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Genetic manipulations reveal dynamic cell and gene functions

Cre-ating a new view of myogenesis

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Development of multicellular organisms is temporally and spatially complex. The Cre/*loxP* and Flp/*FRT* systems for genetic manipulation in mammals now enable researchers to explicitly examine in vivo the temporal and spatial role of cells and genes during development via cell lineage and ablation studies and conditional gene inactivation and activation. Recently we have used these methods to genetically dissect the role of Pax3$^+$ and Pax7$^+$ progenitor populations and the function of β-catenin, an important regulator of myogenesis, in vertebrate limb myogenesis. Our lineage and ablation studies of Pax3$^+$ and Pax7$^+$ progenitors revealed surprising insights into myogenesis not apparent from Pax3 and Pax7 expression and functional studies. In addition, conditional inactivation and activation of β-catenin in different progenitor populations and their progeny demonstrated that β-catenin plays several cell-autonomous roles in myogenesis. Our studies highlight the hierarchical (i.e., genes versus cells), temporal and spatial complexity of development and demonstrate that manipulations of both cells and genes will be required to obtain a full understanding of the development of multicellular organisms.

Key words: Pax3, Pax7, β-catenin, myogenesis, limb, cell lineage, Cre recombinase

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Development of a multicellular organism requires the integration of multiple events occurring at specific times and locations during embryogenesis. Advances in methods for genetic manipulation in mammals now enable researchers to explicitly examine in vivo the temporal and spatial role of genes and cells during development. Recently we have used cell lineage and ablation studies as well as conditional gene inactivation and activation in the mouse to genetically dissect the role of different progenitor populations and the function of β-catenin, an important regulator of myogenesis, in vertebrate limb muscle development.[1] Our studies reveal the hierarchical (i.e., genes versus cells), temporal, and spatial complexity of development and demonstrate that in vivo manipulations of both cells (cell lineage and ablation) and genes (conditional inactivation/activation) will be required to fully understand of the development of multicellular organisms. In this short perspective, we will briefly review the genetic methods now available in mice to manipulate cells and genes and also present our recent findings, using these genetic methods, on the complexity of vertebrate myogenesis.

The ability to manipulate cells and genes in vivo has been made possible by the use of site-specific recombinases derived from bacteriophages and yeast.[2-4] The site-specific recombinases Cre and Flp bind to and recombine specific sequences of DNA termed *loxP* or *FRT* sites, respectively. These have been used as tools to (1) heritably label cells based on their gene expression; (2) ablate cells based on their gene expression; and (3) conditionally inactivate or activate genes. Cell lineage and ablation and conditional gene inactivation or activation all require a Cre or Flp mouse line in which Cre or Flp is expressed under the control of gene-specific promoters. Thus, the temporal-spatial pattern of Cre or Flp expression is determined by the gene into which Cre or Flp has been targeted. Further temporal control of Cre and Flp has been enabled by the development

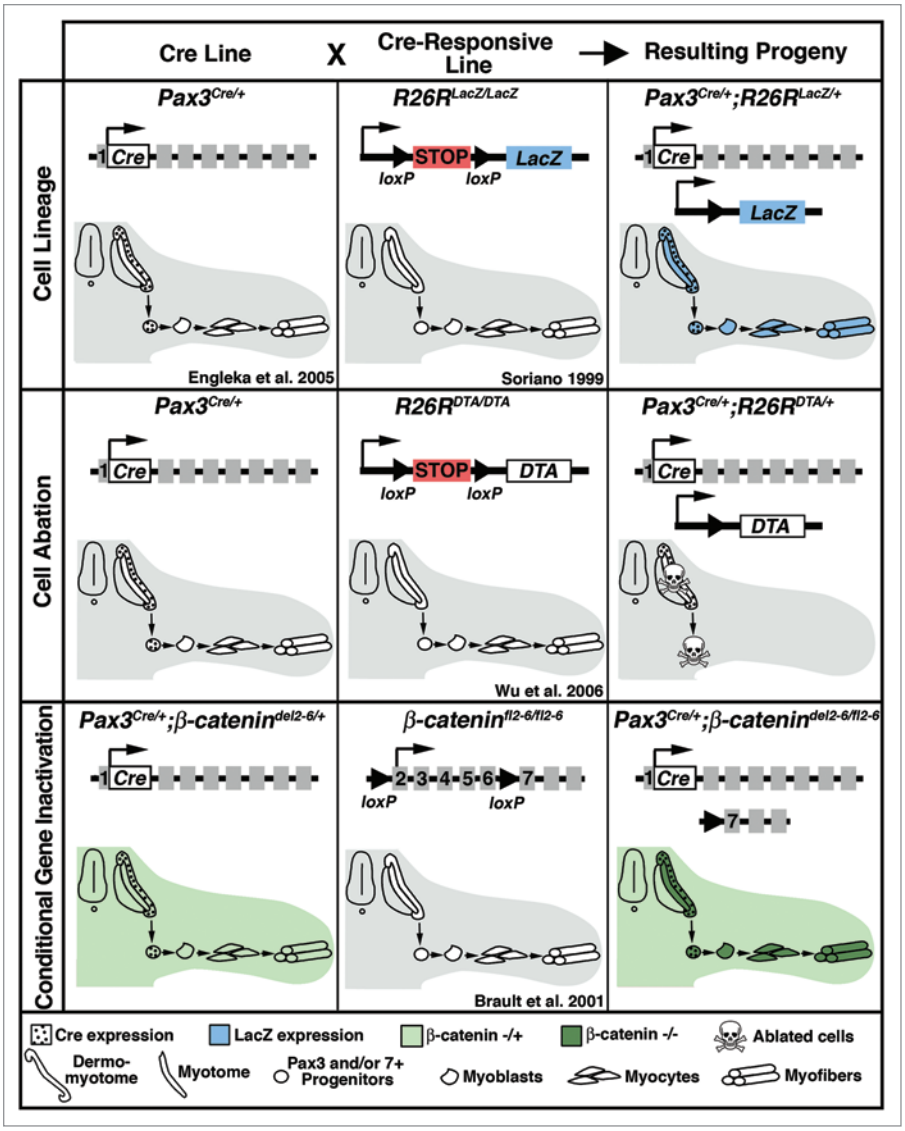

Figure 1. Scheme for using Cre and Cre-responsive mouse lines for cell lineage and ablation studies and conditional gene inactivation. As an example of cell lineage (top row), we show an experiment to genetically label and follow the fate of Pax3^{+} somitic cells migrating into the limb via crossing *Pax3*$^{Cre/+}$ mice with *R26R*$^{LacZ/LacZ}$ Cre-responsive reporter mice. To ablate Pax3^{+} somitic cells and their progeny in the limb (middle row), *Pax3*$^{Cre/+}$ mice are crossed with *R26R*$^{DTA/DTA}$ Cre-responsive ablator mice. To conditionally inactivate the β-catenin gene in Pax3^{+} somitic cell and their progeny in the limb (bottom row), *Pax3*$^{Cre/+}$;*β-catenin*$^{del2-6/+}$ mice (in which one allele of β-catenin is inactivated via deletion of exons 2–6 throughout the embryo) are crossed with mice with two alleles of β-catenin in which exons 2–6 are flanked by *loxP* sites.

of ligand-regulated forms of Cre and Flp, such as tamoxifen-inducible CreER^{T2} and tetracycline-responsive Cre lines.[5-7] Both systems allow the timing of Cre/Flp expression to be tightly regulated.

The Cre/*loxP* and Flp/*FRT* systems can be exploited to genetically label and ablate cells and conditionally manipulate gene expression by crossing Cre or Flp mice to mice harboring Cre-responsive genes (**Fig. 1**). To genetically label cells, Cre or Flp mice are crossed to Cre or Flp-responsive reporters. In these reporter mice, reporters such as β-galactosidase or GFP are placed under the control of a ubiquitous promoter.[8,9] In the absence of Cre or Flp these reporters are not expressed because of the presence of a strong transcriptional stop cassette flanked by *loxP* or *FRT* sites. The presence of Cre or Flp causes recombination of the *loxP* or *FRT* sites and the permanent expression of the reporter. Therefore in mice containing both Cre or Flp and the reporter, cells expressing Cre or Flp and their progeny permanently express the reporter. Such genetic labeling allows the researcher to trace the lineage and fate of cells initially genetically marked by expression of a particular gene, even after the descendent cells no longer express this gene. These genetic lineage experiments are particularly useful for tracing the fate of progenitors that change gene expression profiles, migrate long distances, and/or differentiate into morphologically different cells. The Cre/*loxP* system also has been used to genetically ablate cell lineages. To ablate cells, ubiquitous cell ablator lines have been established in which Cre mediates the expression of cell-death-inducing molecules, such as diphtheria toxin.[10,11] In mice containing both Cre and the ablator, all Cre-expressing cells are killed. Ablation of specific cells and their descendents is critical for testing whether a cell lineage is required for particular aspects of development. Finally, by flanking essential exons with either *loxP* or *FRT* sites particular genes can be conditionally inactivated (or activated, depending on the structure of the genes and exons targeted). In mice containing Cre or Flp and a gene with exons flanked by loxP or FRT sites, the gene will be permanently inactivated/activated in cells expressing Cre or Flp and their progeny. Conditional inactivation of genes is often necessary to circumvent early lethality arising from early loss of function, but also to dissect more subtle temporal or context-dependent changes in gene function within a cell lineage. We have used all three of these techniques to genetically dissect skeletal myogenesis.

Skeletal myogenesis has been intensively studied at the molecular and cellular levels. In development, skeletal muscle arises from somitic progenitor cells, which express the transcription factors Pax3 and/or Pax7.[12,13] These Pax3^{+} and/or Pax7^{+} progenitors either remain in the somitic region to differentiate into axial muscle or migrate into the limb to differentiate into limb muscles. During both axial and limb myogenesis, the progenitors become specified as myoblasts, then differentiate into post-mitotic myocytes, and finally fuse into multinucleate myofibers. In the limb, the earliest embryonic myoblasts give rise

to the initial embryonic musculature and set up the basic pattern of limb muscles.[14,15] Later fetal myoblasts give rise to fetal muscle, which is critical for muscle growth and maturation. Finally, adult progenitors (satellite cells) give rise to adult muscle and are responsible for muscle regeneration. Of intense interest is whether embryonic, fetal, and adult myoblasts derive from common or different progenitor populations and what signaling pathways regulate the proliferation and differentiation of these progenitors. To elucidate the developmental origin of embryonic and fetal muscle, we genetically labeled and ablated Pax3$^+$ and Pax7$^+$ progenitors in vivo to determine whether these progenitors give rise to and/or are required for embryonic and fetal limb muscle. We also tested via conditional inactivation and activation whether β-catenin is required cell-autonomously in these progenitors for embryonic and/or fetal myogenesis.

Our lineage and ablation studies of Pax3$^+$ and Pax7$^+$ progenitors revealed surprising insights into myogenesis not apparent from Pax3 and Pax7 expression and functional studies. Previous research had shown that Pax3$^+$ and Pax7$^+$ somitic cells are progenitors critical for myogenesis.[13,16,17] However, both Pax3 and Pax7 are only transiently expressed in progenitors and downregulated during myogenic differentiation. Therefore a full assessment of the contribution of Pax3$^+$ and Pax7$^+$ progenitors to muscle requires a lineage analysis in which Pax3$^+$ and Pax7$^+$ cells and their progeny are genetically labeled. To do this lineage analysis, we used *Pax3$^{Cre/+}$*,[18] or *Pax7$^{iCre/+}$* mice,[19] in which Cre has been targeted into either the Pax3 or Pax7 genes, crossed with Cre-responsive reporter *R26R$^{LacZ/LacZ}$* mice.[8] We determined that Pax3$^+$ progenitors give rise to embryonic muscle and Pax7$^+$ progenitors, while Pax7$^+$ progenitors give rise to fetal muscle. To test whether Pax3$^+$ and Pax7$^+$ progenitors were required for myogenesis we then crossed *Pax3$^{Cre/+}$* or *Pax7$^{iCre/+}$* mice with Cre-responsive ablator *R26R$^{DTA/DTA}$* mice.[11] In the resulting *Pax3$^{Cre/+}$;R26R$^{DTA/+}$* and *Pax7$^{iCre/+}$;R26R$^{DTA/+}$* mice Cre-mediated recombination activated expression of the Diphtheria toxin subunit A, killing all Pax3$^+$ and Pax7$^+$ cells within 24 hours, but leaving neighboring cells unharmed. In *Pax3$^{Cre/+}$;R26R$^{DTA/+}$* mice there was a complete ablation of both axial and limb embryonic myogenesis (fetal myogenesis could not be evaluated because of the early death of the embryos), demonstrating that Pax3$^+$ progenitors are essential for axial and limb embryonic myogenesis. This contrasts with the finding from *Pax3$^{-/-}$* mice in which all limb muscle is lost, but axial muscle is unaffected.[13] Therefore while Pax3 protein is required for multiple aspects of limb myogenesis but not axial myogenesis, Pax3$^+$ progenitor cells are critical for both axial and limb muscle development. In *Pax7$^{iCre/+}$;R26R$^{DTA/+}$* mice embryonic myogenesis was unaffected, but fetal myogenesis was severely compromised, indicating that Pax7$^+$ progenitors are critical for fetal muscle. Again this contrasts with results from *Pax7$^{-/-}$* mice which show that Pax7 function is only required for survival and proliferation of adult muscle progenitors.[13,20-22] Recent conditional inactivation of Pax7 has further demonstrated that Pax7 function is only required until early adulthood (P21 in mouse) for maintenance of adult progenitors.[23] Thus our findings at one biological level of organization, i.e., function of cells expressing Pax3 or Pax7, are not equivalent to findings at another biological level, i.e., function of the genes Pax3 or Pax7.

These lineage and ablation studies highlight the complexity of myogenic progenitors. Our lineage studies determined for the first time that embryonic and fetal limb muscle are derived from distinct, although related progenitors. Our ablation studies determined that Pax3$^+$ progenitors are required for both axial and limb myogenesis while Pax7$^+$ progenitors are critical for fetal (and presumably adult) limb myogenesis. Furthermore, the myogenic capacity of Pax3$^+$ and Pax7$^+$ progenitors is different. Despite the highly proliferative nature of myogenic progenitors, Pax3$^+$ progenitors were unable to compensate for the loss of Pax7-derived myogenic cells when Pax7$^+$ progenitors were ablated. Thus Pax3-derived embryonic myogenic progenitors and myoblasts are unable to compensate for the Pax7$^+$ population and participate in fetal myogenesis. The limitations of Pax3 embryonic progenitors suggest that this cell type may be of limited therapeutic use in repair of diseased adult muscle.

Using conditional mutagenesis, we have determined that β-catenin plays several cell-autonomous roles in skeletal myogenesis. Multiple earlier studies had demonstrated that Wnt/β-catenin signaling is important for axial and limb myogenesis.[1] However, whether β-catenin signaling is required cell-autonomously in vivo for embryonic and fetal myogenesis was still unanswered. Using multiple myogenic Cre lines crossed with conditional alleles β-*catenin$^{fl2\text{-}6/fl2\text{-}6}$* to inactivate[24] or β-*catenin$^{fl3/fl3}$* to constitutively activate β-catenin,[25] we tested the cell-autonomous role of β-catenin in myogenesis. We used *Pax3^{Cre}* to manipulate β-catenin in somitic cells at the time of somite formation, *MCre^{Tg}* (Cre is expressed under the control of a specific Pax3 enhancer element) to manipulate expression in somitic cells as they delaminate from the dermomyotome,[26] and *Pax7^{iCre}* to manipulate progenitors in the limb that give rise to fetal muscle. Inactivation of β-catenin in Pax3-derived somitic cells completely abolished the somitic compartments of the dermomyotome and myotome and subsequently all axial and limb myogenesis. However, inactivation of β-catenin via *MCre^{Tg}* in delaminating somitic cells and their progeny did not affect limb embryonic myogenesis. Later inactivation of β-catenin via *Pax7^{iCre}* resulted in severe defects in limb fetal myogenesis, with a change in the number of fetal progenitors and fiber type of differentiated muscle. Thus β-catenin is required within the somite for dermomyotome and myotome formation and delamination of limb myogenic progenitors. Subsequently, β-catenin is not required cell autonomously for embryonic myogenesis but later is required for fetal myogenesis in the limb. Only through systematic inactivation and activation were we able to identify these temporally and spatially distinct roles for β-catenin in myogenesis.

β-catenin plays many roles throughout development, and the complexity in β-catenin function we have found in myogenesis also exists in other systems. For instance, in limb development mesodermal β-catenin has multiple functions; β-catenin regulates limb outgrowth, dorsal-ventral patterning, and scapula

development.[27] In the spinal cord, canonical Wnt/β-catenin signaling is critical for proliferation throughout the spinal cord and for dorsal patterning.[28] Interestingly, these functions of β-catenin in the spinal cord are temporally separate, as proliferation requires Wnt activity at an earlier stage than dorsal patterning. In addition, the function of Wnt signaling in regulating proliferation is mediated by the downstream transcriptional effector Tcf3, while the Wnt function in dorsal patterning is mediated by Tcf7. Finally, Wnt/β-catenin signaling has been found to have several temporally distinct cell-autonomous and non-cell-autonomous roles in skeletal development.[29-31] Early in development β-catenin negatively regulates the differentiation of mesenchymal cells into a common skeletal precursor. Later β-catenin promotes osteogenesis at the expense of chondrogenesis. In more mature osteoblasts, β-catenin expression regulates bone growth via non-cell autonomous regulation of neighboring osteoclasts, which resorb bone matrix. Therefore complex temporal and spatial regulation of Wnt/β-catenin signaling (and other signaling pathways) may be a common feature of development. In the case of myogenesis in which three distinct, but related progenitor populations are required to generate embryonic, fetal, and adult muscle changing responsiveness to and requirements for β-catenin may be critical. We propose that differential sensitivity to Wnt signaling may be a mechanism to allow embryonic muscle progenitors to differentiate during development but still maintain a fetal progenitor population for later myogenesis.

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