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The Nuclear Orphan Receptor COUP-TFII Is Required for Limb and Skeletal Muscle Development

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The nuclear orphan receptor *COUP-TFII* is widely expressed in multiple tissues and organs throughout embryonic development, suggesting that COUP-TFII is involved in multiple aspects of embryogenesis. Because of the early embryonic lethality of *COUP-TFII* knockout mice, the role of COUP-TFII during limb development has not been determined. *COUP-TFII* is expressed in lateral plate mesoderm of the early embryo prior to limb bud formation. In addition, *COUP-TFII* is also expressed in the somites and skeletal muscle precursors of the limbs. Therefore, in order to study the potential role of COUP-TFII in limb and skeletal muscle development, we bypassed the early embryonic lethality of the *COUP-TFII* mutant by using two methods. First, embryonic chimera analysis has revealed an obligatory role for COUP-TFII in limb bud outgrowth since mutant cells are unable to contribute to the distally growing limb mesenchyme. Second, we used a conditional-knockout approach to ablate *COUP-TFII* specifically in the limbs. Loss of COUP-TFII in the limbs leads to hypoplastic skeletal muscle development, as well as shorter limbs. Taken together, our results demonstrate that COUP-TFII plays an early role in limb bud outgrowth but not limb bud initiation. Also, COUP-TFII is required for appropriate development of the skeletal musculature of developing limbs.

The muscles of the limbs and ventral body wall originate from the somites, epithelial spheres of paraxial mesoderm that form in a rostral-to-caudal direction along both sides of the neural tube (10, 12, 13, 15). The somites give rise to two separate muscle-forming cell populations. First of all, cells of the dorsomedial dermomyotome give rise to the epaxial myotome, which eventually develops into the muscles of the back. These epaxial muscle precursors express MyoD and other muscle-specific proteins and are dependent on signals arising from the notochord and the floor plate for their differentiation (8, 21, 40, 45, 49). Second, cells of the ventrolateral dermomyotome generate the hypaxial muscle lineage, whose developmental fate is dependent on the rostrocaudal positioning of the somite. At interlimb levels, the lateral dermomyotome generates the thoracic and abdominal muscles (14). For somites that are located adjacent to the limbs, however, the muscle precursor cells of the lateral dermomyotome undergo an epithelial-mesenchymal transition and migrate to the limbs and diaphragm, where they subsequently proliferate and then undergo differentiation (5, 7, 11, 22). These migrating muscle precursors do not express myogenic determination genes until after they have reached their targets in the limbs. Although the signals regulating hypaxial cell specification are not as well understood, it is believed that signals from the surface ectoderm are required for their formation (9, 20, 53, 55).

Once the migrating muscle precursor cells reach the limbs, they populate the dorsal and ventral mesenchyme as two premuscular masses. These premuscular masses consist of two parts, a surface layer of proliferating muscle precursor cells and a deeper layer of differentiating myoblasts that express myogenic differentiation markers such as myogenin. These two populations strike a balance between proliferation and differentiation (42) such that any increase in proliferation leads to an increase in muscle size. However, if terminal differentiation is induced prematurely, there has not been enough time for adequate proliferation to occur and the result is smaller muscles (28). This balancing act is mediated by the activity of several signals. An increase in the proliferative pool can be induced by FGFs, IGFI, and BMPs, whereas a decrease in muscle mass can be achieved through the use of BMP antagonists such as noggin (1, 4, 26, 31). Furthermore, activation of the Notch pathway has been shown to cause muscle precursor cells to continue to express *Pax3* and *Myf5*, downregulating *MyoD* and thereby preventing the onset of terminal differentiation (17). Recently, Sonic hedgehog (SHH) has been demonstrated to play a role in limb skeletal muscle size by acting as a survival and proliferation factor. Loss of SHH activity results in hypoplastic limb muscles, whereas ectopic overexpression in the chick is able to cause muscle hypertrophy, possibly by upregulating the BMP pathway (2, 23, 37).

Chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) is a nuclear orphan receptor of the steroid-thyroid hormone receptor superfamily (57). *COUP-TFII* is most highly expressed during embryonic development, and mutation of this gene results in early embryonic lethality because of defects in angiogenesis and heart development (43, 44, 47). Previous study of cell cultures has indicated a role for COUP-TFII in muscle development by showing that COUP-TFII inhibits the expression and transcriptional function of MyoD, thereby preventing muscle differentiation (3, 41). Fur-

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thermore, COUP-TFII lies downstream of SHH signaling and may therefore be a mediator of the SHH pathway in certain developmental situations (35, 36). Although COUP-TFII is well defined biochemically, its physiological functions during embryogenesis remain largely undefined. This is in large part because of the fact that the *COUP-TFII* mutant is an early embryonic lethal mutant that dies prior to E10 because of angiogenesis and cardiovascular failure, preventing the study of the role of COUP-TFII in the development of tissues and organs at later stages. In this study, we addressed the function of COUP-TFII during embryonic limb development. We used both embryonic chimera and tissue-specific knockout techniques to bypass the early embryonic lethality of the *COUP-TFII* knockout. By embryonic chimera analysis, we demonstrated that COUP-TFII is required for mesenchymal cells to contribute to the distally growing limb bud in chimeric embryos. In addition, tissue-specific knockout of *COUP-TFII* in the limb bud results in hypoplastic musculature. Therefore, COUP-TFII is required for limb bud outgrowth and proper limb muscle development.

MATERIALS AND METHODS

Generation of stable ES cell lines. Stable embryonic stem (ES) cell lines homozygous for the *COUP-TFII* mutation were generated and marked with the ubiquitously expressed *ROSA26-LacZ* allele (27), and *COUP-TFII*$^{+/-}$ mice were naturally mated with *COUP-TFII*$^{+/-}$ *ROSA26*$^{+/-}$ mice (mixed 129Sv-C57BL/6 background). Blastocysts were then isolated by flushing the uterine horns of pregnant females at embryonic day 3.5 (E3.5), where the day of vaginal plug detection is designated E0.5. Uteri were flushed with sterile ES medium (high-glucose Dulbecco modified Eagle medium, 15% fetal calf serum, 1% L-glutamine, 0.5% penicillin-streptomycin, 0.18% β-mercaptoethanol) supplemented with 0.02 M HEPES. Blastocysts were then transferred onto a 60-mm-diameter tissue culture dish containing a feeder layer of mitotically inactivated SNL cells in ES medium and allowed to adhere and grow for approximately 4 to 6 days at 37°C in 5% CO_2. Once the inner cell mass outgrowth had reached an appropriate size (large colonies but prior to any cell death or differentiation), colonies were picked and disaggregated first by treatment with 0.25% trypsin–EDTA and then pipetting up and down. Disaggregated colonies were plated into individual wells of a feeder layer-coated 24-well dish. Cells were fed with new medium daily and passed 1:1 every 3 days to a new well until ES cells became apparent. ES cell lines were genotyped by PCR for *COUP-TFII* and by PCR and β-galactosidase (β-gal) staining for *LacZ*. ES cell lines were also karyotyped for normal chromosome complement as described by Hogan et al. (32). Both wild-type control and mutant ES cell lines were generated in this fashion.

Generation of chimeric embryos. ES cells were plated and grown until they reached 90% confluency; they were then passed 1:3 one time until they reached 90% confluency once again prior to trypsinization and resuspension in injection medium (ES medium) before being placed on ice. After 30 min, most SNL feeder cells and debris have settled to the bottom of the tube. E3.5 C57BL/6 blastocysts were flushed out of uteri with injection medium. Malformed or early stage embryos were sorted out, and the remaining mature blastocysts were washed in M2 medium. Twelve to 14 ES cells were microinjected into the blastocoels of the embryos. After all of the embryos had been microinjected, they were transferred into the uteri of day 2.5 pseudopregnant ICR foster mothers at no more than 10 embryos per uterine horn. Subsequently, embryos were dissected at mid-gestational stages for 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining and analysis. Embryonic age was determined by the uterine age of the foster mothers.

β-Galactosidase staining of embryos. Embryos were dissected into cold 1× phosphate-buffered saline (PBS; Gibco) and fixed in 2% paraformaldehyde (PFA; Sigma) in 1× PBS–0.125 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.9)–1 mM $MgCl_2$–5 mM EGTA (pH 8.0) for 15 to 45 min at room temperature, depending on the size of the embryo (E9.5 to E12.5, respectively). Embryos were then washed three times with PBS for 20 min each time. Embryos were stained in 2 mM $MgCl_2$–0.01% deoxycholate–0.02% NP-40–100 mM phosphate buffer (pH 8.0)–5 mM $K_4Fe(CN)_6$–5 mM $K_3Fe(CN)_6$–1 mg of X-Gal per ml. After staining, embryos were washed in PBS and stored in 4% PFA at 4°C.

Whole-mount in situ hybridization. Whole-mount in situ hybridization was performed with probes to *myogenin* (from Eric Olson) and *Lbx1* (from Krzysztof Jagla) as templates for the generation of digoxigenin-labeled antisense riboprobes. The protocols used were essentially the same as those published previously (46, 48).

Immunohistochemical staining. Embryos were fixed in 2% PFA–PBS (pH 7.4) for 1 h, cryoprotected in 30% sucrose–PBS (overnight, 4°C), and frozen in PBS containing 15% sucrose and 7.5% gelatin. Frozen tissues were sectioned at a thickness of 10 μm in a cryostat. The sections were air dried for 1 h at room temperature and stored at −20°C until used.

The air-dried sections were washed in PBS and incubated in blocking buffer (PBS, 1% bovine serum albumin, 5% serum, 10 μg of Fab fragment donkey anti-mouse IgG [heavy and light chains] per ml, 0.02% Triton X-100) for 30 min prior to incubation with the primary antibody (monoclonal anti-Pax3 [1:500; DSHB], anti-COUP-TFII [1:5,000; kindly provided by Toshiya Tanaka, Department of Molecular Biology and Medicine, The University of Tokyo], or monoclonal anti-phospho-histone H3 [1:200; Cell Signaling Technology]) overnight at 4°C. After being washed three times with PBS, the sections were incubated with biotin-SP-conjugated donkey anti-mouse IgG (heavy and light chains; Jackson ImmunoResearch Laboratories) for 60 min at room temperature. Antibody binding was visualized with TSA kit no. 22 (Molecular Probes) in accordance with the manufacturer's recommendation.

For double immunostaining, the sections were first incubated with the diluted first primary antibody, monoclonal anti-COUP-TFII (1:5,000) as described above and staining was amplified with TSA kit no. 22. Subsequently, sections were incubated with the monoclonal anti-Pax3 second primary antibody and visualized by conventional fluorescent staining. Briefly, upon completion of the first immunostaining of COUP-TFII, the sections were treated with the blocking buffer for 30 min and then incubated with anti-Pax3 antibody overnight at 4°C. Antibody binding was visualized with the Cy3-conjugated donkey anti-mouse IgG (heavy and light chains; Jackson ImmunoResearch Laboratories) secondary antibody.

RESULTS

Expression of COUP-TFII indicates a role in limb and muscle development. To determine the function of COUP-TFII during embryonic limb development, we began by examining the tissue-specific expression pattern of the *COUP-TFII* gene. This was done by using a *LacZ* knock-in mouse generated in our laboratory, in which a nuclear *LacZ* gene was used to replace the *COUP-TFII* gene, thereby being expressed under the control of the *COUP-TFII* regulatory region (56). Therefore, the expression of *COUP-TFII* during limb development was monitored in heterozygotes by measuring β-gal activity by X-Gal staining. When E9.5 embryos were stained with X-Gal, it was found that *COUP-TFII* was expressed in the lateral plate mesoderm (LPM) from the earliest stages of limb bud initiation and outgrowth (Fig. 1A). Since the embryonic limb is initiated and first appears as an outgrowth of the LPM around early E9 (33, 58), this expression suggests that COUP-TFII has an important function during early limb bud development, either during limb bud initiation itself or in events following induction of the limb bud.

Interestingly, *COUP-TFII* is also expressed in the embryonic somites. *COUP-TFII* expression in the somites begins at E8.5 (data not shown) and can be clearly seen by E9.5 (Fig. 1B). This expression in the somites suggests that COUP-TFII plays a role in muscle development. At the limb levels, *COUP-TFII* is highly expressed throughout both the myotome and the dermomyotome of the developing somites (Fig. 1C). Therefore, it is possible that COUP-TFII functions in both epaxial and hypaxial lineages of somite-derived muscle cells (11, 22). First, its expression in the myotome suggests that COUP-TFII is important for development of the muscles of the body proper, including the intercostal muscles, abdominals, and tho-

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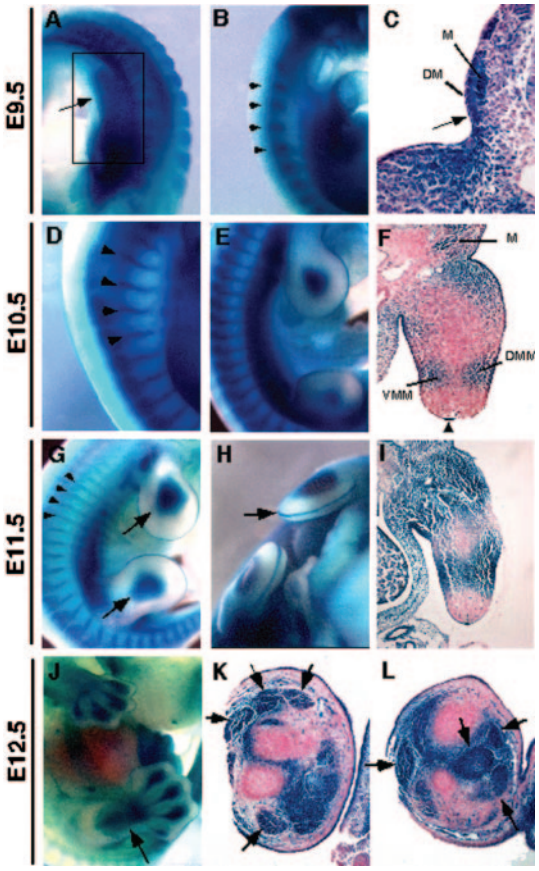

FIG. 1. Expression of *COUP-TFII*. (A and B) Whole-mount X-Gal staining of E9.5 embryos. *COUP-TFII* is expressed in the LPM (arrow and box in panel A) and somites (arrowheads in panel B). (C) Cross section of X-Gal-stained E9.5 embryos. At limb levels, *COUP-TFII* is expressed in the myotome and lateral dermomyotome, which gives rise to migrating muscle precursors (arrow). (D and E) Whole-mount X-Gal staining of E10.5 embryos. *COUP-TFII* is still expressed in somites (arrowheads in panel D) and becomes expressed in the dorsal and ventral muscular masses in the limb, as well as in the AER. (F) Cross section of X-Gal-stained E10.5 embryo. *COUP-TFII* is expressed in the myotomes and dorsal and ventral muscle masses, as well as the AER (arrowhead). (G and H) Whole-mount X-Gal staining of E11.5 embryos. *COUP-TFII* is expressed in somites (arrowheads in panel G), in premuscular masses (arrows in panel G), and in the AER (arrow in panel H). (I) Cross section of an X-Gal-stained E11.5 embryo. *COUP-TFII* is expressed in premuscle masses, as well as in other mesenchymal tissues of the limb. (J) Whole-mount X-Gal staining of an E12.5 embryo. *COUP-TFII* is expressed in the developing muscle bundles (arrow). (K and L) Frontal sections of fore and hind limbs of X-Gal-stained embryos at E12.5. Expression of *COUP-TFII* can be clearly seen in individual muscle bundles (arrows) and nonmuscle mesenchyme. Abbreviations: M, myotome; DM, dermomyotome; DMM, dorsal myogenic mass; VMM, ventral myogenic mass.

racic muscles. Second, its expression in the ventrolateral dermomyotome at limb levels suggests a role in the migrating hypaxial muscle lineage, which consists of cells that migrate from the somites to form the muscles of the limbs and diaphragm (7, 11, 20, 22, 52).

This expression pattern in muscle precursor cells persists in older embryos. At E10.5, *COUP-TFII* is still highly expressed in the somites (Fig. 1D). However, *COUP-TFII* is no longer expressed throughout the mesenchyme as it is at E9.5, but

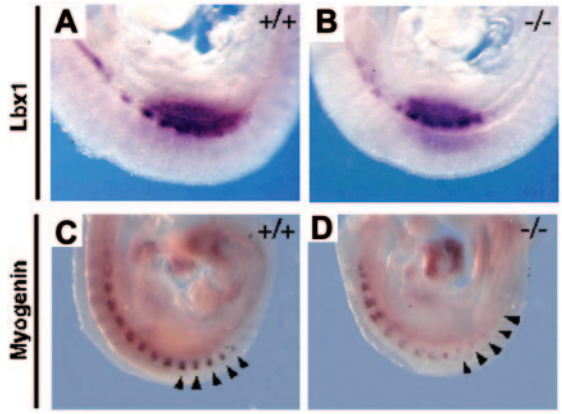

FIG. 2. Expression of marker genes for muscle migration and muscle differentiation. (A and B) Whole-mount in situ hybridization of E9.5 wild-type and mutant embryos for *Lbx1*. *Lbx1* expression is detected in both somites and limbs of *COUP-TFII* embryos, indicating that muscle precursor cells are able to migrate into the limbs. (C and D) Whole-mount in situ hybridization of wild-type and *COUP-TFII* mutant embryos for *myogenin*. Expression of *myogenin* is significantly decreased in caudal somites of *COUP-TFII* mutant embryos compared to that in wild-type embryos (arrowheads).

rather it is expressed in the fore and hind limb buds in a centralized core of the mesenchyme (Fig. 1E). Cross sections of these limbs demonstrate that *COUP-TFII* is expressed in two distinct centralized mesenchymal masses (Fig. 1F). One of these masses is located in the dorsal half of the limb, and the other is located in the ventral half. This expression pattern is highly reminiscent of the location of the dorsal and ventral premuscle masses, which are groups of mesenchymal tissue that eventually differentiate into the skeletal muscle elements of the limbs (11). Additionally, *COUP-TFII* is expressed in the apical ectodermal ridge (AER) at this age (Fig. 1F; also E11.5, in Fig. 1H). By E11.5, *COUP-TFII* is still expressed in the myogenic mesenchyme (Fig. 1G), and by E12.5, *COUP-TFII* is expressed in the individual differentiating muscle bundles of both the fore and hind limbs (Fig. 1J, K, and L). *COUP-TFII* also appears to be expressed in other mesenchymal tissues, which are not associated with developing muscle at both E11.5 and E12.5.

COUP-TFII is required for somitic myogenesis. On the basis of the somitic expression of *COUP-TFII* in the myotomes and limb level dermomyotomes, we hypothesized that COUP-TFII may play a role in embryonic muscle development. To determine if COUP-TFII plays a role in hypaxial muscle precursor cell migration, we analyzed *COUP-TFII* null mutants for the expression of Lbx1, a hypaxial lineage marker (30). Whole-mount in situ hybridization for *Lbx1* in both wild-type and *COUP-TFII* null mutant embryos shows positively expressing cells in both the somites and the limbs, but at a slightly reduced level in *COUP-TFII* mutant embryos (Fig. 2A and B). This result suggests that muscle precursor cells are able to migrate to the limb but may be at a reduced level.

Next, we examined the expression of the myogenic determination factor *myogenin* in wild-type and *COUP-TFII* mutant embryos in order to determine if COUP-TFII plays a role in myogenic differentiation. Since *COUP-TFII* mutants are embryonic lethal prior to E10 (44), we analyzed E9.5 embryos at

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the 22-somite stage of development by whole-mount in situ hybridization. *myogenin* expression in *COUP-TFII* mutant embryos is significantly lower than in wild-type embryos of the same age (Fig. 2C and D). Although *myogenin* is still expressed in the mutant embryos, it does not extend as far in the caudal axis as in wild-type embryos and the level of *myogenin* expression is much reduced at the caudal end. This indicates that COUP-TFII is important for muscle development and suggests that either COUP-TFII is directly involved in the myogenic differentiation pathway or that, in the absence of COUP-TFII, there are fewer differentiating muscle cells.

Chimera analysis reveals an early cell-autonomous role for COUP-TFII in limb bud outgrowth. On the basis of the *COUP-TFII* expression pattern in limbs, we examined the role of COUP-TFII during limb development. To circumvent the early lethality of our mutant, we used an embryonic chimera approach (50). This approach consists of microinjecting wild-type or *COUP-TFII* mutant ES cells into wild-type blastocysts in order to generate chimeric embryos that are composed of a mixture of ES cell-derived and blastocyst-derived cells (32, 34). Since the ES cells were derived from mice containing the ubiquitously expressed *ROSA26-LacZ* transgene (27), they can be differentiated from the wild-type blastocyst-derived cells by detecting β-gal enzyme activity with X-Gal.

With two independently generated mutant ES cell lines, chimeric embryos were stained for β-gal enzyme activity. The percent chimerism of each embryo was visually estimated by examining sections of each embryo for the extent of X-Gal staining. Five mutant chimeras composed of greater than 50% mutant cells analyzed at E10.5 show a distinct white patch at the distal end of the limb bud (Fig. 3A), while chimeric embryos generated with wild-type ES cells show an even distribution of X-Gal-positive tissue (Fig. 3D). This result indicates that the mutant cells are not able to maintain contribution to the limb bud and demonstrates that COUP-TFII has a cell-autonomous function during limb development. By E11.5 and E12.5, it is very clear that *COUP-TFII* mutant cells are unable to contribute to the limb bud, since virtually the entire limb is devoid of blue-staining mutant cells by these ages (Fig. 3B and C). In contrast, wild-type cells have no difficulty populating the entire limb bud at E11.5 and E12.5 (Fig. 3E and F). This suggests that COUP-TFII has a critical cell-autonomous function during limb bud outgrowth.

To analyze this phenotype further, we examined cross sections of chimeric limbs and found that while *COUP-TFII* mutant cells are initially able to contribute to the early stages of limb bud initiation and outgrowth at E9.5 (Fig. 3G), *COUP-TFII* mutant cells do begin to restrict from the very distal tip of the limb bud. By E10.5, the mutant cells are absent from approximately half of the limb bud, and by E11.5 only scattered mutant cells are present in the limb (Fig. 3H and I). In contrast, wild-type ES cell-derived cells can contribute to the whole limb at all of the stages examined without any obvious deficiencies (Fig. 3J to L). These results suggest that COUP-TFII is not required for limb bud initiation, since the early limb bud still contains *COUP-TFII* mutant cells. Rather, it is more likely that COUP-TFII plays an important, cell-autonomous role in later stages of limb bud outgrowth. This observation is consistent with the fact that the conventional *COUP-TFII* knockout, which is embryonic lethal prior to E10, still displays

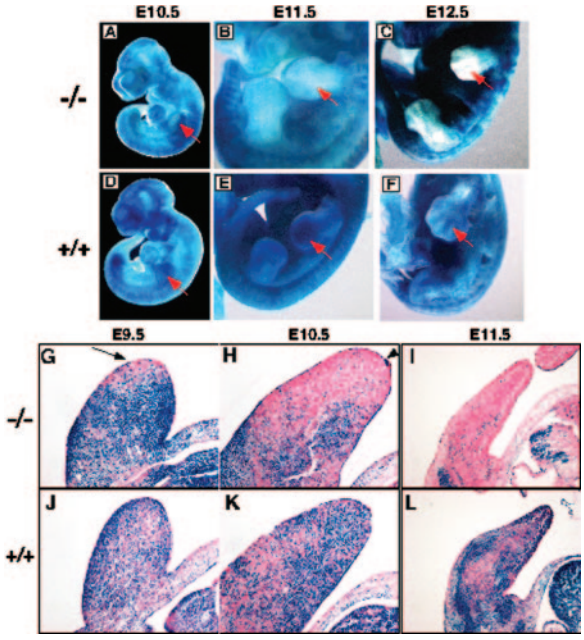

FIG. 3. COUP-TFII is required cell autonomously in limb mesenchyme. Whole-mount X-Gal staining (A to F) and cross sections of X-Gal-stained chimeric embryos (G to L). (A to C) Chimeric embryos restrict mutant cells progressively starting at E10.5, as shown by the white patches visible in chimeric E10.5 limbs (arrow in panel A) and the almost completely white limbs at E11.5 and E12.5 (arrows in panels B and C). (D to F) Wild-type chimeras do not have restriction of ES cell-derived cells from the limbs (arrows), which are uniformly blue. (G to I) Cross sections of X-Gal-stained mutant chimeras show that exclusion of mutant cells begins at E9.5 (arrow in panel G) and is largely complete by E12.5. Mutant cells are able to contribute to the AER (arrowhead in panel H). (J to L) Wild-type ES cell-derived cells are able to contribute to the limbs at all stages.

an early limb bud. Interestingly, COUP-TFII also appears only to be required cell autonomously for contribution to the limb mesenchyme, since both the surface ectoderm of the limb and the AER still contain *COUP-TFII* mutant cells (arrowhead in Fig. 3H).

Conditional ablation of COUP-TFII in limbs reveals a hypoplastic-muscle phenotype. To further assess the function of COUP-TFII in limb outgrowth, the Cre/loxP system was used to ablate *COUP-TFII* in the limbs. We have generated an allele of *COUP-TFII* in which the *COUP-TFII* locus is flanked by *loxP* sites (*COUP-TFII*^{flox}) (56). Upon exposure to the Cre recombinase enzyme, the *COUP-TFII* coding region is excised and replaced with a *LacZ* reporter gene, which is then expressed under the control of the *COUP-TFII* regulatory region. Consequently, tissue-specific deletion of the *COUP-TFII* gene in cells that normally express COUP-TFII can be identified by X-Gal staining. To generate a limb-specific knockout of *COUP-TFII*, we used the *Prx1-Cre* transgenic mouse. This mouse contains a transgenic *Cre* recombinase gene under the control of the *Prx1* enhancer, which directs *Cre* expression specifically throughout the limb bud mesenchyme, body wall tissue, and some craniofacial mesenchyme (38). When this strain was crossed with our *COUP-TFII*^{flox} mice (Fig. 4A and B) or with ROSA reporter mice (54; data not shown), staining for β-gal activity demonstrated that recombination occurs spe-

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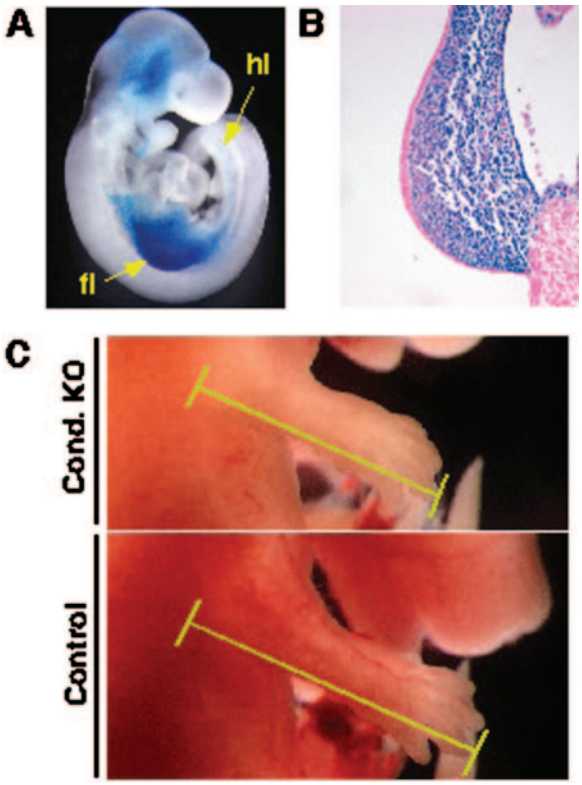

FIG. 4. Conditional mutation of *COUP-TFII* in the limb. (A) Whole-mount X-Gal staining of embryos derived by crossing *Prx1-Cre* mice with *COUP-TFII*^{flox} mice. Significant recombination has occurred in the forelimb at E9.5, but few cells are recombined in the hind limb. (B) Histological sections of an embryo identical to that in Fig. 5A show a high percentage of recombination in the forelimb at this age. (C) Conditional-mutant forelimbs at E15.5 are shorter than those of a wild-type littermate, although individual limb elements appear to be appropriately specified. The bar approximately brackets the limb between the shoulder and the tip of the digits. Abbreviations: fl, forelimb; hl, hind limb; Cond. KO, conditional knockout.

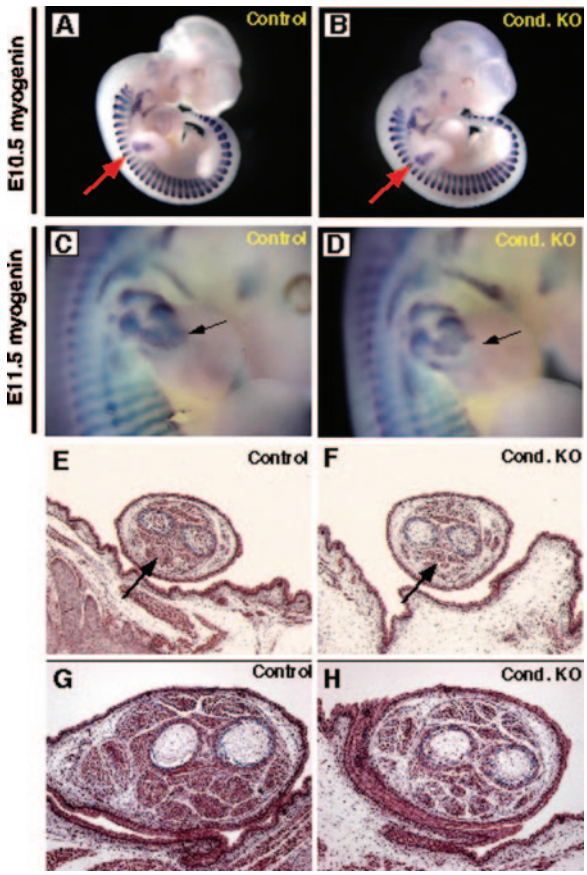

FIG. 5. Loss of *COUP-TFII* leads to hypoplastic muscles. (A and B) Whole-mount in situ hybridization for *myogenin* of control and conditional-knockout (Cond. KO) embryos at E10.5. There is no significant difference in expression (arrows) between control and conditional-knockout embryos. (C and D) Whole-mount in situ hybridization for *myogenin* of control and conditional-knockout embryos at E11.5. The region of *myogenin* expression does not extend as far distally in the conditional-knockout embryo as in the control embryo (arrows). (E to H) Trichrome staining of frontal sections of an E17.5 forelimb. Compared to that of control embryos at E17.5 (E and G), conditional-knockout limb bud musculature is hypoplastic and reduced in size (F and H). Although most individual muscle bundles appear to be appropriately specified, they are significantly smaller (arrows).

cifically in the limb bud (Fig. 4A and B). The recombination induced by the *Prx1-Cre* transgene is initiated around E9.5 in the forelimb and is not completed until E10.5. In the hind limb at E9.5, however, Cre recombinase activity was only detected in a few scattered cells (Fig. 4A).

When embryos containing the *Prx1-Cre* transgene and *COUP-TFII*^{flox} alleles were analyzed, we observed that while patterning and other general developmental features appeared to be unaffected, the mutant limbs were almost always noticeably shorter than those of their controlled littermates (*Prx1-Cre* or *COUP-TFII*^{flox}). This first appeared distinctively at E12.5 and was particularly obvious at E15.5 (Fig. 4C), when the mutant limb was approximately 85% of the length of that of the wild-type littermate. When these embryos were stained for skeletal preps, however, it appeared that all of the skeletal elements were intact (data not shown). Consequently, we believe that COUP-TFII may play a role in limb outgrowth, and this may reflect a function of the early expression of COUP-TFII in the LPM at E9. Since recombination of *COUP-TFII*^{flox} did not take place until E9.5, at a time when *COUP-TFII* is already expressed, the incomplete excision of *COUP-TFII* during this critical time period may contribute to the partial shortening of the limbs seen at later ages. Although there was shortening of the forelimbs, there was no defect in the hind limbs of the conditional knockouts at any of the embryonic development stages; this may have been due to the low Cre recombinase activity in the hind limbs of the *Prxl-Cre* mice used (Fig. 4A).

To analyze whether COUP-TFII plays a role in the development of the skeletal muscle elements of the limbs, we began by looking at the expression of the differentiation marker *myogenin* by in situ hybridization. At E10.5, *myogenin* is expressed in the somitic myotomes, as well as in the mesenchyme of the limb buds. In the limb-specific knockout embryos, *myogenin* was clearly expressed in the limb buds at this age and there appeared to be no detectable difference in expression between the limb-specific *COUP-TFII* knockout mutant (Fig. 5B) and control (Fig. 5A) embryos. By E11.5, however, the *myogenin* expression pattern was distinctively altered in the mutants (Fig.

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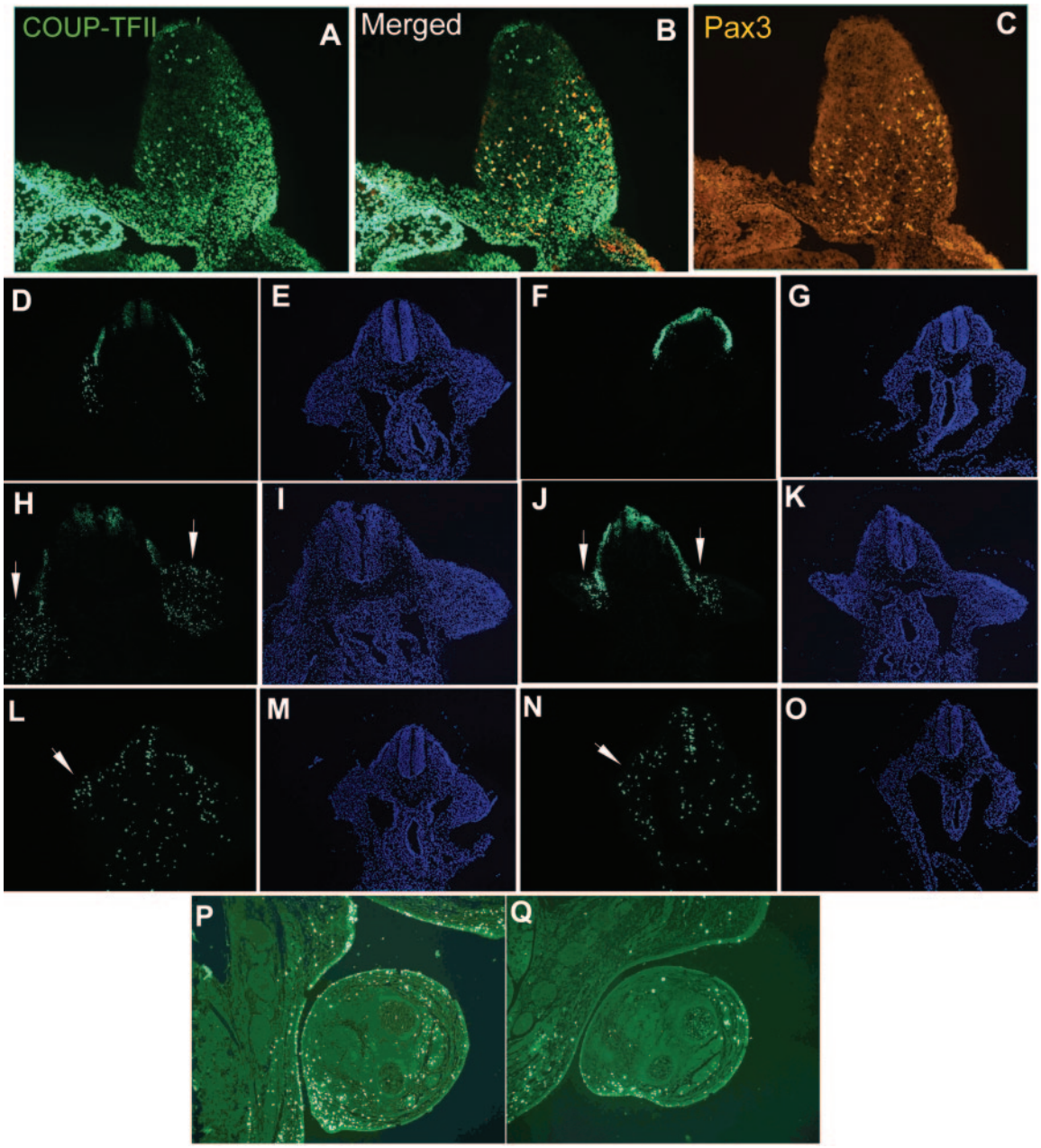

FIG. 6. Cell migration and proliferation. (A to C) Transverse sections through the forelimbs of E10.5 wild-type embryos stained with antibodies to detect COUP-TFII (green) and Pax3 (red). Pax3-expressing cells are seen delaminating from the dermomyotome, and migrating muscle precursors coexpress Pax3 and COUP-TFII. (D to K) Transverse sections through the forelimbs of E9.5 (D to G) and E10.5 (H to K) control embryos (D, E, H, and I) and conditional-knockout littermates (F, G, J, and K) stained with antibody Pax3 (green) and 4′,6′-diamidino-2-phenylindole (DAPI) (blue). (L to O) Transverse section through the forelimbs of an E9.5 control embryo (L and M) and a conditional-knockout littermate (N and O) stained with antibody for phospho-histone H3 (green) and DAPI (blue). (P and Q) Frontal sections of the forelimbs of an E14.5 control embryo (P) and a conditional-knockout littermate (Q) stained with antibody for phospho-histone H3 (green).

5D) in comparison with that in the control (Fig. 5C). At this embryonic age, what was initially a solid mesenchymal core of differentiating muscle has begun to segregate into separate muscle bundles expressing myogenin. When mutant embryos were examined and compared to control littermates, it was clear that the mutant limb bud contained muscle bundles that did not extend distally to the same extent as in the control (Fig. 5C and D). This result was observed consistently in multiple experiments and strongly suggests that COUP-TFII is required for appropriate development of the limb musculature.

When the limb-specific mutant embryos were examined histologically, it was found that the mutant limb buds had underdeveloped, hypoplastic musculature. This first became evident as early as E12.5 but became particularly pronounced by E17.5 (Fig. 5E to H). Both wild-type and mutant E17.5 fore limbs were sectioned frontally and compared to each other, and the

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individual muscle bundles of the mutant embryos were drastically smaller and less well developed. The diameters of the developing bones, however, appeared relatively unchanged. Interestingly, it appeared that all of the individual muscle bundles were present, indicating that COUP-TFII does not play a role in the patterning or specification of muscle tissue. Rather, all of the muscle bundles were hypoplastic in the mutant, suggesting that COUP-TFII is required for the establishment of muscle bundles of the appropriate size.

In order to understand the mechanism underlying the role that COUP-TFII plays in limb development, we carried out marker analysis to determine whether COUP-TFII plays a role in muscle precursor cell proliferation and migration. Previous RNA in situ analyses of *Lbx1* expression showed that *Lbx1* is expressed in the ventral dermomyotome at the limb levels in presumptive migratory muscle precursors, and there is a reduction of *Lbx1* expression in *COUP-TFII* mutant embryos (Fig. 2A and B). This result raises the possibility that COUP-TFII is involved in muscle precursor cell migration. To support this hypothesis, it is important to demonstrate that *COUP-TFII* is coexpressed in the same precursor cells as the myogenic precursor cell marker Pax3. Therefore, the expression of *COUP-TFII* and *Pax3* was examined in wild-type embryos at E10.5 by immunohistochemistry. The COUP-TFII antibody detected high *COUP-TFII* expression in the limb and the adjacent mesenchymal cells (Fig. 6A). Specific Pax3 antibody detected *Pax3* expression in the dorsal neural tube, in dorsal root ganglia, in dermomyotomes (data not shown), and in scattered cells in the forelimb (Fig. 6C). Most importantly, all positively Pax3-stained cells in the forelimb coexpressed *COUP-TFII* (Fig. 6B). Therefore, COUP-TFII is expressed in the myogenic precursor cells and may play a role in muscle precursor cell migration.

Next, we analyzed the muscle precursor cell migration in the conditional knockouts and the control littermate embryos by Pax3 immunostaining. We observed a migration defect in the conditional knockouts at E9.5 and E10.5 (Fig. 6F and J) in which the Pax3-positive cells did not extend as far into the limb axis as in the control embryos (Fig. 6D and H). However, the observed migration defect in the conditional knockouts was no longer detected at later stages (i.e., E11.5 or later; data not shown). This suggested that ablation of *COUP-TFII* delays early muscle precursor cell migration and contributes partly to the defects observed.

To delineate the other factor contributing to the limb defect of the *COUP-TFII* mutant, we analyzed the proliferation activity of control and conditional-knockout embryos at different developmental stages by phospho-histone H3 immunostaining (a mitotic index marker). We found no significant difference in the proliferation index between control (Fig. 6L) and conditional-knockout (Fig. 6N) embryos at E9.5, while a reduction in the mutant (Fig. 6Q) compared to the controls (Fig. 6P) was observed later, at E14.5. Thus, a reduction in cell proliferation in conditional knockouts is likely also responsible for the observed shorter-limb phenotype in addition to delayed migration.

DISCUSSION

The nuclear orphan receptor *COUP-TFII* is highly expressed in multiple tissues throughout embryonic development. In this study, we have investigated the role of COUP-TFII in limb bud outgrowth and muscle development. *COUP-TFII* is expressed throughout the LPM of the limb field prior to limb bud initiation and then becomes highly expressed in the early limb mesenchyme. In embryonic chimera analyses, *COUP-TFII* mutant cells are initially able to contribute to the early stages of limb bud development, but at later ages they become progressively restricted from the distally growing limb bud mesenchyme. This shows that *COUP-TFII* mutant cells are unable to successfully compete against wild-type cells in a chimeric environment during limb bud outgrowth. Furthermore, tissue-specific ablation of *COUP-TFII* in the developing limb bud mesenchyme also results in shorter limbs, providing additional evidence to support a role for COUP-TFII in limb bud outgrowth. Therefore, COUP-TFII is important for limb bud outgrowth but not limb bud initiation.

To determine whether the shorter limb is caused by more cell death in conditional knockouts, we did terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling staining (29); however, we did not detect any significant difference between the conditional knockouts and the controls by this assay at any stage of development (data not shown). This suggests that the shorter limbs in the conditional-knockout mutant are not due to cell death. Similarly, analysis of *COUP-TFII* conditional-knockout embryos at E9.5 has failed to show any significant difference in proliferation compared to that in the littermate control embryos. However, at later ages it becomes obvious that the mesenchyme of limbs lacking COUP-TFII proliferates at a lower rate than in wild-type embryos. This further suggests that COUP-TFII is not involved in limb bud initiation but rather is required for the maintenance of limb bud outgrowth. Similar findings have been reported for the FGF signaling pathway. Loss of FGFR1 or its downstream effector Shp2 also leads to defects in limb bud outgrowth (18, 51). Interestingly, loss of FGFR1 also leads to hypoplastic muscles (25), establishing a link between the two developmental processes. *FgfR1* expression, however, does not appear to be significantly altered in our mutant, nor does the AER-specific growth factor *Fgf8* (data not shown).

In the absence of COUP-TFII, myogenesis does not proceed normally. Expression of *myogenin* is reduced in the somites at the caudal end of *COUP-TFII* mutant embryos. It appears that COUP-TFII is involved in hypaxial muscle precursor cell migration since there is a decrease in the expression of *Lbx1* in the *COUP-TFII* null mutant at E9.5. The muscle precursor cell migration defects are also observed in limb buds of the conditional knockout at E9.5 and E10.5 by immunostaining with Pax3 antibody. Both *Lbx1* and *Pax3* are markers of hypaxial muscle precursor cell migration (6, 16, 19, 24, 39). In addition, the muscles of the limbs fail to develop to the appropriate size when *COUP-TFII* is specifically ablated in the limb mesenchyme. This defect in muscle development is reflected by the altered expression of the myogenic differentiation gene *myogenin* in both somitic and limb muscle lineages. This phenotype is manifested as a reduction in muscle size, although it appears that all of the individual muscle bundles of the limb are appropriately specified and develop in their proper positions. Consequently, it appears that COUP-TFII is not involved in the patterning or specification of the limb muscle but may be involved in the maintenance or expansion of the muscle lin-

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eage. In addition, COUP-TFII is required for the appropriate migration of limb muscle precursor cells from the somites to the limb buds. Since other genes known to be involved in muscle cell migration are still expressed in the *COUP-TFII* mutant, this suggests that COUP-TFII may be involved in a novel pathway of muscle cell migration that has not previously been described. It is important to analyze this defect in the future.

A current model of myogenesis proposes that a balance between muscle growth and differentiation must be maintained in order for proper muscle development to occur (28, 42). COUP-TFII has been shown in cell culture to inhibit both the expression and the posttranscriptional regulation of MyoD (3, 41), which is required to initiate the myogenic program, which inevitably causes muscle precursor cells to exit the cell cycle and differentiate. It is therefore conceivable that COUP-TFII is required to inhibit muscle differentiation, thereby maintaining the pool of undifferentiated cells. Furthermore, a recent study suggests that the mitogen SHH is required for repression of the differentiation of myoblasts in order to stimulate their proliferation. Without SHH, the myoblasts differentiate precociously, which eliminates the pool of proliferating myoblasts, leading to less muscle (37). Our laboratory has previously demonstrated that COUP-TFII lies downstream of SHH signaling in vitro (35, 36). Furthermore, we have recently shown that COUP-TFII may act as an effector of SHH signaling in vivo in the development of the stomach (56). Consequently, it is conceivable that COUP-TFII lies downstream of SHH to function in the maintenance and expansion of muscles by inhibiting terminal myogenesis.

In conclusion, we have established critical roles for COUP-TFII in limb and muscle development. COUP-TFII is required for the distal outgrowth of embryonic limbs and also for appropriate embryonic muscle development. COUP-TFII is required for the maintenance of proliferation in the growing limb bud, as well as the migration of muscle precursor cells to the limbs. COUP-TFII represents a novel player in this complex field, and it will be exciting to further define the exact molecular pathways in which COUP-TFII functions.

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