein is expressed in a minor

fraction of quiescent satellite cells, a hallmark for activated satellite cells is the robust expression of this protein before the first cell division (Kanisicak et al., 2009; Zammit et al., 2002). Systematic detection of Myf5 protein in quiescent and freshly activated satellite cells has been less clear (Beauchamp et al., 2000; Day et al., 2010; Dhawan and Rando, 2005; Gayraud-Morel et al., 2007; Gayraud-Morel et al., 2009; Zammit et al., 2006). *Pax7* expression declines during myogenic commitment, but it is maintained in self-renewing satellite cells, which also lose Myod protein expression (Dhawan and Rando, 2005; Zammit et al., 2004; Zammit et al., 2006).

Heterogeneity among adult muscle stem cells has been suggested by results from different experimental approaches. For example, satellite cells have different developmental origins and consequently, those found in the head have distinct molecular signatures that reflect in part their developmental ontology (Harel et al., 2009; Sambasivan et al., 2009). In vitro heterogeneity of satellite cells was illustrated by their different clonal sizes and capacity to differentiate into myotubes (Cooper et al., 1999; Day et al., 2010; Lagord et al., 1998; Ono et al., 2010). Other studies reported that some, but not all, satellite cells (7–50%) perform biased DNA segregation during mitosis where one daughter cell inherits older template strands and the other daughter cell newly replicated DNA strands (Conboy et al., 2007; Shinin et al., 2006, Rocheteau et al., 2012). Although the precise reason for this phenomenon remains unclear (Tajbakhsh and Gonzalez, 2009), it underscores a phenotypic difference among satellite cells that might have functional consequences. In other studies, Syndecan4, Sca1, Abcg2, (3% satellite cells) (Tanaka et al., 2009) or CXCR4 (80% satellite cells) (Cerletti et al., 2008) were used as markers to distinguish satellite cell subpopulations with reported differences in engraftment potential.

Using $Myf5^{Cre}$ crossed with a $Rosa^{Stop\text{-}YFP}$ reporter mouse, a subset of postnatal *Pax7*-expressing satellite cells were found to be YFP^- (about 10%) suggesting that they were less committed (Kuang et al., 2007). Functional transplantation studies into *Pax7*-null muscle suggested that YFP^- cells have a reduced tendency to form myofibres but a greater capacity to replenish the stem cell niche compared with cells historically expressing *Myf5* (Kuang et al., 2007). Another study showed that YFP^- cells were enriched for mRNA encoding the receptor Tie2, which has been shown to promote cellular quiescence (Abou-Khalil et al., 2009). By contrast, when $Myod^{Cre}$ mice were crossed with $Rosa^{Stop\text{-}YFP}$ reporter mice, virtually no YFP^- stem cells were observed (Kanisicak et al., 2009). Thus some genetic tools have exposed heterogeneities among muscle stem cells, yet the functional consequences of these differences remain unclear.

Collectively, these studies have prompted a closer examination of the skeletal muscle stem cell population to understand the nature of the heterogeneity, and whether their potential can be revealed functionally during tissue repair. Reconciling the reported differences in stem cell properties as well as their biological relevance can provide important insights in how cell fate in this paradigmatic stem cell population is regulated. Here, we show that although heterozygous $Myf5^+$ satellite cells are transcriptionally primed for commitment, they regenerate skeletal muscle and replenish the satellite cell niche more efficiently than wild-type satellite cells after transplantation. This differential effect was not observed with heterozygous *Pax7* stem cells. These findings underscore the importance of using different phenotypic readouts to identify distinct stem cell states, and they provide insights into how Myf5 acts as a modulator of muscle stem cell fate.

Results

Expression of Myf5 protein in quiescent satellite cells

Our previous studies, and those from other laboratories, showed that the majority (~90%), but not all, satellite cells are labelled in $Myf5^{nlacZ/+}$ mice (Beauchamp et al., 2000; Day et al., 2010; Zammit et al., 2002). It remains unclear whether genetic reporters, which are relatively stable compared with transcription factors, reflect endogenous Myf5 protein levels, an important point in assessing the myogenic commitment state of muscle stem cells. Furthermore, the expression of Myf5 protein has not been systematically assessed in quiescent and activated satellite cells, due in part to difficulties in using antibodies against Myf5; hence the role of this protein in quiescent satellite cells remains poorly defined.

To investigate the quiescent satellite cell population, we used two different *Pax7* reporter mice: transgenic *Tg:Pax7-nGFP* mice (Sambasivan et al., 2009) and knock-in mice $Pax7^{nGFP/+}$. Immunostaining of satellite cells with antibodies against Pax7 and GFP on freshly isolated extensor digitorium longus (EDL) fibres showed 100% co-expression between endogenous Pax7 protein and the *nGFP* reporter for both *Tg:Pax7-nGFP* and $Pax7^{nGFP/+}$ mice (Sambasivan et al., 2009) (Fig. 1A). This was also the case for satellite cells on sections of Tibialis anterior (TA) muscle (supplementary material Fig. S1A). Isolated single myofibres can be cultured in suspension for several days and this paradigm permits the monitoring of distinct cell states from the quiescent muscle stem cell, to the generation of myoblasts, and finally self-renewal or commitment cell fates in the absence of cell fusion with the myofibre (Zammit et al., 2004). To validate our findings, this model and directly cultured primary myogenic cells were evaluated. In experiments with floating myofibres at 72 hours, and with 1 week cultured myoblasts, we found that the *nGFP* reporter from *Tg:Pax7-nGFP* mice faithfully reproduced endogenous *Pax7* expression (supplementary material Fig. S1B,C). *GFP* expression was essentially absent, or weakly expressed, in differentiated $myogenin^+$ cells and absent in myonuclei (supplementary material Fig. S1B,C). Isolation of satellite cells by FACS from *Tg:Pax7-nGFP* mice showed that they were phenotypically small in size and exhibited low cellular granulosity, as previously reported for satellite cells isolated using other markers (Fig. 1A) (Bosnakovski et al., 2008; Kuang et al., 2007; Montarras et al., 2005). Under our gating conditions, $Pax7^+$ cells represented about 2% of all mononucleated cells from fore- and hindlimb muscle extracts. In general, 5×10^5–10×10^5 quiescent satellite cells were isolated from *Tg:Pax7-nGFP* fore- and hindlimbs.

Immunolabelling followed by FACS showed that $Pax7^{nGFP/+}$ and *Tg:Pax7-nGFP* populations were negative for CD45, CD31 and Sca1, but positive for CD34 and α7 integrin, as shown previously for freshly isolated satellite cells (supplementary material Fig. S2A) (Bosnakovski et al., 2008; Cerletti et al., 2008; Joe et al., 2010; Montarras et al., 2005). To investigate the purity and myogenic potential of these populations, GFP^+ cells were isolated, plated overnight and stained with antibodies against Pax7 and Myod. All GFP^+ cells were positive for Pax7 and/or Myod ($n=100$ cells, $n=3$ animals for each model; data not shown). Purity and myogenicity of GFP^+ cells were tested further by clonal analysis of single cells in 96-well plates. After 1 week

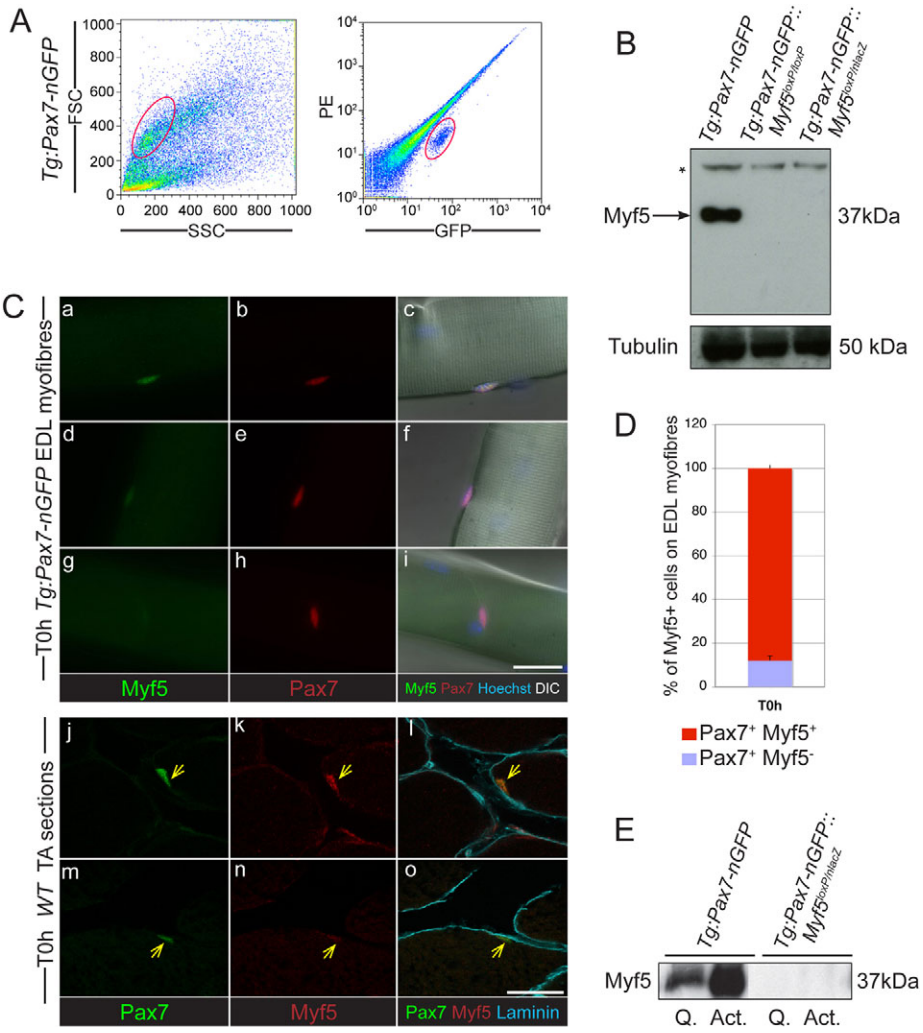

Fig. 1. Myf5 protein is expressed in adult quiescent satellite cells. (**A**) FACS profiles of freshly isolated GFP$^+$ satellite cells from *Tg:Pax7-nGFP* skeletal muscles. GFP$^+$ cells correspond to a low granulosity (low side-scatter; SSC), small size cell (low forward scatter; FSC) population. *Y*-axis on left panel is uncompensated red fluorescence; PE, phycoerythrin, 594 channel. (**B**) Western blot of FACS-isolated quiescent GFP$^+$ satellite cells from control *Tg:Pax7-nGFP* and *Myf5*-null mice, *Tg:Pax7-nGFP:Myf5*$^{loxP/loxP}$ and *Tg:Pax7-nGFP:Myf5*$^{loxP/nlacZ}$. Satellite cells from *Tg:Pax7-nGFP* show a 37 kDa band for Myf5 protein; note its absence in *Myf5* mutants. Asterisk denotes nonspecific band (Gayraud-Morel et al., 2007). (**C**) (a–i) Immunostaining on freshly isolated (T0h) *Tg:Pax7-nGFP* EDL myofibres. Note heterogeneous Myf5 protein expression in Pax7$^+$ population. Examples of robust (a–c), faint (d–f) or negative (g–i) staining obtained with Myf5 antibodies. (j–o) Immunostaining on wild-type TA frozen sections with anti-Myf5, Pax7 and laminin antibodies shows a high (j–l) and a low (m–o) Myf5 expressing cell (arrows). (**D**) Quantification of Myf5$^+$ cells within Pax7$^+$ satellite cells on *Tg:Pax7-nGFP* EDL myofibres at T0h (n=3 mice). (E) Western blot of quiescent and cultured (3 days) satellite cells. Myf5 is present in quiescent (Q.) satellite cells and upregulated in activated myoblasts (Act.). Results are mean ± s.e.m. Scale bars: 20 μm (C).

in culture, clones varied in size, and they all expressed Myod and/or myogenin, with some cells undergoing fusion at this stage (supplementary material Fig. S2B). Therefore, *Pax7*$^{nGFP/+}$ and *Tg:Pax7-nGFP* faithfully recapitulate *Pax7* expression in quiescent satellite cells and they permit the isolation of highly pure muscle stem cells.

Given that only satellite cells are GFP$^+$ in skeletal muscles of *Tg:Pax7-nGFP* adult muscle, quiescent satellite cells were isolated by FACS and examined by western blot for Myf5 protein. Robust expression of Myf5 was observed, and this expression was specific because *Myf5*-null satellite cells isolated by FACS from *Tg:Pax7-nGFP::Myf5*$^{loxP/loxP}$ or *Tg:Pax7-nGFP::Myf5*$^{loxP/lacZ}$ were negative for this protein (Fig. 1B). The specificity of the antibody was confirmed further by immunostaining control and *Myf5*-null cryosections (supplementary material Fig. S3A). Interestingly, immunostaining showed that the level of expression varied in individual wild-type cells either on single myofibres (Fig. 1Ca–i) or frozen tissue sections (Fig. 1Cj–o). Quantification revealed that the majority of satellite cells expressed Myf5 protein (Fig. 1D; n=4 mice, 12–24 myofibres per mouse). A significant upregulation was observed in myoblasts after satellite cell activation and proliferation (Fig. 1E). As expected, Myf5 protein expression was downregulated during myogenic differentiation (supplementary material Fig. S3B, myogenin$^+$ cells).

Myf5 heterozygous muscle stem cells are transcriptionally primed for commitment

The varied expression of Myf5 protein suggested that this might be related to the heterogeneous behaviour of satellite cells. To determine the functional role of Myf5, mice with one compromised allele of *Myf5* were examined using several approaches. Western blot analysis showed that *Myf5*$^{loxP/+}$and *Myf5*$^{GFP\text{-}P/+}$ satellite cells, whether taken as the entire cell population, or a subpopulation based on GFP positivity, expressed approximately half of the levels of Myf5 protein compared with levels in wild-type satellite cells (Fig. 2A,B). This suggests that no significant mono-allelic or cross-allelic regulation of the two *Myf5* alleles takes place in *Myf5* heterozygous mice.

Because *Myf5* acts as a determination gene in embryonic muscle progenitors, we reasoned that in the adult, *Myf5* heterozygous satellite cells would be less committed. To test this notion, we performed quantitative reverse transcriptase PCR (RT-qPCR) for several markers specific to quiescence, activation or differentiation of total quiescent *Myf5* heterozygous satellite cells isolated by FACS. Unexpectedly, using *Tg:Pax7-nGFP*$^+$ mice in comparison to the total wild-type population, significantly higher levels of *Myod* and *Myog* transcripts were observed in *Tg:Pax7-nGFP:Myf5*$^{loxP/+}$ cells (Fig. 2C). This committed status was underscored further by the increased

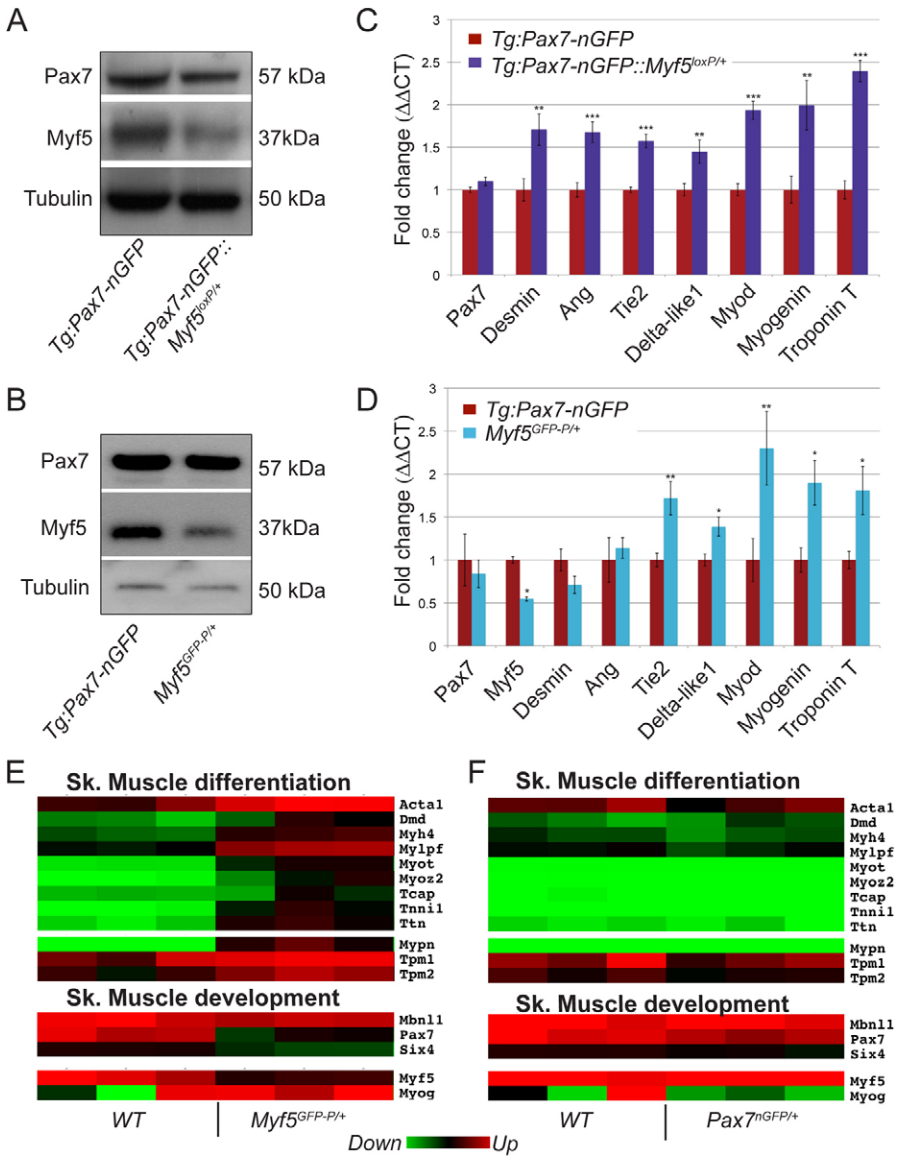

Fig. 2. *Myf5* heterozygous satellite cells are primed for myogenic commitment. (**A**) Western blot with *Tg:Pax7-nGFP:Myf5*$^{loxP/+}$ and *Tg:Pax7-nGFP*, GFP^{+} sorted satellite cells. Note reduced Myf5 protein levels in heterozygous quiescent satellite cells. (**B**) Western blot with *Myf5*$^{GFP\text{-}P/+}$ and *Tg:Pax7-nGFP*, GFP^{+} sorted satellite cells. Expression of Myf5 protein is reduced in heterozygous quiescent satellite cells. (**C**) Quantitative real-time RT-PCR. Results are expressed as a fold change (ΔΔCT) between GFP^{+} sorted cells from *Tg:Pax7-nGFP::Myf5*$^{loxP/+}$ and *Tg:Pax7-nGFP* (n=5 mice). (**D**) Quantitative real-time RT-PCR. Results are expressed as fold change (ΔΔCT) between GFP^{+} sorted cells from *Myf5*$^{GFP\text{-}P/+}$ and *Tg:Pax7-nGFP*. (n) values for RT-qPCR represent number of animals from *Tg:Pax7-nGFP* and *Myf5*$^{GFP\text{-}P/+}$ mouse, respectively: *Myf5* (n=10; 8), *Myod* (n=4; 4), *Myog* (n=10; 8), *Pax7* (n=4; 4), *TnT* (n=8; 7), *Dll1* (n=7; 4), *Des* (n=13; 5), *Tie2* (n=8; 7), *Ang1* (n=8; 7). (**E**) Gene expression profiles of WT (from *Tg:Pax7-nGFP*) and *Myf5*$^{GFP\text{-}P/+}$. Heat map from Affimetrix GeneChip microarray analysis showing a cluster of genes involved in skeletal muscle contractile properties enriched in *Myf5* heterozygous cells (*Myf5*$^{GFP\text{-}P/+}$) compared with control (*Tg:Pax7-nGFP*). (**F**) Gene expression profiles of WT (from *Tg:Pax7-nGFP*) and *Pax7*$^{nGFP/+}$ quiescent satellite cells. Note only minor differences between gene expression profiles of control cells (*Tg:Pax7-nGFP*) and the heterozygous *Pax7*$^{nGFP/+}$ cells. Results are mean ± s.e.m. **P*<0.05, ***P*<0.01, ****P*<0.001.

level of mRNA encoding the structural protein of mature skeletal muscle, Troponin-T, a protein that is expressed well downstream in this lineage, in differentiated cells. Satellite-cell-derived myoblasts were reported to express high levels of members of the Notch–Delta signalling pathway for myoblast proliferation before differentiation (Conboy et al., 2003; Sun et al., 2008; Waddell et al., 2010). Consistent with the notion that *Myf5* heterozygous cells are more committed, we noted higher expression levels of the Delta-like1 ligand, which marks committed myogenic cells (Delfini et al., 2000; Kuang et al., 2007; Schuster-Gossler et al., 2007; Waddell et al., 2010). Pax7, which is expressed in quiescent and activated myogenic cells, did not show any notable difference in expression. Interestingly, angiopoietin and its receptor Tie2, which are involved in satellite cell self-renewal, were both increased in *Myf5* heterozygous cells. These findings were largely confirmed when a subpopulation of heterozygous satellite cells, marked by *GFP* expression from *Myf5*$^{GFP\text{-}P/+}$ mice, was examined (Fig. 2D).

To validate and extend these findings, we performed Affimetrix GeneChip Microarrays of satellite cells from hind- and forelimb skeletal muscles (Fig. 2E). Several genes involved in the myofibre contractility machinery were upregulated in a heterozygous *Myf5*$^{GFP\text{-}P/+}$ subpopulation of quiescent satellite cells, such as skeletal actin (*Acta1*, 3.7-fold), dystrophin (*Dmd*, 2.5 fold), isoforms of myosin heavy and light chains (*Myh4*, *Mylpf*, 3-fold and 3.5-fold, respectively), troponin I (*Tnni1*, 5.1-fold), tropomyosin (*Tpm1*, 2-fold) and titin (*Ttn*, 4.7-fold). Accordingly, *Myog* expression was upregulated in these cells. A similar transcriptome analysis carried out between *Pax7* heterozygous satellite cells from *Pax7*$^{nGFP/+}$ knock-in mice and wild-type satellite cells showed no significant variations in gene expression between the two populations (Fig. 2F), indicating that unlike *Myf5*, haploinsufficiency of *Pax7* does not alter the transcriptional priming state of satellite cells. Taken together, these findings indicate that quiescent muscle stem cells are sensitive to *Myf5* expression levels, and that *Myf5* heterozygous satellite cells are more transcriptionally primed for myogenic commitment.

Transplanted *Myf5* heterozygous and wild-type satellite cells have equivalent regenerative potentials

To determine whether the molecular signatures of cell commitment observed in *Myf5* heterozygous quiescent satellite cells have functional consequences, we performed transplantation experiments with all cells or a subpopulation of quiescent satellite cells heterozygous for *Myf5*. To collect all satellite cells *Tg:Pax7-nGFP* were crossed with $Myf5^{GFP\text{-}P/+}$ mice. Unlike *Tg:Pax7-nGFP* mice, which express a nuclear GFP, the $Myf5^{GFP\text{-}P}$ expresses a brighter cytoplasmic GFP (Fig. 3A) (Kassar-Duchossoy et al., 2004; Sambasivan et al., 2009). Consequently, two GFP populations could be distinguished on the FACS profiles of satellite cells from simple (Fig. 3A) and compound crosses (Fig. 3B). $Myf5^{GFP\text{-}P/+}$ EDL myofibres contain an expected average number of about 10 satellite cells per EDL myofibre and therefore *Myf5* heterozygosity does not appear to affect satellite cell numbers as is the case also with $Myf5^{nlacZ/+}$ mice (Gayraud-Morel et al., 2007). However, only a subset (up to 20%) of $Pax7^{+}$ satellite cells co-expressed this *GFP* reporter (Fig. 3Ca–f), and this was not notably improved by removal of the puromycin resistance cassette (data not shown). This is consistent with fewer fluorescent cells obtained by FACS (0.2 to 0.4%) from $Myf5^{GFP\text{-}P/+}$ limb muscles and a reduced number of GFP^{+} cells counted per EDL myofibre (Fig. 3A,C) compared with that obtained from control mice.

For the transplantation experiments, two additional transgenic mice were used to follow the fate of the transplanted cells in vivo: (1) *Tg:CAG-hPLAP* carrying the human placental alkaline phosphatase gene which is expressed ubiquitously (DePrimo et al., 1996); and (2) *Tg:MLC3F-nlacZ-2E*, which marks

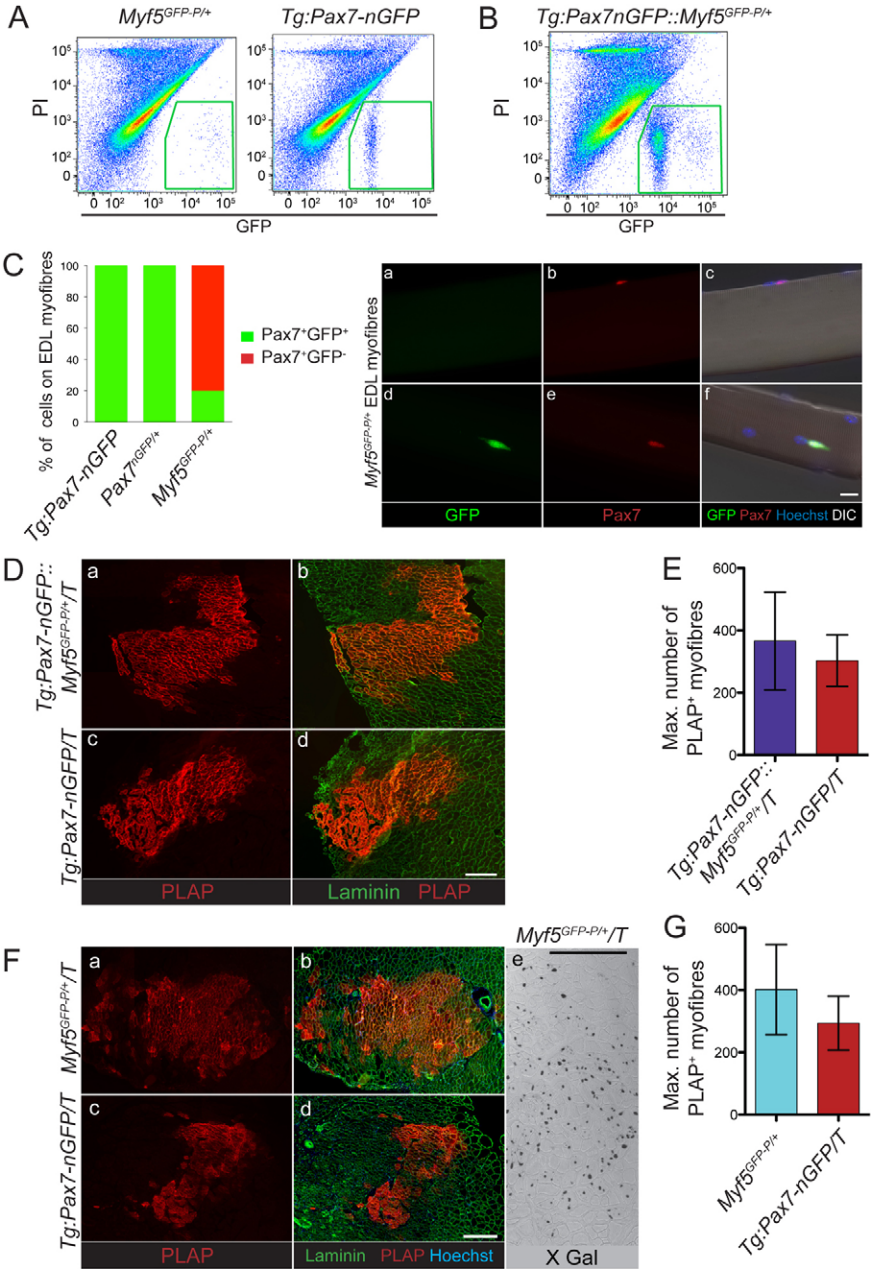

Fig. 3. Equivalent regenerative capacity of transplanted *Myf5* heterozygous satellite cells. (**A**,**B**) FACS profile of satellite cells from $Myf5^{GFP\text{-}P/+}$ or *Tg:Pax7-nGFP* (A) and compound *Tg:Pax7-nGFP::*$Myf5^{GFP\text{-}P/+}$ (B) mouse skeletal muscles. (**C**) Quantification and immunostaining for Pax7 and GFP on EDL myofibres (T0h). In *Tg:Pax7-nGFP* and $Pax7^{nGFP/+}$ mice all $Pax7^{+}$ satellite cells are GFP^{+}. In $Myf5^{GFP\text{-}P/+}$ the majority of $Pax7^{+}$ satellite cells are GFP^{-} (a–c), only 10–20% of $Pax7^{+}$ cells co-express GFP (d–f). (**D**) Cryodamaged $Rag2^{-/-}{:}\gamma C^{-/-}$ mice were transplanted with 10^{4} freshly sorted satellite cells and analysed after 4 weeks. Representative area of *Tg:Pax7-nGFP:*$Myf5^{GFP\text{-}P/+}$*/T* and *Tg:Pax7-nGFP/T* donor-derived $PLAP^{+}$ myofibres stained with anti-laminin antibody to outline the basement membrane. (**E**) Average number of $PLAP^{+}$ myofibres from *Tg:Pax7-nGFP:*$Myf5^{GFP\text{-}P/+}$*/T* (n=4 recipient mice) and *Tg:Pax7-nGFP/T* (n=4 recipient mice). Equivalent myofibre regeneration was noted (P>0.05). (**F**) (a–d) Representative areas of $Myf5^{GFP\text{-}P/+}$*/T* and *Tg:Pax7-nGFP/T* donor derived $PLAP^{+}$ myofibres stained with anti-laminin antibody to outline the basement membrane. (e) Myonuclei generated from the transplanted cells are detected by X-gal^{+} staining. (**G**) Average number of $PLAP^{+}$ myofibres from $Myf5^{GFP\text{-}P/+}$*/T* (n=4 recipient mice) and *Tg:Pax7-nGFP/T* (n=4 recipient mice). Equivalent myofibre regeneration was noted (P>0.05). Results are mean ± s.e.m. Scale bars: 10 µm (C), 300 µm (D, F).

differentiated myonuclei (Kelly et al., 1995). Therefore, triple (T) or quadruple genetically modified mice were generated for the transplantations: *Tg:Pax7-nGFP::Myf5$^{GFP\text{-}P/+}$::Tg:hPLAP::Tg:3F-nlacZ-2E* (hereafter called *Tg:Pax7-nGFP::Myf5$^{GFP\text{-}P/+}$/T*), *Tg:Pax7-nGFP::Tg:hPLAP::Tg:3F-nlacZ-2E* (hereafter called *Tg:Pax7-nGFP/T*).

For the functional assays, 10^4 freshly isolated GFP$^+$ cells were transplanted into the TA muscle of *Rag2$^{-/-}$:γC$^{-/-}$* immunocompromised recipient mice (Colucci et al., 1999) where the muscle was previously injured by cryodamage (Silva-Barbosa et al., 2005). PLAP$^+$ myofibres were enumerated by immunostaining in combination with anti-laminin antibody, which outlines the surrounding basement membrane of each myofibre (Fig. 3D). The majority of myogenic cells localised close to the site of injection, as reported previously (Schultz et al., 1988). Although the number of PLAP$^+$ myofibres generated from engrafted cells varied (from 40–700) between host animals as reported previously (Collins et al., 2005), individual animals responded similarly for the *Tg:Pax7-nGFP::Myf5$^{GFP\text{-}P/+}$/T* and *Tg:Pax7-nGFP/T* engrafted populations. On average, *Tg:Pax7-nGFP::Myf5$^{GFP\text{-}P/+}$/T* and *Tg:Pax7-nGFP/T* satellite cells generated 366 and 300 PLAP$^+$ myofibres respectively (Fig. 3E; $n=4$ animals, $P>0.05$). These findings show that *Myf5* heterozygous and wild-type satellite cells contributed equivalently to regenerating myofibres.

Similar results were obtained with the subpopulation of *Myf5* heterozygous satellite cells isolated from *Myf5$^{GFP\text{-}P/+}$/T*. The myogenic specificity of these cells was also evaluated by FACS and clonal analysis as described above for *Tg:Pax7-nGFP* mice (supplementary material Fig. S2A,B). The number of PLAP$^+$ myofibres generated from engrafted cells varied (from 20 to 700), yet individual animals responded similarly for the *Myf5$^{GFP\text{-}P/+}$/T* subpopulation compared with the total *Tg:Pax7-nGFP/T*-engrafted population (Fig. 3F). On average, *Myf5$^{GFP\text{-}P/+}$/T* and *Tg:Pax7-nGFP/T* satellite cells generated 400 and 300 PLAP$^+$ myofibres, respectively (Fig. 3G; $n=4$ animals, $P>0.05$). Four weeks after transplantation, cross sections of muscle contained centrally located X-gal$^+$ myonuclei and large clusters of PLAP$^+$ regenerating myofibres (Fig. 3Fe). Therefore, using either the total *Myf5* heterozygous cells, or a subpopulation, we found no significant difference in myofibre contribution compared with wild-type satellite cells.

Higher self-renewal capacity of *Myf5* heterozygous compared with wild-type muscle stem cells

As regeneration is completed and muscle homeostasis is re-established, the skeletal muscle niche is replenished with new quiescent muscle stem cells. Self-renewal of satellite cells was assayed for *Tg:Pax7-nGFP::Myf5$^{GFP\text{-}P/+}$/T* (Fig. 4A) or *Myf5$^{GFP\text{-}P/+}$/T* (Fig. 4B) mice to investigate the effect of *Myf5* haploinsufficiency on stem cell behaviour. Engrafted satellite cells that were located under the basement membrane were positive for Pax7 staining (Fig. 4Bd,i) and were scored as GFP$^+$ and PLAP$^+$ (Fig. 4A,B). Only rare GFP$^+$ satellite cells were found associated with PLAP$^-$ myofibres, in keeping with our findings above that the transplanted satellite cells did not disperse extensively. Quantification showed that, unexpectedly, a higher number of GFP$^+$ cells was associated with newly formed myofibres for *Myf5* heterozygous satellite cells (*Tg:Pax7-nGFP::Myf5$^{GFP\text{-}P/+}$/T* donors) compared with wild-type cells (*Tg:Pax7-nGFP/T* donors) (Fig. 4C; $P<0.001$).

Similarly, quantification of GFP$^+$/PLAP$^+$ satellite cells engrafted from *Myf5$^{GFP\text{-}P/+}$/T* mice (Fig. 4B), where only the GFP$^+$ subpopulation of satellite cells was transplanted, showed that haploinsufficiency of *Myf5* affected the frequency of self-renewed cells (Fig. 4D; $P<0.001$). To determine whether the regenerative potential was proportional to the number of cells injected, similar experiments were done with five times fewer transplanted cells (2000 cells instead of 10^4 cells, Table 1). For both donor populations, about half the number of PLAP$^+$ myofibres and four times fewer satellite cells were obtained. Thus, contribution to the number of regenerating myofibres by the injected cells was not proportional to the number of cells injected, suggesting that a limited number of cells is required for myofibre contribution, and this could also be limited by the extent to which satellite cells can migrate. Notably, also in these experiments, more self-renewing cells were observed with *Myf5* heterozygous transplanted satellite cells compared with the wild-type control (Table 1).

Long term self-renewal capacity of *Myf5* heterozygous and *Tg:Pax7-nGFP* satellite cells

To evaluate the capacity of *Myf5* heterozygous and *Tg:Pax7-nGFP* satellite cells to self-renew after a second round of stem cell mobilisation, 3 weeks after the initial transplantation, the TA muscle was re-injured with notexin to provoke myofibre destruction and to trigger satellite cell activation. Neonatal myosin heavy chain (nMyHC), which marks regenerating myofibres (Collins et al., 2005), was co-expressed with PLAP, indicating that muscle regeneration took place after the notexin injury (Fig. 5A). Large areas of PLAP$^+$ myofibres were observed after the reinjury (Fig. 5Ba,b), and their quantification showed an equivalent number of PLAP$^+$ myofibres for *Myf5$^{GFP\text{-}P/+}$/T* and *Tg:Pax7-nGFP/T* ($P>0.05$; Fig. 5C). Quantification of GFP$^+$ satellite cells associated with PLAP$^+$ myofibres, showed more GFP$^+$ cells per section with *Myf5$^{GFP\text{-}P/+}$/T* compared with *Tg:Pax7-nGFP/T* (Fig. 5D; $P>0.01$, $n=5$ mice). Therefore, *Myf5* heterozygous satellite cells continue to contribute robustly to muscle repair, and they yield more GFP$^+$ satellite cells compared with wild-type satellite cells.

Discussion

How stem cells regulate the balance between self-renewal and differentiation is a central question in stem cell biology. A variety of experimental approaches are used as readouts to assess these endpoints, such as cell transplantation and niche occupancy, as well as determination of the cell state using molecular readouts (Claudinot et al., 2005; Collins et al., 2007; Enver et al., 2009; Osawa et al., 1996; Sacco et al., 2008; Snippert and Clevers, 2011; Van Keymeulen et al., 2011). Skeletal muscle stem cells provide a model to address these questions, because the stem cell entity can be readily isolated and manipulated. Similar to many other tissues, however, the extent of heterogeneity in this population, and its functional relevance is not clear. To investigate these questions, we used genetic tools that permit a detailed phenotypic analysis of muscle stem cells. We present evidence that the levels of a determination gene *Myf5* impact on muscle stem cell behaviour, and unexpectedly, with divergent phenotypic consequences depending on the assay employed to examine stem cell potential for commitment and self-renewal.

Although satellite cells play a crucial role in muscle growth and repair, outstanding questions remain regarding how

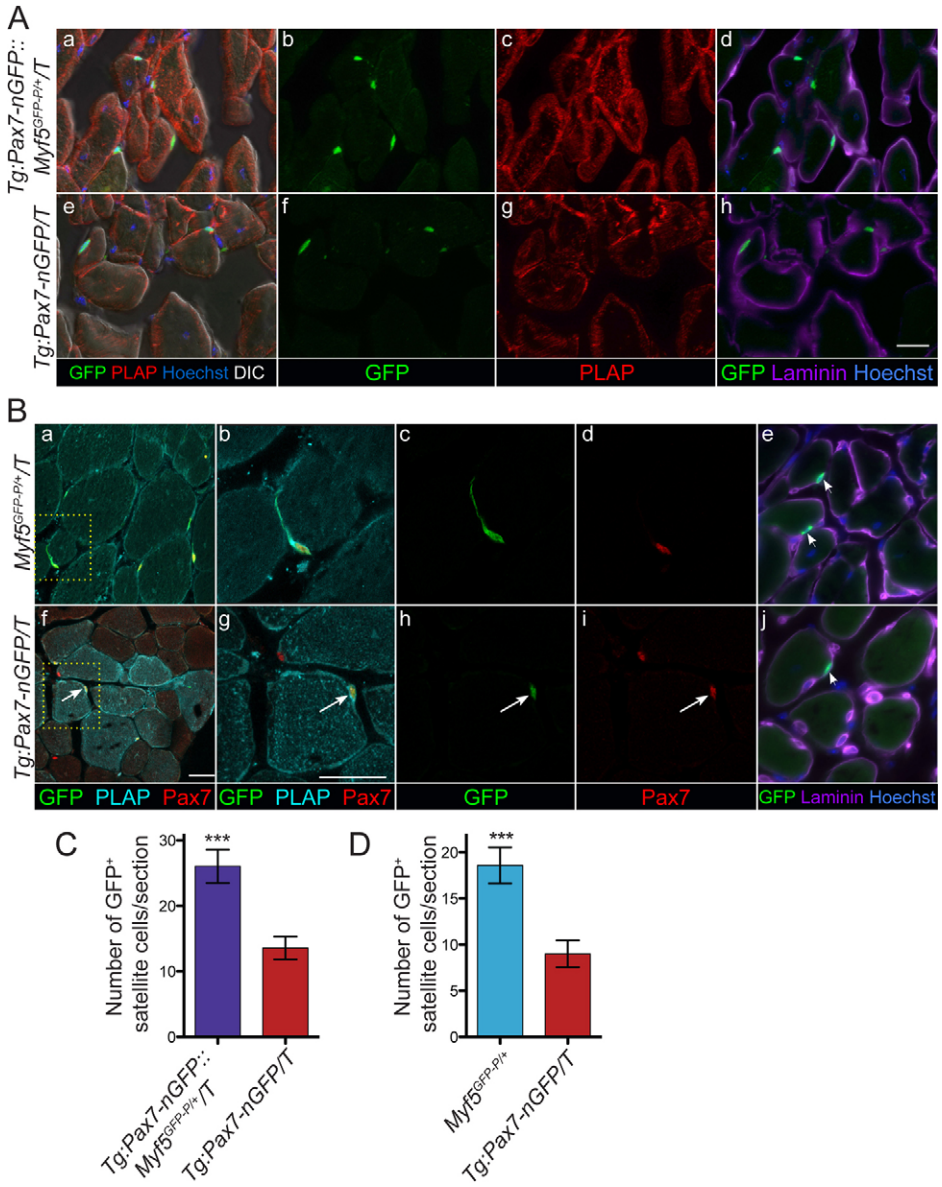

Fig. 4. Higher self-renewal capacity of transplanted *Myf5*$^{GFP/+}$ satellite cells. (**A**) Immunostaining on cryosections of transplanted TA muscle showing PLAP$^+$GFP$^+$ engrafted satellite cells from *Tg:Pax7-nGFP::Myf5*$^{GFP\text{-}P/+}$*/T* (a–d) and *Tg:Pax7-nGFP/T* (e–h) located in the periphery of newly formed PLAP$^+$ myotubes. GFP$^+$ cells are located under the laminin$^+$ basement membrane (d,h). (**B**) Immunostaining of cryosections of transplanted TA muscle showing PLAP$^+$GFP$^+$ engrafted satellite cells from *Myf5*$^{GFP\text{-}P/+}$*/T* (a–e) and *Tg:Pax7-nGFP/T* (f–j) located in the periphery of newly formed PLAP$^+$ myofibres. (a,f) Low magnification of representative areas of PLAP$^+$ regenerated myofibres. (b–d, g–i) High magnification of PLAP$^+$ myofibres with Pax7$^+$GFP$^+$ associated satellite cells (arrows). GFP$^+$ cells are located under the laminin$^+$ basement membrane (e, j; arrowheads). (**C**) Quantification of GFP$^+$ cells 4 weeks after engraftment represented as mean number of GFP$^+$ cells enumerated for all sections (*Tg:Pax7-nGFP::Myf5*$^{GFP\text{-}P/+}$*/T; n*=4 animals, 24 sections; *Tg:Pax7-nGFP/T*; *n*=4 animals, 24 sections). ***$P<0.001$. (**D**) Quantification of GFP$^+$ cells 4 weeks after engraftment represented as mean number of GFP$^+$ cells enumerated for all sections (*Myf5*$^{GFP\text{-}P/+}$*/T; n*=4 animals, 43 sections; *Tg:Pax7-nGFP/T*; *n*=4 animals, 35 sections). ***$P<0.001$. Scale bar: 20 µm (A,B).

heterogeneities reported in this population (Beauchamp et al., 2000; Cerletti et al., 2008; Collins et al., 2005; Ieronimakis et al., 2010; Kuang et al., 2007; Montarras et al., 2005; Ono et al., 2010; Tanaka et al., 2009) correlate with self-renewal and commitment decisions. The two genetic models characterised here, heterozygous *Pax7*$^{nGFP/+}$ and transgenic *Tg:Pax7-nGFP* mice, faithfully recapitulate the expression of the endogenous *Pax7* gene and express cell surface markers reported previously (Bosnakovski et al., 2008; Kuang et al., 2007; Montarras et al., 2005). Interestingly, unlike *Myf5*, heterozygosity of *Pax7* did not show notable differences with wild-type stem cells by transcriptome analysis. This was unexpected given that haploinsufficiency has been noted for the paralogue of Pax7, Pax3, at least in neural crest derivatives (Goulding et al., 1991).

Genetic studies have underscored the importance of threshold levels of the crucial myogenic determination factors in the

Table 1. Comparative engraftment of 2000 and 10,000 cells injected in pre-injured TA muscle

	Injection of 2000 cells (*n*=3 mice)			Injection of 10,000 cells (*n*=4 mice)		
	Myf5$^{GFP\text{-}P/+}$*/T*	*Pax7-nGFP/T*	*P* value	*Myf5*$^{GFP\text{-}P/+}$*/T*	*Pax7-nGFP/T*	*P* value
Max. number of PLAP$^+$ myofibres	226±91	122±24	>0.05	401±145	293±86	>0.05
Mean number of GFP$^+$ satellite cells per section*	4.7±0.94	2.1±0.39	0.01	18.5±1.96	9±1.46	<0.001
Range of max. number of PLAP$^+$ myofibres per animal	55–366	95–172		90–690	177–548	

*, *n*=33 sections for the injection of 2000 cells; *n*=40 sections for the injection of 10,000 cells.

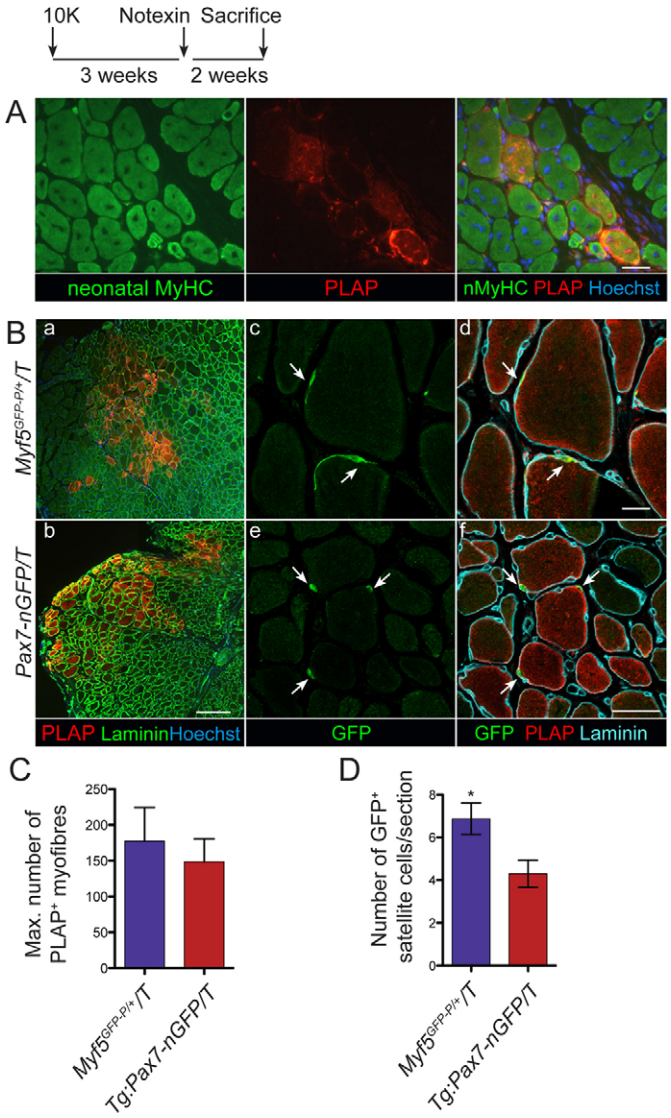

Fig. 5. Higher self-renewal capacity of *Myf5* heterozygous satellite cells after second cycle of regeneration. (**A**) Neonatal myosin heavy chain (nMyHC) staining indicates newly regenerated myofibres from PLAP$^+$ engrafted cells (*Myf5*$^{GFP\text{-}P/+}$/*T*). (**B**) Immunostaining for PLAP, laminin and GFP shows myofibres (a,b) and associated satellite cells (c–f; arrows), generated by engrafted satellite cells. (**C**) Quantification of PLAP$^+$ myofibres 14 days after reinjury with notexin in TA engrafted with *Myf5*$^{GFP\text{-}P/+}$/*T* (n=5 recipient mice) and *Tg:Pax7-nGFP/T* (n=5 recipient mice). P>0.05. (**D**) Quantification of GFP$^+$ satellite cells after notexin reinjury. Engrafted satellite cells from *Myf5*$^{GFP\text{-}P/+}$/*T* (n=5 recipient mice, 55 sections) and *Tg:Pax7-nGFP/T* (n=5 recipient mice, 46 sections) mice scored as GFP$^+$PLAP$^+$. Higher potential of *Myf5*$^{GFP\text{-}P/+}$/*T* cells to self-renew after notexin reinjury. *P>0.01. Results are mean ± s.e.m. Scale bars: 200 μm (A,B).

regulation of cell fate during prenatal development (Kassar-Duchossoy et al., 2004; Rudnicki et al., 1993; Weintraub, 1993). However, the role of these genes in quiescent adult stem cells remained unknown. We report the unexpected finding that muscle stem cells respond differentially to Myf5 for cell commitment, because heterozygous cells, either as a subpopulation from *Myf5*$^{GFP\text{-}P}$ reporter mice, or the entire population, are more primed for myogenic commitment compared with total wild-type muscle stem cells. An additional unexpected outcome from our study was the observation that in spite of their more transcriptionally committed state, *Myf5* heterozygous satellite cells assume a more stem-like phenotype after transplantation, because they show a higher frequency of self-renewal and niche occupancy. These findings indicate that for self-renewal after engraftment, the dose of Myf5 protein is crucial for modulating this cell state, because it is biased towards a stem-like phenotype in the heterozygous situation. Similar results were obtained after a second round of injury where engrafted donor muscle stem cells are mobilised from their niche to effect tissue repair, and subsequently self-renewing once again during homeostasis. Interestingly, this does not affect their ability to generate new myofibres. Consistent with these findings, *Myf5*-null muscle stem cells have a higher self-renewal and niche occupancy capacity than wild-type stem cells; however, their ability to generate differentiated myofibres is reduced (B.G.M. and S.T., unpublished observations). These observations underscore the importance of the cell fate regulator *Myf5* in modulating the balance between self-renewal and commitment. They also highlight unexpected differences in stem cell fate potential depending on the assay used, as was demonstrated recently for mammary stem cells (Van Keymeulen et al., 2011).

In differentiated cultures of the transformed muscle cell line C2C12, Myf5 protein is observed in mononucleated reserve cells, which share some characteristics with satellite cells, including cellular quiescence, loss of Myod, and expression of Pax7 (Kitzmann et al., 1998; Yoshida et al., 1998). However, the expression of Myf5 protein in quiescent satellite cells had not been examined in detail and it has been debated extensively (Dhawan and Rando, 2005; Kuang et al., 2008; Tajbakhsh and Gonzalez, 2009; Zammit et al., 2002; Zammit et al., 2006). In the present study, western blot and immunolabelling on tissue section and myofibres showed that Myf5 protein is present in the majority of quiescent satellite cells, unlike the other MRFs. By contrast, Myod and myogenin proteins are detected in only a subset (7–11% and 1%, respectively) of quiescent satellite cells (Beauchamp et al., 2000; Fukada et al., 2007; Zammit et al., 2002) (data not shown). The third muscle determination factor Mrf4 is absent during quiescence, and it is expressed after differentiation (Gayraud-Morel et al., 2007). These observations can now be considered in light of the present findings, where we show that reducing the gene dose of Myf5 to heterozygous levels in quiescent satellite cells promoted the transcriptional upregulation of commitment genes. Moreover, Myf5 expression is markedly downregulated during differentiation (Beauchamp et al., 2000), and cells triple mutant for *Myf5*, *Mrf4* and *Myog* do not differentiate (Valdez et al., 2000). The duality in the functional role of Myf5, as a promoter of muscle fate, and also as incompatible with differentiation, raises questions on the precise role of this transcription factor during different cell states in a myogenic lineage progression. Our findings with endogenous *Myf5* heterozygous quiescent satellite cells indicates that Myf5 protein expression maintains the ‘stemness’ state, and lowering the levels of this cell fate determinant genetically results in a myogenic commitment phenotype in this cell state. It appears that its role is distinct in activated satellite cells as the levels of Myf5 protein increase dramatically in the transit amplifying myoblast population.

Interestingly, the number of satellite cells negative for Myf5 protein is in the same range as that reported for YFP$^-$ cells in a

previous study (≤10%) obtained by crossing *Myf5*^{cre} mice with *Rosa*$^{STOP\text{-}YFP}$ (Kuang et al., 2007). After satellite cell activation, Myf5 expression is upregulated and it is co-expressed with Pax7 and Myod. Notably, some quiescent and activated cells remain unstained for Myf5 protein (data not shown) as reported also using *Myf5*$^{nlacZ/+}$ mice (Cooper et al., 1999). Taken together, satellite cell heterogeneity is clearly suggested by numerous studies, yet the mechanisms responsible for this heterogeneity have remained elusive. We show here that heterozygous levels of *Myf5* can have an impact on cell fate for self-renewal, but not for cell commitment. One possibility is that cell fate decisions are deterministic and influenced by Myf5 protein levels when additional environmental cues are favourable for myogenic commitment. Another possibility is that all satellite cells exist in a state of flux with respect to the levels of this transcription factor, and cell fate decisions to commit or self-renew are decided stochastically. It is likely that in both scenarios, the level of Myf5 protein is a nodal point for how this decision is executed, as is suggested by our findings.

Other studies have shown that the level of Nanog transcription factor in ES cells is crucial for determining the state of the cell, either for favouring pluripotency (Nanog-high) or commitment (Nanog-low) (Chambers et al., 2007). In another study, the Sca1 receptor in a blood cell line was used to show the stochastic behaviour of cells and fluctuations of the expression of this gene at a population level. Interestingly, a Gaussian distribution of expression of Sca1 was re-established irrespective of the levels of expression of the isolated starting population (Chang et al., 2008). These and other studies looking at Pax6 in eye development and aniridia (Hill et al., 1991), Tbx1 in pharyngeal arch arteries and in DiGeorge syndrome (Lindsay et al., 2001), Tcf4 in CNS and Pitt–Hopkins Syndrome (Brockschmidt et al., 2007), or RUNX2 in osteoprogenitors and cleidocranial dysplasia (Cohen, 2009) have highlighted the notion that cell fates are assumed based on the expression levels of crucial factors when the opportunity arises. We propose that this is the case for Myf5 protein levels in quiescent muscle stem cells.

Previous studies showed that the number of satellite cells was not altered significantly in mice heterozygous or null for *Myf5* up to 1 year of age, both in vitro and in vivo (Gayraud-Morel et al., 2007, Ustanina et al., 2007), indicating that the self-renewal and commitment decisions that are reported here are not due to overt alterations in cellular proliferation. Although a germ line null mutation in *Myod* results in twice as many myogenic cells in vivo (Gayraud-Morel et al., 2007; Macharia et al., 2010), their self-renewal capacity after transplantation remains to be determined. Other factors that can affect regeneration efficiency are the type of injury model used (Gayraud-Morel et al., 2009), the number of transplanted cells, because fewer transplanted cells yield proportionally more self-renewing and differentiated cells, and the donor and recipient mouse strains used (Collins et al., 2005; Gross et al., 1999).

A recent study reported that satellite cell self-renewal is regulated by FGF (Shea et al., 2010) and Bmp (Wang et al., 2010) signalling, as well as the Angiopoietin receptor Tie2. Notably, less satellite cell self-renewal was reported in the absence of Tie2 (Abou-Khalil et al., 2009). Our study is in keeping with this observation because the levels of the Tie2 receptor, and its ligand Angiopoietin 1, were elevated in *Myf5* heterozygous satellite cells, which is consistent with their tendency to self-renew more efficiently.

In summary, our findings point to a crucial role for *Myf5* in regulating muscle stem cell self-renewal after transplantation during regeneration, in spite of their capacity to assume a more committed state in the resident niche. These observations point to unexpected opposing phenotypes that underlie the flexibility in the muscle stem cell state, and they suggest that Myf5 is a key modulator of these cell states. As such, they provide insights into how self-renewal and differentiation are differentially modulated in muscle stem cells.

Materials and Methods

Ethics statement

All animal work was performed according to national and European guidelines.

Mice

Myf5$^{GFP\text{-}P}$, *Myf5*^{loxP} and *Myf5*^{nlacZ} were described earlier (Kassar-Duchossoy et al., 2004; Tajbakhsh et al., 1996). Briefly, *Myf5*^{nlacZ} and *Myf5*$^{GFP\text{-}P}$ comprise a 122 amino acid deletion in exon 1 and thus lack the bHLH domain. The *Myf5*^{loxP} allele contains about 124 bp in the first exon of *Myf5* and this insertion does not affect *Mrf4* expression in muscle progenitors in the embryo (Kassar-Duchossoy et al., 2004). For the transgenic Pax7–nGFP reporter mice, *Tg:Pax7-nGFP,* a BAC containing approximately 200 kbp of mouse genomic DNA including the locus encoding *Pax7* and sequences both upstream (~55 kbp with respect to *Pax7* initiator ATG) and downstream (~60 kbp from terminator codon) was recombined in *E. coli* with a nuclear-localised EGFP (nGFP). The targeting vector was designed to introduce *nGFP* into the first exon of the *Pax7* gene (Sambasivan et al., 2009). The *Pax7*^{nGFP} mouse was derived from a parental *Pax7*$^{nGFP\text{-}Puro/nlacZ}$ knock-in mouse (*nlacZ* flanked by frt sites) that was crossed with a universal Flippase deleter mouse to place the *nGFP* reporter gene in the first exon of *Pax7* (R.S. and S.T., unpublished results). To isolate pure populations of satellite cells, based on GFP epifluorescence by FACS, *Myf5*$^{loxP/+}$, *Myf5*$^{GFP\text{-}P/+}$ and *Myf5*$^{nlacZ/+}$ were crossed to *Tg:Pax7-nGFP*. To permanently mark engrafted cells, *Myf5* and *Pax7* reporter mice were crossed to the *Tg:CAG-hPLAP* carrying the human placental alkaline phosphatase gene that is expressed ubiquitously (DePrimo et al., 1996); and to the *Tg:MLC3F-nlacZ-2E*, which marks differentiated myonuclei (Kelly et al., 1995). For simplicity, mice carrying three to four genetic modifications, for example, *Myf5*$^{GFP\text{-}P}$*::Tg:CAG-hPLAP ::Tg:MLC3F-nlacZ-2E* or *Tg::Pax7-nGFP::Myf5*$^{GFP\text{-}P}$*::Tg:CAG-hPLAP ::Tg:MLC3F-nlacZ-2E* or *Tg:Pax7-nGFP::Tg:CAG-hPLAP ::Tg:MLC3F-nlacZ-2E*, are indicated as *Myf5*$^{GFP\text{-}P}$*/T*, *Tg:Pax7-nGFP::Myf5*$^{GFP\text{-}P}$*/T* or *Tg:Pax7-nGFP/T* (T, Triple). Engraftment experiments were performed with immunocompromised *Rag2*$^{-/-}$*:γC*$^{-/-}$recipient mice (Colucci et al., 1999).

Satellite cell preparation and fluorescence-activated cell sorting and cytometry

Satellite cells were prepared from mouse hindlimb and forelimb muscles. After removal of the major tendons, nerves and adipose tissue, muscle tissue was minced with scissors, then digested with a mixture of 0.1% collagenase D (Roche) and 0.2% trypsin (Invitrogen) in DMEM (Invitrogen) for five consecutive cycles of 25 minutes at 37℃. For each round, the supernatant was filtered through a 100 and then 70 μm cell strainer and trypsin was blocked with 10% FBS (Invitrogen) on ice. Pooled supernatants from each digestion cycle were centrifuged at 515 ***g*** for 15 minutes at 4℃. Pellets were washed at least four times with cold DMEM. Immediately before FACS, the cell suspension was filtered through a 40 μm cell strainer and resuspended in 2% FCS-DMEM. When necessary, propidium iodide was added at 1 μM final concentration to eliminate dead cells during sorting. Isolation of cells was performed on a FACSAria (BD Biosciences) or Moflow (Beckman Coulter) FACS machine. Satellite cells for single-cell clonal analysis were sorted directly into 96-well plates with a 100 μM nozzle on the FACSAria. For cytometry, single-cell suspension from muscle extract was incubated for 30 minutes on ice, with fluorescent primary antibodies (supplementary material Table S1), washed once with PBS and analysed by flow cytometry (CyAn, Beckman Coulter). Quadrants were established by the negative threshold based on isotype controls. Data was analysed post-acquisition by FlowJo.

Muscle injury and satellite cell transplantation

Immunocompromised *Rag2*$^{-/-}$*:γC*$^{-/-}$ mice were subjected to freeze injury 2 days before cell engraftment. Briefly, mice were anesthetised with 0.5% Imalgene and 2% Rompun. The TA muscle was frozen with three consecutive cycles of freeze-thawing by applying a liquid nitrogen cooled metallic rod. The skin was sutured and mice were kept on a warm plate until recovery. Satellite cells collected by FACS were centrifuged for 15 minutes in an Eppendorf centrifuge at 550 ***g***. The supernatant was carefully eliminated and the pellet was resuspended in a minimal volume to inject 5–10 μl of cell suspension per TA. Cell suspensions were enumerated using a Malassez counting chamber to adjust the concentration of cells

injected to 2000 or 10,000 satellite cells in pre-injured TA with a 10 µl Hamilton syringe. Four weeks after transplantation, mice were sacrificed by cervical dislocation and the tissue analysed. For reinjury experiments, 3 weeks after the transplantation, the TA are injected with 10 µl of notexin (Latoxan) and mice were sacrificed 14 days later for analysis.

Immunofluorescence and X-gal staining

The TA muscle was fixed in 1% paraformaldehyde and 0.1% Triton X-100 in PBS at 4℃ for 2 hours, followed by an overnight incubation in 15% sucrose at 4℃. The muscle was frozen in liquid nitrogen in OCT and processed essentially as described (Gayraud-Morel et al., 2007). Cryosections (10 µM) were collected on Superfrost slides (Thermo Scientific). Sections were washed in PBS, incubated in 0.5% Triton X-100 in PBS for 5 minutes, washed with PBS and blocked with 10% heat-inactivated goat serum. Primary antibodies (see supplementary material Table S1) were incubated overnight at 4℃. After three PBS washes, Alexa-Fluor- or Cy3-conjugated secondary antibodies were added for 2 hours at room temperature, followed by several PBS washes and mounting of slides in 25% PBS and 75% glycerol. For Pax7 staining on frozen sections, the primary monoclonal antibody was incubated overnight in 0.5% Triton X-100 in PBS, which replaces the unmasking protocol described previously (Gayraud-Morel et al., 2007). Myoblasts and single fibres were immunostained as described (Gayraud-Morel et al., 2007). For X-Gal staining, frozen sections were rinsed with PBS and stained for 2 hours with X-gal solution (Tajbakhsh et al., 1997) at 37℃. Pictures were taken with a Leica SPE confocal or Zeiss Observer microscope equipped with a Zeiss camera.

Quantification of myofibres and engrafted satellite cells

For quantification, the entire TA muscle was sectioned and at least four different evenly spaced sections were stained and used for $PLAP^+$ myofibre and GFP^+ satellite cell enumerations after immunostaining. For myofibre quantification, the section with the maximum number of $PLAP^+$ myofibres counted was considered for each animal. Graphs display the mean for all animals tested. Quantification of the GFP^+ satellite cells were expressed as the average number of GFP^+ satellite cells counted in 3–4 cryosections obtained from four different levels in $n=4$–5 mice. Graphs display average values of all animals tested ± s.e.m. Student's *t*-test or Mann–Whitney tests were performed to evaluate the significance of the values ($*P<0.05$, $**P<0.01$, $***P<0.001$).

Single-fibre preparations and cell culture

Single fibres were isolated as described previously (Zammit et al., 2002). Briefly, EDL muscles were dissected and treated for 1 hour with 0.1% collagenase (C-0130, Sigma) at 37℃. For time zero experiments, fibres were fixed for 5–10 minutes with 4% paraformaldehyde (PFA) in PBS at room temperature immediately after single-fibre isolation. When required, single myofibres were incubated for up to 72 hours in 20% FCS (Invitrogen) in DMEM (Invitrogen):MCDB201 (Sigma) (1:1) medium with penicillin and streptomycin. For myoblast cultures, sorted cells were plated on Matrigel (BD Biosciences) coated dishes, and grown in medium containing 20% FCS (Invitrogen) and 2% Ultroser in DMEM (Invitrogen):MCDB201 (Sigma) (1:1) medium with penicillin and streptomycin.

Quantitative real-time reverse transcription polymerase chain reaction

Extraction and preparation of RNA for QRT-PCR were performed as described previously (Jory et al., 2009). Briefly, total mRNA were extracted with the RNAeasy Micro Plus purification kit (Qiagen) from 10,000 to 100,000 GFP^+ satellite cells collected during FACS directly into the lysis buffer containing 1% β-mercaptoethanol as suggested by the manufacturer. RNAs were processed for random-primed reverse transcription using Superscript II protocol of Invitrogen (Carlsbad, CA). cDNAs were analysed using *power*SYBR Green Universal Mix or Taqman universal Master Mix. Primers are listed in supplementary material Table S2.

Western blotting

Satellite cells were collected in 10% FCS in DMEM from the cell sorter and centrifuged for 10 minutes at 6000 ***g*** at 4℃. The pellet was resuspended in a minimal volume of lysis buffer (5 mM EDTA, 50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.5% NP40, 0.1% SDS) with 2 × protein inhibitor cocktail (Roche). Protein extracts were separated in 4–12% Nu-Page precast gels (Invitrogen). After protein transfer [(20% methanol, 80% Tris-glycine-SDS buffer (Bio-Rad)], the membrane was incubated with antibodies as described (Gayraud-Morel et al., 2007). To evaluate Myf5 content in quiescent and activated (3days in culture) cells, 5×10^4 cells were loaded per lane.

GeneChip microarrays

Briefly, satellite cell populations from *Myf5*$^{GFP/+}$ and *Tg:Pax7-nGFP,* were collected from adult (6- to 8-week-old) mice based on GFP expression and isolation by FACS. RNA extraction was done using a Qiagen RNeasy Micro kit. cDNA obtained from 100 ng of RNA was amplified by using the GeneChip Expression Two-Cycle 3′ amplification system (Affymetrix). Fragmented biotin-labelled cRNA samples were hybridized on GeneChip Mouse Genome 430_2 arrays (Affymetrix 430.2.0 mouse array that contains 45000 probe sets). For each experimental group, three biological replicates were hybridized. The generation of cell intensity files and the quality control of hybridizations were performed with GeneChip Operating Software (Affymetrix). Raw data were pre-processed using the GC-Robust Multichip Analysis (GCRMA) algorithm in order to correct the background, to adjust the intensity distribution over the arrays and to convert probe intensity summarisation into a unique probe set signal. Local Pooled Error (Jain et al., 2003) tests were performed to identify significant differences in gene expression between groups. The Benjamini–Hochberg (Benjamini and Hochberg, 1995) multiple correction test was applied to control for the number of false positives with an adjusted 5% statistical significance threshold. Unbiased analysis of comparative enrichment of functionally related gene ontology categories were performed using DAVID (Huang da et al., 2009). Selected candidates from two relevant enriched categories (skeletal muscle differentiation and development) are presented in this article.

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