Pitx2 in Embryonic and Adult Myogenesis

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Skeletal muscle is a heterogeneous tissue that represents between 30 and 38% of the human body mass and has important functions in the organism, such as maintaining posture, locomotor impulse, or pulmonary ventilation. The genesis of skeletal muscle during embryonic development is a process controlled by an elaborate regulatory network combining the interplay of extrinsic and intrinsic regulatory mechanisms that transform myogenic precursor cells into functional muscle fibers through a finely tuned differentiation program. However, the capacity of generating muscle still remains once these fibers have matured. Adult myogenesis resembles many of the embryonic morphogenetic episodes and depends on the activation of satellite cells that have the potential to differentiate into new muscle fibers. Pitx2 is a member of the bicoi family of homeodomain transcription factors that play an important role in morphogenesis. In the last decade, Pitx2 has emerged as a key element involved in the fine-tuning mechanism that regulates skeletal-muscle development as well as the differentiation and cell fate of satellite cells in adult muscle. Here we present an integrative view of all aspects of embryonic and adult myogenesis in which Pitx2 is involved, from embryonic development to satellite-cell proliferation, fate specification, and differentiation. Those new Pitx2 functions on satellite-cell biology might open new perspectives to develop therapeutic strategies for muscular disorders.

Keywords: Pitx2, myogenic precursor cells, embryonic myogenesis, adult myogenesis, satellite cell and regeneration

INTRODUCTION

Skeletal muscle is a heterogeneous tissue that represents between 30 and 38% of the human body mass (Janssen et al., 2000). It is composed of individual muscle fibers, diversified in size, shape, and contractile protein content, to fulfill the different functional needs of the vertebrate body such as maintaining body posture, locomotor impulse, or pulmonary ventilation. The genesis of skeletal muscle during embryonic development and postnatal life is a process controlled by an extremely elaborate regulatory network that combines the interplay of extrinsic (e.g., morphogens, neurohormonal input, muscle damage, etc.) and intrinsic elements (gene regulatory elements). The intrinsic elements form hierarchical interactions between transcriptional regulators, regulatory RNAs, and chromatin-remodeling factors. In this sense, during embryogenesis, muscle progenitors are specified by the sequential expression of a network of transcription factors composed of PAX3 and PAX7, and the basic helix-loop-helix (bHLH) myogenic regulatory factors (MRFs) MYOD,
MYF5, MYF6 (also called MFR4), and MYOG (Bentzinger et al., 2012; Moncaut et al., 2013). In addition, during adult life the skeletal muscle fibrillar network development mechanisms that compensate for the physiological turnover and damage in order to maintain tissue homeostasis (Schmalbruch and Lewis, 2000; Pelletier and Alvarado, 2007). This adult myogenesis depends on the activation of satellite cells (SCs), that have the potential to proliferate, differentiate, and generate new fibers, or repair existing ones (Chargé and Rudnicki, 2004). It has been well-established that SCs are closely related to progenitors of embryonic origin (Gros et al., 2005; Relaix et al., 2005; Schienda et al., 2006; Hutcheson et al., 2009; Lepper and Fan, 2010). Thus, many similarities have been discovered between prenatal myogenesis and regeneration in the mature skeletal musculature, such as common transcription factors and signaling molecules (Tajbakhsh, 2009).

During the last two decades the homeobox transcription factor Pitx2 has emerged as a key element in the fine-tuning mechanism that regulates skeletal-muscle development. Concurrently, several recent experimental pieces of evidence point to the role of Pitx2 in SC biology. Here, we present an integrative view of the role of Pitx2 in prenatal and adult myogenesis (from embryonic development to SC proliferation), fate specification, and differentiation. Finally we discuss the potential therapeutic use of Pitx2 in the future.

PRENATAL AND ADULT MYOGENESIS

In vertebrates, skeletal-muscle development is a biphasic process. A primary (embryonic) myogenesis takes place to generate primary muscle fibers, between embryonic day (E) 9.5 and E14.5 in the mouse. This is followed during fetal stages by a secondary myogenesis which gives rise to the bulk of skeletal-muscle fibers present at birth (Kelly and Zacks, 1969; Biressi et al., 2007; Tajbakhsh, 2009; Deries and Thorsteinsdóttir, 2016). All skeletal-muscle cells have the same underlying functions, although their progenitors within the paraxial mesoderm are spread throughout the embryo at the onset of myogenesis. This bears emphasizing since the genetic networks that control myogenesis present differences depending on the location of those myogenic precursors in the embryo.

Embryonic Myogenesis: The Trunk and Limb Muscles

The muscles of the trunk and limbs derive from somites (Figure 1A), which are transient paraxial mesodermal structures that form pairwise on either side of the neural tube, following an anterior-posterior developmental gradient. The somite is initially a spherical unit of polarized epithelial cells that soon after subdivides into two compartments, the ventral mesenchymal sclerotome and the dorsal epithelial dermomyotome. Shortly afterwards, myogenic precursor cells from the epaxial and hypaxial lips of the dermomyotome undergo an epithelial-mesenchymal transition (EMT) and accumulate underneath, where they differentiate and elongate to form the myocytes of the myotome, the first myogenic structure to develop in the body (Buckingham and Relaix, 2015; Deries and Thorsteinsdóttir, 2016). The epaxial region of the myotome gives rise to the deep back muscles, whereas the hypaxial myotome is the source of body wall muscles and most other trunk muscles (Buckingham and Relaix, 2015; Deries and Thorsteinsdóttir, 2016). In segments adjacent to the limb-region cells of the hypaxial dermomyotome undergo an EMT, leave the epithelial structure, and migrate toward the fore and hind limbs to form dorsal and ventral muscle masses in the limb-bud mesenchyme, where they begin to differentiate and express muscle-specific genes (Biressi et al., 2007; Deries and Thorsteinsdóttir, 2016).

Cell commitment in the somite is highly dependent on a number of transcription factors which act in a hierarchical molecular cascade to orchestrate the specification, determination, and differentiation of myogenic precursors. In the genetic hierarchy that regulates the onset of trunk myogenesis, Pax3 and Myf5 play a dominant role (Buckingham and Relaix, 2015). Pax3 is already transcribed in pre-somatic mesoderm adjacent to the first somite (Schubert et al., 2001) and then throughout the newly formed somites. As somites mature Pax3 expression becomes confined to the dermomyotome (Goulding et al., 1991) and persists in myogenic progenitor cells that delaminate and migrate from the somite to more distant sites of myogenesis such as the limb (Buckingham and Relaix, 2015). Myogenic cells that have activated the myogenic determination genes Myf5/Myf6 and MyoD downregulate Pax3 and delaminate from the edges of the dermomyotome (Buckingham and Relaix, 2015). The epaxial myotome then start to form. This depends on the early epaxial activation of Myf5, which is driven by Wnt and Shh signaling, without any Pax3 and/or Pax7 requirement (Borello et al., 2006; Buckingham and Relaix, 2015). These cells do not activate MyoD but rather Myog and differentiate (Kablar et al., 2003). On the other hand, the activation of Myf5 in the hypaxial somite as well as in the limb depends on Pax3 (Bajard et al., 2006; Buckingham and Relaix, 2015). At this stage MYF6 also acts as a myogenic determination factor (Kassar-Duchossoy et al., 2004). The Myod gene is activated after the onset of Myf5 expression in the rest of the dermomyotome and limbs (Hu et al., 2008). Finally, the transcription factor MYOG is required for the onset of the expression of terminal differentiation genes needed for the fusion of myocytes and the formation of myotubes (Bentzinger et al., 2012).

Embryonic Myogenesis: The Head Muscles

Although, all skeletal muscle throughout the body originates within paraxial mesoderm, in the head, identifiable compartments such as the somites in the trunk are not evident histologically or by most molecular criteria. This unsegmented head mesoderm is remodeled at the early stages of embryonic development (Figures 1B,C). The unsegmented head mesoderm gives rise to all craniofacial skeletal muscles, which can be cataloged as four distinct populations: extra-ocular (EOMs), branchial, laryngoglossal, and axial neck muscles (Noden and Francis-West, 2006; Tzahor, 2015). EOMs are formed by cells from the cranial paraxial mesoderm that migrate through the first branchial arch (FBA) as well as from the prechordal mesoderm (Jacob et al., 1984; Evans and Noden, 2006; Tzahor,
FIGURE 1 | Embryonic myogenesis (A) Schematic representation of somite maturation. Somites mature following an anterior to posterior developmental gradient (Modified from Gray’s Anatomy. The Anatomical Basis of Clinical Practice, 40th Edition, Standring, 2008): myogenic precursor cells arise from the epaxial and hypaxial lips of the dermomyotome after archive epithelial-mesenchymal transition (EMT) and migrate toward the limbs to form dorsal and ventral muscle masses where they begin to differentiate. (B,C) Head frontal and transverse planes of a mouse embryo between stages of development E7.5–8.75 and E8–9.25 in mouse. At an open neural plate stage, head mesoderm in a frontal plane includes the prechordal mesoderm and the paraxial mesoderm. When the neural tube closes dorsally and the endoderm ventrally, the prechordal mesoderm is integrated within the remaining paraxial mesoderm, which is located anterior to the somites. Dashed line illustrates the cutting plane. (D) Origins of skeletal muscles: Myogenic precursors arise from different paraxial mesoderm compartments. (E) Pitx2 expression domains at the E10.5 stage of development in mouse. NT, neural tube; NC, notochord; SM, somites; DMT, dermomyotome; ST, sclerotome; MT, myotome; LMP, limb muscle precursors; FL, forelimb; PAM, head paraxial mesoderm; PCM, paraxial mesoderm; PROS, prosencephalon; MES, mesencephalon; MET, metencephalon; SPM, splanchnic mesoderm; OFT, outflow tract of heart; HT, heart tube; EOM, extra-ocular muscles; BRM, branchial muscles; LGM, laryngoglossal muscles; HGC, hypoglossal cord; ANM, axial neck muscles; BM, back muscles; BWM, body wall muscles; FLM, forelimbs muscles; HLM, hind limbs muscles.
Branchial arch muscles are formed mainly by migrating cells from the cranial paraxial mesoderm and the lateral splanchnic mesoderm (Harel et al., 2009; Sambasivan et al., 2009; Tzahor, 2015). Laryngoglossal muscles develop from migratory myoblasts arising from occipital somites that form a condensed mesenchymal band, the hypoglossal cord, which elongates and similarly brings myoblasts ventral to pharynx (Hammond, 1965; Hazelton, 1970; Tzahor, 2015). Finally, in the transition zone between the head and the trunk are the axial neck muscles. They arise from medio-dorsal and latero-ventral domains of occipital and cervical somites (Noden, 1983; Couly et al., 1992; Matsuoka et al., 2005).

The genetic hierarchy governing primary myogenesis in the trunk does not appear to operate for head-muscle formation. Activation of the myogenic program in the head therefore depends on different upstream factors, responds differently to signaling pathways and also displays site-dependent regulation. Branchial-arch-derived muscles depend on Myf5/Myf6/Myod, whereas extra-ocular muscle formation is initiated by Myf5/Myf6 and in their absence cannot be restored by Myod (Tajbakhsh and Buckingham, 1999).

### Fetal Myogenesis

During fetal myogenesis, secondary fibers in trunk, limbs, and head are generated by the fusion of fetal myoblasts. Secondary fibers form initially at the site of innervation of the primary fiber and are surrounded by the same basal lamina as the primary fiber on which they lie (Duxson et al., 1989). The secondary myotubes remain attached for a short period to primary fibers and subsequently elongate and become independent fibers, which can be distinguished from primary fibers by their relative small size (Kelly and Zacks, 1969). Although, the genetic networks that rule this second stage of prenatal myogenesis is less understood, it is known that the MRFs MYF5, MYOD, and MYOG are also crucial, since in Myog−/− as well as Myf5−/−;MyoD−/− double-mutant secondary myogenesis is completely inhibited (Venuti et al., 1995; Kassar-Duchossoy et al., 2004).

### Adult Myogenesis

The regulatory inputs that orchestrate myogenesis during prenatal myogenesis are partially reactivated in adult muscle repair. In adulthood, the maintenance as well as the repair of muscle tissue are both directed mainly by SCs. These cells, originally identified via electron microscopy in 1961 by Alexander Mauro, are located underneath the basal lamina and adjacent to the plasma membrane of the skeletal-muscle myofiber (Mauro, 1961; Figure 2). In their quiescent state, SC express the transcription factor Pax7 and represent a genuine stem-cell population indispensable for skeletal-muscle repair (Lepper et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011; Miersch et al.,

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**FIGURE 2 | Adult myogenesis.** The overall myogenic differentiation pathway includes the activation of quiescent SCs, commitment to differentiation, proliferation, fusion to form myotubes and ultimately maturation into myofibers, SC, satellite cell; MN, myonucleus.
2017; Stuelsatz et al., 2017). It has been established that SCs in adult muscle represent a lineage continuum of the embryonic myogenic progenitor cells. Thus, while SCs of the body and limbs arise from somites, in common with the muscle that they are associated with (Armand et al., 1983; Gros et al., 2005; Relaix et al., 2005; Schienda et al., 2006), the SCs located in head muscles also originate from the cranial mesoderm (Harel et al., 2009). Within a context of physiological stimuli (physical exercise or pathological conditions) SCs become activated, proliferate, differentiate and fuse to form multinucleated myofibers in order to undergo proper myogenesis (Lepper et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011; Miersch et al., 2017; Stuelsatz et al., 2017; Figure 2). In this regard, numerous studies have revealed a striking similarity between adult and embryonic myogenesis, where the core regulatory network composed of the MRFs MYF5, MYOD, MYOG, and MYF6 is mainly required (Bentzinger et al., 2012; Segalès et al., 2016; Figure 2).

THE PITX2 GENE

The Pitx gene family includes three vertebrate paralogues, Pitx1, Pitx2, and Pitx3, which have been cloned in multiple organisms (Gage et al., 1999b; Knopp et al., 2013). These three genes encode transcription factors that belong to the bicoid-related subclass of homeodomain proteins (Gage et al., 1999b). The members of this family share an almost identical protein sequence within their homeodomains, varying mainly in the N-terminal region (Gage et al., 1999b; Knopp et al., 2013). Mutations or misregulation of Pitx1, Pitx2, and Pitx3 result in developmental disorders in humans, such as Facioscapulohumeral Muscular Dystrophy (FSHD; Dixit et al., 2007), Axenfeld-Rieger syndrome (Semina et al., 1996), and Anterior Segment Mesenchymal Dysgenesis (ASMD; Semina et al., 1998), respectively. Muscle expression of these genes during development has been systematically studied. Thus, Pitx1 is highly expressed in developing hind-limb-bud mesenchyme and is shown to determine hind-limb identity in mice (Lancôt et al., 1999; Szeto et al., 1999), chicks (Logan and Tabin, 1999), and fish (Shapiro et al., 2004). On the other hand, Pitx3 is widely expressed in all skeletal muscles of the head, trunk and limbs (Semina et al., 1998; L’honore et al., 2007). Curiously, despite its apparent importance in muscle development, the investigation of Pitx3−/− mice indicates that Pitx3 on its own is not required for myogenesis (L’honore et al., 2007). In this scenario Pitx2, the third Pitx family member is strongly upregulated and appears to fully compensate for the loss of Pitx3 during muscle formation (L’honore et al., 2007). Pitx2 is also able to control the growth ability of hind-limb mesenchyme together with Pitx1 (Marcil et al., 2003), indicating the importance of Pitx2 in the control of skeletal myogenesis during development.

In mice, the Pitx2 (Pituitary homeobox 2 or Paired-like homeodomain transcription factor 2) gene is mapped on chromosome 3 (3G3; 357.84 cm) (Gage and Camper, 1997) and is transcribed into three distinct isoforms: Pitx2a, Pitx2b, and Pitx2c. Pitx2a and Pitx2b share the same promoter while Pitx2c uses an alternative one upstream of exon 4 (Schweickert et al., 2000). In human, PITX2 is mapped on chromosome 4 (4q25) and maintains a similar genetic structure, but presents a fourth isoform (Arakawa et al., 1998; Cox et al., 2002). This fourth isoform is generated by the PITX2C alternative promoter and differential splicing, being able to suppress the transcriptional activity of the other PITX2 isoforms (Cox et al., 2002). All Pitx2 isoforms share a K50 DNA-binding homeodomain which binds to the consensus sequence TAATCC (Amendt et al., 1998; Chaney et al., 2005), thus being able to induce a transcriptional activation of Prl (Amendt et al., 1998) or Anf (Ganga et al., 2003) promoters. The Pitx2 gene was isolated independently by several research groups and designated as Otx2 (Muccielli et al., 1996), Rieg (Semina et al., 1996), Pitx2 (Gage and Camper, 1997), Brxl (Kitamura et al., 1997), and Arp1 (Arakawa et al., 1998). Although, most of these works focused on the role of this gene in the development of brain structures, the authors reported the expression of Pitx2 in the mesenchyme of the eye, the first and second branchial arches, the fore and hind limbs as well as the dermomyotome at somite stages E8.5 and 10.5 in mouse, and its equivalent stages in chicken (Figure 1E). Soon afterwards, a role for Pitx2 was also described in left-right asymmetry, being proposed as the molecular transducer of embryonic left-right signaling during early developmental stages at the level of organs such as heart, gut, and/or stomach (Logan et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998; Campione et al., 1999).

PITX2 WITHIN THE GENETIC HIERARCHIES THAT CONTROL MUSCLE DEVELOPMENT

Pitx2 Function during Embryonic Myogenesis

Pitx2 in Trunk and Limb Muscle Development

The first evidence involving Pitx2 in the molecular process controlling myogenesis was provided by Kitamura et al. (1999). These authors reported Pitx2 expression co-localizing in dermomyotomes with Pax3, a muscle specification marker playing a key role in delamination and migration of the somitic muscle progenitor cells to the limb buds (Goulding et al., 1994; Tajbakhsh et al., 1997). Later, Marcil et al. demonstrated the presence of PITX2 protein in the myoblasts of the limb bud, displaying an expression pattern similar to that of PAX3 and MYOD (Marcil et al., 2003). All these data suggested that PITX2 was involved in muscle patterning. A more detailed temporal and spatial analysis during initial muscle-cell-cluster formation, by using lacZ expression from a Pitx2 gene insertion, revealed the presence of a Pitx2-expressing cell cluster lateral to the dermomyotome (Shih et al., 2007b). This cluster first appeared at the forelimb level at E10.25. After E10.5, Pitx2(+/lacZ)-expressing cells were then also detected on sections of the limbs. Curiously, Pax3 and the muscle-regulatory factors (MRFs) stained only subsets of Pitx2+ cells within these areas, and virtually all Pitx2+ cells in these areas express at least one of these known myogenic markers (Shih et al., 2007b). These observations led the authors to conclude that Pitx2 marks the muscle
lineage more completely than any of the known markers does. In agreement with the interpretation that muscle progenitors express Pitx2, L’Honoré et al. (2007) found extensive co-labeling of myotome- and dermomyotome-proliferating cells with PITX2, Pax3, and with Pax7. Notably, they also observed Pax3-positive cells that have completed migration at the proximal limb bud also express PITX2 while not all PITX2-positive cells expressed Pax3. All these data suggest that Pitx2 might be a player within the molecular pathways controlling muscle-progenitor fate.

Sometime afterwards, additional information regarding the hierarchical position occupied by Pitx2 within the genetic cascade that control somite-derived myogenesis was inferred by using Pitx2−/−; Myf5lacZ/−nlacZ and Pitx2−/−;Myf5lacZ/−nlacZ double-mutant mice (L’Honoré et al., 2010). In this work, the authors showed that PITX2 protein directly regulates Myod expression through binding to its core enhancer in wild-type limbs. In agreement, the authors described a delayed myogenic differentiation and a Myod down regulation in Pitx2−/− limb buds and proposed that this phenotype appears to be due to the failure to activate the Myod core enhancer. However, although the inactivation of Myf5 and Myf6 in Myf5lacZ/−nlacZ mutant embryos (Myf6 is inactivated in cis in this mutant; Tajbaksh et al., 1997) did not affect Myod expression in limb buds, this inactivation in a Pitx2−/− background (Pitx2−/−;Myf5lacZ/−nlacZ) induced a synergic effect that resulted not in a downregulation but in almost a complete loss of Myod expression compared with Pitx2−/−;Myf5lacZ/+ embryos, where the presence of one active Myf5 allele prevented Myod loss in about 60% of myogenic precursors cells. These results imply that Myf5 and/or Myf6 cooperate with Pitx2 to control Myod expression during early limb-bud myogenesis (Figure 3A2). In contrast to limb-muscle cells, myotome expression of Myod was not delayed in Pitx2−/− embryos. Nevertheless, in Myf5lacZ/−nlacZ mutant embryos, Myod expression was delayed by ~2 days. Therefore, the onset of Myod expression in the myotome does not appear to depend on PITX2 but mostly on MYF5/MYF6. Nonetheless the inactivation of Myf5 and/or Myf6 in a Pitx2−/− background (Pitx2−/−;Myf5lacZ/−nlacZ) led to an almost complete loss of Myod expression in myotome, as happened in limbs (L’Honoré et al., 2010). These results indicate that Myf5 and/or MYF6 also cooperate with PITX2 to control Myod expression during myotome development (Figure 3A1).

In addition, it should be stressed that the analysis of Pax3 mutant Splotch mice revealed a deficit of Pitx2 expression restricted to the myotome (L’Honoré et al., 2010). This deficit is not observed in neighboring mesenchyme, indicating that Pitx2 is downstream of Pax3 during myotome myogenesis. This is also supported by transcriptanalyses of Pax3 (GFP) /Pax3−/−transgenic mice carried out by Lagha et al. (2010) since, in gain-of-function screens for Pax3 targets, they found an up-regulation of Pitx2 in somites but not in limb buds. Although, all these seminal works suggest that Pitx2 could be acting downstream of Pax3 and in parallel with Myf5, at least in the myotome, as noted above, not all PITX2-expressing cells were positive for Pax3, and limb expression of Pitx2 preceeded Myf5 (L’Honoré et al., 2010). Therefore, additional studies using conditional Pitx2 inactivation in specific myogenic cell populations would help to elucidate the function of Pitx2 in embryonic myogenesis.

Scientific evidence also relates Pitx2 to cell proliferation in myogenic cells and somite derivates. Notably, Pitx2 has been reported to be a target gene in the Wnt/Dvl2-beta-catenin pathway and operates in specific cell types to control proliferation by regulating expression of the growth-control genes Ccn1, Ccn2, and c-Myc (Kioussi et al., 2002; Baek et al., 2003). These authors established that the PITX2 N-terminal domain is required for its effects on proliferation in a myoblast cell line. We have previously demonstrated that Pitx2c is the main Pitx2 isoform expressed in Sol8 myoblasts and that overexpression of Pitx2c in Sol8 cells led to an increase in proliferative capacity and completely blocked terminal differentiation, mainly because high levels of Pax3 expression were maintained (Martínez-Fernández et al., 2006). Additional data in vivo have supported the role of Pitx2 in cell proliferation during myogenesis. In this sense, Abu-Elmagd et al. (2010) showed that Pitx2 loss of function in chicken embryos decreased the number of differentiated myocytes/myofibers in the somites, whereas Pitx2 overexpression increased myocyte/myofiber numbers, particularly in the epaxial region of the myotome. In agreement with Abu-Elmagd et al. and by using Pitx2 c−/− mutant embryos, we have reported that Pitx2c plays a pivotal role in the control of the subtle equilibrium between proliferation and differentiation during trunk and limb myogenesis. This control is exercised by balancing Pax3+/Pax7+ myogenic population in vivo as well as regulating key myogenic transcription factors such as Pax3 through the repression of miR-27 (Lozano-Velasco et al., 2011; Figures 3A1, A2). This new function of Pitx2c mediated by miRNAs introduces a new level of complexity in the intricate regulatory network that governs myogenesis in the embryo.

Pitx2 during Head-Muscle Development

As mentioned above, Pax3 controls the myogenic specification of muscle embryonic progenitors in trunk and limbs (Tajbaksh et al., 1997). However, it has been proposed that, instead of Pax3, Pitx2 plays a major role as an upstream regulator of craniofacial myogenesis (Zacharias et al., 2011; Buckingham and Rigby, 2014). This is supported by the fact that EOM development is impaired in Pitx2 null mice (Gage et al., 1999a; Kitamura et al., 1999). However, in the early studies it was not evident whether this muscle dysgenesis in Pitx2 mutant mice resulted from an intrinsic defect in the developing myoblasts or was secondary to the loss of Pitx2 expression in the pericranial mesenchyme. Other authors have subsequently suggested that this phenotype could be due to the Pitx2 effect on proliferation rate of myogenic precursors (Noden and Francis-West, 2006), in agreement with previously reported data (Kioussi et al., 2002; Martinez-Fernandez et al., 2006). The hypothesis that Pitx2 plays a part in controlling cell proliferation in myogenic cells in this context is also supported by the fact that conditional inactivation of Pitx2 in neural-crest-derived cells does not affect the early differentiation of eye muscles (Evans and Gage, 2005), while conditional Pitx2 deletion in the mesoderm induces a down-regulation of Myf5, Myf6, Myod1, and Myog expression and, therefore, blocks the onset of myogenesis of EOM (Zacharias...
During embryonic stages, Pitx2 contribution is different depending on the initial muscle-cell clusters (myotome myogenesis (A1), limb myogenesis (A2), EOM myogenesis (A3), or branchial myogenesis (A4)). First myocytes of the myotome differentiate through Myf5 and/or Myf6 directly to Myog without turning on MyoD. This is represented by dashed arrows. Dotted arrows represent direct molecular relationships that still remain elusive.

Proposed model for Pitx2 in adult myogenesis promoting activation and commitment of SCs.

In this regard, in 2009, Sambasivan et al. by analyzing double defective mutant mouse embryos Myf5(Myf6) (Myf5\textsuperscript{nlacZ/+}, Myf5\textsuperscript{nlacZ/nlacZ}) and Myf4\textsuperscript{−/−} mutants, showed that Pitx2 cannot ensure survival and activation of Myod expression in EOM in the absence of both Myf5 and Myf6 (Sambasivan et al., 2009). Shortly afterwards, Zacharias et al. were able to inactivate the expression of Pitx2 in mesodermal EOM precursors by using a tamoxifen inducible UBC- CreER\textsuperscript{T2} promoter (Zacharias et al., 2011). This inactivation clearly showed that Pitx2 is required for EOM precursor specification and survival, acting as an anti-apoptotic factor in the pre-myogenic mesoderm and subsequently activating the myogenic program in these cells through direct binding to Myf5 and Myod promoters (Zacharias et al., 2011). Taken together, all these data clearly suggest that Pitx2 is an upstream regulator of Myf5, Myf6, and Myod in EOM embryonic myogenesis (Figure 3A3).

Pitx2 is also expressed in the myogenic precursors of the FBA. Tbx1 expression on FBA premyoblast is required for specification leading to Myf5 and Myod1 activation in those cells (Kelly et al., 2004). Notably, systemic Pitx2 mutants, whether Pitx2\textsuperscript{−/−} (Dong et al., 2006) or Pitx2\textsuperscript{LacZ/LacZ} (Shih et al., 2007a), display a down regulation of Tbx1 expression in this structure, although Pitx2 expression is unaffected in Tbx1 null mutants (Dong et al., 2006). These data, together with the fact that Pitx2 directly interacts with Tbx1 regulatory elements (Shih et al., 2007a) suggest that Pitx2 is an upstream activator of Tbx1 in FBA. A fuller analysis of both systemic Pitx2 mutants reveals that the inactivation of Pitx2 in FBA results in increased cell death in the mesodermal core and loss of early premyoblast specification markers such as Six2.
Since Pitx2 and Myogenesis

Although, the role that Pitx2 in SCs isolated from EOM slowed their proliferation rate, and a similar trend was seen for SCs isolated from tibias anterioris muscle. These data led to the conclusion that Pitx2 helps maintain a proliferating pool of myogenic precursor cells. Finally, the authors highlight that this greater proliferative capacity may facilitate the repair of damaged EOM tissue, thereby contributing to the sparing of EOM in muscular dystrophies (Herbet et al., 2013).

More recently, a study conducted in our laboratory has provided additional information about the molecular mechanisms by which the Pitx2 transcription factor regulates cell proliferation in SCs (Lozano-Velasco et al., 2015). We have reported that Pitx2 expression is higher in early-activated SCs than in long-term activated ones, and our in vitro Pitx2 gain-of-function experiments have revealed that Pitx2 stimulates Ccnd1 and Ccnd2 expression, accelerating cell proliferation during early satellite-cell activation. Moreover, we have demonstrated that such Pitx2 effect on SCs proliferation is due to Pitx2-mediated downregulation of the miRNAs miR-15b, miR-106b, miR-23b, and miR-503 (Figure 3B). The existence of the Pitx2-miRNA pathway controlling the expression of key regulatory cell-cycle genes in early-activated SCs revealed a role of Pitx2 in satellite-cell activation. Although, muscle SCs are promising targets for cell therapies, the paucity of SCs that can be isolated or expanded from adult muscle tissue is limiting; thus these findings provide new molecular tools to overcome such a bottleneck. It bears noting that our analyses also showed that Pitx2c can increase Myf5 expression by down-regulating miR-106b (Figure 3B), thus expanding the Myf5+ satellite-cell population and revealing a role for Pitx2c in promoting satellite-cell populations more primed for myogenic commitment (Lozano-Velasco et al., 2015). In this context it should be highlighted that in several muscular disorders such as muscular dystrophies, the progressive muscle wasting and weakness is often associated with exhaustion of muscle-regeneration potential. Therefore, the progressive loss of muscle mass has been attributed, at least partly, to the inability of muscle stem cells to efficiently regenerate tissue loss as the result of the disease (Berardi et al., 2014). Thus, critical for the development of effective strategies to treat muscle disorders is the optimization of approaches targeting muscle stem cells and capable of regenerating tissue loss as the result of the disease or as the result of normal muscle turnover (Bertoni, 2014). Notably, very recent reports have been pointed out that muscle stem cells should be considered as a therapeutic target for restoring muscle function in individuals with DMD (Chal et al., 2015; Dumont et al., 2015). Therefore, identification of new Pitx2 functions in the context of SC biology may significantly contribute to the clarification of the molecular and cellular mechanisms of skeletal-muscle regeneration and may help to develop therapeutic strategies for muscular disorders.

Notably, the analysis of adult single and double Pitx2:Pitx3 conditional mutant mouse lines targeted to the muscle stem-cell compartment revealed that double mutant SCs undergo senescence with impaired regeneration after injury, suggesting...
that Pitx2-mediated changes in ROS levels are required for differentiation of SCs (L'honoré et al., 2014b).

All these data provide new insight into the function of Pitx2 in the molecular mechanisms that control SC behavior and might thus have future application to enhance the regenerative capacity of these myogenic precursor cells. Further analysis using in vivo models could aid in understanding how the Pitx2-mediated effects on SCs can influence the kinetics of muscle regeneration.

CONCLUSIONS AND FUTURE CHALLENGES

The data reviewed above show that Pitx2 is a comprehensive marker for cells undergoing myogenic progression, more so than any of the MRFs. This supports models that include a Pitx2-dependent pathway in virtually all skeletal muscles. Many pieces of experimental evidence have pointed out that Pitx2 is the first molecular signal specifying all myogenic precursors in the head muscles. However, although several works have characterized Pitx2 as a key transcription factor in the molecular cascade regulating trunk- and limb-muscle progenitors, additional work is needed to elucidate the function of Pitx2 in specification vs. determination during trunk and limb myogenesis. In addition, since seminal works have revealed that Pitx2 functions on myogenic cells may be due to Pitx2-mediated regulation of miRNAs, the role of Pitx2 in the post-transcriptional control of myogenesis should be further explored.

In parallel, the role of Pitx2 during adult myogenesis is beginning to be explored. Skeletal muscle has the ability to repair and regenerate due to the presence of resident SCs. SC function in adults requires redeployment of many of the regulatory networks fundamental to developmental myogenesis. Currently, SCs are considered potential therapeutic targets for restoring muscle function in muscle degenerative disorders such as muscular dystrophies. Recent works indicate that Pitx2 is expressed in proliferating SCs and can promote differentiation of satellite-cell-derived myoblasts. Moreover, the identification of Pitx2-miRNA pathways that regulate satellite-cell behavior as well as the impact of Pitx2 on redox condition during satellite-cell differentiation may open insights toward future applications to modulate satellite-cell fate during muscle regeneration. Therefore, these findings propose Pitx2 as a new player on skeletal-muscle satellite-cell biology and may help to develop therapeutic strategies for muscular disorder.

AUTHOR CONTRIBUTIONS

FH, LR, and AA conceived of the structure and content. FH wrote the first draft document. FH and LR designed and produced the figures. LR and DF critically revised the manuscript for intellectual content. AA corrected, edited, and approved the final version of the document to be published.

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