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Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage

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Summary

Following their discovery in 1961, it was speculated that satellite cells were dormant myoblasts, held in reserve until required for skeletal muscle repair. Evidence for this accumulated over the years, until the link between satellite cells and the myoblasts that appear during muscle regeneration was finally established. Subsequently, it was demonstrated that, when grafted, satellite cells could also self-renew, conferring on them the coveted status of 'stem cell'. The emergence of other cell types with myogenic potential, however, questioned the precise role of satellite cells. Here, we review recent recombination-based studies that have furthered our understanding of satellite cell biology. The clear consensus is that skeletal muscle does not regenerate without satellite cells, confirming their pivotal and non-redundant role.

Key words: Muscle regeneration, Pax7, Satellite cells, Skeletal muscle, Stem cells

Introduction

Skeletal muscle has evolved to allow precise movement in animals. By some estimates, there are around 640 skeletal muscles in the human body, which together account for ~38% of total body mass for men and 30% for women (Janssen et al., 2000). The functional unit of skeletal muscle is the long cylindrical muscle fibre that generates force by contraction. Each myofibre is packed with myofibrils composed of thousands of sarcomeres that contain the actin and myosin filaments that interact to produce the force (Fig. 1A). Myofibres are multinucleated, often containing hundreds of myonuclei, and are formed by the fusion of many myoblasts during embryonic and foetal development (Mintz and Baker, 1967).

Skeletal muscle has a robust regenerative capacity, with rapid re-establishment (by 3 weeks) of full power occurring even after severe damage that causes widespread myofibre necrosis (Rosenblatt, 1992). Indeed, regeneration is so efficient that function is restored even when a muscle is removed, minced and replaced back in situ (Studitsky, 1964). As myonuclei are post-mitotic, muscle repair and regeneration parallels developmental myogenesis, with myoblasts again fusing together for de novo myotube formation, or fusing to damaged myofibres to replace lost myonuclei. Furthermore, skeletal muscle will continue to regenerate even after repeated injury, requiring the generation of thousands of myoblasts on each occasion (Luz et al., 2002).

The cell responsible for generating myoblasts in postnatal skeletal muscle is the satellite cell, which is located in a niche on the surface of the myofibre (Katz, 1961; Mauro, 1961) (Fig. 1A-E). Satellite cells initially provide myoblasts for muscle growth, before becoming mitotically quiescent as the muscle matures. In adults, satellite cells can be recruited to supply myoblasts for routine muscle fibre homeostasis, or for the more sporadic demands of myofibre hypertrophy or repair (Zammit, 2008). In addition to producing progeny destined for differentiation, satellite cells also maintain their own population by self-renewal, thus fulfilling the defining criteria of a stem cell (Collins et al., 2005).

Although satellite cells had long been thought of as the primary source of postnatal myoblasts, the description of bone marrow cells with myogenic potential (Ferrari et al., 1998) opened the floodgates to a series of high-profile papers describing various non-satellite cell myogenic precursors (reviewed by Tedesco et al., 2010; Zammit et al., 2006). The controversy surrounding the relative input of satellite cells versus these 'unorthodox' myogenic precursors to skeletal muscle growth and repair has thus become a major pre-occupation of many researchers in the field.

Satellite cells have become inextricably linked to the paired box transcription factor Pax7, since a defining study by Michael Rudnicki and colleagues showed that satellite cells express *Pax7* and that inactivation of *Pax7* results in severe depletion of these muscle stem cells (Seale et al., 2000). Indeed, *Pax7* expression is maintained in virtually all quiescent satellite cells in adult mouse muscle (Gnocchi et al., 2009) (Fig. 1B-E) and in many other species as diverse as salamander, chicken and human (Morrison et al., 2006; Yablonka-Reuveni, 2011). Thus, the *Pax7* gene also provides a valuable target locus to facilitate genetic manipulation of the satellite cell genome (see Box 1, Fig. 2). Here, we review how such sophisticated recombination-based technology has helped resolve some questions that are central to satellite cell biology, with a particular focus on the seminal observations that muscle regeneration fails after the genetic ablation of satellite cells (Lepper et al., 2011; McCarthy et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011b).

An overview of the muscle satellite cell

A cell on the edge

Skeletal muscle regeneration was first properly described in the 1860s, but almost a century elapsed before the cellular mechanisms of this process were resolved (Scharner and Zammit, 2011). A series of pioneering papers published between 1960 and 1961 provided compelling evidence that multinucleated myofibres in both developing and regenerating muscle arise from the fusion of multiple myoblasts (Bintliff and Walker, 1960; Capers, 1960; Konigsberg et al., 1960; Pietsch, 1961; Stockdale and Holtzer, 1961). Controversy surrounded the source of these myoblasts in regenerating muscle, with theories that they emanated from amitotic division of surviving myonuclei, from de-differentiation of viable myonuclei back into myoblasts, or from cells in the interstitium and/or circulation (reviewed by Scharner and Zammit, 2011). Concurrent with the confirmation of cell fusion as the mechanism of myotube formation, the satellite cell was discovered and proposed as a new candidate for providing such myoblasts (Katz, 1961; Mauro, 1961).

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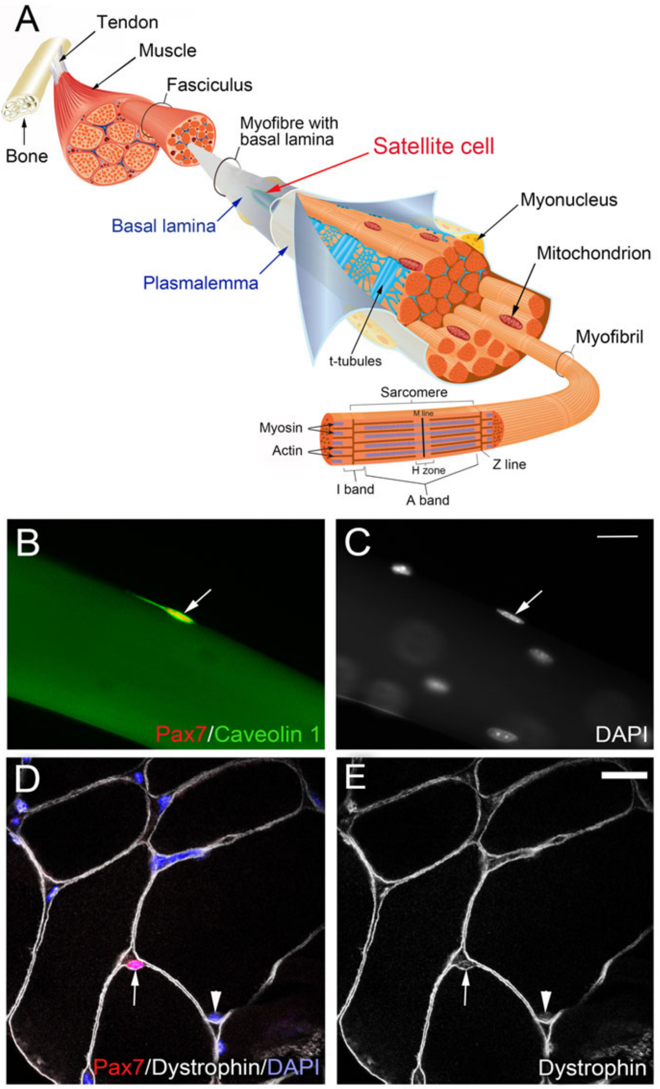


Fig. 1. Muscle structure and the satellite cell niche. (**A**) The structure and ultra-structure of skeletal muscle [adapted, with permission, from Shahragim Tajbakhsh (Tajbakhsh, 2009)]. The satellite cell niche is on the surface of the myofibre, beneath the surrounding basal lamina, as indicated. (**B**,**C**) A quiescent murine satellite cell retained in its niche on a myofibre isolated from the extensor digitorum longus muscle of an adult mouse. The preparation has been co-immunostained for Pax7 (B; red-nuclear) and caveolin 1 (B; green) to reveal the satellite cell (indicated by an arrow). DAPI counterstaining (C) reveals both the nucleus of the same satellite cell (arrow) and the myonuclei of the myofibre. (**D**,**E**) Confocal image of a transverse section of an adult mouse extensor digitorum longus muscle co-immunostained for Pax7 (D; red), together with dystrophin (D,E; white) to delimit the plasmalemma of the myofibre, and counterstained with DAPI (D; blue). The arrow indicates a *Pax7*-expressing satellite cell located on the surface of a myofibre; the arrowhead highlights a myonucleus. Scale bars: 20 μm in B,C; 100 μm in D,E.

Satellite cells reside in a niche on the surface of the muscle fibre, beneath the ensheathing basal lamina (Fig. 1A-E), and are found in a similar location in many vertebrate species (Yablonka-Reuveni, 2011). Studies throughout the 1960s indicated that satellite cells were the likely myogenic precursors for muscle regeneration (e.g. Church et al., 1966; Shafiq and Gorycki, 1965), and they were seen to undergo cell division in regenerating muscle (Reznik, 1969). It was not until the culture of isolated myofibres, however, that it was unambiguously demonstrated that satellite cells generate progeny that become myoblasts (Bischoff, 1975; Konigsberg et al., 1975).

Much as haematopoietic stem cells have been tested by transplantation into hosts whose own bone marrow has been destroyed (e.g. by irradiation), the function and fate of myogenic precursors has been assayed by grafting them into skeletal muscle. Such transplantation studies showed that satellite cells provide myoblasts for muscle growth and repair in vivo (Collins et al., 2005; Lipton and Schultz, 1979; Snow, 1977; Snow, 1978). The contribution of endogenous satellite cells to muscle regeneration is clearly illustrated using recombination-based lineage tracing (see Box 1); when *Cre-ERT2* is activated by tamoxifen in either adult $Pax7^{CE/+}$: $R26R^{lacZ/+}$ or $Pax7^{CreER/+}$: $R26R^{lacZ/+}$ mice, only satellite cells have β-galactosidase activity. After muscle damage, however, many of the regenerated myofibres also exhibit robust reporter expression, because of the incorporation of satellite cell-derived myoblasts carrying the recombined *Rosa* locus (Lepper et al., 2009; Shea et al., 2010).

Satellite cells are muscle-resident myogenic stem cells

Satellite cells not only generate myoblasts, but also re-appear in their niche as myotubes reform (Church et al., 1966). That this was self-renewal was implied from lineage tracing in growing muscle, where it was observed that a satellite cell division could lead to one progeny that differentiated into a myonucleus, while the other remained a satellite cell (Moss and Leblond, 1971). In adults, grafting an isolated myofibre with a small number of associated satellite cells (Collins et al., 2005), or even just a single fluorescence-activated cell sorting (FACS)-isolated satellite cell (Sacco et al., 2008), produces many more donor-derived satellite cells in the host muscle than originally transplanted. Such amplification requires extensive proliferation from the donor satellite cells and/or their progeny, showing that self-renewal had occurred. Importantly, donor satellite cells remain viable, being able to again participate in regeneration if the muscle is damaged (Collins et al., 2005). Donor-derived satellite cells can also be recovered from muscles after transplantation, and serially transplanted/recovered several more times, showing the extent of their potential for self-renewal (Rocheteau et al., 2012). Thus, as satellite cells not only generate differentiated progeny, but also maintain their own population by self-renewal, they can be classified as myogenic stem cells.

The term 'stem cell', however, also evokes thoughts of multi-potency. In vitro, evidence has been presented that satellite cells can be pushed towards the adipogenic and osteogenic lineages (e.g. Asakura et al., 2001), but contamination of such cultures from non-myogenic cells is sometimes hard to dismiss as the underlying cause of this observed multipotency (Day et al., 2010; Starkey et al., 2011). Recent examination using recombination-based lineage tracing indicates that, although satellite cells can be stimulated to accumulate lipid, they do not undergo terminal adipogenic differentiation in vitro (Starkey et al., 2011). Furthermore, whereas exposure to bone morphogenetic proteins (BMPs) inhibits myogenic differentiation in satellite cells in vitro, it does not result in any overt change to the osteogenic lineage (Ono et al., 2011). In vivo, there is also a negligible (<5%) contribution of satellite cells to BMP-mediated ectopic osteogenesis (Lounev et al., 2009). Therefore, satellite cells can be considered monopotent muscle-resident myogenic stem cells.

Satellite cell heterogeneity

Multiple lines of evidence point to functional heterogeneity of satellite cells, which indicates that they do not all have stem cell characteristics. Not only do satellite cell populations from different

Box 1. Recombination-based technology: genetic tools to examine satellite cell function

Recombination-based technology generally uses the enzymatic activity of Cre recombinase to target loci that contain engineered *loxP* sites – the *Cre-lox* system. The cellular distribution of Cre is dictated by creating a transgene or by targeting *Cre* to a particular genetic locus. When targeted to a locus, *Cre* can be placed in the reading frame (usually to create a null allele of the targeted gene), such as in $Pax3^{Cre}$ (Engleka et al., 2005). Alternatively, the use of an internal ribosome entry site (IRES) to drive *Cre* in the 3′UTR allows the endogenous locus to remain functional, as in $Pax7^{iCre}$ (Keller et al., 2004). In cells where Cre recombinase is present in the nucleus, it excises sequences flanked by *loxP* sites and recombines the cut ends (termed 'floxing') to cause irreversible rearrangement at the 'floxed' locus to produce a heritable change in the genome.

The regulatory elements of the transgene or targeted locus define the spatiotemporal expression of *Cre*, so the expression of the locus engineered to contain *loxP* sites does not need to be restricted. The ubiquitously expressed *Rosa* locus has been targeted with numerous constructs that only express after blocking sequences have been floxed (Soriano, 1999). Of relevance here are examples in which the *Rosa* locus drives reporter genes (e.g. $Rosa26^{lacZ}$ and $Rosa^{YFP}$) after recombination, so that all progeny of the cell in which recombination was induced will continue to express the reporter, regardless of whether Cre remains active (Soriano, 1999). In another example, Cre-mediated recombination of $R26R^{DTA}$ results in expression of diphtheria toxin fragment A (DTA) (Wu et al., 2006), a potent inhibitor of protein translation that kills the cell in which it is produced (see Fig. 2).

More sophisticated genetic tools allow temporal control of Cre recombinase activity in those cells that express *Cre*, by fusing Cre to the mutated ligand-binding domain of the human oestrogen receptor (Cre-ERT) (Metzger and Chambon, 2001). Cre-ERT, or the more efficient Cre-ERT2, protein is produced in a cell-restricted distribution, as controlled by the transgene or locus to which it is targeted but, as it remains cytoplasmic, it does not recombine *loxP* sites. The ability of Cre-ERT to recombine is then dictated by administration of the oestrogen receptor agonist tamoxifen (or its derivatives). Tamoxifen binds to the mutated ligand-binding domain of the human oestrogen receptor and causes Cre-ERT to enter the nucleus, where it can then recombine *loxP* sites and excise intervening sequences (Metzger and Chambon, 2001). Of interest here, *Cre-ERT2* has been inserted into the *Pax7*-coding sequence to create a knock-in/knockout conditional allele called $Pax7^{CE}$ (Lepper and Fan, 2012), which produces Cre-ERT2 (but not *Pax7*) in cells expressing *Pax7*, but which only recombines target sequences on administration of tamoxifen (Fig. 2). An *IRES-CreERT2* cassette has also been inserted into the 3′UTR of the *Pax7* gene to express *Cre-ERT*, while preserving *Pax7* expression, as in $Pax7^{iCreERT2}$ (Murphy et al., 2011) and $Pax7^{CreER}$ (Nishijo et al., 2009).

Finally, it is important to note some of the caveats of using recombination-based technologies. First, loci or transgene-driven *Cre* expression is not always restricted to the intended target cells, and constructs vary in the degree that they have off-target expression – referred to as 'leakiness'. Furthermore, Cre-mediated recombination can be less than 100% efficient, meaning that a failure of recombination does not always imply a lack of *Cre* expression, as expression may be low and/or some *loxP* sites are less accessible and/or easily recombined than others. Last, for conditional *Cre* alleles, careful testing of the tamoxifen administration regime is needed to ensure widespread recombination, which can, for example, be particularly difficult when dosing the mother to activate *Cre* in embryos.

muscles exhibit heterogeneity in their proliferation rate, clonogenic capacity, extent and rate of differentiation, and ability to self-renew, these variations also exist between satellite cells of the same muscle (Day et al., 2010; Lagord et al., 1998; Molnar et al., 1996; Ono et al., 2010; Ono et al., 2012; Schultz, 1996). Heterogeneity is also revealed by transplantation studies, as only a limited number of grafts result in large numbers of new satellite cells being produced, so extensive self-renewal does not appear to be a universal feature of satellite cells (Collins et al., 2005; Sacco et al., 2008).

Myogenic progression and self-renewal in satellite cells can be modelled ex vivo (Halevy et al., 2004; Olguin and Olwin, 2004; Zammit et al., 2004). The transcription factor myogenic differentiation 1 (Myod1; previously MyoD) [which, together with myogenic factor 5 (Myf5), myogenic factor 6 (Myf6; previously MRF4) and myogenin, makes up the myogenic regulatory factors] is rapidly induced in virtually all satellite cells during activation (Yablonka-Reuveni and Rivera, 1994; Zammit et al., 2002). After proliferation as Pax7/MyoD-expressing myoblasts, most cells maintain MyoD but downregulate Pax7 and commit to differentiation via activation of myogenin. Other myoblasts, however, maintain Pax7 but downregulate MyoD and eventually withdraw from the cell cycle, regaining markers that characterise myogenic quiescence (Day et al., 2007; Nagata et al., 2006). These observations suggest that all satellite cells pass through a common stage of co-expressing Pax7 and MyoD, before the decision to either self-renew or differentiate is made. However, whether such uniform induction of MyoD occurs in all activated satellite cells in vivo remains unknown (Cooper et al., 1999; Grounds et al., 1992) and awaits further examination; for example, by using an inducible $MyoD^{CreERT}$ allele.

Various markers distinguish between satellite cell populations [e.g. activity of the *Pax3* locus (Relaix et al., 2006)], but it is often difficult to link this to different functional abilities. For example, although different regenerative potentials are ascribed to satellite cell subpopulations isolated by FACS (Conboy et al., 2010), it is sometimes difficult to confirm their provenance in vivo, or the size of any putative satellite cell subpopulation, as the antibodies used for FACS are often not effective for immunocytochemistry.

Recombination-based lineage tracing has also been used to try and identify any putative 'satellite stem cell'. Most satellite cells in adult $Myf5^{cre/+}$: $Rosa^{YFP/+}$ mice have undergone recombination, but ~10% of satellite cells are yellow fluorescent protein (YFP) negative yet can produce both YFP-negative and YFP-positive progeny (Kuang et al., 2007). It has been proposed that these YFP-negative cells are a dedicated subset of satellite stem cells, as they have never activated the myogenic program, whereas the YFP-positive cells are their transit-amplifying progeny (Kuang et al., 2007). However, all satellite cells have a degree of *Myf5* locus activity when reported by β-galactosidase activity in $Myf5^{nlacZ/+}$ mice, although this activity is variable, with some satellite cells requiring prolonged exposure to X-gal (Day et al., 2010). Levels of Myf5 protein are also variable, with ~10% of satellite cells not immunostaining for Myf5 at all (Gayraud-Morel et al., 2012). Alternatively, YFP-negative satellite cells in $Myf5^{cre/+}$: $Rosa^{YFP/+}$ mice could reflect the sensitivity of YFP as a readout, as the same $Myf5^{cre}$ allele in $Myf5^{cre/+}$: $Rosa26^{nlacZ/+}$ mice results in 96% of satellite cells with β-galactosidase activity (Brack et al., 2009). Crucially though, if $MyoD^{icre/+}$ is used instead of $Myf5^{cre/+}$ to drive recombination, then virtually all satellite cells express the reporter gene (Kanisicak et al., 2009). As quiescent satellite cells do not generally contain MyoD protein (Yablonka-Reuveni and Rivera, 1994; Zammit et al., 2002), this clearly indicates that they, or their predecessors, have expressed MyoD at some point and have had a 'myogenic experience', but then downregulated MyoD before becoming quiescent.

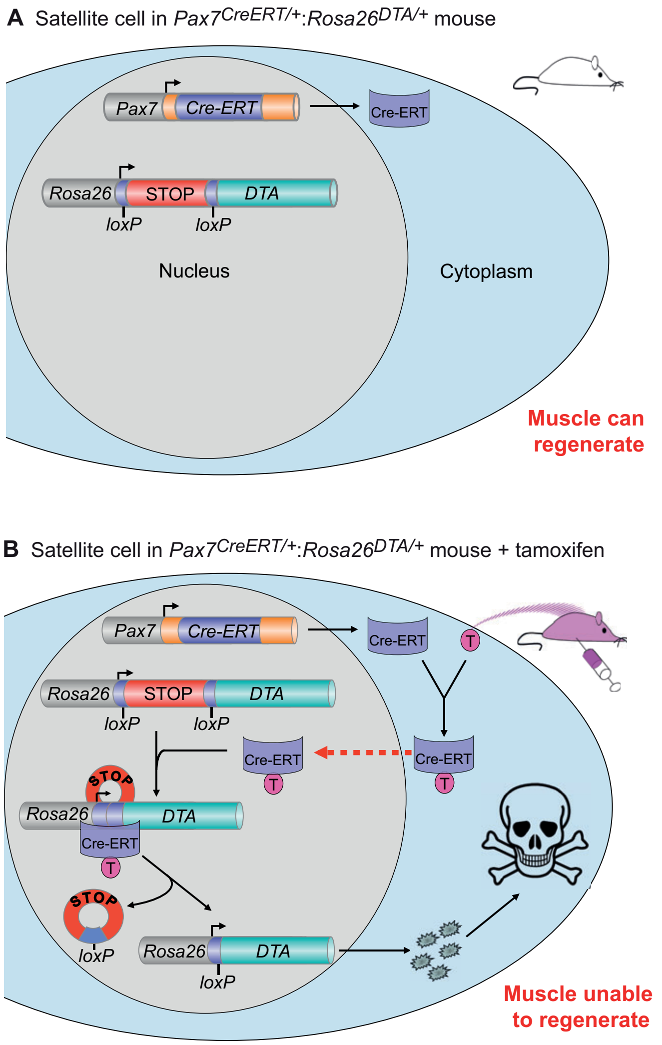


Fig. 2. Satellite cell ablation strategy using *Cre-lox* recombination. The *Pax7* locus was targeted with *Cre-ERT*. *Pax7*$^{CreERT2/+}$ mice were crossed to a line in which the *Rosa26* locus was engineered to contain a stop cassette flanked by *loxP* sites, upstream of sequences encoding diphtheria toxin fragment A (DTA). (**A**) *Pax7*$^{CreERT/+}$: *Rosa26*$^{DTA/+}$ mice generate Cre-ERT2 in all *Pax7*-expressing satellite cells, but it remains in the cytoplasm if tamoxifen is not present. Owing to the stop cassette in the modified *Rosa26* gene, there is no DTA produced without recombination at the locus. In such untreated mice, satellite cells are viable and muscle regenerates effectively after acute injury. (**B**) When tamoxifen (T) is administered systemically to *Pax7*$^{CreERT/+}$: *Rosa26*$^{DTA/+}$ mice, it binds to the cytoplasmic Cre-ERT encoded by the *Pax7* locus. Tamoxifen-bound Cre-ERT enters the nucleus and recombines the engineered *Rosa26* locus between the *loxP* sites and excises the intervening stop cassette. The *Rosa26* gene is then able to drive expression of DTA, which inhibits protein translation and kills the satellite cell it is expressed in. When satellite cells are genetically ablated in this way, muscle regeneration fails following severe injury.

Therefore, the satellite-cell population may be composed of both lineage-based satellite ‘stem’ cells together with more committed myogenic precursors, or satellite cells may acquire variable stem-cell characteristics over time, perhaps because some cells have been activated fewer times, or have undergone fewer divisions. Alternatively, satellite cells could be a more uniform population, with environmental cues dictating cell fate following activation.

Satellite cell specification and function during muscle growth

Developmental origins of satellite cells

In vertebrates, skeletal muscles of the trunk and limb are derived from cells of the somite. These paraxial mesoderm-derived pairs of transient epithelial balls flank the neural tube and form in an anterior-posterior progression during the process of somitogenesis (Pourquie, 2003). Somites undergo maturation into the sclerotome and dermomyotome. Cells in the dermomyotome are then specified to the myogenic lineage by Pax3 (Fig. 3). Later, *Pax7* is activated within these *Pax3*-expressing myogenic precursors, which produce progenitor cells of the embryonic and foetal body muscles (Gros et al., 2005; Kassar-Duchossoy et al., 2005; Relaix et al., 2005). *Pax3* is also expressed in cells that migrate from the somite to the limb, tongue and diaphragm, providing the muscle progenitor cells for these locations, with *Pax7* induced once migration is complete (Kassar-Duchossoy et al., 2005; Relaix et al., 2004; Schienda et al., 2006). Indeed, Pax genes directly control activation of the myogenic programme in the limb by binding and activating the myogenic regulatory factors *Myf5* and *Mrf4*, followed by *MyoD* (Bajard et al., 2006; Buckingham and Relaix, 2007; Hu et al., 2008; McKinnell et al., 2008). *Pax7* is maintained in foetal myogenic precursors and satellite cells in adults, whereas *Pax3* is downregulated during the foetal period (Horst et al., 2006), although the *Pax3* locus remains active in a subset of satellite cells of particular muscles in the adult, as shown by reporter gene expression in *Pax3*$^{eGFP/+}$ mice (Montarras et al., 2005; Relaix et al., 2006).

Only when the basal lamina forms around myotubes towards the end of foetal development, however, can morphology and location be first used to classify cells as satellite cells (Kelly and Zacks, 1969; Ontell and Kozeka, 1984). Both grafting quail somites into chick embryos (Armand et al., 1983) or tracing cells after dye injection (Gros et al., 2005; Schienda et al., 2006) show that myogenic progenitors of the somite give rise to satellite cells. Lineage tracing in *Pax3*$^{Cre/+}$: *Rosa26*$^{lacZ/+}$ and *Pax7*$^{CE/+}$: *R26R*$^{lacZ/+}$ mice reveal that it is specifically the *Pax3*- and *Pax7*-expressing cells of the somite that not only contribute to both the trunk and limb musculature, but also to their satellite cell populations (Engleka et al., 2005; Lepper et al., 2009; Lepper and Fan, 2010; Schienda et al., 2006).

Pax3 acts as an early survival factor in the dermomyotome, as *Pax3*-null mice display trunk muscle defects, while limb and diaphragm muscles fail to form owing to loss of the long-distance migrating cells (Buckingham and Relaix, 2007). Inactivation of *Pax7* has no obvious effects on embryogenesis or foetal development, but loss of both *Pax3* and *Pax7* leads to defective muscle specification and little muscle formation (Fig. 3), revealing redundancy between these two transcription factors (Relaix et al., 2005). The importance of these *Pax3/7*-expressing progenitors is further confirmed after they are ablated in *Pax3*$^{Cre/+}$: *R26R*$^{DTA/+}$ embryos, where myogenic cells are lost in the embryonic limbs and trunk (Hutcheson et al., 2009). Although ablation of *Pax7*-expressing cells in *Pax7*$^{iCre/+}$: *R26R*$^{DTA/+}$ mice has little effect on embryonic myogenesis (up to E14.5), there is a complete absence of foetal myogenic progenitors and myofibres (Hutcheson et al., 2009).

Inactivation of the Notch/Delta pathway in these *Pax3*-expressing cells reveals that they also contribute to satellite cells found in the perinatal period. *Pax3*$^{Cre/+}$: *RBP-Jk*$^{flox/flox}$ mice have severe foetal muscle hypoplasia owing to disproportionate myogenic differentiation (Vasyutina et al., 2007), with a similar phenotype observed in hypomorphic Delta-like-1 mutant mice (Schuster-Gossler et al., 2007). Although these mice die just after

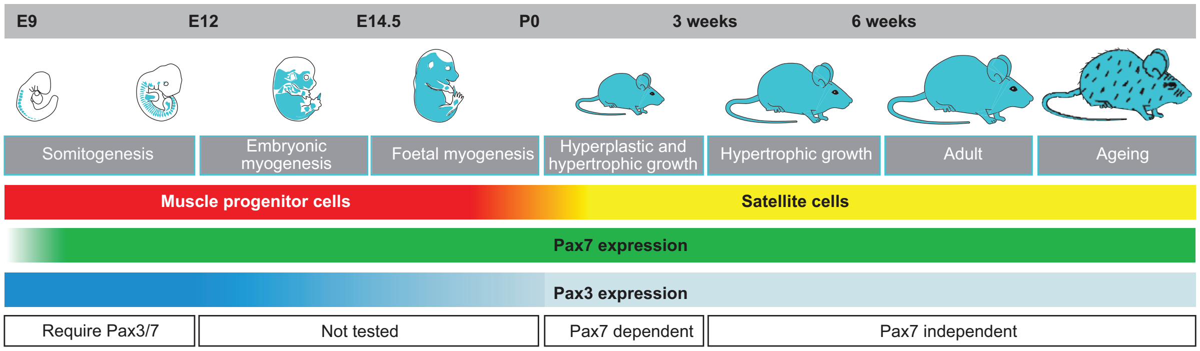


Fig. 3. The dependence of myogenic stem cell populations on Pax genes. The timing of the embryonic and postnatal periods of muscle development in mouse is indicated, with the distribution of skeletal muscle within the developing embryo shown in blue. The times when embryonic and foetal muscle progenitor cells are the dominant myogenic stem cells are indicated in red, whereas the periods during which satellite cells predominate is highlighted in yellow. The expression dynamics of *Pax7* (green) and *Pax3* (blue) in muscle progenitors and satellite cells are shown. Finally, the time points at which embryonic and foetal muscle progenitor and satellite cells require *Pax3* and *Pax7* gene function are indicated.

birth, the satellite cell niche is unoccupied in foetal/newborn mice, implying that the excessive myogenic differentiation causes depletion of the *Pax3*-expressing myogenic progenitor cells that would normally become, or generate, satellite cells (Vasyutina et al., 2007).

Unlike body and limb muscles, the musculature of the head derives from non-somitic cranial mesoderm (Noden and Francis-West, 2006; Sambasivan et al., 2011a), and Pax genes are not part of the transcriptional networks that control formation of this tissue (Bismuth and Relaix, 2010). $Mesp1^{Cre/+}$- or $Isl1^{Cre/+}$-mediated lineage tracing shows that, again, both muscle and satellite cells in the head are derived from a common progenitor, but instead located in the cranial mesoderm (Harel et al., 2009). Interestingly, despite the distinct genetic regulation of muscle and satellite cell development in the head, satellite cells still activate *Pax7* in the foetal period and maintain expression in adult (Sambasivan et al., 2009; Gnocchi et al., 2009).

Postnatal muscle growth is perturbed by the loss of satellite cells

Despite having no obvious phenotype when born (Mansouri et al., 1996), *Pax7*-null mice fail to thrive and have retarded growth, with most dying within 2 weeks of birth (Seale et al., 2000). The extent to which this growth defect and early death is linked to the lack of Pax7 function in skeletal muscle, or in other sites such as the central nervous system, remains unclear. Satellite cell numbers fall rapidly in $Pax7^{-/-}$ mice postnatally, with a severe reduction already evident by P10/11 (<80%). Muscle weakness has been reported, with muscle fibres of smaller calibre containing fewer myonuclei present (Kuang et al., 2006; Relaix et al., 2006; Seale et al., 2000), although others find the juvenile musculature to be overtly normal (Oustanina et al., 2004).

In the conditional $Pax7^{CE}$ allele, *Cre-ERT2* is inserted into the *Pax7*-coding sequence, and so $Pax7^{CE}$ is a null allele for *Pax7* (Lepper et al., 2009). A combination of $Pax7^{CE}$ with a *Pax7* allele that can be flox inactivated ($Pax7^{f}$) generates heterozygous $Pax7^{CE/f}$ mice in which the functional *Pax7* allele can be inactivated by Cre from the $Pax7^{CE}$-null allele. Administering tamoxifen to $Pax7^{CE/f}$ mice at different stages of postnatal growth (P7-11 and P14-18) established that regeneration was compromised when muscle was damaged up to P21. However, satellite cells have a decreasing requirement for Pax7, as regeneration was normal if *Pax7* was deleted after P21 (Lepper et al., 2009), defining a crucial period of Pax7 requirement in postnatal satellite cells (Fig. 3). Unfortunately, the condition of undamaged growing muscle with postnatal *Pax7* inactivation was not reported.

What is the significance of P21 in mouse? The number of myofibres does not change after birth, so postnatal muscle growth is achieved by both an increase in myofibre size and the addition of further myonuclei (Enesco and Puddy, 1964), with an approximate fivefold (from ~50 to ~250) increase in myonuclear content per myofibre between P3 and P21 (White et al., 2010). Satellite cells proliferate in growing muscle to supply these new myonuclei (Moss and Leblond, 1971; Shafiq et al., 1968), with the extent readily visualised in $Pax3^{Cre/+}$: $Rosa26^{lacZ/+}$ and $Pax7^{CE/+}$: $R26R^{lacZ/+}$ mice (Lepper et al., 2009; Lepper and Fan, 2010; Schienda et al., 2006). However, there are at least two populations of satellite cells identifiable with respect to the length of their cell cycle (Schultz, 1996), which indicates that not all satellite cells produce myonuclei at the same rate. Furthermore, the overall number of satellite cells gradually falls during this early postnatal period and so not all satellite cells contribute to the adult pool (Schultz, 1974; White et al., 2010). The supply of myonuclei from satellite cells gradually decreases, so that by around P21, further muscle growth is achieved by myofibre hypertrophy (Lepper et al., 2009; White et al., 2010), with the remaining satellite cells becoming mitotically quiescent (Moss and Leblond, 1971; Schultz et al., 1978).

Therefore, satellite cells are clearly required for muscle growth. Surprisingly, deletion of *Pax7* (and of both *Pax7* and *Pax3*) in satellite cells after P21 does not affect their function, with robust and efficient muscle regeneration maintained (Lepper et al., 2009). This requirement of Pax7 for satellite cell function only during muscle growth demonstrates clear differences between adult quiescent satellite cells and their embryonic, foetal or postnatal counterparts.

Satellite cell depletion compromises muscle regeneration

In the rare (5-10%) constitutive *Pax7*-null mice that survive to adulthood, satellite cell numbers are very low, with muscle reported as being weaker with myofibre loss (Kuang et al., 2006), or muscle size being moderately reduced and containing more small-calibre muscle fibres (Oustanina et al., 2004). The few remaining satellite cells exhibit proliferation and differentiation defects ex vivo (Kuang et al., 2006; Oustanina et al., 2004; Relaix et al., 2006). This lack of satellite cells correlates with a general failure of muscle regeneration.

Other interventions that deplete satellite cells and/or compromise their function are also associated with defective muscle regeneration. For example, loss of Notch signalling in satellite cells in either *Tg:Pax7-CT2*: *Rbpj*$^{flox/-}$: *Rosa26*$^{mTomato\text{-}STOP\text{-}mGFP/+}$ (Mourikis et al., 2012) or *Pax7*$^{CreER/+}$: *RBP-J*$^{f/f}$: *Rosa*$^{YFP/+}$ (Bjornson et al., 2012) adult mice leads to their spontaneous exit from quiescence and rapid myogenic differentiation, often without an intervening phase of cell division. Importantly, self-renewal is reduced without Notch signalling and the quiescent satellite cell pool is quickly depleted. Again, muscle regeneration is drastically perturbed (Bjornson et al., 2012; Mourikis et al., 2012). Likewise, inhibiting Notch signalling by simultaneous constitutive inactivation of both Notch target genes *Hesr1* and *Hesr3* also results in satellite cells differentiating rather than self-renewing, and, again, depletion of the satellite cell pool and impaired muscle regeneration (Fukada et al., 2011).

Finally, prevention of mature miRNA production in satellite cells via targeted inactivation of the miRNA-processing enzyme Dicer, causes most satellite cells to exit quiescence and undergo apoptosis in *Pax7*$^{CreER/+}$: *Dicer*$^{flox/flox}$ mice. The near complete loss of satellite cells prevents muscle regeneration, with no recovery in muscle morphology even 6 months after disruption of the *Dicer* gene (Cheung et al., 2012).

These studies, which show defective muscle regeneration after satellite cell loss, are complementary to others that indicate that there is no obvious contribution of cells from elsewhere in the body to muscle regeneration. An example is the lack of effective regeneration after high local doses of irradiation to a limb to prevent cell division (Heslop et al., 2000; Wakeford et al., 1991).

Unorthodox myogenesis: non-satellite cells with myogenic potential

Although satellite cells were generally accepted as a major source of myoblasts for muscle regeneration in adult, the description of bone marrow cells with myogenic potential (Ferrari et al., 1998) suggested that these cells could also contribute to muscle regeneration. This report was followed by descriptions of other non-satellite cell myogenic precursors (reviewed by Tedesco et al., 2010; Zammit et al., 2006). To date, many cells with myogenic potential have been described that are either in muscle tissue, including side population (Gussoni et al., 1999; Jackson et al., 1999), Sk-34 (Tamaki et al., 2002), mesangioblasts (Sampaolesi et al., 2003), CD45^{+}/Sca1^{+} cells (Polesskaya et al., 2003) and PW1^{+}/Pax7^{-} interstitial cells (PICs) (Mitchell et al., 2010), or in the circulation, such as AC133-expressing stem cells (Torrente et al., 2004). The inherent myogenic potential of cells responsible for such 'unorthodox' myogenesis is not understood, with most expressing muscle genes only after undergoing myogenic reprogramming following interaction/fusion with myoblasts and/or myofibres (e.g. Asakura et al., 2002; Kirillova et al., 2007). Some of these cell populations can also be found in the satellite cell niche following grafting in adult muscle (Asakura et al., 2002; LaBarge and Blau, 2002) and during muscle regeneration (Mitchell et al., 2010), leading to the suggestion that they could act as satellite cell precursors. Finally, de-differentiation of mammalian myonuclei to generate myogenic cells has been observed following certain genetic manipulations (Odelberg et al., 2000; Pajcini et al., 2010), but it is highly unlikely that this occurs normally during muscle regeneration.

Although there is evidence that mesangioblasts can contribute to muscle growth and the satellite cell pool during the postnatal period (Dellavalle et al., 2011), whether non-satellite cell myogenic progenitors have a physiological role in muscle regeneration in adult is unclear. This role is often affirmed by cell grafting, but the sensitivity of modern techniques to follow labelled cells often has single cell resolution, so in some cases may be detecting non-physiological levels of engraftment owing to cells merely being passively incorporated into regenerating myofibres. Even if cells do incorporate, they can fail to fully activate, or sustain, the myogenic programme (Lapidos et al., 2004; Wernig et al., 2005). Furthermore, it cannot be discounted that modification of cell properties by their preparation and grafting, then influences their fate in vivo.

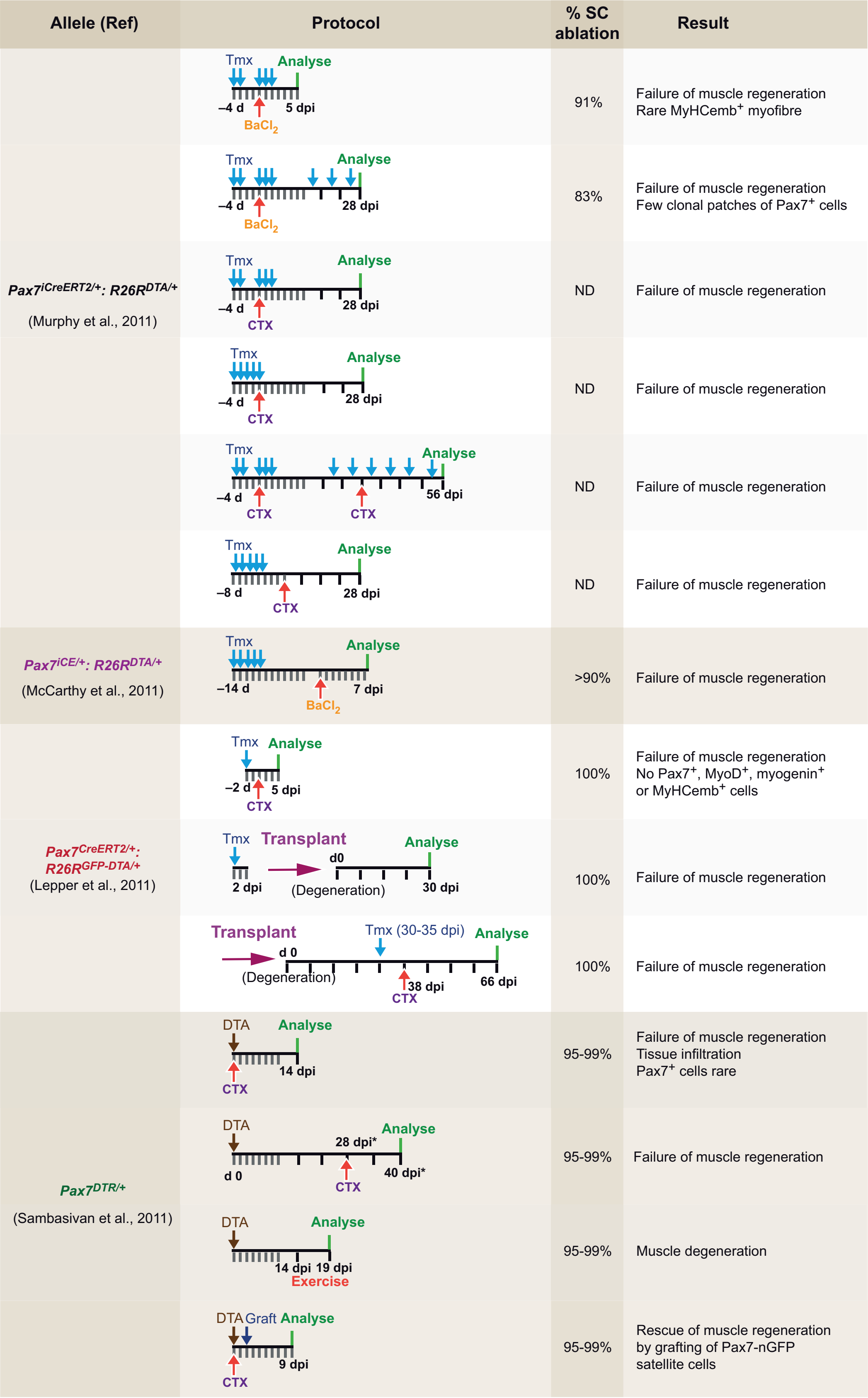
Satellite cells are indispensable for muscle regeneration

Genetic strategies to ablate satellite cells

The universal expression of *Pax7* in satellite cells (Seale et al., 2000; Gnocchi et al., 2009) means that *Pax7*^{Cre} alleles now provide an effective means to genetically ablate satellite cells in a defined temporal manner. Four papers using this strategy have recently been published (Lepper et al., 2011; McCarthy et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011b). A comparative analysis of the main experiments performed in these studies is presented in Fig. 4.

Fan and co-workers used their *Pax7*^{CE} allele (Lepper et al., 2009), while the Kardon and Peterson groups used independent mouse models in which an *IRES-CreERT2* cassette was inserted into the 3′UTR of the *Pax7* gene, thus preserving Pax7 function [*Pax7*^{iCreERT2} in the Kardon study (Murphy et al., 2011), and *Pax7*^{CreER} from the Keller laboratory (Nishijo et al., 2009) for the Peterson work (McCarthy et al., 2011)]. All three groups crossed their mice with *Pax7*^{CreERT} alleles with mice carrying *R26R*^{DTA} (Wu et al., 2006) or *Rosa26*$^{eGFP\text{-}DTA}$ (Ivanova et al., 2005) to constitutively express diphtheria toxin fragment A (DTA) once blocking sequences are floxed.

All satellite cells are eliminated within 36 hours of a single tamoxifen dose in *Pax7*$^{CE/+}$: *R26R*$^{eGFP\text{-}DTA/+}$ mice, such that even *Pax7* or *Cre* transcripts are no longer detectable. Interestingly, the *Pax7*$^{CE/+}$: *R26R*$^{eGFP\text{-}DTA/+}$ mice die within 7-10 days of tamoxifen treatment (Lepper et al., 2011) (Fig. 4). With *Pax7*^{CreER} or *Pax7*^{iCreERT2} alleles, about 90% of the satellite cells are ablated after five daily tamoxifen doses, but both *Pax7*$^{CreER/+}$: *R26R*$^{DTA/+}$ and *Pax7*$^{iCreERT2/+}$: *R26R*$^{DTA/+}$ mice then survive for several months at least (McCarthy et al., 2011; Murphy et al., 2011) (Fig. 4). This clear difference in lifespan is most likely because *Pax7* is also expressed in muscle spindles, which are lost in *Pax7*$^{CE/+}$: *R26R*$^{eGFP\text{-}DTA/+}$ mice, and specific regions of the brain, so the extent to which these other cell types are killed presumably correlates with survival. This probably also relates to the level of *Cre* expression, which is influenced by where *Cre* is inserted into the *Pax7* locus; higher levels may be achieved in *Pax7*^{CE} with *Cre* in the *Pax7*-reading frame, when compared with placing an *IRES-CreERT2* cassette in the 3′ UTR as in *Pax7*^{iCreERT2} and *Pax7*^{CreER}. There is also a difference in the potency of the DTA isoforms used in the *R26R*^{DTA} and *Rosa26*$^{eGFP\text{-}DTA}$ alleles, as *R26R*^{DTA} contains a slightly less toxic, attenuated form of fragment A (DTA176), which is designed to minimise any potential off-target effects due to 'leakiness' (Ivanova et al., 2005; Wu et al., 2006).



Allele (Ref)	Protocol	% SC ablation	Result
Pax7$^{iCreERT2/+}$: R26R$^{DTA/+}$ (Murphy et al., 2011)	Tmx Analyse –4 d 5 dpi BaCl$_2$	91%	Failure of muscle regeneration Rare MyHCemb$^+$ myofibre
	Tmx Analyse –4 d 28 dpi BaCl$_2$	83%	Failure of muscle regeneration Few clonal patches of Pax7$^+$ cells
	Tmx Analyse –4 d 28 dpi CTX	ND	Failure of muscle regeneration
	Tmx Analyse –4 d 28 dpi CTX	ND	Failure of muscle regeneration
	Tmx Analyse –4 d CTX CTX 56 dpi	ND	Failure of muscle regeneration
	Tmx Analyse –8 d CTX 28 dpi	ND	Failure of muscle regeneration
Pax7$^{iCE/+}$: R26R$^{DTA/+}$ (McCarthy et al., 2011)	Tmx Analyse –14 d 7 dpi BaCl$_2$	>90%	Failure of muscle regeneration
Pax7$^{CreERT2/+}$: R26R$^{GFP-DTA/+}$ (Lepper et al., 2011)	Tmx Analyse –2 d 5 dpi CTX	100%	Failure of muscle regeneration No Pax7$^+$, MyoD$^+$, myogenin$^+$ or MyHCemb$^+$ cells
	Tmx Transplant Analyse d0 2 dpi (Degeneration) 30 dpi	100%	Failure of muscle regeneration
	Transplant Tmx (30-35 dpi) Analyse d 0 (Degeneration) 38 dpi CTX 66 dpi	100%	Failure of muscle regeneration
Pax7$^{DTR/+}$ (Sambasivan et al., 2011)	DTA Analyse 14 dpi CTX	95-99%	Failure of muscle regeneration Tissue infiltration Pax7$^+$ cells rare
	DTA Analyse 28 dpi* d 0 CTX 40 dpi*	95-99%	Failure of muscle regeneration
	DTA Analyse 14 dpi 19 dpi Exercise	95-99%	Muscle degeneration
	DTA Graft Analyse 9 dpi CTX	95-99%	Rescue of muscle regeneration by grafting of Pax7-nGFP satellite cells

Fig. 4. Comparative analysis of satellite cell ablation studies. The main experiments performed in the four studies examining muscle regeneration in the absence of satellite cells are summarised (Lepper et al., 2011; McCarthy et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011b). Within the 'Protocol' column, time is represented by a vertical grey bar for each day and by a vertical black bar for each week. Administration of tamoxifen (Tmx) is indicated by blue arrows and intramuscular injection of diphtheria toxin fragment A (DTA) is shown by brown arrows. The day of injury is designated with a red arrow, with the method noted (BaCl$_2$ or cardiotoxin; CTX). The day of muscle injury and transplantation is designated as Day 0, with days before injury indicated by –*n* d, while days after injury are represented by dpi (days post injury). The percentage of satellite cells ablated (% SC ablated) in each approach is indicated and the final outcome is summarised. MyHCemb, embryonic myosin heavy chain isoform; ND, not determined.

The Tajbakhsh/Galy groups used a different approach and targeted the diphtheria toxin receptor to the *Pax7* locus (*Pax7^{DTR}*). Intramuscular injection of DTA leads to the ablation of *Pax7*-expressing cells only in the locality of the injection site (Sambasivan et al., 2011b) and not throughout the mouse, as systemic administration of tamoxifen in *Pax7^{CreERT}* mice does. The number of satellite cells remaining after DTA treatment is estimated to be between 1% and 5%, but these surviving cells do not generate any functional myogenic cells ex vivo, suggesting that DTA may impair cell function without leading to cell death (Fig. 4). Unhelpfully, intramuscular injection of DTA in $Pax7^{DTR}$ mice also leads to a mild inflammatory response and cellular infiltration, with a sustained (assayed up to 8 weeks later) loss in muscle weight of between 20% and 40%, although vasculature, innervation and neuromuscular junctions are unaffected. Probably owing to nonspecific cross-reactivity with the mouse receptor, the precise cause of this muscle mass loss (e.g. myofibre hypotrophy or degeneration) was not reported (Sambasivan et al., 2011b).

Muscle regeneration fails in the absence of satellite cells

The main conclusion of these four studies is that in the absence of most, or all, *Pax7*-expressing cells, a profound failure of muscle regeneration occurs (Figs 2, 4). Muscle injury was induced by intramuscular injection of either cardiotoxin (a snake venom with membrane-damaging activity inducing tissue necrosis) or $BaCl_2$ (which causes muscle depolarisation and myofibre death by stimulating exocytosis while blocking the efflux of Ca^{2+}). Such acute muscle injury in tamoxifen-treated $Pax7^{CE/+}$: $Rosa^{eGFP\text{-}DTA/+}$, $Pax7^{CreER/+}$: $R26R^{DTA/+}$ or $Pax7^{iCreERT2/+}$: $R26R^{DTA/+}$ mice results in negligible myotube formation after 5-7 days, a time when control muscle already has myotubes present (Lepper et al., 2011; McCarthy et al., 2011; Murphy et al., 2011). Similarly, DTA injection combined with cardiotoxin-mediated injury in $Pax7^{DTR/+}$ mice also causes a near-complete lack of myogenic cells and an absence of regenerating myofibres after 4 or 8 days (Sambasivan et al., 2011b).

Apart from $Pax7^{CE/+}$, the other *Pax7* alleles fail to completely ablate satellite cells, although the few survivors would presumably be further reduced by cardiotoxin, which is known to also kill satellite cells [probably more than $BaCl_2$-induced injury (Gayraud-Morel et al., 2009)]. However, any *Pax7*-expressing cells that survived Cre-mediated DTA ablation and cardiotoxin are unable to significantly regenerate muscle (Fig. 4). It may be argued that more time is required before effective regeneration could begin, to permit the few remaining satellite cells to proliferate sufficiently, and/or to allow satellite cells to be replenished from another source or to let non-satellite cell populations establish themselves. However, regeneration is prevented, and not merely delayed, as even 28 or 56 days later, no visible muscles reform in satellite cell-ablated tibialis anterior muscle of $Pax7^{iCreERT2/+}$: $R26R^{DTA/+}$ mice, even in response to a second cardiotoxin injury (Murphy et al., 2011).

Snakebites (especially in Northern Europe!) or injuries that lead to complete muscle degeneration are unusual. A more common cause of muscle damage in man is strenuous resistance exercise (Brentano and Martins Kruel, 2011). Modelling such vigorous exercise in mice with forced daily running of 30 minutes for 5 days in satellite cell-ablated $Pax7^{DTR/+}$ mice, led to a striking loss of myofibres, with inflammatory cell and adipocyte infiltration. It needs to be remembered, though, that this effect could have been exacerbated by the muscle damage directly elicited by the DTA injection used to ablate the satellite cells (Sambasivan et al., 2011b), and so should be confirmed using Pax^{CreERT} alleles that do not directly affect myofibres.

Collectively, these studies clearly demonstrate that satellite cells are required for skeletal muscle regeneration following a variety of acute myotoxic injuries (Figs 2, 4). It also appears that a threshold number of satellite cells may be needed to even partially regenerate such severely damaged muscle. Crucially, unorthodox myogenic precursors are unable to substitute for this regenerative function performed by satellite cells.

Ablated satellite cells are not replaced

It is possible that satellite cell ablation, quickly followed by massive injury, does not allow the satellite cell population time to recover. It is estimated that induction of Cre is finished within 24 hours of the final tamoxifen dose, but there were still no satellite cells present in $Pax7^{CE/+}$: $Rosa^{eGFP\text{-}DTA/+}$ mice 6.5 days later (Lepper et al., 2011). Tamoxifen-treated $Pax7^{iCreERT2/+}$: $R26R^{DTA/+}$ mice also had a near-complete absence of satellite cells on day 5 of regeneration, with fewer than 15% present after 30 days (Murphy et al., 2011). It is untested, however, whether satellite cell-ablated uninjured or injured muscle might gain more satellite cells in the longer term.

If satellite cell precursors within the muscle, or elsewhere in the body, also express *Pax7*, they too would be ablated by systemic administration of tamoxifen, and so would not be available to restore the satellite cell pool. As DTA is injected intramuscularly in $Pax7^{DTR/+}$ mice, it can be assumed that *Pax7*-expressing cells distant from the site of injection would not be ablated, leaving the possibility that these cells could be mobilised to replace the satellite cells in the DTA-injected muscle. However, functional compensation by other cell types did not occur, as muscle was unable regenerate following cardiotoxin-induced injury, even with an intervening 14- to 35-day recovery period after satellite cell ablation (Sambasivan et al., 2011b). Although the muscle environment is clearly affected by DTA treatment, it was not rendered completely hostile to satellite cells, as wild-type satellite cells grafted into cardiotoxin/DTA-treated $Pax7^{DTR/+}$ muscle can still effectively regenerate areas of myotubes (Sambasivan et al., 2011b).

The contribution of unorthodox myogenic progenitors to muscle regeneration was also assayed using transplantation of entire muscles (Lepper et al., 2011). A grafted muscle initially undergoes near-complete degeneration, followed by myofibre regeneration and re-establishment of both vasculature and innervation, with the process complete within 1 month. When a satellite cell-ablated extensor digitorum longus (EDL) muscle of a $Pax7^{CE/+}$: $Rosa^{eGFP\text{-}DTA/+}$ donor mouse is transplanted, it degenerates in the wild-type host mouse, but then fails to regenerate. However, a grafted EDL regenerates well if from a non-tamoxifen treated $Pax7^{CE/+}$: $Rosa^{eGFP\text{-}DTA/+}$ donor. If a host mouse carrying a regenerated donor EDL muscle is given tamoxifen, then only *Pax7*-expressing cells in the transplanted muscle are ablated, not those of the wild-type host. If such grafted, satellite cell-ablated, regenerated EDL muscles are subsequently injured with cardiotoxin, they then fail to re-regenerate (Lepper et al., 2011). Thus, even with access to the circulation of the host for 1 month, and then for several days after satellite cell ablation, the donor muscle is not repopulated with host-derived unorthodox myogenic precursors (Fig. 4).

Conclusions and future perspectives

Once *Pax7*-expressing cells are ablated locally or systemically, muscle is unable to regenerate and, importantly, does not recover this ability. Ablation of *Pax7*-expressing cells clearly destroys satellite cells, which are generally agreed to uniformly express *Pax7*. Other proposed muscle-resident or non-resident myogenic stem cell populations do not express *Pax7*, and so would be spared ablation using targeted *Pax7* alleles. Therefore, muscle does not regenerate without satellite cells, and other potential myogenic stem cells do not compensate for their loss. Furthermore, as myonuclei do not express *Pax7*, they too would be immune from ablation, yet the absence of measurable regeneration indicates that, as expected, myonuclear de-differentiation does not occur to any significant degree under normal circumstances.

These studies also confirm that satellite cells are responsible for maintaining their own population via the closed loop of self-renewal. Satellite cell precursors that do not express *Pax7* are no longer present in adult, or cannot be effectively recruited to the satellite cell pool. This assumes that *Pax7* is not expressed in any of these precursors, but even if it was, the recovery periods after tamoxifen treatment should have allowed for further differentiation of non *Pax7*-expressing cells into new Pax7-positive satellite cell precursors, which failed to happen. Some satellite cells clearly remain after tamoxifen treatment in either $Pax7^{CreER/+}$ or $Pax7^{iCreERT2/+}$ mice, yet it is striking that regeneration fails in both genetic models, suggesting a threshold number for efficient satellite cell function. However, effective regeneration can occur following transplantation of only a few or even just one satellite cell (Collins et al., 2005; Sacco et al., 2008). In these grafting experiments though, the host muscle retains its endogenous satellite cell pool (even if irradiated), suggesting a community effect and support activity to the grafted satellite cells. Indeed, reciprocal support between both satellite cells and endothelial cells (Christov et al., 2007) and satellite cells and fibroblasts (Murphy et al., 2011) has been demonstrated. It is also possible that unorthodox myogenic precursors can not regenerate muscle without paracrine/physical support from satellite cells, as has been observed for mesoangioblasts or PICs in vitro (Tedesco et al., 2010) or that dying satellite cells release factors that directly compromise non-satellite cell precursors.

Questions remain regarding the role of satellite cells in skeletal muscle homeostasis, hypertrophy and ageing. Uninjured muscles that are depleted of satellite cells following *Dicer* gene disruption in $Pax7^{CreER/+}$: $Dicer^{flox/flox}$ mice still appear overtly normal 6 months later, but do exhibit a mild muscle fibre atrophy over time (Cheung et al., 2012). Mice with satellite cells ablated using the $Pax7^{CreER}$ or $Pax7^{iCreERT2}$ alleles remain alive for at least several months, but the condition of muscles in the longer term was not reported, other than to state that the endothelial ($CD31^{+}$) and haematopoietic ($CD45^{+}$) compartments of the muscle were unaffected (McCarthy et al., 2011; Murphy et al., 2011). Ablation of satellite cells in young mice with long-term follow up is necessary to see how muscle ages without satellite cells. Studying the effects of the loss of satellite cells in geriatric muscle would also be interesting.

Hypertrophy was examined after satellite cell ablation in one study, where the plantaris muscle in tamoxifen-treated $Pax7^{CreER/+}$: $R26R^{DTA/+}$ mice was forced to hypertrophy by removing synergistic muscles. Hypertrophy still occurred in the short term (2 weeks), despite the absence of the majority of satellite cells (McCarthy et al., 2011). Does this mean that satellite cells are not initially required for hypertrophy, or that the few remaining cells were sufficient (yet do not seem able to mount a regenerative response to acute injury)? A detailed analysis of myonuclear content per myofibre could resolve whether hypertrophy was accompanied by an increase in myonuclei. Examination of whether muscle hypertrophy is maintained longer term (>6 weeks) without satellite cells needs to be addressed. Additionally, the deleterious effects on muscle of strenuous exercise in the absence of satellite cells, as revealed by DTA intramuscular injection in $Pax7^{DTR/+}$ mice (Sambasivan et al., 2011b), should be confirmed using the $Pax7^{CreERT}$ alleles that can be used to ablate satellite cells without also causing overt myofibre damage.

These experiments demonstrate that satellite cells alone are required for supplying myoblasts during acute skeletal muscle regeneration. It would be interesting to ablate satellite cells at various points during muscle regeneration to examine the dynamics of *Pax7* locus activity and the profile of differentiation and self-renewal. The four studies discussed above concentrated on hind limb muscle, but satellite cells throughout the body express *Pax7*, so the relative role of satellite cells and other non-satellite cell populations in muscle homeostasis and regeneration can readily be assessed for many other muscles, including those of the head.

It is also necessary to determine the effects of satellite cell ablation on the chronic degeneration/regeneration cycles seen in some muscle diseases. The phenotype in the *mdx* mouse model of Duchenne muscular dystrophy is more pronounced if teleomerase activity is deleted (Sacco et al., 2010), although in this study, the inactivation of telomerase was not restricted to satellite cells. Ablating satellite cells in conditional $Pax7^{cre}$: $R26R^{DTA/+}$ mice on an *mdx* background would assay the function of satellite cells in chronic regeneration and also test whether non-satellite cell types make an effective contribution in this situation.

The possibility remains that unorthodox myogenic progenitors could be useful for cell therapy-based strategies. For example, it will be interesting to test whether cell types such as the PICs (Mitchell et al., 2010) or mesoangioblasts (Sampaolesi et al., 2003) are able to contribute to myogenesis after local or systemic delivery into muscle lacking satellite cells, as grafted satellite cells can (Sambasivan et al., 2011b). Furthermore, specific ablation of these individual non-satellite cell populations would show whether satellite cells are also able to function in their absence, considering that interactions with cell populations such as macrophages and connective tissue fibroblasts are required for efficient satellite cell function (Murphy et al., 2011).

Now that satellite cells are established as being responsible and absolutely required for muscle regeneration, there is a need to resolve the issue of whether there are subpopulations of satellite cells within a common niche. Ultimately, resolution of the composition and nature of the satellite cell pool probably awaits single cell-based analyses and prospective endogenous markers that are able to directly identify any satellite ‘stem cell’.

In summary, these recent studies on the depletion or genetic ablation of satellite cells using complementary approaches (Fig. 4) clearly demonstrate that satellite cells are responsible for skeletal muscle regeneration after acute injury. Under such conditions, non-satellite cell populations are unable to substitute for the function of satellite cells, which are indispensable for muscle regeneration. The cell on the edge has now returned centre stage!

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Competing interests statement

The authors declare no competing financial interests.

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