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Connective tissue fibroblasts and Tcf4 regulate myogenesis

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SUMMARY

Muscle and its connective tissue are intimately linked in the embryo and in the adult, suggesting that interactions between these tissues are crucial for their development. However, the study of muscle connective tissue has been hindered by the lack of molecular markers and genetic reagents to label connective tissue fibroblasts. Here, we show that the transcription factor Tcf4 (transcription factor 7-like 2; Tcf7l2) is strongly expressed in connective tissue fibroblasts and that *Tcf4*^{GFPCre} mice allow genetic manipulation of these fibroblasts. Using this new reagent, we find that connective tissue fibroblasts critically regulate two aspects of myogenesis: muscle fiber type development and maturation. Fibroblasts promote (via Tcf4-dependent signals) slow myogenesis by stimulating the expression of slow myosin heavy chain. Also, fibroblasts promote the switch from fetal to adult muscle by repressing (via Tcf4-dependent signals) the expression of developmental embryonic myosin and promoting (via a Tcf4-independent mechanism) the formation of large multinucleate myofibers. In addition, our analysis of Tcf4 function unexpectedly reveals a novel mechanism of intrinsic regulation of muscle fiber type development. Unlike other intrinsic regulators of fiber type, low levels of Tcf4 in myogenic cells promote both slow and fast myogenesis, thereby promoting overall maturation of muscle fiber type. Thus, we have identified novel extrinsic and intrinsic mechanisms regulating myogenesis. Most significantly, our data demonstrate for the first time that connective tissue is important not only for adult muscle structure and function, but is a vital component of the niche within which muscle progenitors reside and is a critical regulator of myogenesis.

KEY WORDS: Connective tissue, Myogenesis, Tcf4, Tcf7l2, Myosin, Mouse

INTRODUCTION

Development of the vertebrate musculoskeletal system requires the coordinated development and morphogenesis of muscle, muscle connective tissue, tendon and bone. Both in the adult and during development, muscle and its connective tissue are intimately linked. Adult muscle connective tissue consists of a small number of fibroblasts that produce, and are embedded in, a protein- and carbohydrate-rich extracellular matrix (ECM) (Sanes, 2004). The connective tissue ensheaths individual multinucleate myofibers, bundles myofibers into fascicles and groups fascicles into whole anatomical muscles (Sanes, 2004), and is essential for muscle structure and function (Borg and Caulfield, 1980). In the embryo, the developing connective tissue is composed of a large number of fibroblasts surrounded by relatively little ECM. The morphogenesis of muscle and its connective tissue is tightly coordinated (Kardon et al., 2003) and suggests that interactions between these tissues might be crucial for their development.

During development, muscle connective tissue of the limb develops from the lateral plate mesoderm of the limb bud (Chevallier et al., 1977; Christ et al., 1977). The connective tissue forms a muscle-like pattern in the lateral plate mesoderm, independent of the muscle (Grim and Wachtler, 1991; Jacob and Christ, 1980; Kardon et al., 2003; Lanser and Fallon, 1987). Subsequently, connective tissue and muscle development is closely associated both temporally and spatially (Kardon et al., 2003). However, a detailed study of the development of connective tissue has been hindered by the lack of molecular markers and genetic reagents to label and manipulate the connective tissue fibroblasts.

Limb muscle originates from somitic progenitors that migrate into the limb and give rise to muscle by a complex process (Emerson and Hauschka, 2004). Myogenesis occurs in successive phases (see Table S1 in the supplementary material) (Biressi et al., 2007; Stockdale, 1992). Embryonic myogenesis establishes the basic muscle pattern [embryonic day (E)10.5-12.5 in mouse] (Biressi et al., 2007); fetal and neonatal myogenesis are crucial for muscle growth and maturation [E14.5-postnatal day (P)0 and P0-P21]; and adult myogenesis is necessary for postnatal growth and repair of damaged muscle. Each one of these phases involves specification of myoblasts from progenitors, differentiation of committed myocytes and fusion of myocytes into multinucleate myofibers. During embryonic myogenesis, embryonic myoblasts differentiate into primary fibers, whereas during fetal myogenesis, fetal myoblasts both fuse to primary fibers and fuse to one another to make secondary myofibers. During neonatal myogenesis, myofiber growth occurs by a rapid increase in myonuclear number, whereas in the adult, myofiber growth is not accompanied by changes in myonuclear number (White et al., 2010).

Embryonic, fetal/neonatal and adult progenitors, myoblasts and myofibers are distinctive. Two related, but distinct, progenitor populations give rise to embryonic versus fetal and adult myoblasts (Hutcheson et al., 2009). Embryonic, fetal and adult myoblasts (satellite cells) differ in vitro in their appearance, media requirements, response to extrinsic signaling molecules and sensitivity to drugs (Biressi et al., 2007; Stockdale, 1992). Furthermore, these myoblast classes are specified by different combinations of transcription factors (Kassar-Duchossoy et al., 2004; Kassar-Duchossoy et al., 2005; Relaix et al., 2005), express different genes (Biressi et al., 2007) and have different cell-

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autonomous requirements for β-catenin and BMP (bone morphogenetic protein) signaling (Hutcheson et al., 2009; Wang et al., 2010). Finally, primary, secondary and adult myofibers differ in their morphology and in the muscle contractile proteins, including the myosin heavy chain (MyHC) isoforms, that they express (Gunning and Hardeman, 1991; Schiaffino and Reggiani, 1996; Wigmore and Evans, 2002). The intrinsic and/or extrinsic signals that regulate the switch from embryonic to fetal, neonatal and adult myogenesis are largely unknown.

An important component of muscle development is the diversification of myofibers into different fiber types (Gunning and Hardeman, 1991; Rubinstein and Kelly, 2004; Schiaffino and Reggiani, 1996). Adult skeletal muscles are composed of two classes of myofibers that differ in their contractile and metabolic properties: slow oxidative fibers that are fatigue-resistant, and fast glycolytic fibers that contract quickly but fatigue rapidly. Based on MyHC isoform expression, myofibers are classified as slow MyHCI (Myh7 – Mouse Genome Informatics) or fast MyHCIIa (Myh2 – Mouse Genome Informatics), MyHCIId/x (Myh1 – Mouse Genome Informatics) and MyHCIIb (Myh4 – Mouse Genome Informatics) myofibers, with MyHCIIa exhibiting oxidative metabolism and MyHCIId/x and MyHCIIb exhibiting glycolytic metabolism. Fiber type diversification occurs gradually (see Table S1 in the supplementary material) (Agbulut et al., 2003; Lu et al., 1999). In mouse, all primary myofibers express MyHCI and the developmental (fast) isoform MyHCembryonic (MyHCemb; Myh3 – Mouse Genome Informatics). Secondary myofibers begin to diversify; all myofibers express the MyHCemb and either the developmental (fast) isoform MyHCperinatal (MyHCperi; Myh8 – Mouse Genome Informatics) or MyHCI. Later in fetal myogenesis, MyHCIIb and then MyHCIIa and MyHCIIx begin to be expressed (Lu et al., 1999). During neonatal myogenesis, MyHCemb and MyHCperi are repressed until, by P21, they are no longer expressed. By 6 weeks, the fiber type of most myofibers is established; myofibers express either MyHCI, MyHCIIa, MyHCIIx or MyHCIIb (Agbulut et al., 2003; Lu et al., 1999). The mechanisms controlling specification of fiber type, particularly in mammals, are not well understood. Fiber type, at least in birds, is partially regulated by intrinsic factors present in somitic progenitors prior to their migration into the limb (Nikovits et al., 2001; Van Swearingen and Lance-Jones, 1995). However, fiber type is also regulated extrinsically by innervation and thyroid hormone (Gunning and Hardeman, 1991; Schiaffino and Reggiani, 1996). In addition, the lateral plate mesoderm appears to regulate fiber type (Butler et al., 1988; Duprez et al., 1999; Kardon et al., 2002; Nikovits et al., 2001; Robson et al., 1994; Van Swearingen and Lance-Jones, 1995). Potentially, the connective tissue component of the lateral plate mesoderm could be a source of extrinsic signals regulating fiber type.

In this study, we use genetic lineage tracing, cell ablation and conditional mutagenesis in vivo in mouse, as well as transwell fibroblast/myoblast cultures in vitro, to test the role of connective tissue fibroblasts and the transcription factor Tcf4 (Tcf7l2 – Mouse Genome Informatics) in the regulation of myogenesis. We show that Tcf4 is strongly expressed in muscle connective tissue fibroblasts during development and in the adult, and we have made $Tcf4^{GFPCre}$ mice that allow for the genetic manipulation of these fibroblasts. Using this new reagent, we find that the connective tissue fibroblasts regulate two aspects of myogenesis: muscle fiber type development and maturation. In addition, our analysis of Tcf4 function reveals a novel mechanism of intrinsic regulation of muscle fiber type development. Thus, we have identified novel extrinsic and intrinsic mechanisms regulating muscle fiber type development and maturation. Most significantly, our data demonstrate for the first time that connective tissue is important not only for adult muscle structure and function, but is a critical regulator of muscle development.

MATERIALS AND METHODS

Mice

$Tcf4^{GFPCre+neo}$ mice were generated (in collaboration with M. R. Capecchi, University of Utah, Salt Lake City, UT, USA) by replacing exon1 of *Tcf4* with a *GFPCre* cassette (Le et al., 1999) and GCSF polyA using standard gene targeting techniques (Nagy et al., 2002). $Tcf4^{GFPCre-neo}$ were derived by crossing $Tcf4^{GFPCre+neo}$ mice with *ACTBFlpe* mice (Rodriguez et al., 2000) to remove the neomycin selection cassette. $Tcf4^{fl}$ mice were generated by M.A.-H. and M. R. Capecchi by flanking exon1 by *loxP* sites, and $Tcf4^{del}$ mice were derived by crossing $Tcf4^{fl}$ mice with ubiquitous Cre-expressing $Hprt^{Cre}$ mice (Tang et al., 2002). Cre-driver $Pax3^{Cre}$ and $Pax7^{Cre}$; Cre-responsive reporter $R26R^{lacZ}$, $R26R^{YFP}$ and $R26R^{mTmG}$; and Cre-responsive ablater $R26R^{DTA}$ mice were previously reported (Engleka et al., 2005; Keller et al., 2004; Muzumdar et al., 2007; Soriano, 1999; Srinivas et al., 2001; Wu et al., 2006).

β-Galactosidase staining, immunofluorescence and microscopy

The whole-mount β-galactosidase (β-gal) staining protocol is available online at http://genepath.med.harvard.edu/~cepko/protocol/xgalplapstain.htm. For section immunofluorescence, OCT (optimal cutting temperature)-embedded (embryonic) or flash-frozen (neonatal and adult) tissues were sectioned, fixed for 5 minutes in 4% paraformaldehyde (PFA), washed in PBS and then, if needed, subjected to antigen retrieval, a method consisting of heating slides in citrate buffer (1.8 mM citric acid, 8.2 mM sodium citrate in water) in a 2100 PickCell Retriever. Cells were washed in F10 or F12 tissue culture media and fixed for 5 minutes in 4% PFA or cold methanol. Tissue sections and cells were blocked for 30-60 minutes in 5% goat serum in PBS, incubated overnight at 4°C in primary antibody, washed in PBS, incubated for 2 hours at room temperature in secondary antibody, washed in PBS and stained for 5 minutes with DAPI to label nuclei. Primary antibodies are listed in Table 1. Secondary antibodies used were: Alexa 488- or Alexa 594-conjugated goat anti-mouse IgG1 (Invitrogen) or Cy2- or Cy3-conjugated goat-anti mouse, anti-rabbit, anti-rat or anti-chick IgG (Jackson ImmunoResearch). Sections and cells were imaged on a Zeiss TCS SP5 or a Nikon AR1 confocal microscope. Each image is a composite of maximum projections derived from stacks of optical sections.

Cell counts and statistics

For counts of $MyHCI^+$ myofibers, total $laminin^+$ myofibers or $Tcf4^+$ cells on sectioned control and mutant limbs, three sections in the center of each muscle were identified. Counts of three sections were averaged for three individuals of each genotype and analyzed using a two-tailed *t*-test. For counts of $MyHCI^+$ and GFP^+ cells and nuclei in culture experiments, equivalent regions (50-650 cells) of two coverslips for each condition (myoblasts cultured alone, with $Tcf4^{+/+}$ fibroblasts or with $Tcf4^{del/del}$ fibroblasts) were counted, averaged and analyzed using a *t*-test. Fusion index was quantified as (total number of nuclei within myofibers with ⩾3 nuclei/total number of nuclei within all myofibers) ×100.

qPCR and western blots

For qPCR, total RNA was extracted using the TissueLyser II (Qiagen) and the RNeasy Lipid Tissue Mini Kit (Qiagen) and reverse transcribed with Superscript III (Invitrogen). qPCR was performed using an ABI Prism 7900HT instrument (Applied Biosciences) with SYBR Green chemistry and ROX internal reference. Each reaction was performed in triplicate and normalized by GAPDH levels. For each paired set of mutant and wild-type muscles, values were normalized to wild-type levels (set at one). Primer sequences are in Table S2 in the supplementary material. For all qPCR data, three replicates for each muscle were averaged for eight individuals of each genotype and analyzed using a *t*-test.

Table 1. Antibodies used for immunofluorescence or western blots in this study

Antibody	Type	Source	Product number	Working concentration (μg/ml)	Sodium citrate antigen retrieval for sections
Pax7	Mouse IgG1	Developmental Studies Hybridoma Bank (DSHB)	PAX7	2.4	No
MyoD	Mouse IgG1	Santa Cruz Biotechnology	Sc-32758 (5.8A)	4	No
MyHCemb	Mouse IgG1	DSHB	F1.652	3 (IF, western)	Yes
MyHCI	Mouse IgG1	Sigma	M8421 (NOQ7.5.4D)	1.5	Yes
MyHCperi + MyHCII	Mouse IgG1	Sigma	M4276 (MY-32)	10	Works with or without
Laminin	Rabbit polyclonal	Sigma	L-9393	2.5 (IF, western)	Works with or without
CD31	Rat monoclonal	BD Biosciences	557355	0.5	No
F4/80	Rat monoclonal	eBioscience	14-4801	2	No
α-SMA	Mouse IgG2a	Sigma	A 2547 (Clone 1A4)	5.2	No
Vimentin	Chick polyclonal	Aves Labs	VIM	3	No
GFP	Chick polyclonal	Aves Labs	GFP-1020	20	No
Tcf4	Mouse IgG2a	Millipore	05-511 (Clone 6H5-3)	10	Yes
Tcf4	Rabbit monoclonal	Cell Signaling	2569 (C48H11)	0.7 (IF), 0.07 (western)	Yes
NG2	Rabbit polyclonal	Millipore	AB5320	2	No
Cleaved caspase 3	Rabbit monoclonal	Cell Signaling	9664 (5A1E)	0.02	No
β-Actin	Mouse monoclonal	Cell Signaling	3700 (810D10)	1.25	Western only

IF, immunofluorescence.

For western blots, total protein lysates from whole brain or hind limb muscles of P0 animals were made by homogenizing tissue in 3× volume of lysis buffer using the TissuelyserII. Lysates were quantified using the Bradford method (Bradford, 1976) and equal amounts separated using SDS-PAGE. The separated proteins were transferred onto a PVDF membrane and detected using the Odyssey imaging system (LI-COR Biosciences). Primary antibodies are listed in Table 1 and secondary antibodies used were 1RDye 800 CW-conjugated goat anti-mouse IgG (LI-COR Biosciences) and Alexa 680-conjugated goat anti-rabbit IgG (Invitrogen).

Fluorescence-activated cell sorting (FACS) and cell culture

Myogenic cells were isolated from limb muscles of P0-7 *Pax7*$^{iCre/+}$;*R26*$^{RYFP/+}$ mice. Muscles were stripped of tendons, digested with 1000 U/ml Collagenase I (Worthington) for 20 minutes at 37°C, passed through 70-μm and 40-μm filters, spun at 2500 rpm (1258 ***g***) for 20 minutes, and sorted based on low side-scatter and high YFP on a FACSVantage (BD Biosciences). Myogenic cells were plated at a density of 17,500 cells/cm^2 on gelatin-coated coverslips (in 24-well plates) for 24 hours in myoblast proliferation media [20% fetal bovine serum (FBS) in F10 with 0.5 nM fibroblast growth factor 2 (FGF2)] and gradually changed to myoblast differentiation media [5% pre-tested horse serum in Dulbecco's modified eagle medium (DMEM)] by 48 hours after initial isolation. Connective tissue fibroblasts were isolated from limb muscles of P0-5 *Tcf4*$^{+/+}$, *Tc4*$^{GFPCre+neo/+}$;*R26R*$^{YFP/+}$ or *Tcf4*$^{del/del}$ mice. Muscles were processed similarly to myogenic cells (see above) and then plated at 25,000 cells/cm^2 on 6 cm plastic dishes for 2 hours at 37°C in fibroblast proliferation media (10% FBS in F12 Hams). Cells in the supernatant were discarded and adherent fibroblasts were detached with trypsin-EDTA, washed and plated in fibroblast proliferation media either on gelatin-coated coverslips at 25,000 cells/cm^2 in 24-well plates or at 80,000 cells/cm^2 in PET transwell cell culture inserts with 1-μm pores (BD Falcon). For transwell experiments with myoblasts, fibroblasts in inserts were gradually changed to myoblast differentiation media by 24 hours after initial isolation and allowed to condition media 24 hours before transfer of insert, cells and conditioned media to wells with myoblasts. Myoblasts alone or with fibroblasts were cultured for 24 hours in proliferation media, 24 hours in proliferation/differentiation media and 7 days in differentiation media. Four transwell experiments with two replicates of each condition were conducted comparing myoblasts cultured alone or with Tcf4$^+$ fibroblasts. One transwell experiment with three replicates of each condition was conducted comparing myoblasts cultured alone, with Tcf4$^+$ fibroblasts or with Tcf4$^-$ fibroblasts. For cultures of pure fibroblasts or total cells derived from muscle, at least two experiments culturing cells for 48-72 hours were conducted for each variable measured.

RESULTS

Tcf4 is highly expressed in connective tissue fibroblasts associated with skeletal muscles during development and in the adult

Our previous analysis of limb development in chick (Kardon et al., 2003) revealed that Tcf4 is expressed in lateral plate-derived limb mesodermal cells, closely associated with muscle and presumed to be precursors of muscle connective tissue fibroblasts. To determine whether Tcf4 is similarly expressed in the mouse during development and also expressed in adult connective tissue fibroblasts, we analyzed expression of Tcf4 by immunofluorescence in mouse. Similar to our data in chick, we found that during embryonic myogenesis in mouse (E12.5), Tcf4 was highly expressed in cells closely associated with all limb muscles, but was not expressed in the myogenic cells themselves (Fig. 1A-C). By the end of fetal myogenesis (P0), Tcf4$^+$ cells were interspersed between and surrounding the myofibers of all limb muscles (Fig. 1D-F). These Tcf4$^+$ cells were located within the connective tissue, which, by P0, is enriched in collagen and strongly stained by Sirius Red (Fig. 1D-G). In the adult, Tcf4$^+$ cells were present in the Sirius Red$^+$ connective tissue of all limb muscles (Fig. 1H-K). These data demonstrate that Tcf4$^+$ cells are closely associated with all limb muscles throughout development and present within the adult muscle connective tissue.

We characterized Tcf4$^+$ cells further by isolating and growing muscle connective tissue fibroblasts in culture. Connective tissue fibroblasts were isolated by plating on plastic culture dishes cells freshly dissociated from neonatal whole limb muscles. After two hours, myogenic cells, which do not readily adhere to plastic (Richler and Yaffe, 1970), were discarded and adherent cells were detached and plated onto coverslips. The adherent cells were highly

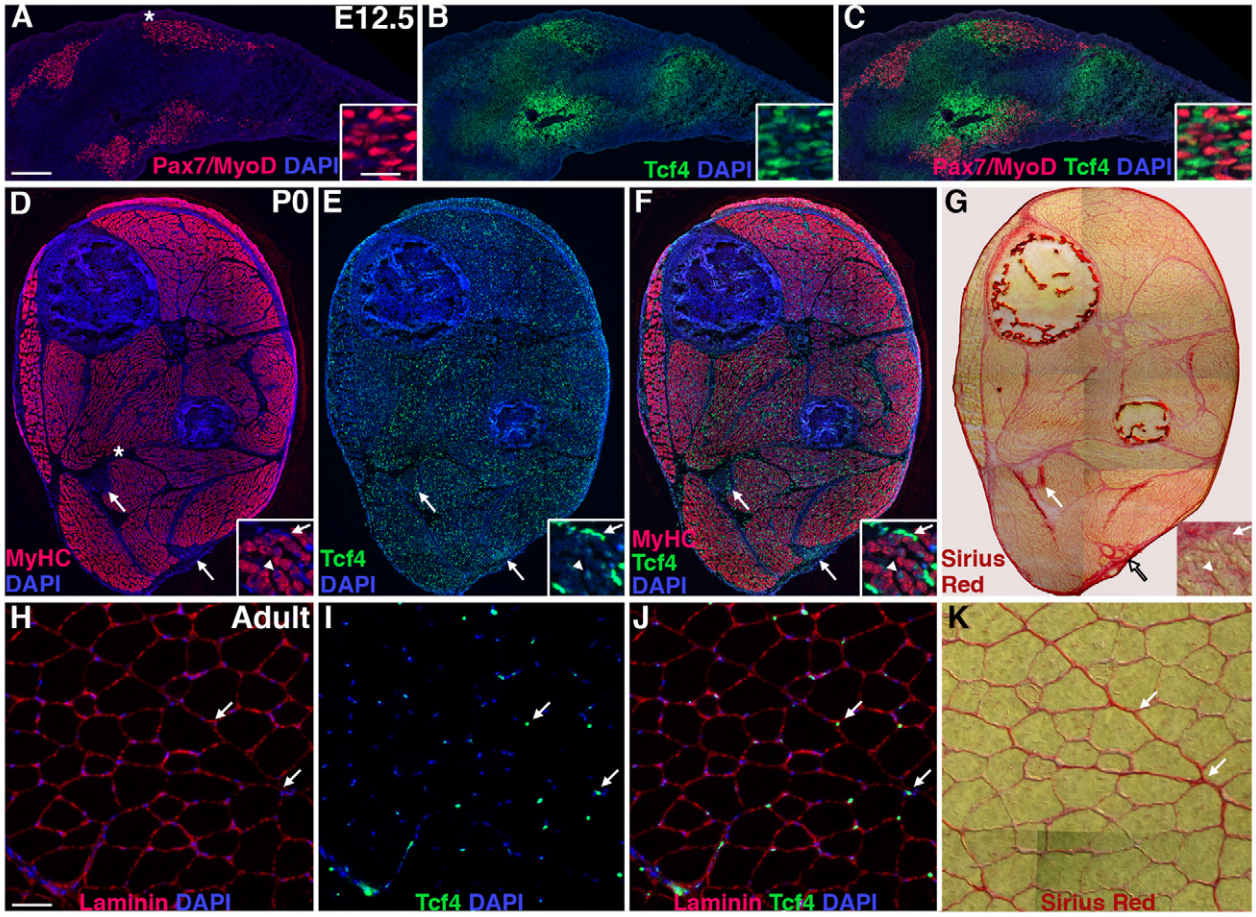

Fig. 1. Tcf4 is highly expressed in muscle connective tissue fibroblasts. In the embryonic (**A-C**), neonatal (**D-G**) and adult (**H-K**) limb, Tcf4$^+$ fibroblasts are associated with Pax7/MyoD$^+$ myoblasts (A-C), MyHC$^+$ myofibers (D-F) and laminin-ensheathed myofibers (H-J). By P0 and in the adult mouse, Tcf4$^+$ fibroblasts (arrows) lie within the Sirius Red$^+$ connective tissue (G,K) outside the MyHC$^+$ myofibers (D-F) and laminin$^+$ muscle basal lamina (H-J). In the neonate, some myonuclei (arrowheads) express low levels of Tcf4. Asterisks in A and D indicate enlarged inset regions in A-G. Scale bars: in A, 200 μm in A-G; in A inset and H, 50 μm in A-G insets and H-K.

enriched for fibroblasts and contained a variable percentage of contaminating myogenic cells (5-22% MyoD$^+$/total cells), and low numbers of endothelial cells (<1% CD31$^+$/total cells) and macrophages (<1% F4/80$^+$/total cells). Ninety-seven percent of the cells were stained by NG2, a marker of pericytes, but NG2 has also been shown to label multiple cell types besides pericytes (Stallcup, 2002). Most of the adherent cells had pseudopodia (Fig. 2B-D) and 87% expressed Tcf4 (Fig. 2A). In addition, all of the adherent cells expressed the intermediate filament vimentin (Fig. 2D), which is characteristic of muscle connective fibroblasts (Zou et al., 2008). Another marker of fibroblasts is α-smooth muscle actin (αSMA) (Tomasek et al., 2002); 94% of the adherent cells expressed αSMA and 97% of Tcf4$^+$ cells were also αSMA$^+$ (Fig. 2A-C). The antibody ER-TR7 (originally isolated as an antibody that recognizes thymus reticular fibroblasts) (Van Vliet et al., 1984) has also been reported to recognize muscle connective tissue fibroblasts (Brack et al., 2007). However, in our hands ER-TR7 did not label these fibroblasts in culture or in tissue sections.

To establish that Tcf4 does not label other cell types associated with muscle, we analyzed Tcf4 expression in cells isolated from whole neonatal limb muscles and in tissue sections. We found that neither endothelial cells (labeled with CD31) nor macrophages (labeled with F4/80) expressed Tcf4 in culture or in tissue sections (data not shown).

To determine whether Tcf4 is expressed in myogenic cells, we examined Tcf4 expression in myogenic cells isolated from neonatal limb muscles and in tissue sections. We found that in culture Tcf4 was expressed in MyoD$^+$ cells (although the small Tcf4$^+$ myoblast nuclei were readily distinguishable from the larger Tcf4$^+$ fibroblast nuclei; see Fig. S1A-C in the supplementary material). Similarly, the fibroblast markers αSMA and vimentin were also expressed in myoblasts in culture (although fibroblasts expressed higher levels than myoblasts of both proteins; see Fig. S1D-F in the supplementary material; data not shown). Therefore, at least in culture, all reported fibroblast-specific markers were also expressed in myoblasts. Based on our culture results, we re-examined limb tissue sections. Although we never found Tcf4 expressed in Pax7$^+$ progenitors or MyoD$^+$ myoblasts at E12.5 (Fig. 1A-C), we did find that Tcf4 was expressed at low, near-background levels in some myofibers at P0 (Fig. 1D-F; see Fig. S3G-I in the supplementary material).

In summary, we show that Tcf4 is highly expressed in and robustly labels mouse muscle connective tissue fibroblasts in the adult and their precursors during development both in vivo and in vitro. However, similar to other reported fibroblast markers, Tcf4 is also expressed in myogenic cells in vitro as well as at low levels in some fetal myonuclei in vivo.

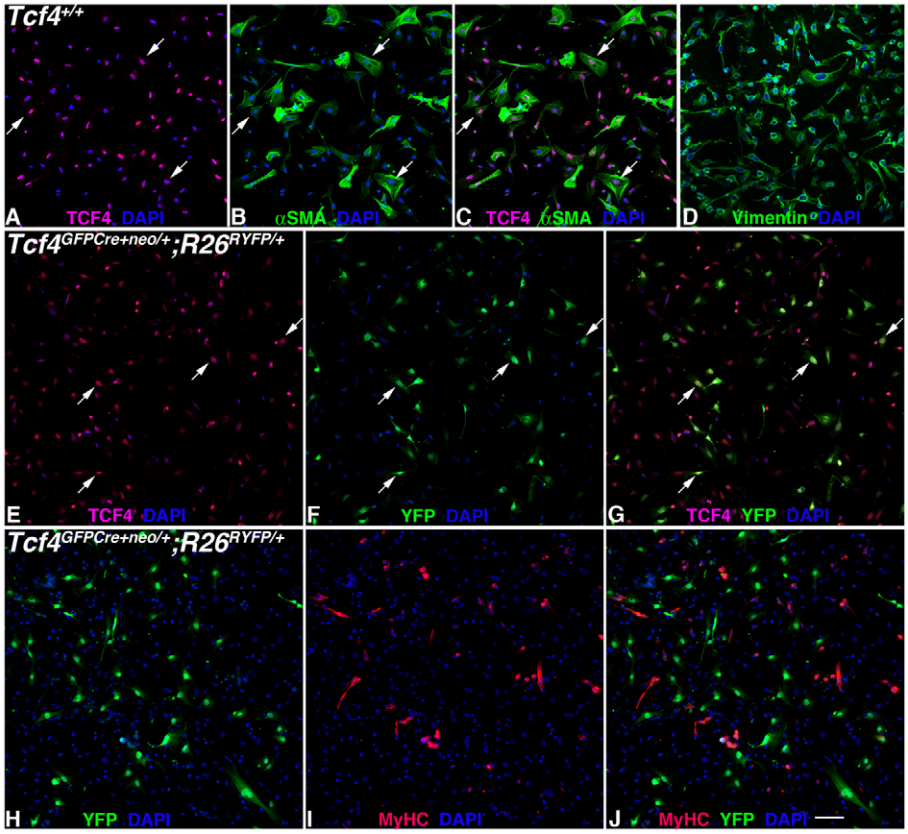

Fig. 2. Tcf4 is highly expressed and *Tcf4*$^{GFPCre+neo}$ genetically labels muscle connective tissue fibroblasts in culture. (**A-D**) Adherent fibroblasts derived from neonatal limb muscles express Tcf4 and αSMA (arrows, A-C) and vimentin (D). (**E-G**) In a preparation of adherent cells from limb muscles from *Tcf4*$^{GFPCre+neo/+}$;*R26R*$^{YFP/+}$ mice, 58% of Tcf4$^+$ fibroblasts are genetically labeled with YFP (arrows, E-G). (**H-J**) In a preparation of total cells from limb muscles from *Tcf4*$^{GFPCre+neo/+}$;*R26R*$^{YFP/+}$ mice, MyHC$^+$ myogenic cells are not labeled by YFP. Scale bar: in J, 100 μm for A-J.

Tcf4$^{GFPCre+neo}$ genetically labels muscle connective tissue fibroblasts but not myogenic cells

As Tcf4 is strongly expressed in muscle connective tissue fibroblasts and their precursors, we engineered *Tcf4*^{GFPCre} mice in order to genetically manipulate connective tissue fibroblasts. *Tcf4*^{GFPCre} mice were created by replacing exon 1 and its splice donor with a *GFPCre* cassette (Le et al., 1999) placed at the endogenous *Tcf4* ATG (see Fig. S2A in the supplementary material). Two versions of *Tcf4*^{GFPCre} mice were made: *Tcf4*$^{GFPCre+neo}$ mice, which contain the PGK neomycin selection cassette, and *Tcf4*$^{GFPCre-neo}$ mice, in which the FRT-flanked PGK neomycin selection cassette was deleted by crosses to *ACTBFlpe* mice (see Fig. S2A in the supplementary material) (Rodriguez et al., 2000). In both *Tcf4*$^{GFPCre+neo}$ and *Tcf4*$^{GFPCre-neo}$ mice, the replacement of exon 1 by *GFPCre* resulted in a 'knock-in/knock-out' allele such that Tcf4 was not expressed from the *Tcf4*^{GFPCre} allele (see Fig. S2B-G in the supplementary material). Similar to other *Tcf4* heterozygous mice (Korinek et al., 1998), *Tcf4*$^{GFPCre+neo/+}$ and *Tcf4*$^{GFPCre-neo/+}$ mice were phenotypically normal.

We first analyzed whether *Tcf4*$^{GFPCre+neo/+}$ mice genetically label Tcf4$^+$ muscle connective tissue fibroblasts in vivo. *Tcf4*$^{GFPCre+neo/+}$ mice were crossed to *R26R*^{lacZ}, *R26R*^{YFP} or *R26R*^{mTmG} reporter mice, in which *lacZ*, YFP or membrane-bound GFP (mGFP), respectively, are permanently expressed in response to Cre (Muzumdar et al., 2007; Soriano, 1999; Srinivas et al., 2001). At E12.5 in *Tcf4*$^{GFPCre+neo/+}$;*R26R*$^{lacZ/+}$ mice, β-gal$^+$ cells lie closely associated with, but interstitial to, the developing limb muscles (Fig. 3A-F). At P0 in *Tcf4*$^{GFPCre+neo/+}$;*R26R*$^{mTmG/+}$ mice, mGFP$^+$ cells are found interstitial to all limb muscles in regions of Sirius Red$^+$ connective tissue (Fig. 3G-J; cytoplasmic GFP from the *GFPCre* cassette is only weakly expressed in *Tcf4*$^{GFPCre+neo}$ mice). As expected, myogenic cells were rarely labeled (<1% myogenic cells were GFP$^+$). Strikingly, P0 limbs of *Tcf4*$^{GFPCre+neo/+}$;*R26R*$^{lacZ/+}$ mice stained for β-gal activity in whole-mount preparations revealed that β-gal$^+$ cells were not only interspersed between the muscle myofibers, but concentrated at the aponeuroses (ECM-rich regions of connective tissue linking muscle with tendon) of various muscles (Fig. 3K-N).

Tcf4$^{GFPCre+neo/+}$ mice also genetically label Tcf4$^+$ muscle connective tissue fibroblasts in culture. Analysis of connective tissue fibroblasts derived from limb muscles of *Tcf4*$^{GFPCre+neo/+}$;*R26R*$^{YFP/+}$ mice and isolated by their adherence to plastic culture dishes revealed that Tcf4$^+$ connective tissue fibroblasts were YFP$^+$ (Fig. 2E-G). Fifty-eight percent of Tcf4$^+$ fibroblasts were YFP$^+$, demonstrating that Cre-mediated recombination occurred in many, but not all Tcf4$^+$ fibroblasts. The presence of the PGK neomycin cassette probably hindered the levels of Cre expressed from the *Tcf4* locus, and so not all Tcf4$^+$ fibroblasts were genetically labeled. Importantly, analysis of myogenic cells isolated from *Tcf4*$^{GFPCre+neo/+}$;*R26R*$^{YFP/+}$ mice showed that no Pax7$^+$ myogenic precursors, MyoD$^+$ myoblasts or MyHC$^+$ myofibers were YFP$^+$ (Fig. 2H-J; data not shown). This indicates that the Cre from the *Tcf4*$^{GFPCre+neo}$ allele is not active in any myogenic cells.

We then compared *Tcf4*$^{GFPCre+neo}$ mice with *Tcf4*$^{GFPCre-neo}$ mice, in which the FRT-flanked PGK neomycin selection cassette was deleted. In *Tcf4*$^{GFPCre-neo/+}$;*R26R*$^{mTmG/+}$ mice, mGFP was expressed in all connective tissue fibroblasts, but was also expressed in myogenic cells (see Fig. S3A-F in the supplementary material). Therefore, removal of the PGK neomycin cassette in the

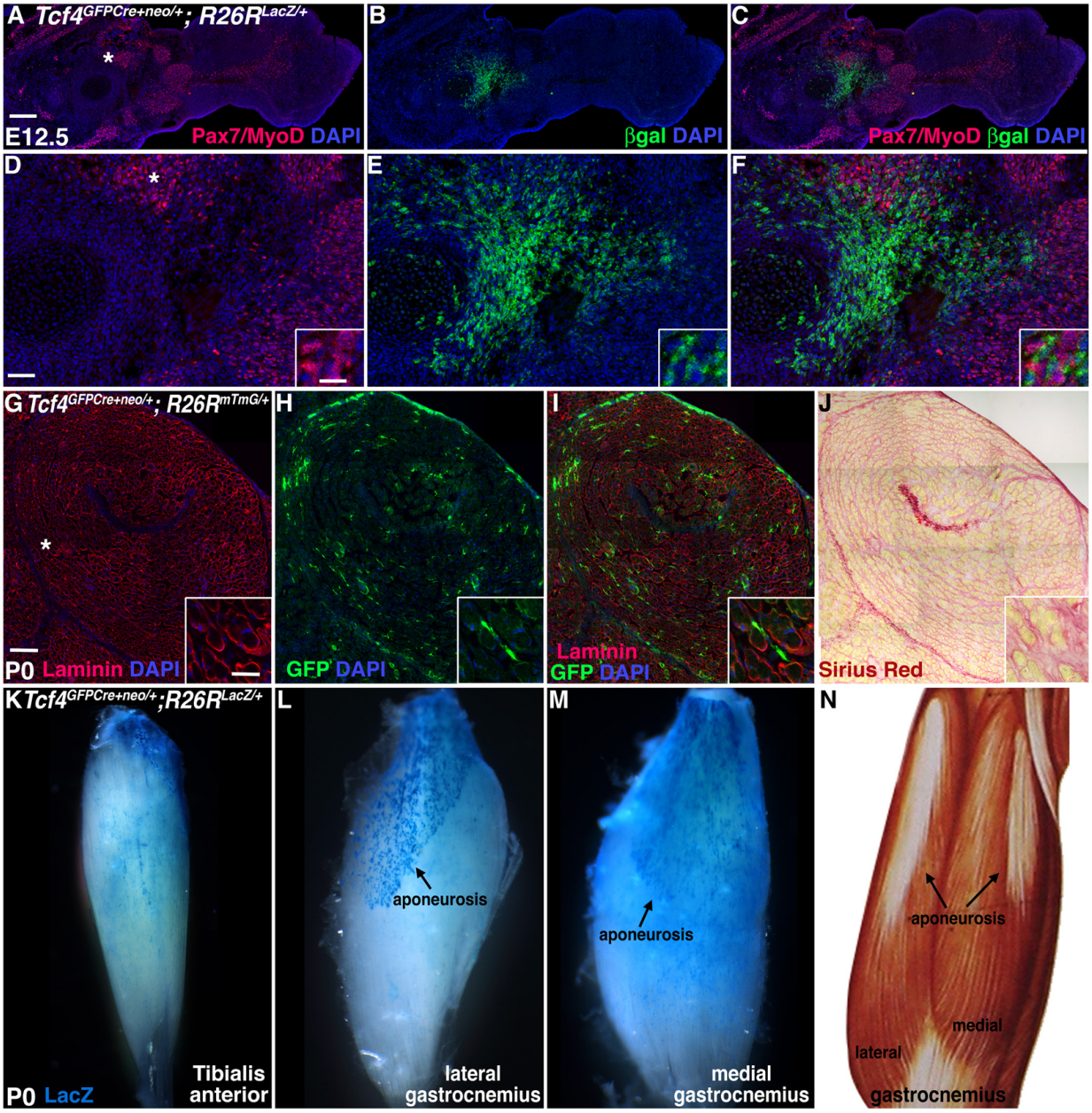

Fig. 3. *Tcf4*$^{GFPCre+neo}$ genetically labels muscle connective tissue fibroblasts in vivo. (**A-J**) In the embryonic limb of *Tcf4*$^{GFPCre+neo/+}$;*R26R*$^{lacZ/+}$ mice (A-F) and neonatal limb of *Tcf4*$^{GFPCre+neo/+}$;*R26R*$^{mTmG/+}$ mice (G-J) β-gal^{+} (A-F) or membrane bound GFP^{+} (G-I) Tcf4-derived cells lie associated with, but interstitial to, Pax7/MyoD^{+} myoblasts (A-F) or laminin-ensheathed myogenic cells (G-I) and in Sirius Red^{+} regions (J). (**K-M**) In whole-mount preparations, β-gal^{+} cells (blue) are present throughout the tibialis anterior and gastrocnemius muscles and concentrated in the gastrocnemius aponeurosis of *Tcf4*$^{GFPCre+neo/+}$;*R26R*$^{lacZ/+}$ mice. (**N**) Drawing of the gastrocnemius muscle. Asterisks in A, D and G indicate enlarged regions in D-F, insets in D-F and insets in G-J, respectively. Scale bars: in A, 50 μm for A-C; in D, 12.5 μm for D-F; in D inset, 3.125 μm for D-F insets; in G, 100 μm for G-J; in G inset, 25 μm for G-J insets.

Tcf4$^{GFPCre\text{-}neo/+}$ mice allowed Cre to be active in fibroblasts, which express high levels of Tcf4, and also in fetal myogenic cells, which express low levels of Tcf4.

Thus, we have engineered two lines of mice, *Tcf4*$^{GFPCre+neo}$ and *Tcf4*$^{GFPCre\text{-}neo}$, which are the first reagents to enable muscle connective tissue fibroblasts to be genetically labeled and manipulated. In *Tcf4*$^{GFPCre+neo}$ mice, Cre is expressed only in cells expressing high levels of Tcf4, and so Cre is specifically active in fibroblasts and not in myogenic cells. However, because not all fibroblasts express uniformly high levels of Tcf4, Cre is active in only 58% of fibroblasts. In *Tcf4*$^{GFPCre\text{-}neo}$ mice, Cre is expressed in cells expressing high and low levels of Cre, and so Cre is active in both fibroblasts and myogenic cells. Although Cre is not exclusively active in fibroblasts in *Tcf4*$^{GFPCre\text{-}neo}$ mice, Cre is active in all fibroblasts.

Tcf4 regulates the expression of MyHC isoforms

The strong expression of Tcf4 in connective tissue fibroblasts suggested that Tcf4 might be functionally important. We tested Tcf4 function by generating *Tcf4*$^{GFPCre/GFPCre}$ mice, in which Tcf4

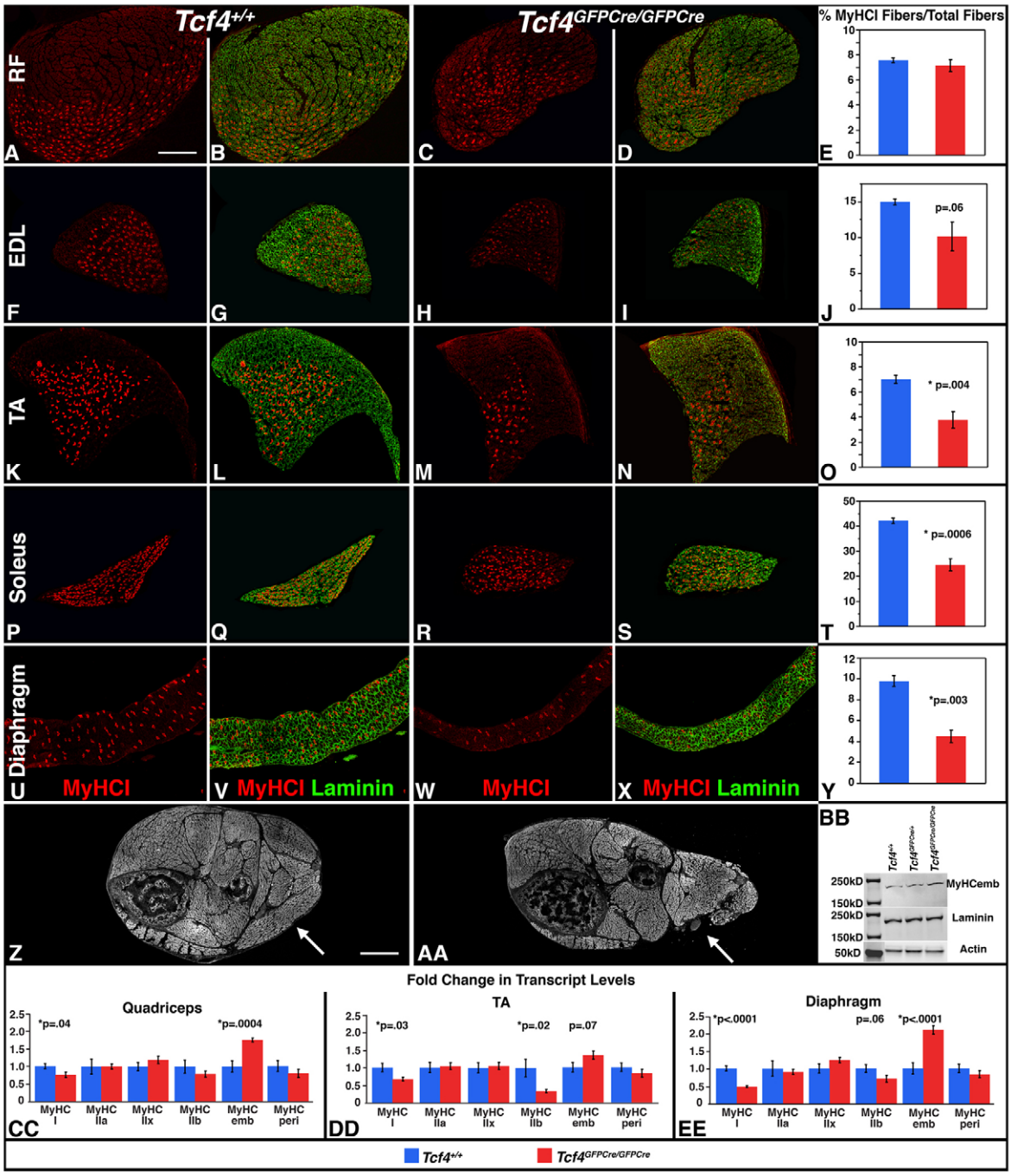

Fig. 4. Germline loss of *Tcf4* leads to a reduction in slow MyHCI and fast MyHCIIb and an increase in developmental MyHCemb in many muscles. (**A-Y**) Loss of *Tcf4* in $Tcf4^{GFPCre/GFPCre}$ leads to a reduction in the percent MyHCI^{+}/total laminin^{+} myofibers in the extensor digitorum longus (EDL; F-J), tibialis anterior (TA; K-O), soleus (P-T) and diaphragm (U-Y), but not in the rectus femoris (RF; A-E). (**Z-BB**) All muscles in $Tcf4^{+/+}$ and $Tcf4^{GFPCre/GFPCre}$ mice express MyHCemb (Z,AA), but overall MyHCemb protein levels (by western blot of P0 hind limbs) are increased with loss of Tcf4 (BB). The medial head of gastrocnemius is truncated with loss of *Tcf4* (arrows, Z,AA). (**CC-EE**) By qPCR, MyHCI transcript levels are significantly reduced, whereas MyHCemb levels are increased in $Tcf4^{GFPCre/GFPCre}$ quadriceps (CC), TA (DD) and diaphragm (EE). MyHCIIb levels are significantly reduced in the $Tcf4^{GFPCre/GFPCre}$ TA and diaphragm (DD,EE). Data are expressed as mean ± s.e.m. Scale bars: in A, 200 μm for A-D, F-I, K-N, P-S, U-X; in Z, 400 μm for Z,AA.

expression was completely absent, as determined by quantitative RT-PCR (qPCR), immunofluorescence and western blot (see Fig. S2B-G in the supplementary material). Consistent with previously generated *Tcf4*-null mice (Korinek et al., 1998), $Tcf4^{GFPCre/GFPCre}$ mice died within 24 hours of birth, probably from respiratory failure (data not shown), and suffered multiple gastrointestinal defects (M.A.-H. and M. R. Capecchi, unpublished).

Analysis of $Tcf4^{GFPCre/GFPCre}$ mice revealed defects in the musculoskeletal system. At birth, $Tcf4^{GFPCre/GFPCre}$ mice had reduced limb mobility, with a loss of flexion at the elbow and knee joints. The pattern of multiple muscles was abnormal; several muscles were aberrantly split or truncated, and origins and insertions of muscles crossing the knee or elbow joints were altered (Fig. 4Z,AA).

Strikingly, the expression of MyHC isoforms was altered in *Tcf4*$^{GFPCre/GFPCre}$ mice (Fig. 4; see Table S3 in the supplementary material). Sections through P0 hind limbs of *Tcf4*$^{GFPCre/GFPCre}$ mice revealed a global reduction in the number of myofibers expressing slow MyHCI. Quantification of individual muscles (with different MyHC isoform profiles) demonstrated that the percentage of MyHCI^{+} myofibers was significantly reduced in the tibialis anterior (TA) and soleus, decreased in the extensor digitorum longus (EDL) and unchanged in the rectus femoris (RF) component of the quadriceps (Fig. 4A-T). In addition, the percentage of MyHCI^{+} myofibers was significantly reduced in the diaphragm (Fig. 4U-Y). In none of these muscles was the overall number of myofibers significantly changed in the mutants (data not shown), nor was apoptosis of myofibers significantly increased (see Fig. S2I-L in the supplementary material). To determine whether other MyHC isoforms were altered, we quantified MyHC transcript levels of P0 quadriceps, TA and diaphragm (EDL and soleus were too small to be quantified without pooling samples) using qPCR. MyHCI transcript levels were significantly reduced in the quadriceps, TA and diaphragm of *Tcf4*$^{GFPCre/GFPCre}$ muscles (48-76% of control levels; Fig. 4CC-EE; see Table S3 in the supplementary material). No accompanying changes in fast MyHCIIa, MyHCIIx or MyHCperi were found. However, fast MyHCIIb was decreased in the TA and diaphragm (34-71% of control levels) and the developmental isoform MyHCemb was increased in all three muscles of *Tcf4*$^{GFPCre/GFPCre}$ mice (134-211% of control levels). In addition, whereas immunofluorescence for MyHCemb showed that all myofibers on sections through Tcf4$^{+/+}$ and *Tcf4*$^{GFPCre/GFPCre}$ muscles expressed MyHCemb, a western blot revealed that the overall amount of MyHCemb was significantly increased with loss of Tcf4 (Fig. 4Z-BB). These changes in fiber type occurred during fetal myogenesis, as no changes in MyHC1 or MyHCemb were apparent during embryonic myogenesis (at E12.5, data not shown) and MyHCIIb is not expressed until E15 (Lu et al., 1999). Thus, Tcf4 normally positively regulates the expression of slow MyHCI and fast MyHCIIb, and negatively regulates developmental MyHCemb during fetal myogenesis of most limb muscles and the diaphragm.

Tcf4 intrinsically regulates MyHCI and MyHCIIb expression in limb and diaphragm myofibers

Because our Tcf4 antibody and lineage analysis revealed that Tcf4 is expressed at high levels in the fibroblasts but also at low levels in fetal myogenic cells, Tcf4 might extrinsically (via its expression in fibroblasts) and/or intrinsically (via its expression in myogenic cells) regulate MyHC expression. To test whether Tcf4 intrinsically regulates MyHC expression, we conditionally deleted Tcf4 in myogenic cells using two other alleles of Tcf4. *Tcf4*^{fl} mice contain *loxP* sites flanking exon 1 and its splice donor site, and *Tcf4*^{del} mice

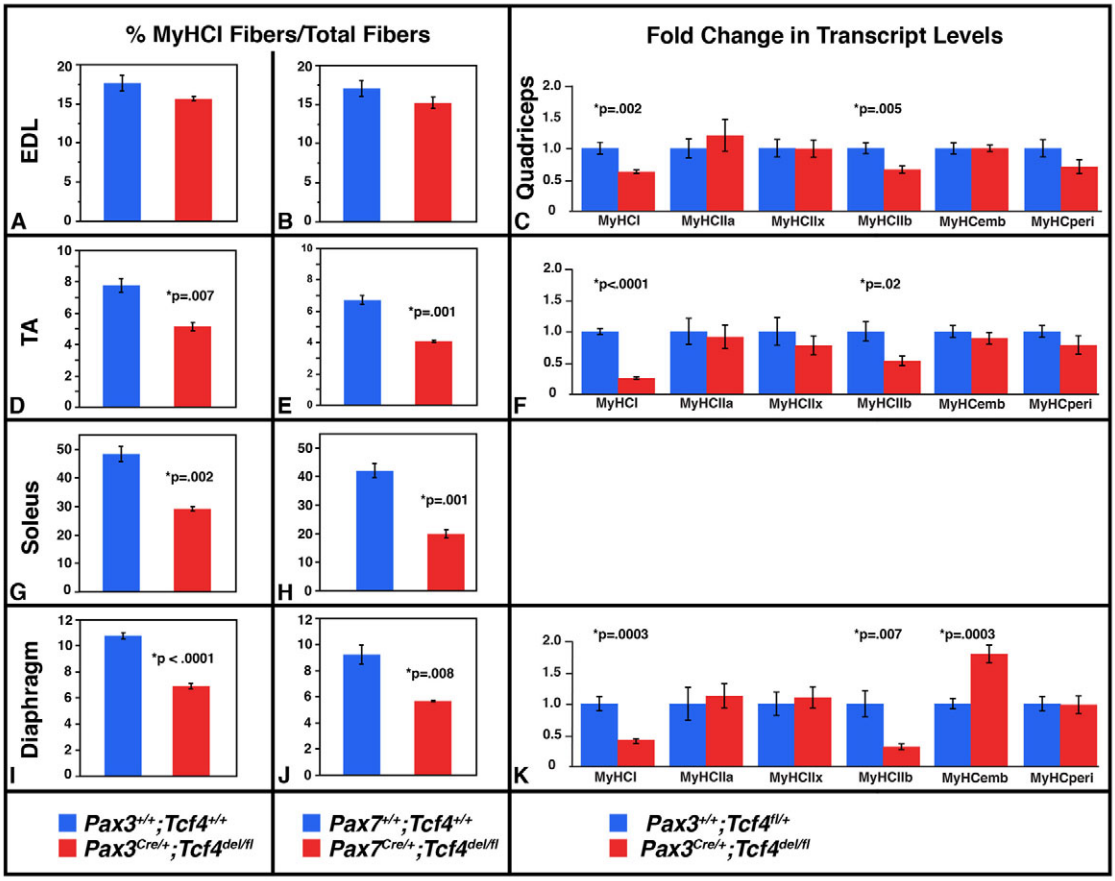

Fig. 5. Myogenic loss of Tcf4 leads to a reduction in slow MyHCI and MyHCIIb in limb and diaphragm muscles. (**A**,**B**,**D**,**E**,**G-J**) Loss of Tcf4 in embryonic and fetal muscles (*Pax3*$^{Cre/+}$;*Tcf4*$^{del/fl}$) or fetal muscles only (*Pax7*$^{Cre/+}$;*Tcf4*$^{del/fl}$) leads to a reduction in the percent MyHCI^{+}/total laminin^{+} myofibers in the TA (D,E), soleus (G,H) and diaphragm (I,J), but not in the EDL (A,B). (**C**,**F**,**K**) By qPCR, MyHCI and MyHCIIb levels are reduced in the quadriceps, TA and diaphragm of *Pax3*$^{Cre/+}$;*Tcf4*$^{del/fl}$ mice (C,F,K). MyHCemb levels are increased in the diaphragm of *Pax3*$^{Cre/+}$;*Tcf4*$^{del/fl}$ mice (K). Data are expressed as mean ± s.e.m.

(generated by crossing $Tcf4^{fl}$ mice with $Hprt^{Cre}$ mice) (Tang et al., 2002) contain no exon 1 or its splice donor site (M.A.-H. and M. R. Capecchi, unpublished). Similar to the $Tcf4^{GFPCre/GFPCre}$ mice, $Tcf4^{del/del}$ mice died at birth and had muscle patterning defects (data not shown). Using these *Tcf4* alleles, we genetically deleted Tcf4 in embryonic and fetal myogenic cells by generating $Pax3^{Cre/+};Tcf4^{del/fl}$ mice or in just fetal myogenic cells by generating $Pax7^{Cre/+};Tcf4^{del/fl}$ mice (see Fig. S2H in the supplementary material) (Hutcheson et al., 2009). No effects on MyHC levels were detected in $Tcf4^{del/+}$ mice (data not shown). However, deletion of Tcf4 in muscle resulted in a decreased percentage of MyHCI$^+$ myofibers in the TA, soleus and diaphragm, but not the EDL at P0 (Fig. 5A,B,D,E,G-J). By qPCR, the levels of MyHCI and MyHCIIb were significantly reduced in the quadriceps, TA and diaphragm of $Pax3^{Cre/+};Tcf4^{del/fl}$ P0 mice (Fig. 5C,F,K; see Table S3 in the supplementary material). In addition, MyHCemb levels were increased in the diaphragm of $Pax3^{Cre/+};Tcf4^{del/fl}$ mice. This Tcf4 intrinsic regulation of MyHC isoforms is probably occurring during fetal, not embryonic, myogenesis. First, MyHCIIb is not expressed in embryonic muscle. Second, MyHCI levels were normal in embryonic muscle with germ-line deletion of Tcf4. Finally, the percentage of MyHCI$^+$ myofibers was equivalent or slightly more reduced when Tcf4 was deleted in just fetal fibers ($Pax7^{Cre/+};Tcf4^{del/fl}$) versus embryonic and fetal fibers ($Pax3^{Cre/+};Tcf4^{del/fl}$). Together, these data demonstrate that, despite low levels of Tcf4 in myogenic cells, Tcf4 intrinsically positively regulates both MyHCI and MyHCIIb, the two most abundant mature isoforms in mice, in most hind limb muscles and the diaphragm during fetal myogenesis.

Tcf4 in connective tissue fibroblasts extrinsically regulates MyHCI and MyHCemb expression in limb myofibers

The high level of Tcf4 in connective tissue fibroblasts suggests that loss of Tcf4 in fibroblasts might extrinsically regulate MyHC expression in myogenic cells. To test the role of Tcf4 in connective tissue fibroblasts, we generated $Tcf4^{GFPCre+neo/fl}$ mice. In $Tcf4^{GFPCre+neo/fl}$ mice, the *GFPCre+neo* did not cause deletion of the floxed allele in myogenic cells (and thus they maintained one functional allele of *Tcf4*), but did cause deletion of the floxed allele in fibroblasts (see Fig. S2H in the supplementary material). Because of the lower efficiency of the *GFPCre+neo*, only ~60%

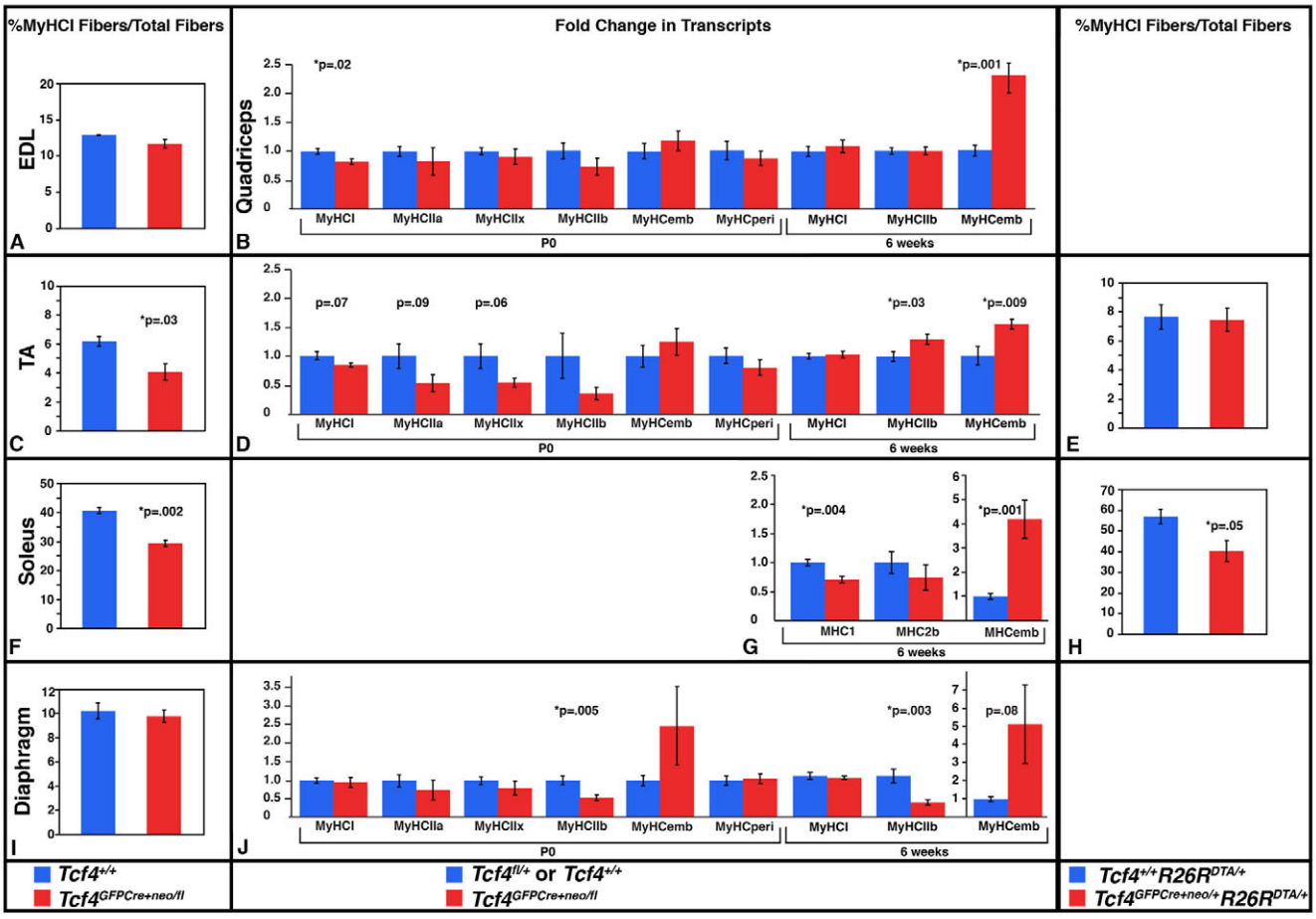

Fig. 6. Reduction of Tcf4 in connective tissue fibroblasts leads to a reduction in slow MyHCI in fetal limb muscles and an increase in MyHCemb in neonatal limb and diaphragm muscles. (**A**,**C**,**F**,**I**) Deletion of Tcf4 in ~60% of connective tissue fibroblasts in $Tcf4^{GFPCre+neo/fl}$ mice leads to a reduction in the percent MyHCI$^+$/total laminin$^+$ myofibers in the TA (C) and soleus (F), but not in the EDL (A) or diaphragm (I). (**B**,**D**,**G**,**J**) By qPCR, MyHCI levels are reduced in neonatal quadriceps and TA and adult soleus (B,D,G), whereas MyHCemb levels are increased in adult quadriceps, TA, soleus and diaphragm muscles of $Tcf4^{GFPCre+neo/fl}$ mice (B,D,G,J). (**E**,**H**) Ablation of ~50% of the Tcf4$^+$ fibroblasts in $Tcf4^{GFPCre+neo/+};R26R^{DTA/+}$ mice leads to a reduction in the percent MyHCI$^+$ myofibers in the soleus (H) but not the TA (E). Data are expressed as mean ± s.e.m.

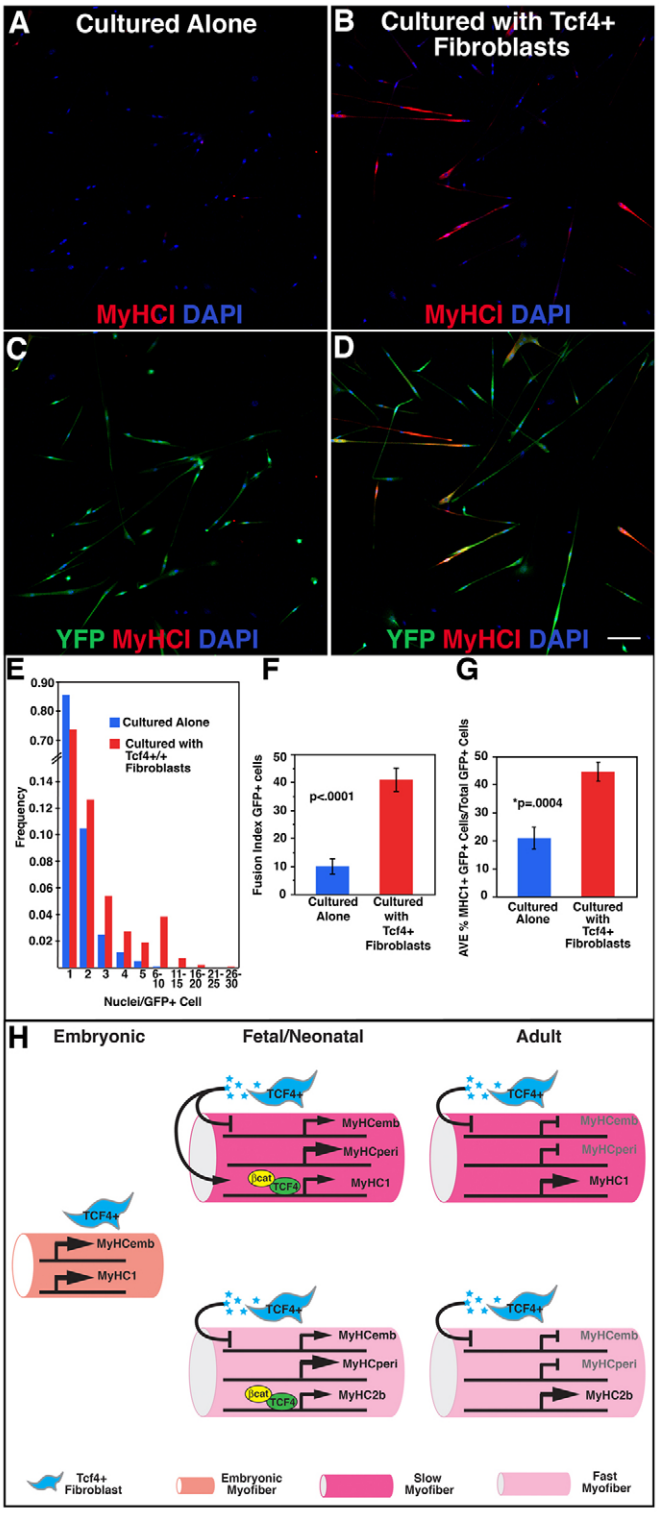

Fig. 7. Myoblasts cultured in the presence of Tcf4$^+$ fibroblasts differentiate into myofibers with increased slow MyHCI expression and more nuclei. (**A-G**) After 7 days of differentiation, myoblasts cultured in the presence of Tcf4$^+$ fibroblasts had a greater percent MyHCI$^+$/total YFP$^+$ myofibers (A-D,G) and an increased frequency of more multinucleate myofibers (E) and a higher fusion index (F). Data in F and G are expressed as mean ± s.e.m. (**H**) Model of the role of Tcf4 and Tcf4$^+$ fibroblasts in regulating muscle fiber type during development. Tcf4 intrinsically regulates MyHCI and MyHCIIb, probably by direct binding of Tcf4 to their enhancers and activation by β-catenin in fetal myofibers. Tcf4-dependent signals in fibroblasts extrinsically promote MyHCI and repress MyHCemb in myofibers.

of the fibroblasts are completely null for Tcf4. Loss of Tcf4 in most fibroblasts did not result in loss of muscle connective tissue fibroblasts, as fibroblasts from limb muscles of *Tcf4$^{GFPCre+neo/fl}$* mice could still be isolated in culture by their adherence to plastic. Nevertheless, by P0, muscle fiber type was altered (Fig. 6; see Table S3 in the supplementary material). The percentage of MyHCI$^+$ myofibers in the TA and soleus, but not the EDL or diaphragm, was reduced (Fig. 6A,C,F,I). By qPCR, the levels of MyHCI were significantly reduced at P0 in the quadriceps and TA, but not the diaphragm (Fig. 6B,D,J). Interestingly, at 6 weeks in *Tcf4$^{GFPCre+neo/fl}$* mice (which survive past the P0 lethality of *Tcf4$^{GFPCre/GFPCre}$* mice), MyHCI levels are no longer significantly different in the quadriceps or TA (Fig. 6B,D) but are still reduced in the MyHCI-enriched soleus (Fig. 6G). By contrast, MyHCemb levels were somewhat (but not significantly) elevated at P0, but were significantly elevated by 6 weeks in *Tcf4$^{GFPCre+neo/fl}$* mice in all muscles tested (Fig. 6B,D,G,J). This increase in MyHCemb could possibly result from increased regeneration in adult *Tcf4$^{GFPCre+neo/fl}$* mice, but this is unlikely because levels of MyHCperi (which are normally also upregulated during regeneration) were unchanged in these mice (data not shown). MyHCIIa and MyHCIIx levels were not significantly changed in *Tcf4$^{GFPCre+neo/fl}$* mice and MyHCIIb levels were variably reduced (diaphragm at P0 and 6 weeks) or elevated (TA at 6 weeks). These data demonstrate that Tcf4 in fibroblasts extrinsically promotes MyHCI expression in several limb muscles in the fetal mouse and in the MyHCI-enriched soleus in the adult. In addition, Tcf4 in fibroblasts is crucial for repressing developmental MyHCemb, beginning at the end of fetal myogenesis and continuing in the adult. Together, these data provide the first evidence that muscle fiber type is regulated extrinsically, via Tcf4-dependent signals, by the connective tissue fibroblasts that surround muscle.

Muscle connective tissue fibroblasts extrinsically regulate muscle fiber type and maturation

To further explore how connective tissue fibroblasts regulate muscle, we genetically ablated Tcf4$^+$ fibroblasts using *Tcf4$^{GFPCre+neo/+}$;R26R$^{DTA/+}$* mice. In *Tcf4$^{GFPCre+neo/+}$;R26R$^{DTA/+}$* mice Cre activates expression of diphtheria toxin A (DTA) and kills connective tissue fibroblasts specifically. Because of the inefficiency of the *GFPCre+neo*, only 45-64% of Tcf4$^+$ fibroblasts were ablated. *Tcf4$^{GFPCre+neo/+}$;R26R$^{DTA/+}$* mice were born, but died between 3 and 6 weeks of age. The variability in the number of MyHCI$^+$ and total myofibers was much greater than in the *Tcf4$^{GFPCre+neo/fl}$* mice (probably due to variable ablation and compensating proliferation of Tcf4$^+$ fibroblasts). Nevertheless, although the total number of myofibers was unchanged in the TA and soleus, there was a significant reduction in the percentage of MyHCI$^+$ myofibers in the soleus at P0 (Fig. 6H). This indicates that connective tissue fibroblasts are necessary to achieve normal numbers of MyHCI fetal/neonatal myofibers.

To test directly whether connective tissue fibroblasts regulate myogenic cells, we developed a fibroblast/myoblast transwell culture system. Fibroblasts were isolated from limb muscles of

P0-P5 $Tcf4^{+/+}$ mice by their adherence to plastic and then cultured in transwell cell culture inserts. Pure myoblasts were isolated by FACS of YFP^+ cells from P0-P7 limb muscles of $Pax7^{Cre/+};R26R^{YFP/+}$ mice and cultured on gelatin-coated coverslips in proliferation media and then switched to differentiation media in the presence or absence of $Tcf4^+$ fibroblasts in transwell culture inserts. After 7 days in differentiation media, YFP^+ myoblasts readily differentiated into multinucleate myofibers when cultured alone (Fig. 7A,C). However, YFP^+ myoblasts cultured in the presence of $Tcf4^+$ fibroblasts differentiated into myofibers containing significantly more myonuclei (skewed distribution of myofibers with more myonuclei and increased fusion index of 41 versus 10; Fig. 7E,F). Because ablation of $Tcf4^+$ fibroblasts led to a reduction in the percentage of $MyHCI^+$ myofibers in the soleus, we predicted that $Tcf4^+$ fibroblasts would increase the percentage of $MyHCI^+$ myofibers if myoblasts were cultured with fibroblasts. Indeed, the percentage of $MyHCI^+$ myofibers was significantly increased when myoblasts were differentiated in the presence of $Tcf4^+$ fibroblasts (Fig. 7A-D,G). In addition, we examined whether levels of MyHCemb were reduced in the presence of fibroblasts. However, all myofibers cultured alone or with fibroblasts were $MyHCemb^+$ (determined by immunofluorescence) during the timecourse of the experiment, indicating that either $Tcf4^+$ fibroblasts were not sufficient to repress MyHCemb in culture or that differences in MyHCemb levels were not detectable by immunofluorescence (similar to our finding that all myofibers were $MyHCemb^+$ in sections of $Tcf4^{+/+}$ or $Tcf4^{GFPCre/GFPCre}$ mice; Fig. 4Z-AA). In summary, our fibroblast/myoblast transwell experiments demonstrate that $Tcf4^+$ fibroblasts are sufficient, via non cell-contact mediated signals, to promote both increased myoblast fusion and the expression of MyHCI in myofibers.

In a more limited set of experiments, we tested whether the effect of fibroblasts on myoblast fusion and muscle fiber type required Tcf4. To test this, we cultured YFP^+ myoblasts from $Pax7^{Cre/+};R26R^{YFP/+}$ neonatal limb muscles alone or in the presence of $Tcf4^+$ or $Tcf4^-$ fibroblasts derived from P0 $Tcf4^{+/+}$ or $Tcf4^{del/del}$ limb muscles. In these experiments, myoblasts cultured in the presence of either $Tcf4^+$ or $Tcf4^-$ fibroblasts had an increased fusion index (50.3 ± 2.4 s.e.m. and 52.2 ± 3.5 s.e.m., respectively) compared with myoblasts cultured alone (43.7 ± 2.4 s.e.m.), although the high variability between replicates precluded statistical significance. We also tested whether the increased percentage of $MyHCI^+$ myofibers when myoblasts were cultured with fibroblasts was dependent on Tcf4. However, an analysis of the percentage of $MyHCI^+$ myofibers when myoblasts were cultured alone or with $Tcf4^+$ or $Tcf4^-$ fibroblasts was inconclusive because of the high variability between replicates. Nevertheless, from these limited experiments, we can conclude that fibroblast signals promoting myoblast fusion depend neither on cell contact nor on Tcf4.

DISCUSSION

Connective tissue is the anatomical partner of muscle, connecting muscle to tendon and bone, and constitutes an important component of the niche within which muscle and its progenitors reside. Using a new molecular marker, Tcf4, and genetic reagents to label and manipulate connective tissue fibroblasts, we now show that connective tissue is an important regulator of myogenesis. In addition, our study also reveals a new mechanism by which development of myofiber type is intrinsically regulated.

Tcf4 is a marker of muscle connective tissue fibroblasts and $Tcf4^{GFPCre}$ mice allow for genetic manipulation of these fibroblasts

To facilitate study of the connective tissue, we have shown that the transcription factor Tcf4 is strongly expressed in connective fibroblasts, in vivo and in vitro, both during development and in the adult. Our analysis of Tcf4 function reveals that Tcf4 is not required for specification of connective tissue fibroblasts (as these cells can be isolated in mice null for *Tcf4*), but does regulate their signaling properties. Detailed examination of Tcf4 expression also shows that although Tcf4 is expressed at high levels in connective tissue fibroblasts, it is not exclusively expressed in these cells. Similar to other markers expressed in fibroblasts, Tcf4 is expressed in myogenic cells in vitro. In addition, Tcf4 is expressed at low (near background) levels in some fetal myonuclei in vivo. Nevertheless, particularly in tissue sections, Tcf4 is an excellent marker of muscle connective tissue fibroblasts.

Based on the high level of Tcf4 expression in muscle connective tissue fibroblasts, we engineered $Tcf4^{GFPCre+neo}$ and $Tcf4^{GFPCre-neo}$ mice, the first reagents to enable these fibroblasts to be genetically labeled, manipulated and ablated. In $Tcf4^{GFPCre+neo}$ mice, Cre is specifically active in fibroblasts and not myogenic cells, although only ~60% of fibroblasts express Cre. In $Tcf4^{GFPCre-neo}$ mice, Cre is active in all fibroblasts, but Cre is also active in myogenic cells. Both lines will be useful for studying the function of muscle connective tissue fibroblasts. For functional studies of genes expressed exclusively in fibroblasts (and not in myogenic cells), $Tcf4^{GFPCre-neo/+}$ mice will be the appropriate Cre line for driving Cre-mediated recombination in all fibroblasts. For studies of genes expressed in both fibroblasts and myogenic cells, $Tcf4^{GFPCre+neo/+}$ mice will allow for manipulation of gene function specifically in muscle connective tissue fibroblasts.

Tcf4-regulated paracrine signals from connective tissue fibroblasts extrinsically regulate muscle fiber type

An important component of myogenesis is the diversification of myofibers into different fiber types. Development of fiber type diversity is essential for the functional specialization of muscle and is regulated by both intrinsic and extrinsic factors. Innervation and thyroid hormones have been identified as important extrinsic regulators of fiber type development (Schiaffino and Reggiani, 1996). In addition, previous in vitro studies and avian surgical manipulations had hinted that the connective tissue might modulate fiber type (Cooper et al., 2004; Kusner et al., 2010; Robson et al., 1994), but the role of connective tissue has never been rigorously established by loss-of-function or in vivo experiments.

Our in vivo genetic manipulations and in vitro transwell cultures demonstrate for the first time that fibroblasts extrinsically regulate development of muscle fiber type (Fig. 7H). During fetal myogenesis, fibroblasts promote increased expression of MyHCI. Ablation of $Tcf4^+$ fibroblasts led to decreased levels of $MyHCI^+$ myofibers, and myoblast/fibroblast transwell experiments showed that fibroblast signals are sufficient to promote increased levels of $MyHCI^+$ myofibers. Furthermore, these paracrine signals from fibroblasts are Tcf4-dependent, as genetic deletion of Tcf4 in fibroblasts led to decreased numbers of $MyHCI^+$ myofibers and MyHCI transcript levels in multiple muscles at P0. Interestingly, this Tcf4-dependent fibroblast regulation of MyHCI levels continued in the adult only in the MyHCI-enriched soleus muscle, but not in the other muscles measured. This lack of effect in the adult in most muscles might reflect the dominant role of

innervation and hormones in controlling MyHCI levels in these muscles, and the need for additional connective tissue promotion of MyHCI in the soleus, which contains a particularly high level (greater than 50%) of MyHCI (Agbulut et al., 2003). Finally, our transwell culture experiments also demonstrate that the paracrine signals from fibroblasts are not cell-contact mediated, but instead secreted. Taken together, our experimental results show that Tcf4 in fibroblasts regulates (directly or indirectly) the secretion of signaling molecules or ECM components, which in turn modulate MyHCI expression levels in adjacent myofibers during fetal myogenesis. Thus, our analysis reveals that the connective tissue is an important extrinsic regulator of myofiber type (Fig. 7H).

Tcf4, via β-catenin activation, intrinsically promotes muscle fiber type maturation

Our analysis of the function of Tcf4 has also unexpectedly revealed the complexity of intrinsic regulation of muscle fiber type development. The fiber type of individual muscles is established gradually beginning during fetal myogenesis, and the intrinsic mechanisms regulating fiber type have been extensively studied. Surprisingly, although many molecular mechanisms have been identified that intrinsically modulate fiber type in the adult, several of these pathways do not appear to regulate the development of muscle fiber type (Issa et al., 2006; Oh et al., 2005). Only recently have some of the intrinsic factors regulating development of muscle fiber type in mammals begun to be elucidated (Hagiwara et al., 2007; Niro et al., 2010). We now show that Tcf4, despite being expressed at low levels in myogenic cells, intrinsically regulates myofiber type development in mouse.

Conditional deletion of Tcf4 in myogenic cells demonstrates that Tcf4 intrinsically regulates MyHCI and MyHCIIb in most limb muscles and the diaphragm during fetal myogenesis. Tcf4 is likely to intrinsically regulate MyHCI and MyHCIIb levels via direct binding and activation of MyHCI and MyHCIIb enhancers by Tcf4/β-catenin in fetal myofibers (Fig. 7H). Tcf and Lef proteins are the most downstream components of the canonical Wnt/β-catenin signaling pathway (Komiya and Habas, 2008). In response to the binding of Wnts to Frizzled and LRP receptors, stabilized β-catenin binds Tcf and Lef proteins, which activate expression of Wnt-responsive genes. Previous research in chick found a role of Wnt signaling in the generation of fiber type (Anakwe et al., 2003). Also, consistent with our results for Tcf4, we have shown that β-catenin positively regulates MyHCI during fetal mouse myogenesis (Hutcheson et al., 2009). Thus, Tcf4 intrinsic regulation of MyHCI is likely to occur via Wnt/β-catenin signaling. Tcf4 might directly regulate MyHCI by binding to the 3.5 kb promoter/enhancer of MyHCI (Hagiwara et al., 2007), as one conserved Tcf binding site is present in this region. In addition, we found that Tcf4 intrinsically and positively regulates MyHCIIb levels. This regulation of MyHCIIb is direct, as recently a conserved Tcf binding site in the MyHCIIb enhancer has been found to be necessary to achieve normal MyHCIIb levels and activation of this Tcf binding site is regulated by β-catenin (Shanely et al., 2009). Altogether, these data demonstrate that in fetal myofibers Tcf4 intrinsically regulates MyHCI and MyHCIIb, probably by direct binding of Tcf4 to their enhancers and activation by β-catenin.

Our analysis of multiple muscles, with different MyHC expression profiles, reveals that development of fiber type is not equivalently regulated in all muscles. In most muscles tested, Tcf4 positively regulates the expression of MyHCI and MyHCIIb during fetal myogenesis. However, loss of neither Tcf4 (shown in this paper) nor β-catenin (Hutcheson and Kardon, 2009) in fetal EDL myofibers affected the development of MyHCI$^+$ myofibers, indicating that the EDL is regulated differently from the other limb muscles. In addition, unlike limb muscles, MyHCemb in the diaphragm is intrinsically (as well as extrinsically) regulated by Tcf4. Thus, in some muscles Tcf4 intrinsically regulates MyHCI or MyHCemb, whereas in other muscles this is not the case. Potentially, differential sensitivity to Wnt signals during development might be a molecular mechanism to generate fiber type diversity in adult muscles.

Surprisingly, we find that Tcf4 intrinsically positively regulates both MyHCI and MyHCIIb, myosins associated with slow and fast myofibers, respectively, during myogenesis (Fig. 7H). Previous studies showed that Tcf/β-catenin signaling positively regulated MyHCI (Hutcheson and Kardon, 2009) or MyHCIIb (Shanely et al., 2009) expression. However, only when we comprehensively examined all MyHC isoforms, was it apparent that Tcf4/β-catenin is intrinsically regulating MyHCI and MyHCIIb simultaneously and so does not control diversification of muscle into slow or fast fiber types. Instead, Tcf4 more generally promotes the expression of mature MyHCs (as opposed to MyHCemb and MyHCperi), as MyHCI and MyHCIIb are the mature MyHCs expressed early and most broadly in limb myofibers (Lu et al., 1999). Thus, our data demonstrate that intrinsic regulation and development of fiber type is more complex than previously thought. Specification of fiber type is not simply regulated by binary slow/fast switches, but involves molecular regulation of fiber type maturation.

Connective tissue fibroblasts promote the switch from fetal to adult muscle

Vertebrate myogenesis occurs in successive embryonic, fetal, neonatal and adult phases. The transition from one phase to the next involves both the repression of developmental genes and the activation of genes expressed in more mature muscle. Regulation of these transitions is crucial for muscle maturation and probably involves both intrinsic and extrinsic factors, but the identity of these factors has been unknown. Recently, the transcription factor Nfix has been found to intrinsically regulate the transition from embryonic to fetal myogenesis (Messina et al., 2010). Here, we identify the connective tissue fibroblasts as an extrinsic source of signals that promotes the switch from fetal to adult muscle (Fig. 7H).

Beginning during fetal myogenesis and continuing into the adult, Tcf4-dependent signals from fibroblasts repress the expression of the developmental MyHCemb isoform. During normal muscle development, MyHCemb is highly expressed in embryonic and fetal myofibers, but then gradually declines until it is no longer detectable 3 weeks after birth (Agbulut et al., 2003; Lu et al., 1999). Tcf4 expressed in fibroblasts is crucial for regulating MyHCemb levels. Deletion of Tcf4 in fibroblasts led to a dramatic increase of MyHCemb levels at 6 weeks in all muscles examined. This indicates that Tcf4 within fibroblasts regulates downstream genes, the products of which non-cell-autonomously repress MyHCemb in neighboring myofibers. These Tcf4-regulated paracrine signals from fibroblasts begin to repress MyHCemb levels just prior to birth and maintain repression of MyHCemb in the adult. Thus, Tcf4-dependent signals from fibroblasts promote the transition from fetal to adult muscle, by repression of the developmental MyHCemb isoform, an isoform not repressed by Nfix (G. Messina, personal communication).

Our myoblast/fibroblast transwell experiments also revealed that connective tissue fibroblasts secrete factors, independent of Tcf4, that promote the formation of large multinucleate myofibers. We

found that myoblasts differentiated in the presence of connective tissue fibroblasts had a significantly higher fusion index than myoblasts cultured alone. This effect was neither contact-mediated nor Tcf4-dependent, and this increased fusion could result from increased numbers of myoblasts and/or increased differentiation and fusion. Thus, fibroblasts secrete Tcf4-independent signals that generally promote muscle maturation by promoting formation of more multinucleate myofibers, characteristic of adult tissue. Interestingly, classic quail-chick chimera studies had hinted that lateral-plated derived tissue (which includes the connective tissue) might regulate the rate of muscle maturation during development (Armand et al., 1983). Also, several in vitro studies (Cooper et al., 2004; Joe et al., 2010; Kusner et al., 2010; Melone et al., 2000; Quinn et al., 1990) show that postnatal fibroblasts are a source of secreted trophic signals for myogenic cells. Recently, the molecular nature of some of these postnatal fibroblast signals has begun to be revealed. Neonatal connective tissue fibroblasts express nitric oxide synthase, and nitric oxide is a known stimulator of myoblast fusion (Dahlman et al., 2010). Potentially, during development fibroblasts could be a source of nitric oxide, which promotes myoblast fusion.

In summary, our study reveals that connective tissue fibroblasts are an important source of both Tcf4-dependent and Tcf4-independent signals that extrinsically regulate the switch from fetal to adult muscle. The connective tissue promotes muscle maturation both by repressing developmental MyHCemb and promoting the formation of multinucleate myofibers.

Connective tissue-muscle interactions in development and disease

During development and adulthood, the connective tissue provides the molecular and cellular niche within which muscle resides. This close association of muscle and its connective tissue has long suggested that interactions between these two tissues are crucial for development. Previously, we and others (Hasson et al., 2010; Kardon et al., 2003) showed that the developing connective tissue is important for muscle morphogenesis, by determining the pattern of muscles formed. Here, we now show, using new genetic reagents, that the connective tissue is also an important regulator of myogenesis by regulating muscle fiber type and maturation. Our findings demonstrate that myogenesis, generally thought to be intrinsically controlled by a network of transcription factors, is critically regulated extrinsically by its connective tissue niche.

The importance of interactions between muscle and connective tissue are revealed further in several human diseases, in which disruptions in these interactions have severe consequences. A hallmark of Duchenne muscular dystrophy, a fatal disease caused by mutations in the muscle protein dystrophin, is increased connective tissue fibrosis (Duchenne, 1968). In turn, this fibrosis inhibits proliferation of myogenic progenitors and contributes to the progressive loss of muscle repair (Melone et al., 2000). Even more striking is the finding that Ullrich and Bethlem congenital muscular dystrophies are caused by mutations in collagen VI, which is expressed exclusively by connective tissue fibroblasts and not by myogenic cells (Zou et al., 2008). Therefore, these muscular dystrophies result from the non-cell-autonomous effects of fibroblasts on muscle. Understanding the cellular and molecular nature of this connective tissue-muscle dialog will be crucial for understanding normal muscle development and the etiology and progression of muscle diseases.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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