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Developmental Dynamics

Characterization of Genomic Structures and Expression Profiles of Three Tandem Repeats of a Mouse Double Homeobox Gene: *Duxbl*

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We identified and cloned a mouse double homeobox gene (*Duxbl*), which encodes two homeodomains. *Duxbl* gene, a tandem triplicate produces two major transcripts, *Duxbl* and *Duxbl-s*. The amino acid sequences of Duxbl homeodomains are most similar to those of human DUX4 protein, associated with facioscapulohumeral muscular dystrophy. In adult tissues, *Duxbl* is predominantly expressed in female reproductive organs and eyes, and slightly expressed in brain and testes. During gonad development, *Duxbl* is expressed from embryonic to adult stages and specifically expressed in oocytes and spermatogonia. During embryonic development, *Duxbl* is transcribed in limbs and tail. However, Duxbl proteins were only detected in trunk and limb muscles and in elongated myocytes and myotubes. In C2C12 muscle cell line, *Duxbl* expression pattern is similar to differentiated marker gene, *Myogenin*, increased in expression from 2 days onward in differentiating medium. We suggest that Duxbl proteins play regulatory roles during myogenesis and reproductive developments. *Developmental Dynamics 239:927–940, 2010*. © **2010 Wiley-Liss, Inc.**

Key words: double homeobox gene; Duxbl; myogenesis; reproductive development; mouse embryo

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INTRODUCTION

Homeobox genes encode transcription factors that regulate embryonic development programs including organogenesis, axis formation, and limb development (McGinnis and Krumlauf, 1992; Boncinelli, 1997). Their products regulate the expressions of target genes in tissue- and spatiotemporal-specific manners through conserved DNA-binding motifs called homeodomains. Homeodomains have three α-helical segments of which the third constitutes the main DNA recognition site and binds to the major groove of DNA (Gehring et al., 1994). This sequence-specific binding allows homeodomain proteins to activate or repress the expression of a battery of downstream target genes. The correct expressions of homeodomain proteins in adult tissues including liver, kidney, and intestine are important for the regulations of cellular morphogenesis, growth, and differentiation (Cillo et al., 2001).

Different homeobox genes are classified through similarities in amino acid sequences within their homeodomains and the other coding regions of their gene products (Galliot et al., 1999; Holland and Takahashi, 2005). The *paired* (PRD) class is divided into two subclasses: the *PAX* subclass and the *PAXL* subclass (Holland et al., 2007). The *PAX* subclass homeodomain proteins have a conserved 130-amino-acid DNA-binding domain, the paired domain, upstream of their homeodomains (Bopp et al., 1986). The *PAX* gene family is an ancient and remarkably conserved gene family, which plays key roles in the formations of tissues and organs during embryogenesis. *PAX3* and *PAX7* mark myogenic progenitor cells and

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regulate their behaviors and entries into the program of skeletal muscle differentiation (Buckingham and Relaix, 2007). *PAX6* is required for eye formation in vertebrates and its homologues in invertebrates, such as *eyeless* in *Drosophila* also play a crucial role in eye formation (Kozmik, 2005). The *PAXL*-subclass homeodomain proteins show significant sequence similarities (55–75%) to PRD-class homeodomain proteins but lack the paired domain, and contain a glutamine residue at position 9 of the third helix in their homeodomains (Burglin, 1994). Another common feature of *PAXL* homeobox genes is the presence of an additional intron within their homeoboxes, between the region that encodes position 46 and 47 homeodomain amino acid residues. Many *PAXL* homeobox genes, such as *Rax* (retinal homeobox), *Arx* (aristaless-related homeobox), and *Vsx* (visual system homeobox), are expressed in the nervous system and during brain or eye morphogenesis (Mathers et al., 1997; Miura et al., 1997; Ohtoshi et al., 2001). They play critical roles during embryonic developments.

The human double homeobox (*DUX*) genes encode two *PAXL*-subclass homeodomains. The *DUX* genes are present in multiple polymorphic copies with a 3.3-kilobase (kb) tandem repeat scattered in human heterochromatins (Ding et al., 1998; Gabriels et al., 1999; Beckers et al., 2001). The 3.3-kb dispersed *DUX* repeats in the D4Z4 locus of chromosome 4 (*DUX4*) have been found to be associated with the facioscapulohumeral muscular dystrophy (FSHD), the third most common form of inherited muscular dystrophy (Wijmenga et al., 1992; van Deutekom et al., 1993; Hewitt et al., 1994). It has been hypothesized that the larger *DUX4* copy numbers in nonaffected individuals are associated with an inhibitory chromatin structure preventing gene expressions, and the inhibition is relieved by the shorter *DUX4* arrays found in FSHD patients (Winokur et al., 1994; Tupler and Gabellini, 2004). The coding region of human *DUX4* gene shows evolutionary conservation (Clapp et al., 2007) and DUX4 protein can be detected in primary myoblasts extracted from FSHD patients (Belayew, 2004; Kowaljow et al., 2007). Previously, DUX4 protein is identified to have pro-apoptotic activity (Kowaljow et al., 2007) and is found to be a transcriptional activator of *PITX1* gene (Dixit et al., 2007). DUX4 expression recapitulates key features of FSHD molecular phenotype, including repression of MyoD and its target genes, and then diminished myogenic differentiation (Bosnakovski et al., 2008). However, the mechanism(s) that causes FSHD phenotype remain unclear. Furthermore, the in vivo functions of DUX4 protein, especially in normal tissues, remain unknown. Other human double homeobox genes are previously identified using *PRD*-class homeoboxes as query sequences to search human genome sequences, and they have been assigned into four paralogous groups including *DUXA*, *DUXB*, *DUXC*, and *DUXB*-like (*Duxbl*; Booth and Holland, 2007; Clapp et al., 2007). The molecular structures of their transcripts and the expression patterns and functions of their protein products have not been provided.

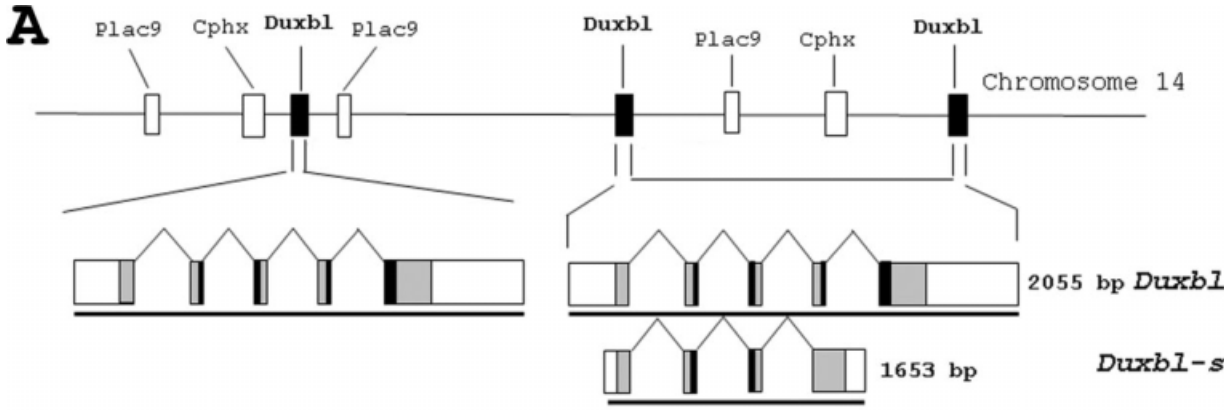
Recently, a novel mouse double homeobox gene has been reported as *Duxl* (Kawazu et al., 2007) and *Duxbl* (Clapp et al., 2007), respectively. This gene has been shown to play a critical role in CD4/CD8 double negative thymocyte development (Kawazu et al., 2007), but its detailed genomic structure, major transcript(s), expression patterns, and protein product(s) are not available. Here, we characterize the genomic structure of this mouse double homeobox gene, *Duxbl*, and suggest that the *Duxbl* gene is the mouse ortholog of human *DUX4* gene. The Duxbl protein is found expressed in adult tissues, including reproductive tissues, eyes, brain, but not in muscle. However, during embryo development, Duxbl is seen expressed in differentiated myocytes. The spatiotemporal expression patterns of *Duxbl* are also analyzed during gonad developments, and *Duxbl* is specifically expressed in germ cells, including oocytes and spermatogonia. Duxbl is predicted to play important regulatory roles during myogenesis and reproductive developments.

RESULTS AND DISCUSSION

Genomic Structure and RNA Transcripts of *Duxbl* Genes

To identify novel homeobox genes involved in early embryonic development, we screened a murine embryonic stem cell cDNA library by degenerate RT-PCR (Wang et al., 2003; Li et al., 2006). One of the genes identified contained a sequence similar to a Riken full-length cDNA clone (1110051B16 Rik) previously reported as *Duxl* (Kawazu et al., 2007) and *Duxbl* (Clapp et al., 2007), respectively. However, the genomic structure, expression profiles, protein product(s) of this gene, and in vivo function(s) of this gene product(s) are all unknown. Based on the sequence in the database (1110051B16Rik), the putative translated protein of this gene, *Duxbl*, contains two helix-turn-helix domains weakly similar to the known homeodomains. Analysis of the genomic structure of *Duxbl* gene based on information in Ensembl and NCBI databases localized it to the mouse chromosome 14A3, downstream of the *Cphx* gene (previously named as *Eso-1*, Li et al., 2006) and mapped to 24.82m (Gene ID: 48502; Fig. 1A). However, we discovered two other downstream *Duxbl* genes mapped to 24.96m (Gene ID: 72675) and 25.1m (Gene ID: 72672; Fig. 1A). These two downstream *Duxbl* genes are identical to each other, and share approximately 95% sequence identity with the upstream one (Gene ID: 48502). During analysis of the genomic structure of *Duxbl*, we observed that in addition to *Duxbl*, *Plac9*, and *Cphx* genes in mouse chromosome 14A3 are all duplicated. Previous reports suggested that *Plac9* and *Cphx* genes play important roles in mouse reproduction (Galaviz-Hernandez et al., 2003; Li et al., 2006). This near-telomeric cluster of reproductive genes in rodents may constitute an evolutionary advantage (Paillisson et al., 2005) and may come from a mechanism for protecting genes against mutation (Clapp et al., 2007). The location of these *Duxbl* genes in a near-telomeric cluster neighboring two reproductive genes (*Plac9* and *Cphx*) suggests that *Duxbl* genes also play a role in mouse reproduction.

According to our data of rapid amplification of cDNA ends (RACE) and the sequences of expressed sequence tag (EST) clones in databases, the upstream *Duxbl* gene (Gene ID: 48502) is composed of five exons spanning 6,390 bp. More than one transcriptional start sites for this *Duxbl* were identified by 5′ RACE analysis. The major



B

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                                   Homeodomain I (H1)
Duxbl      1 MELSCSTGLLEKEARRRRIILTQSQKDTLRVWFEKNPNPDLATRGHLAKELGISESQIMT  60
Duxbl-s    1 MELSCSTGLLEKEARRRRIILTQSQKDTLRVWFEKNPNPDLATRGHLAKELGISESQIMT  60
                                                  Homeodomain II (H2)
Duxbl     61 WFQKHRKIRKQAEFACCSEESQEQEQDKPRVKEARRSRTHFTKFQTDILIEAFEKNRFPG 120
Duxbl-s   61 WFQKHRKIRKQAEFACCSEESQEQEQDKPRVKEARRSRTHFTKFQTDILIEAFEKNRFPG 120
                              Helix 3                 Helix 1
Duxbl    121 IVTREKLAQQTGIPESRIHIWFQNRRARHPDPGQNTQKTPHPPQSSQGPTQKTVGKLAPS 180
Duxbl-s  121 IVTREKLAQQTGIPESRIHVRCASLCLWQPTCPYHKPLFSRAVDGQSQATHGCPCALDTG 180
               Helix 2
Duxbl    181 KTLTSSASVILPLSPPHTPNGPLDLSKGRQKQLPGTTLLQSSQVVQQRSDDQNPNKGHLS 240
Duxbl-s  181 CKYLVFPATVWRNSHIRPAES*                                       202

Duxbl    241 PTTTPGEQGFHSQPPLQLLTQNRGHNPRESGGLAVPRLEDCTQVPAVNQHFRKLDQNDSS 300
Duxbl-s

Duxbl    301 FLQHWDEWFGSMLAEWMPDKEYWSEKAELHPWQVQLRQLASVSPQAHQTP*          351
Duxbl-s
```

C

```
                          Helix 1          Helix 2          Helix 3          Identity
Duxbl-H1  1 ARRRRIILTQSQKDTLRVWFEKNPNPDLATRGHLAKELGISESQIMTWFQKHRKIRKQAE  60  100% (Mm)
Duxbl-H1  1 --------N-------------------------K---------------------VE  60   93% (Rn)
DUX4-H1   1 G-P--LVW-P--SEA--AC--R--Y-GI---ER--QAI--P-PRVQI---NE-SRQLRQH  60   42% (Hs)
DUXA-H1   1 H--C-TKF-EE-LKI-INT-NQK-Y-GY--KQK--L-INTE--R-QI---NR-ARHGFQK  60   35% (Hs)
DUXA-H1   1 H--C-TKF-EE-LKI-INT-NQK-Y-GY--KQK--L-INTE--R-QI---NR-ARHGFQK  60   35% (Pt)
Rax       1 H--N-TTF-TY-LHE-ERA---SHY--VYS-EE--GKVNLP-VRVQV---NR-AKWRRQE  60   32% (Mm)
Mixl      1 Q--K-TSFSSE-LQL-ELV-RQTMY--IHL-ER--ALTLLP--R-QV---NR-AKSRRQS  60   30% (Mm)
Duxbl-H2  1 ---S-THF-KF-T-I-IWA----RF-GIV--EK--QQT--P--R-HI---NR-ARHPDPG  60   43% (Mm)
                                                    *  *    *

                          Helix 1          Helix 2          Helix 3          Identity
Duxbl-H2  1 ARRSRTHFTKFQTDILIWAFEKNRFPGIVTREKLAQQTGIPESRIHIWFQNRRARHPDPG  60  100% (Mm)
Duxbl-H2  1 -G----------I----E-----------------E----------------------K  60   91% (Rn)
DUX4-H2   1 G--K--AV-GS--AL-LR----D-----AA--E--RE--L-----Q-----------GQ-  60   67% (Hs)
DUXA-H2   1 ---C--TYSAS-LHT--K--M--PY---DS--E--KEI-V----VQ-------S-LLLQR  60   53% (Hs)
DUXA-H2   1 ---C--TYSAS-LHT--K--M--PY---DS--E--KEI-V----VQ-------S-LLLQR  60   53% (Pt)
Rax       1 H--N--T--TY-LHE-ER----SHY-DVYS--E--GKVNL--V-VQV-------KWRRQE  60   45% (Mm)
Mixl      1 Q--K--S-SSE-LQL-ELV-RQTMY-D-HL--R--AL-LL-----QV-------KSRRQS  60   45% (Mm)
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D

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                          Helix 1          Helix 2          Helix 3          K + R
Duxbl-H1  1 ARRRRIILTQSQKDTLRVWFEKNPNPDLATRGHLAKELGISESQIMTWFQKHRKIRKQAE  60    8
Duxbl-H2  1 ARRSRTHFTKFQTDILIWAFEKNRFPGIVTREKLAQQTGIPESRIHIWFQNRRARHPDPG  60    6
DUX4-H1   1 GRPRRLVWTPSQSEALRACFERNPYPGIATRERLAQAIGIPEPRVQIWFQNERSRQLRQH  60    6
DUX4-H2   1 GRRKRTAVTGSQTALLLRAFEKDRFPGIAAREELARETGLPESRIQIWFQNRRARHPGQG  60    7
```

Fig. 1. Genomic structure and RNA transcripts of *Duxbl* genes. **A**: The tandem triplicate of mouse *Duxbl* genes and their surrounding genes (*Plac9* and *Cphx*) are present on mouse chromosome 14A3. The lengths of spaces between each *Duxbl* gene and RNA transcripts are indicated. The ORFs of *Duxbl* and *Duxbl-s* transcripts are shown in gray and the regions encoding homeodomains are in black. **B**: Comparison of the predicted amino acid sequences of Duxbl and Duxbl-s proteins. The identical amino acid residues are in bold and the homeodomains are boxed. The different amino acid residues in helix 3 of homeodomain II (H2) are in italics. **C**: Alignment of amino acid sequences of two Duxbl homeodomains (in bold) with those of other predicted homeodomains of double and paired-like homeobox genes. The names of gene products and their homeodomains are listed in the left. Dashes represent sequences identical to those of Duxbl homeodomains. Identity indicates the percentage of sequence identity to Duxbl homeodomain. The asterisks (*) indicate amino acid residues predicted to be involved in sequence-specific DNA binding. **D**: Amino acid sequences of homeodomains of human DUX4 and mouse Duxbl proteins. The basic amino acid residues in two terminal regions of homeodomains are shown in bold. The total numbers of lysine and arginine residues in two ends are shown on the right.

transcript is 2,055 bp in length (Fig. 1A) and was deposited in the NCBI GeneBank, accession number EF472598. Although there are different transcriptional initiation sites found in these *Duxbl* transcripts, they contain the same open reading frame (ORF) of 1,053 bp and a putative polyadenylation signal, AATAAA, at position 2,037 to 2,042. For the two downstream *Duxbl* genes (Gene ID: 72675 and 72672), minor differences in the intron 4 sequences between them and the upstream *Duxbl* gene (Gene ID: 48502) result in producing an additional minor transcript, *Duxbl-s*, composed of four exons (Fig. 1A). More than one transcriptional start sites for *Duxbl-s* transcripts were also identified by 5′ RACE, and they also encode one identical protein. The most abundant *Duxbl-s* transcript is 1,653 bp in length with an ORF of 606 bp, and a putative polyadenylation signal, AATGAA, at position 1,566 to 1,571 (accession number EU257807). The proposed translational start sites for *Duxbl* and *Duxbl-s* transcripts, both correspond to the Kozak consensus site surrounding their start codons (Kozak, 1996). Generations of *Duxbl* and *Duxbl-s* transcripts may result from different promoter usage and/or alternative splicing during development.

Analyzing ORFs of the major *Duxbl* and minor *Duxbl-s* transcripts indicates that the putative Duxbl and Duxbl-s proteins contain 350 and 201 amino acids, respectively (Fig. 1B). The N-terminal 139 amino acid residues of Duxbl and Duxbl-s proteins are identical and contain one domain similar to known homeodomains (homeodomain I, H1). The C terminus of Duxbl protein contains the other homeodomain (homeodomain II, H2); however, Duxbl-s protein does not because it lacks helix 3, the sequence-specific recognition helix (Fig. 1B). Thus, the major product (Duxbl protein) of these three *Duxbl* genes contains two homeodomains, so they are double homeobox genes. Furthermore, the coding regions of all homeodomains for Duxbl and Duxbl-s proteins contain an interrupting intron between homeodomain codon 46 and 47. This is a common feature of *PAXL*-subclass homeodomain proteins (Burglin, 1994). In addition, the amino acid sequences of Duxbl homeodomains share the highest similarities with known *PAXL* homeodomains (Fig. 1C). Therefore, these three *Duxbl* genes belong to *PAXL*-subclass homeobox gene family and they are also mouse double homeobox genes.

Searching for Putative *Duxbl* Ortholog

Although the genomic structure of *Duxbl* is more similar to human *DUXA* gene than human *DUX4* gene, the predicted amino acid sequences of homeodomains for Duxbl and DUX4 proteins are more similar (H1: 42% identity; H2: 67% identity) than those of Duxbl and DUXA proteins (H1: 35% identity; H2: 53% identity; Fig. 1C). Recently, a mouse representative of D4Z4 on chromosome 10 was identified and named as *Dux* (Clapp et al., 2007) and *mDUX* (Bosnakovski et al., 2009), respectively. However, sequence identities of the predicted protein and DUX4 homeodomains (H1: 36% identity; H2: 56% identity) are also lower than those of Duxbl and DUX4. Based on these sequence identities, we suggest that the human ortholog of *Duxbl* gene is *DUX4* gene but not *DUXA* gene as previously reported (Kawazu et al., 2007). Furthermore, comparison of total amino acid sequences of Duxbl protein with those of its predicted ortholog shows that Duxbl protein shares the highest similarity with its rat ortholog (RGD1311053; 80% identity; data not shown). The homeodomain sequences of Duxbl H1 and H2 also exhibit the highest similarity with those of its rat ortholog (93% and 91% identities) followed by its human ortholog (42% and 67% identities; Fig. 1C). Comparisons of homeodomain sequences of Duxbl and other *PAXL*-subclass homeodomain proteins show identities of only 30 to 45% (Fig. 1C). This result indicates that *Duxbl* is a special member of the *PAXL*-subclass homeobox gene family.

Otherwise, the sequence identity between Duxbl H1 and H2 is only 43% (Fig. 1C). We found that Duxbl H1 binds DNA in a nonspecific manner but Duxbl H2 specifically binds a palindromic sequence (Tsai et al., unpublished data). The large differences in amino acid residues and DNA binding properties between Duxbl H1 and H2 suggest that two Duxbl homeodomains may bind and regulate different downstream genes through different mechanisms.

Duxbl Expression Pattern in Adult Tissues

Although many double homeobox genes are found in humans, only DUX4 proteins have been detected in vivo in myoblasts from FSHD patients (Belayew, 2004; Kowaljow et al., 2007). However, the in vivo expression pattern of DUX4 protein is still unknown. Accordingly, we first detected their major and minor transcripts, *Duxbl* and *Duxbl-s*, of these three *Duxbl* genes in various adult tissues by reverse transcriptase-polymerase chain reactions (RT-PCRs). Both *Duxbl* and *Duxbl-s* transcripts are predominantly expressed in adult eye, brain, and reproductive organs including ovary, uterus, placenta and testis (Fig. 2A). We next performed Western blotting to decipher in vivo expressions of Duxbl proteins in adult mouse tissues using affinity-purified homemade Duxbl polyclonal antibodies. A 38-kDa protein band indicating Duxbl protein is predominantly detected in adult eye, brain and ovary (Fig. 2B). The results of *Duxbl* expression patterns in adult tissues by RT-PCRs (Fig. 2A) and Western blotting (Fig. 2B) are complementary, because we only detected Duxbl proteins in adult tissues with strong *Duxbl* transcript signals. However, Duxbl-s protein is not detected in these adult tissues by our polyclonal antibodies. This may result from the much lower expression levels of *Duxbl-s* transcripts than *Duxbl* transcripts in these tissues (Fig. 2A).

Blocking assays for preincubations of polyclonal antibodies with purified glutathione S-transferase (GST) proteins or GST-H1 and GST-H2 fusion proteins were used to verify the specificity and activity of our Duxbl polyclonal antibodies. After preincubation with GST-H1 and GST-H2 fusion proteins, the purified Duxbl polyclonal antibodies did not recognize the previously identified Duxbl proteins in adult ovary and overexpressed Duxbl-V5 fusion proteins (Fig. 2C). However, antibodies preincubated with GST proteins could recognize Duxbl proteins in adult ovary, overexpressed Duxbl-V5 fusion proteins, and purified 6His-Duxbl fusion proteins. This result verifies the activity and specificity of our

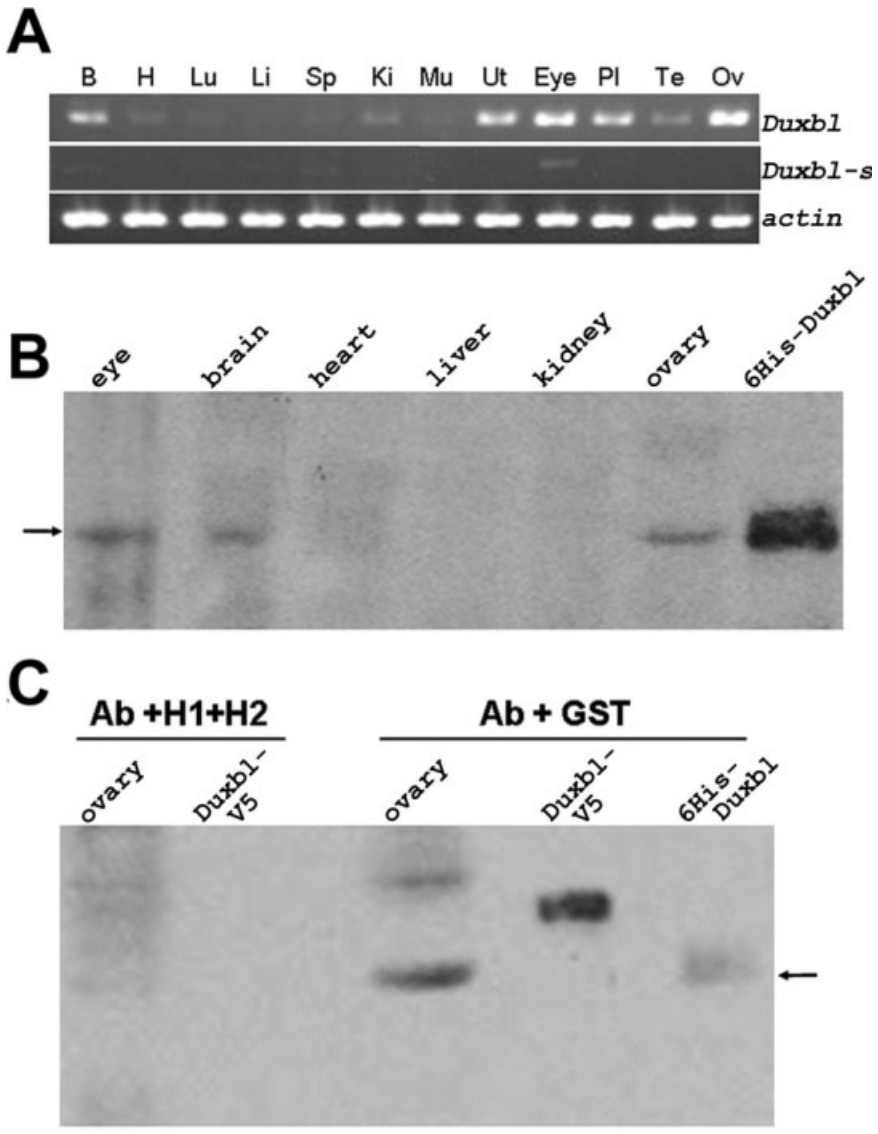

Fig. 2. Expression profiles of *Duxbl* and *Duxbl-s* transcripts and Duxbl protein. **A**: The expression levels of *Duxbl* and *Duxbl-s* transcripts in adult mouse tissues were analyzed by reverse transcriptase-polymerase chain reactions (RT-PCRs). Expression level of *actin* was used as an internal control. B, brain; H, heart; Lu, lung; Li, liver; Sp, spleen; Ki, kidney; Mu, skeletal muscles; Ut, uterus; Eye, eye; Pl, placenta; Te, testis; Ov, ovary. **B**: Purified Duxbl fusion protein containing N-terminal 6 histidine residues (6His-Duxbl) and equal-amount of total proteins extracted from indicated adult tissues were analyzed by Western blotting using affinity-purified Duxbl polyclonal antibodies. **C**: Analyzing the specificity of purified Duxbl polyclonal antibodies. The purified Duxbl polyclonal antibodies were preincubated with purified GST-H1 and GST-H2 fusion proteins (Ab +H1+H2) or with GST proteins only (Ab +GST). Total proteins extracted from adult ovaries and HeLa cells transfected with Duxbl-V5 fusion protein expression vectors (Duxbl-V5), or purified 6His-Duxbl fusion proteins were analyzed by Western blotting using two different pretreated antibodies. Position of 38-kDa protein bands representing Duxbl proteins was indicated by arrow.

homemade affinity-purified Duxbl polyclonal antibodies.

Duxbl Expression Patterns in Gonads

Because *Duxbl* is predominantly expressed in adult ovary and slightly expressed in adult testis (Fig. 2A), we examined *Duxbl* expression levels in embryonic and postnatal gonads by RT-PCRs. In female gonads, *Duxbl* transcripts are observed from embryonic day 12.5 (E12.5) until birth (Fig. 3A). After birth, *Duxbl* expressions in the ovaries are found to be high until adulthood. In male gonads, *Duxbl* transcripts are also detected from E12.5 until birth (Fig. 3B). After birth, *Duxbl* expressions peaked at day 7 and returned to low levels from day 21 until adulthood. Furthermore, the expression patterns of *Duxbl-s* transcripts are similar to but lower than those of *Duxbl* transcripts (Fig. 3A,B). In addition, low levels of *Duxbl* transcripts are also detected in the mesonephros (data not shown). Because no *Duxbl* expression is detected in mouse Leydig (TM3), Sertoli (TM4), and spermatocyte (GC-2) cell lines (data not shown), we suggest that *Duxbl* expresses in germ cells. To verify this suggestion, we performed quantitative RT-PCRs in male gonads. Results of real-time RT-PCRs confirm the increases of *Duxbl* expressions after birth. *Duxbl* expressions in male testes peak at day 7 (Fig. 3C), while male gonocytes exit cell cycle arrest and enter a wave of spermatogonia proliferation. *Duxbl* expressions were seen decreasing from day 15 to adulthood. Before birth, there is a *Duxbl* expression peak with a moderate expression level observed around E13.5, a time for rapid proliferations of male germ cells. After this time, the proliferations of male germ cells slow down and enter mitotic arrest at around E16.5 (Olaso and Habert, 2000). Results of quantitative RT-PCRs suggest high *Duxbl* expressions in proliferating male germ cells including gonocytes and spermatogonia.

We further characterized *Duxbl* expressions in adult testis by in situ hybridizations to decipher the cellular localizations of *Duxbl* transcripts. In adult testis sections, strong *Duxbl* transcript signals are only detected in spermatogonia from stage X to XII seminiferous tubules (Fig. 4A). However, we did not detect any *Duxbl* signal in postmeiotic cells of testis including spermatocytes and spermatids. The *Duxbl* expressions in spermatogonia and the absence in other postmeiotic cells are also confirmed by RT-PCRs of germ cells isolated from distinctive stages (data not shown). Results of in situ hybridizations in adult testis (Fig. 4A) are identical to results of RT-PCRs from testis cell lines, because we could not detect *Duxbl* expression in Leydig and Sertoli cells, and in round spermatids (data not shown). Pang and his coworkers also identified differential expressions of 1110051B16Rik gene in

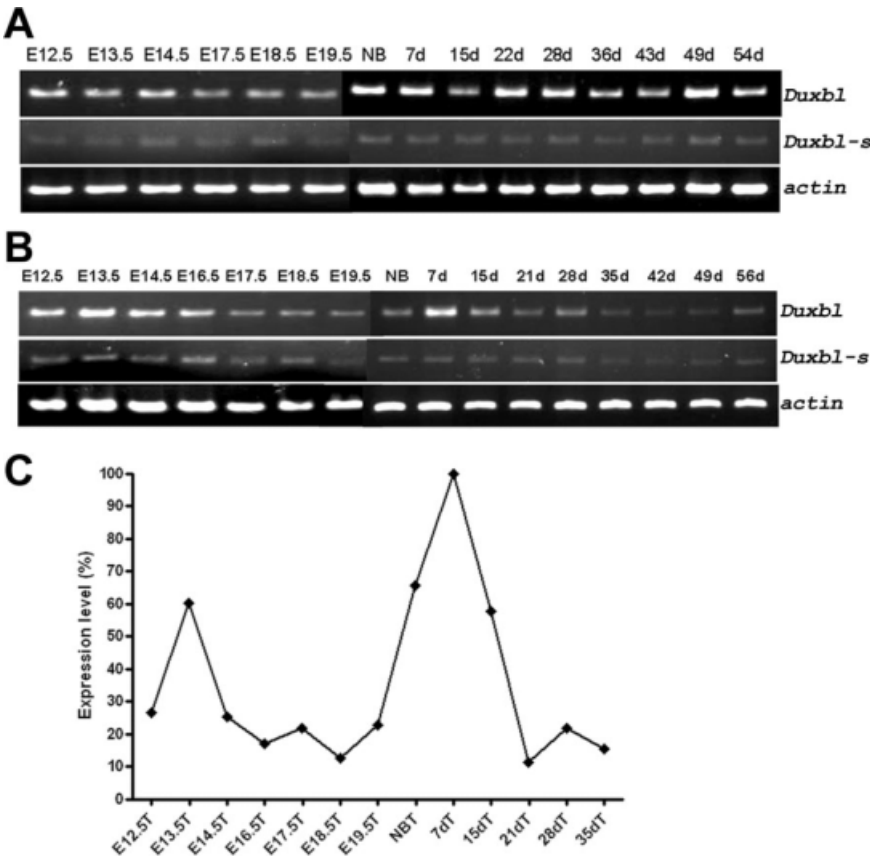

Fig. 3. Analyses of expression levels and profiles of *Duxbl* and *Duxbl-s* transcripts during gonad developments. **A,B**: The expression levels of *Duxbl* and *Duxbl-s* transcripts in female (A) and male (B) gonad developments were analyzed by reverse transcriptase-polymerase chain reactions (RT-PCRs). Total RNAs were extracted from ovaries (A) or testes (B) from different embryonic days (E12.5 to E19.5), newborn mice (NB), and mice at different postnatal days (7d to 56d). Expression levels of *actin* were used as internal controls. **C**: Analysis of *Duxbl* expression levels in testes by real-time RT-PCRs. The relative expression levels of *Duxbl* to *GADPH* transcripts in testes from different embryonic and postnatal days were determined. The relative expression levels were shown after comparison with the expression level of 7-day-old testes.

spermatogonia by microarray and quantitative RT-PCR at different stages of spermatogenesis (Pang et al., 2003). Furthermore, we performed in situ hybridizations to find cells that specifically express *Duxbl* in adult ovary. In adult ovary sections, weak expressions of *Duxbl* transcripts are found in the oocytes of primordial follicles, but strong expressions are seen in the oocytes of primary and secondary follicles (Fig. 4B). These results indicate that *Duxbl* are specifically expressed in oocytes during oogenesis and in spermatogonia during spermatogenesis.

We next examined the in vivo localizations of Duxbl proteins in 2-week-old and adult ovaries by immunohistochemistry using homemade affinity-purified Duxbl polyclonal antibodies. Results of immunostained ovary sections show that Duxbl proteins are specifically present in oocytes of primordial, primary, secondary, and antral follicles (Fig. 5). These results of in vivo Duxbl protein localizations are consistent with results of in situ hybridizations (Fig. 4B), because we obtain oocyte-specific signals of *Duxbl* transcripts and Duxbl proteins in the same types of follicles in both cases.

Subcellular Localizations of Duxbl and Duxbl-s Proteins

We used the same homemade Duxbl polyclonal antibodies to examine the subcellular localizations of overexpressed epitope-tagged Duxbl and Duxbl-s proteins, respectively. Results of immunofluorescence reveal that the overexpressed Duxbl-V5 and FLAG-Duxbl-s fusion proteins are both restricted to the nuclei of transfected cells (Fig. 6). We observed the same subcellular localizations of Duxbl and Duxbl-s fusion proteins using commercial anti-V5 and anti-FLAG monoclonal antibodies, respectively (data not shown). These results indicate that both overexpressed Duxbl and Duxbl-s proteins contain the nuclear localization signal (NLS). Although the amino acid residues of homeodomains from various homeodomain proteins show a high degree of conservation, NLSs of homeodomain proteins are not identical. A common theme of their NLSs is the basic amino acid residues at two ends of homeodomains (Ploski et al., 2004; Ostlund et al., 2005). The first (H1) and the second (H2) homeodomains of Duxbl protein contain 8 and 6 lysine/arginine residues at their two ends, respectively (Fig. 1D). In addition, the human DUX4 protein also contains 6 and 7 lysine/arginine residues at two ends of its H1 and H2, respectively (Fig. 1D). Previously, DUX4 protein has been shown to transactivate *PITX1* expression (Dixit et al., 2007). Because Duxbl and Duxbl-s proteins contain similar lengths of basic residues as DUX4 protein at two ends of their homeodomains (Fig. 1D) and both of them localize in the nuclei (Fig. 6), they might transactivate downstream gene(s) as DUX4 protein. However, overexpressing Duxbl proteins in C2C12 cells could not transactivate *Pitx1* expression (data not shown).

Duxbl Expression Patterns in Embryonic Development

Because *Duxbl* transcripts are predominantly restricted in oocytes of ovary (Fig. 4B), *Duxbl* expressions during embryonic development were further determined. *Duxbl* transcripts are first detected in unfertilized eggs then continued to blastocysts but not in cumulus cells (Fig. 7A). After implantation, *Duxbl* expressions in embryos are seen decreased from embryonic day 11.5 (E11.5) to 17.5 (E17.5), and *Duxbl-s* shows similar expression pattern (Fig. 7B). Furthermore, whole-mount in situ hybridizations were used to identify *Duxbl* transcripts in developing mouse embryos from E9.5 to E12.5. The *Duxbl* transcripts are detected in forelimb, hindlimb, and tail beginning from E9.5 and maintained to E12.5 (Fig. 7C–E). *Duxbl* expressions in limbs were

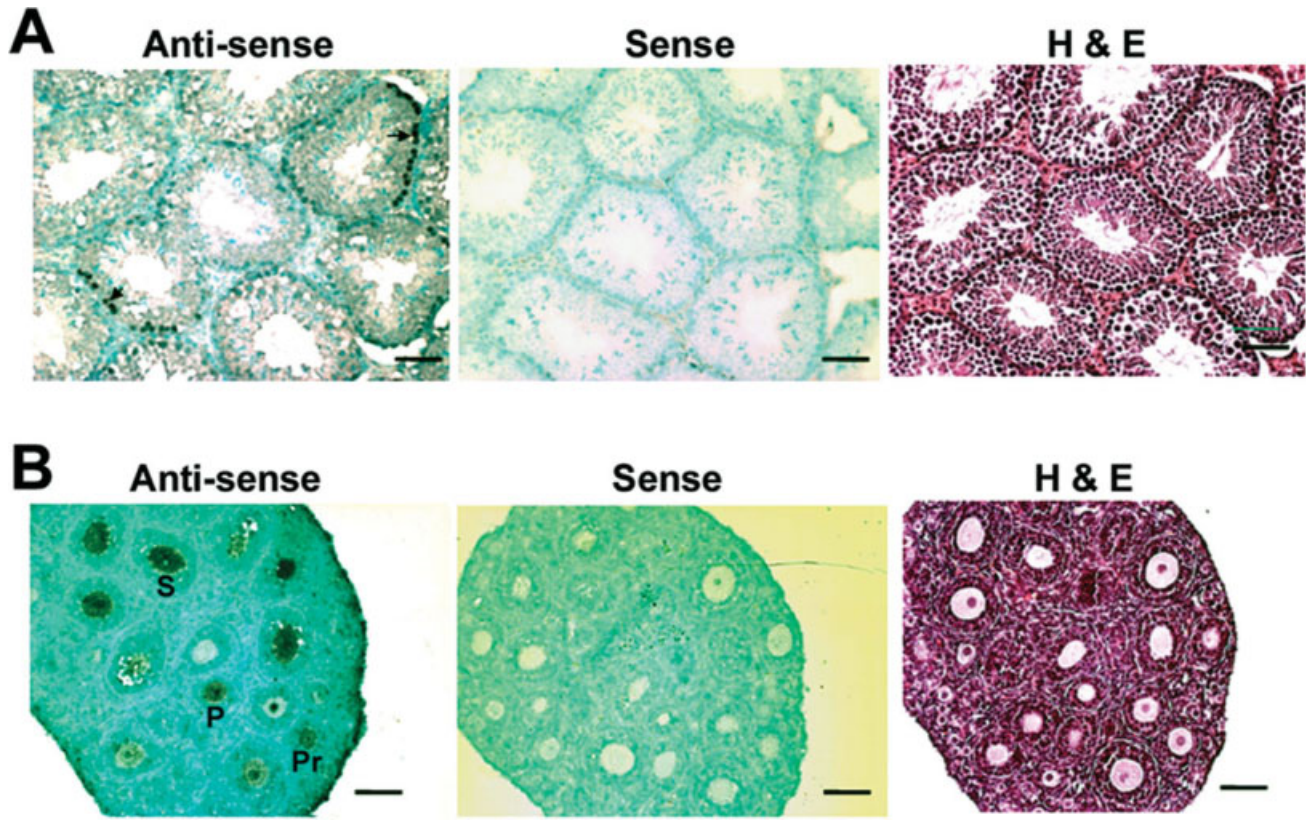

Fig. 4. Identifying *Duxbl* transcripts in gonads by in situ hybridizations. **A,B**: The localizations of *Duxbl* transcripts were analyzed in adult testis (A) and 10-day-old ovary (B). Adjacent sections were labeled with digoxigenin-labeled anti-sense (left panel) and sense probes (middle panel), or stained with hematoxylin and eosin (H&E, right panel). *Duxbl* transcripts were only detected in spermatogonia (indicated by arrows) of testis (A) and in oocyte of primordial (Pr), primary (P), and secondary (S) follicles in ovary (B). Scale bars = 50 μm.

further detected by section in situ hybridization and Alcian blue staining. *Duxbl* signals are localized in muscle cells (data not shown). Furthermore, in vivo Duxbl protein expressions were analyzed by immunohistochemistry. Strong Duxbl protein signals are detected in muscle cells of limbs at E13.5 embryo (Fig. 7F), while myotubes begin to form. Especially, Duxbl protein signals are detected in the fiber-like muscle cells but not in the mononuclear cells expressing MyoD proteins (Fig. 7G). These results indicate that Duxbl proteins are expressed in limbs and tail during embryo development and in differentiated myocytes but not in myoblasts.

Duxbl Expressions During Limb Development

During muscle development in limbs, muscle progenitor cells from somite migrate into limb bud, where they proliferate, express myogenic determination factors and subsequently

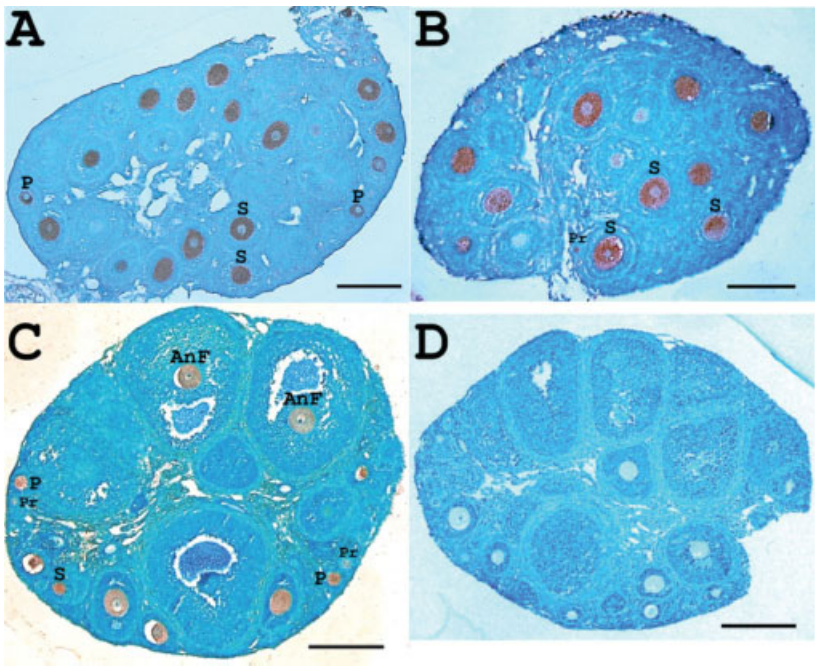

Fig. 5. Identifying Duxbl proteins in 2-week-old and adult ovaries by immunohistochemical analysis. **A–D**: Duxbl proteins in 2-week-old (A,B) and adult (C,D) mouse ovary sections were probed using affinity-purified Duxbl polyclonal antibodies (A–C) and normal rabbit immunoglobulin G (D), respectively. Duxbl proteins are specifically present in oocytes of primordial (Pr), primary (P), secondary (S), and antral follicles (AnF) as indicated. Scale bars = 50 μm.

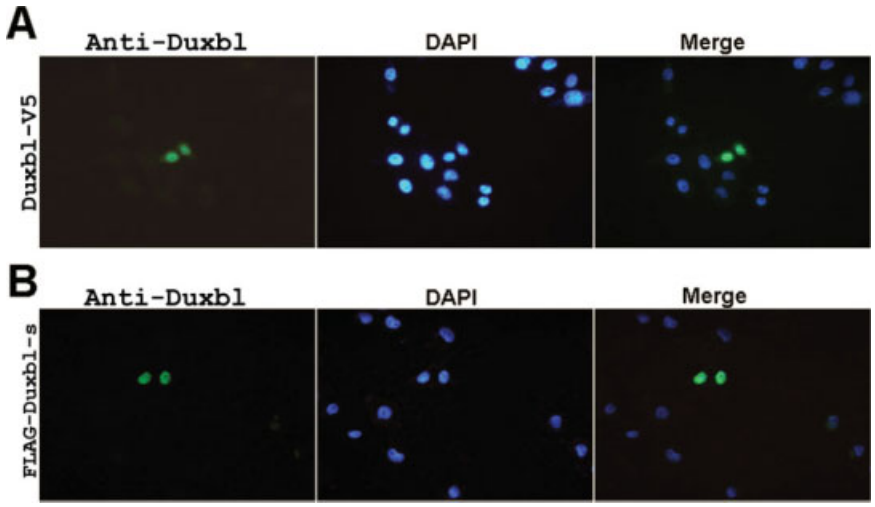

Fig. 6. **A,B**: Subcellular localizations of overexpressed Duxbl and Duxbl-s proteins. Duxbl-V5 (A) and FLAG-Duxbl-s (B) fusion proteins were overexpressed in HeLa cells by transfection of Duxbl-V5 and FLAG-Duxbl-s expression vectors, respectively. The overexpressed Duxbl-V5 or FLAG-Duxbl-s proteins were detected by affinity-purified anti-Duxbl polyclonal antibodies (left panel). Cell nuclei were stained with DAPI (middle panel). Images of left and middle panels were merged (right panel).

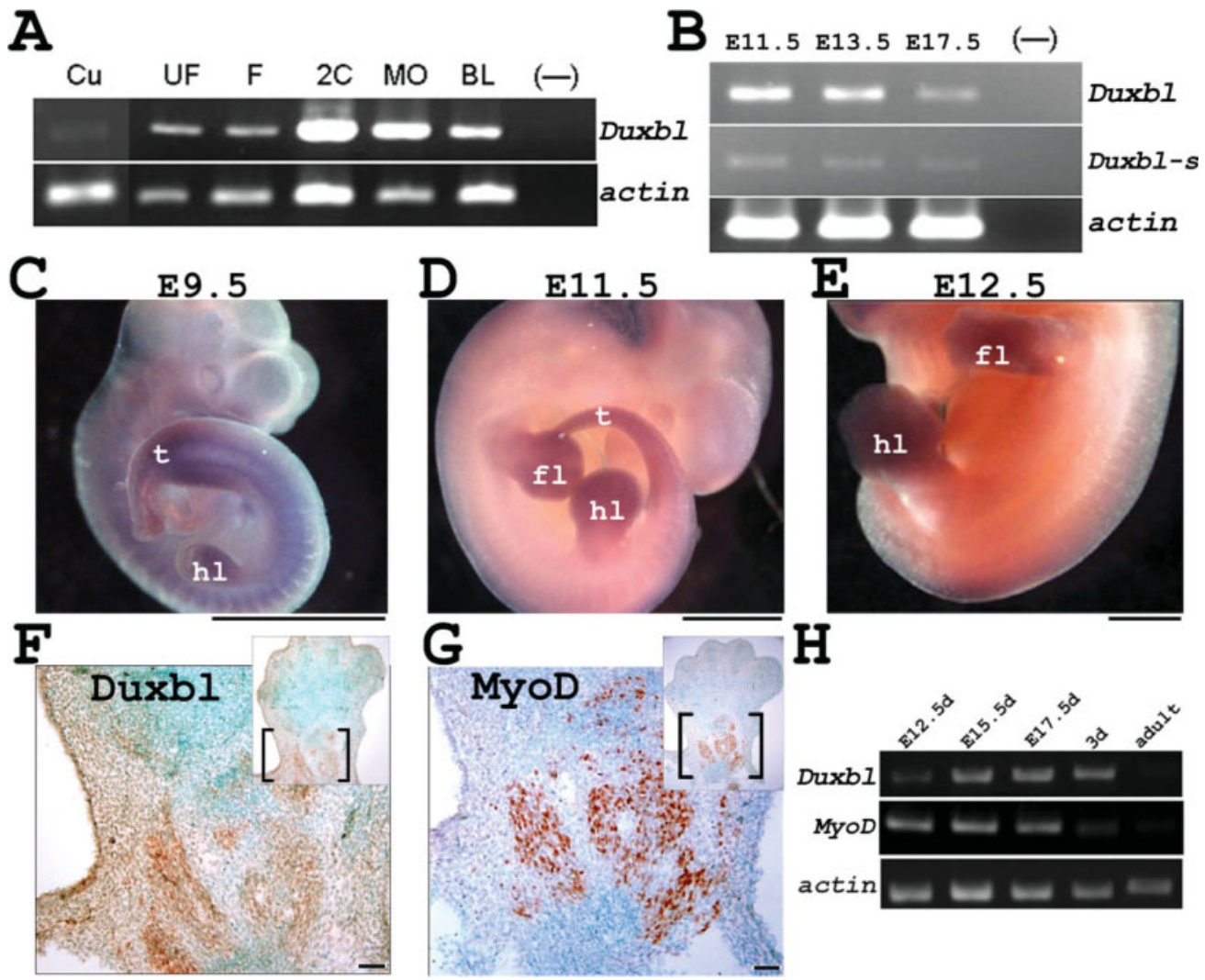

Fig. 7. Identifying *Duxbl* expressions during mouse development by reverse transcriptase-polymerase chain reaction (RT-PCR), whole mount in situ hybridization, and immunohistochemical analysis. **A,B**: Expressions of *Duxbl* and *Duxbl-s* transcripts in preimplantation (A) and postimplantation (B) embryos were analyzed by RT-PCRs. A: Cu, cumulus cells; UF, unfertilized eggs; F, fertilized eggs; 2C, two-cell embryos; MO, morula; BL, blastocysts. B: Total RNAs were extracted from postimplantation embryos at embryonic day 11.5 (E11.5), 13.5 (E13.5), and 17.5 (E17.5), respectively. Amplifications without RNA served as negative controls (−). **C–E**: Identifying *Duxbl* expressions in different embryos at embryonic day 9.5 (E9.5), 11.5 (E11.5), and 12.5 (E12.5) by whole-mount in situ hybridizations, respectively. t: tail; fl: forelimb; hl: hindlimb. **F,G**: Identifying Duxbl and MyoD protein expressions in dorsal sections through forelimb of 13.5-day embryo by immunohistochemical analysis using anti-Duxbl (F) and anti-MyoD (G) antibodies, respectively. The boxed regions in small figures were amplified. **H**: Analyzing *Duxbl* and *MyoD* expression levels by RT-PCRs during embryonic and postnatal limb developments. Total RNAs were extracted from limbs at embryonic day 12.5 (E12.5), 15.5 (E15.5), and 17.5 (E17.5), and at postnatal day 3 (3d) and adult, respectively. Expression levels of *actin* were used as internal controls. Scale bars = 50 μm.

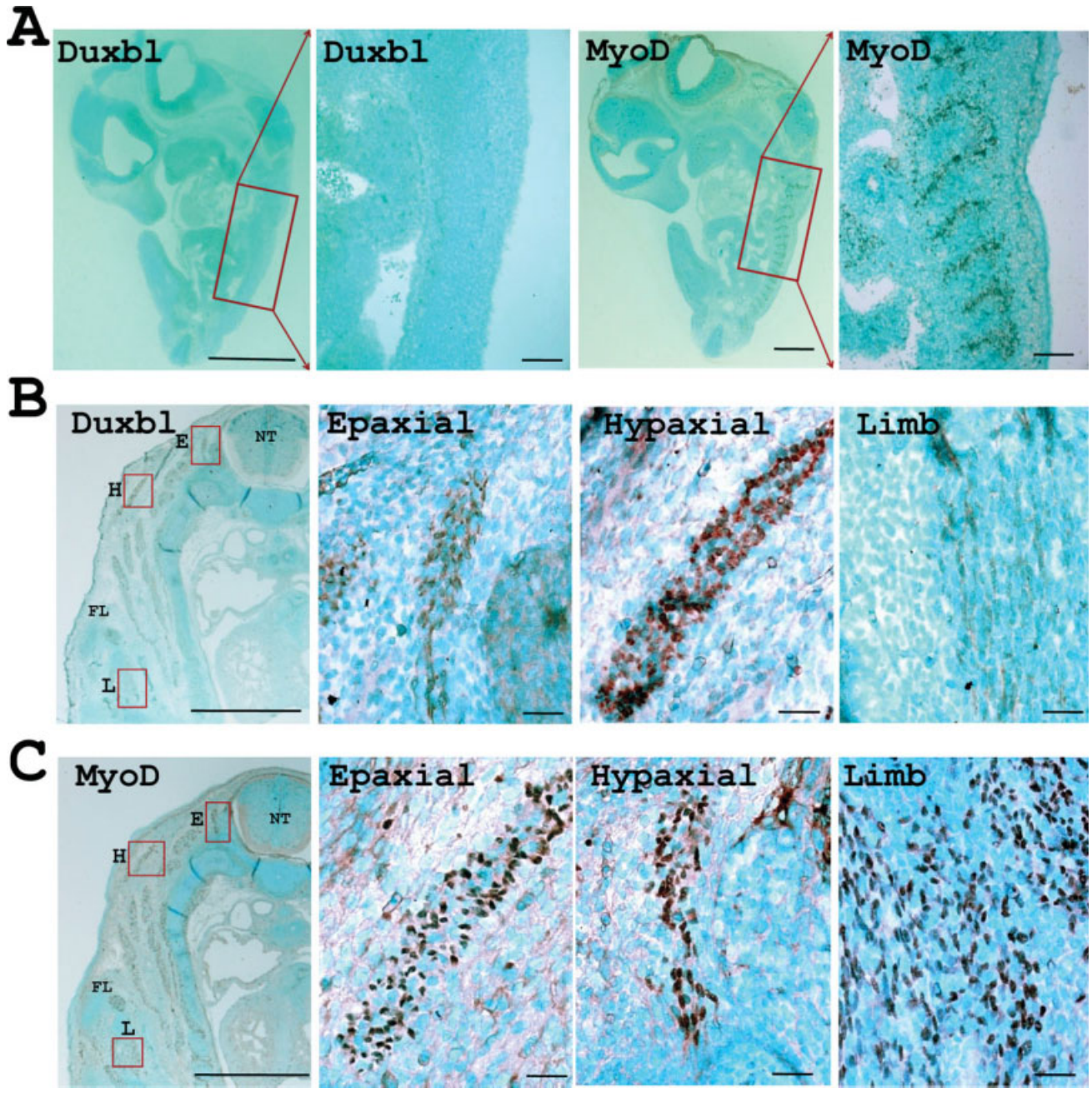

Fig. 8. Identifying Duxbl and MyoD protein expressions in embryonic muscles. A–C: Duxbl and MyoD proteins were detected on parasagittal sections of 11.5-day embryo (A) and transverse sections of 12.5-day embryo (B,C) by immunohistochemistry. **A**: High magnifications of the boxed regions in the left panels were shown in the right. **B,C**: High magnification of the boxed regions in the left panels of epaxial (E), hypaxial (H), and limb (L) muscles were shown in the middle and right panels as indicated. FL, forelimb; NT, neural tube. Scale bars = 50 μm.

differentiate into skeletal muscles. MyoD and myogenin (MyoG) are first detected in the proximal regions of both hindlimb and forelimb at embryonic day (E) 11.5 embryo and later accumulated in the differentiated muscle masses, continuously expressed in fetal skeletal muscles (Sassoon et al., 1989). Although expressions of *Duxbl* and *MyoD* are both continuous from E12.5 to postnatal stage (3d) in limbs by RT-PCR analyses (Fig. 7H), *Duxbl* expressions in limbs are largely increased from E12.5 to E15.5 and maintained in high level to postnatal (3d) stage, but almost undetectable in adult muscles by RT-PCR analyses (Fig. 7H). These results suggest that *Duxbl* expressions are low in myoblast proliferation stage but increase largely following myotube formation, so Duxbl may play a role in myogenesis during embryonic limb development.

Duxbl Protein Expressions in Trunk Myogenesis

During embryogenesis, skeletal muscles in the trunk and limbs are both derived from somites (Tajbakhsh and

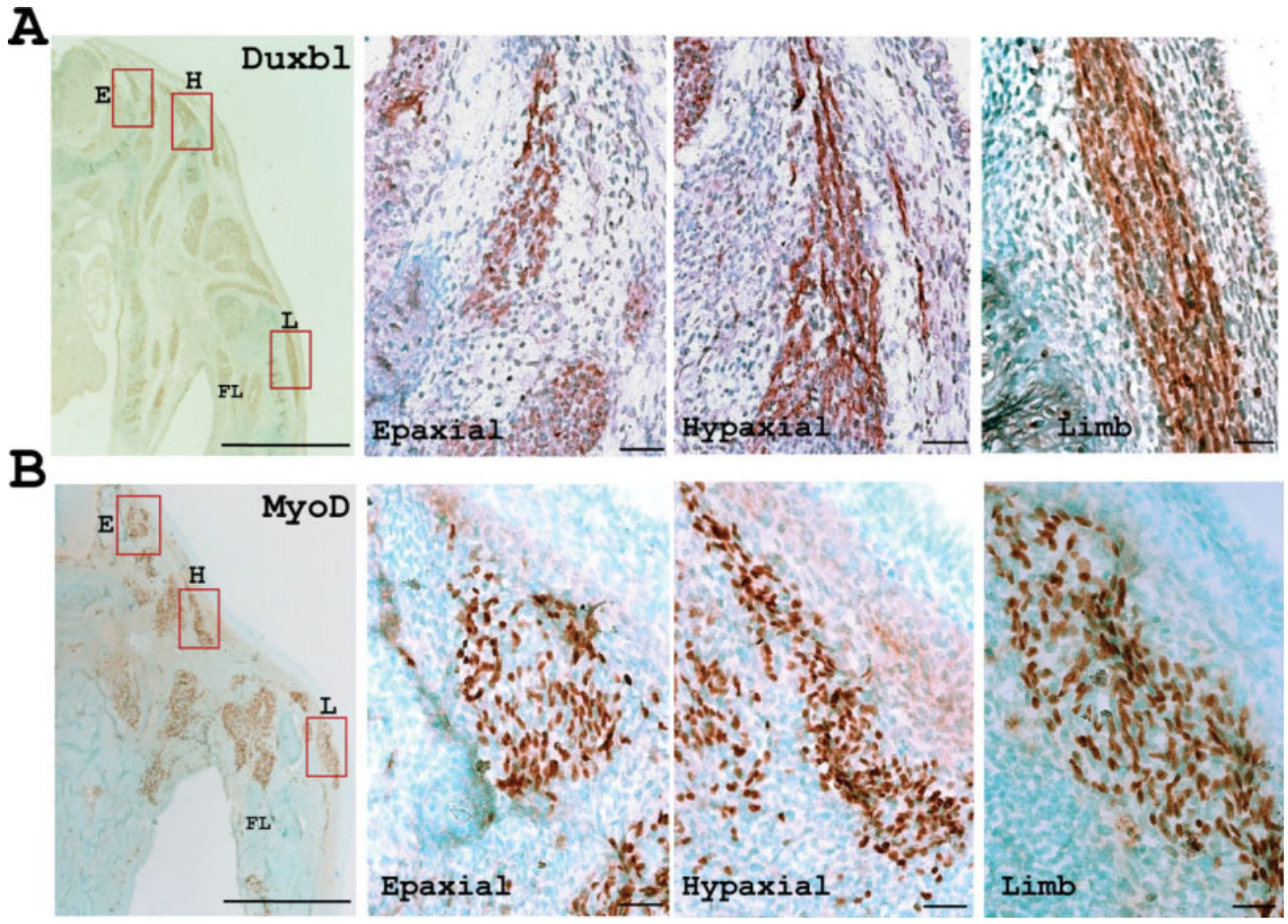

Fig. 9. Identifying Duxbl and MyoD protein expressions in developing muscles. Duxbl (A) and MyoD (B) proteins were detected on transverse sections of 13.5-day embryo by immunohistochemistry. **A,B**: High magnification of the boxed regions in the left panels of epaxial (E), hypaxial (H), and limb (L) muscles were shown in the middle and right panels as indicated. FL, forelimb. Scale bars = 50 μm.

Buckingham, 2000). During myotome development, MyoG and MyoD are detectable at E10.5 (Cusella-De Angelis et al., 1992), while terminally differentiated muscle cells could already be identified in the myotome. To decipher the Duxbl protein expressions during myotome development in advance, we used immunohistochemistry on parasagittal sections of E11.5 embryos. Duxbl protein is not detected in somite (Fig. 8A). However, the myoblast marker gene products, MyoD proteins, are expressed in the myotomes. To further confirm the expressions of Duxbl proteins during skeletal myogenesis in the trunk and limb, we used immunohistochemistry on transverse sections of embryo at E11.5 with antibodies react with MyoD and Duxbl proteins, respectively. In transverse sections of the same embryo, MyoD-positive cells are also observed in myoblasts of developing limbs and somite. However, no Duxbl-expressing cells are present in transverse sections of the same E11.5 embryos (data not shown). In further embryonic development, Duxbl is seen expressed in the epaxial, hypaxial, and forelimb muscles in E12.5 embryo (Fig. 8B), similar to MyoD (Fig. 8C). However, the intensities of Duxbl proteins are less than MyoD proteins in all muscle-forming regions, especially in limb muscles. After comparing stained cell morphology, Duxbl proteins are obviously stained in fewer elongated myocytes and immature myofibrils (Fig. 8B). However, MyoD proteins are detected in mononuclear myocytes (Fig. 8C). These results suggest that Duxbl expressions are low in limb myoblast proliferation stage, embryonic day 12.5. After myoblast proliferation stage, at E13.5 embryo, myoblasts have clearly acquired a spindle-shaped morphology, and considerably more and longer myofibrils have now been formed. In E13.5 embryonic transverse sections, Duxbl proteins are expressed in increased number of cells, virtually including all of the newly formed primary fibers, present in both body wall and limbs (Fig. 9A). Strong Duxbl signals are detected in elongated myocytes and multinuclear myotubes (Fig. 9A). The distribution and intensity of Duxbl expressions are both similar to those of myosin heavy chain (MHC; Kablar et al., 1997). MHC is expressed exclusively in terminally differentiated myotubes and is detectable in the intercostal muscles of the trunk and the muscle anlagen of forelimb. The expressions of both Duxbl and MHC are increased in E13.5 embryo, in which stage the myotube formations are observed.

Duxbl Expressions During C2C12 Cell Differentiation

To verify that *Duxbl* is expressed in differentiated myocytes but not in

myoblasts during skeletal muscle formation, we examined *Duxbl* expressions during in vitro differentiations of C2C12 cells. *Duxbl* transcripts are slightly detected in confluent myocytes, and seen significantly increased in differentiated cells following 2 days in differentiation medium as *MyoG* (Fig. 10A). Because *Duxbl* exhibits similar expression patterns as *MyoG* during C2C12 cell differentiation, we suggest that Duxbl also play a role in myoblast differentiation and/or fusion. Our double immunofluorescence data also show the presence of Duxbl protein signals in differentiating cells and clearly located in the nuclei of multinuclear myotubes (Fig. 10B), similar to results of in vivo immunohistochemistry analyses (Figs. 7F, 8B, 9A). However, MyoD proteins are stained strongly in small nuclear myoblasts and myocytes, while only slightly in myotubes (Fig. 10B). From the above results, we conclude that Duxbl is expressed in myotubes but not in proliferating myoblasts during in vitro differentiation of myoblasts and in vivo skeletal muscle formation. Because the expression of *Duxbl* is downstream of *MyoD* and seems parallel to *MyoG*, whether expression of Duxbl influences *MyoD* or *MyoG* or other muscle specific genes to affect myogenesis need further characterizations. C2C12 can be used as a model cell line to identify the molecular mechanism of Duxbl influencing myoblast differentiation or how Duxbl interact with the myogenic regulatory factors to regulate myogenesis.

In brief, *Duxbl* is a mouse double homeobox gene that contains introns. The Duxbl homeodomain exhibits the maximum identities to those of human DUX4 gene. From the homeodomain similarity, we suggest that *Duxbl* is the ortholog of human *DUX4*, which is the candidate gene to cause FSHD. However, the exact expression pattern of DUX4 in human development or during myogenesis is not detected and might be undetectable, because DUX4 protein cannot be obtained from normal adult tissues. Studying the expression profile of a mouse double homeobox gene, *Duxbl*, can facilitate the understanding of the functions of double homeobox genes including *DUX4* during development. In addition, the characterization of Duxbl expression during myogenesis might help in understanding the molecular mechanism of FSHD. During

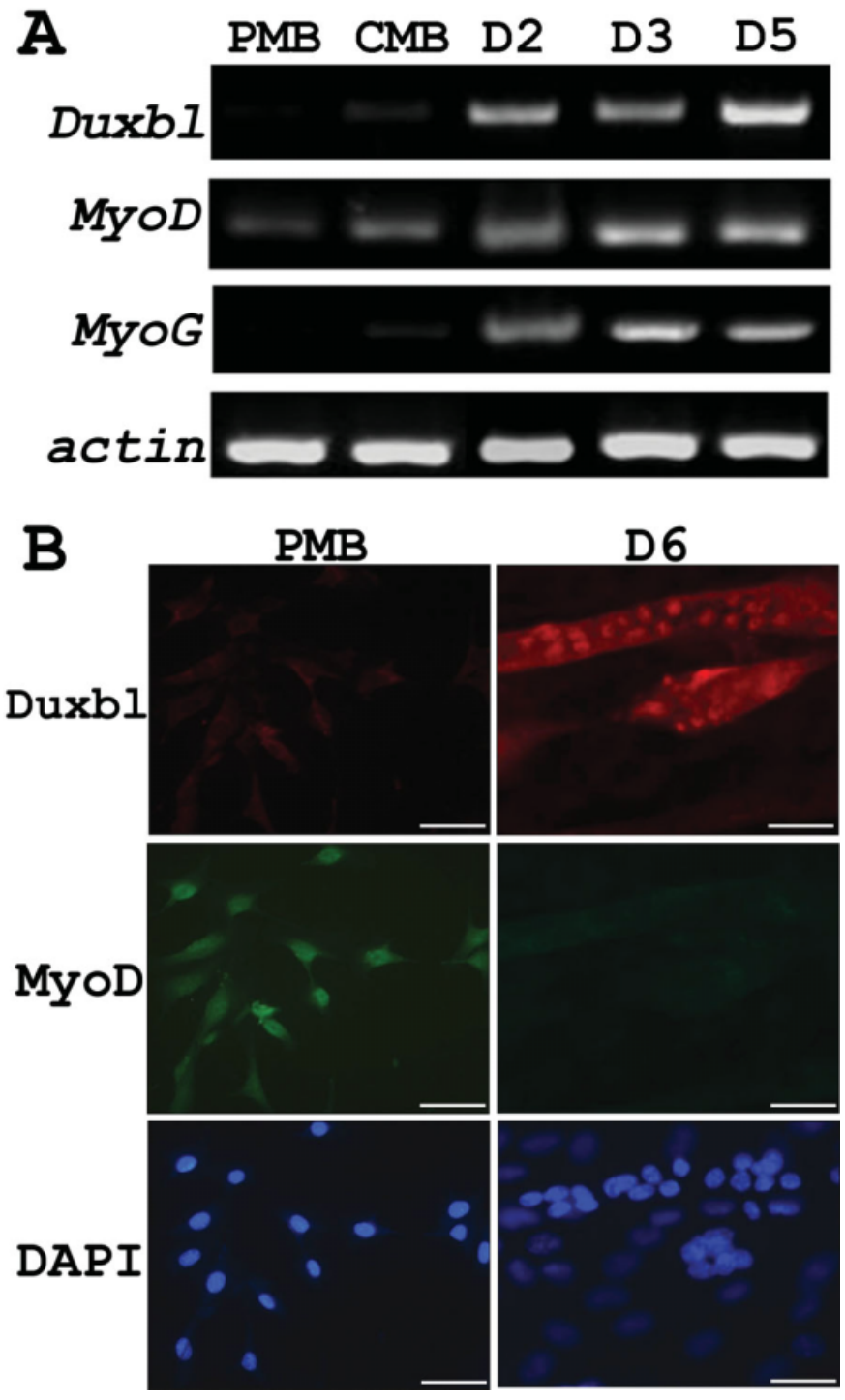

Fig. 10. Identifying *Duxbl*, *MyoD*, and *MyoG* expressions in differentiated and undifferentiated C2C12 cells. **A**: Expression levels of *Duxbl*, *MyoD*, and *MyoG* transcripts were determined by reverse transcriptase-polymerase chain reactions (RT-PCRs) during C2C12 differentiation. Total RNAs were extracted from proliferating myoblasts (PMB), confluence myoblasts (CMB), and differentiating myocytes cultured in differentiation medium for 2, 3, and 5 days (D2, D3, D5), respectively. Expression levels of *actin* were used as internal controls. **B**: Double immunofluorescence analysis of Duxbl and MyoD expressions in C2C12 cells. C2C12 cells were incubated in growth medium as proliferating myoblasts (PMB) and in differentiation medium for 6 days (D6) as differentiating myocytes, respectively. They were analyzed by double immunofluorescence with anti-Duxbl and anti-MyoD antibodies. Signals of Duxbl proteins were detected with Texas red-conjugated secondary antibodies (Duxbl) and signals of MyoD were detected with fluorescein isothiocyanate (FITC) -conjugated secondary antibodies (MyoD). Cell nuclei were stained with 4′-6-diamidino-2-phenylindole (DAPI). Scale bars = 50 μm.

embryo development, Duxbl is predominantly expressed in differentiated myocytes during embryo skeletal myogenesis. The myofibers of epaxial, hypaxial, and limb express Duxbl proteins, first, from E12 embryo. However, Duxbl is barely detectable in the adult limb muscle. Because DUX4 protein can be detected in primary myoblasts extracted from FSHD patients but not from normal people, we suggest that an increase in the expression of embryo protein, mDux in adult tissue may develop a muscular dystrophy with features characteristic of the human disease. In addition to skeletal myogenesis, Duxbl is also expressed in germ cells, especially in oocytes and spermatogonia, during gonad development. Our results suggest that Duxbl is important in germ cell development. Our future experiments would be directed toward characterizing the exact function of this gene using transgenic or conditional knockout analysis.

EXPERIMENTAL PROCEDURES

Animals

Mice were maintained in a specific pathogen-free environment at the animal housing in Chung Shan Medical University. All experimental procedures were conducted in accordance with the guidelines of the institutional animal committee and received protocol number 401.

RNA Isolations and RT-PCRs

Total RNAs were extracted using Trizol reagent (Life Technologies) from various mouse tissues, embryos at different stages, and various mouse cell lines. Quantities and purities of RNAs were determined by ultraviolet absorbance (DU 800; Beckman Coulter) and by gel electrophoresis. Five or two micrograms each of total RNAs were reverse transcribed using Superscript II system (Life Technologies) and oligo-dT primer. P1/GSP1 (P1, 5′-GAGCTGCAGTACTGGCCTACTG-3′; GSP1, 5′-CTGGGAGGACTGAAGTAGTGTGGT-3′) and P1/GSP2 (GSP2, 5′-ATGATTATGCAGGTCTGATGTG-3′) primer pairs were used to determine the expressions of *Duxbl* and *Duxbl-s* transcripts (Fig. 1A), respectively. As positive controls, the expression of β-*actin* gene was detected using the following primers: Actin F: 5′-GAGACCTTCAACACCCCAGC-3′ and Actin R: 5′-AGGAAGGCTGGAAAAGAGCC-3′.

Cloning the Full-Length cDNAs

Because *Duxbl* and *Duxbl-s* transcripts are predominantly expressed in adult ovary, total ovary RNAs were used to characterize the full-length *Duxbl* and *Duxbl-s* transcripts. The 5′ and 3′ end sequences of *Duxbl* and *Duxbl-s* transcripts were obtained by 5′ and 3′ RACE-PCRs using the SMART RACE cDNA amplification kit (Clontech), respectively. Total RNAs were reversely transcribed using 5′ or 3′ CDS primers. The resultant cDNA products were subjected to PCR for generating the 5′ and 3′ *Duxbl* and *Duxbl-s* cDNA fragments, respectively, using transcript-specific primers (GSP1, GSP2, or GSP3: 5′-AGCAGGAGCAGGATAAACCTAGAGTTAAAGA-3′) and UPM primer. The PCR products were then subjected to nested PCR using transcript-specific primers (GSP1 or GSP2) and nested universal primer A. Finally, the PCR products were analyzed on 1.2% agarose gels, subcloned into pGEM-T Easy vectors (Promega) and then sequenced.

Real-Time PCRs

Total RNAs were extracted from testes at different stages using TRI Reagent (Sigma-Aldrich). Primers for real-time PCRs were designed for mouse *Duxbl* and *GAPDH* transcripts. The forward primer for *Duxbl* was 5′-GCATCTCTGAGTCTCAAATTATGACTTG-3′, and the reverse primer was 5′-GCGTTCTGCTCCTTCTAGCTTCT-3′. The forward primer for *GAPDH* was 5′-TGTGTCCGTCGTGGATCTGA-3′, and the reverse primer was 5′-CCTGCTTCACCACCTTCTTG-3′. Complementary DNAs were synthesized from RNA samples using Superscript II RNase H-minus reverse transcriptase (Invitrogen) according to the manufacturer's protocol, and then cDNAs were used as templates for real-time PCR assays using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). Threshold (Ct) values for *Duxbl* and *GADPH* transcripts were determined using Prism SDS software version 1.0 (Applied Biosystems).

Expressions and Purifications of Duxbl Homeodomain Fusion Proteins

The coding regions of Duxbl N-terminal homeodomain (H1) and C-terminal homeodomain (H2) were PCR amplified, gel-purified, and then subcloned into pGEX-6P-2 expression vectors (Amersham). After induction, the expressed glutathione-S-transferase (GST) fusion proteins, GST-H1 and GST-H2, were purified using Glutathione Sepharose beads (Amersham). The forward primer for H1 homeodomain was 5′-CGGAATTCTTGAGCTGAGCTGCAGT-3′, and the reverse primer was 5′-CCCTCGAGCTATGCAAACTCTGCTTG-3′. The forward primer for H2 homeodomain was 5′-CGGAATTCTTAGAGTTAAAGAAGCTAGAAG-3′, and the reverse primer was 5′-GCGGCC-GCTTAAGTTTTCTGAGTGTTCTGTCC-3′.

Production of Anti-Duxbl Polyclonal Antibodies and Western blotting

The *Duxbl* coding region was PCR amplified, gel-purified, and then inserted into the pAE expression vector (Ramos et al., 2004). The Duxbl fusion proteins containing 6 histidine residues at the N-terminus (6His-Duxbl) were overexpressed and then purified using His-bind resin (Novagen), before being used to immunize rabbits for generation of anti-serum against Duxbl proteins. These anti-Duxbl polyclonal antibodies were purified by affinity chromatography using 6His-Duxbl fusion proteins. To test the immunospecificity of purified anti-Duxbl antibodies, we performed a blocking assay using antibodies pretreated with purified GST-H1 and GST-H2 proteins or GST protein only. To detect Duxbl protein in various mouse tissues, equal amount of total proteins extracted from various adult mouse tissues were separated in 12% sodium dodecyl sulfate-polyacrylamide gels and electro-transferred onto polyvinylidene fluoride membranes (Millipore). Expression levels of Duxbl proteins were then determined by Western blotting using purified anti-Duxbl polyclonal antibodies.

Whole-Mount In Situ and Section In Situ Hybridizations

Expressions of the *Duxbl* gene were analyzed by in situ hybridizations using a 660-bp *Duxbl* cDNA fragment (265-925), which encodes the coding region of Duxbl protein. The 660-bp *Duxbl* cDNA fragments (position 265-925) were subcloned into pGEM-T Easy vectors. The sense and anti-sense riboprobes were prepared by in vitro transcriptions using SP6 and T7 RNA polymerases with digoxigenin (Dig)-UTP (Boehringer Mannheim), respectively. Some serial sections were stained with hematoxylin and eosin (Sigma-Aldrich).

Testes from adult mice and ovaries from 10-day-old mice were collected and fixed in 4% paraformaldehyde for in situ hybridizations. They were dehydrated, embedded in paraffin, and then serially sectioned. Five- or 7-μm sections were cut and counterstained with methyl green. These sections were then mounted before observation. For whole-mount in situ hybridizations, embryos were rehydrated and bleached in PBT containing 6% hydrogen peroxide. Whole-mount in situ hybridizations of embryos was performed as previously described (Correia and Conlon, 2001).

Immunohistochemistry

Embryos of FVB mice and adult ovaries were fixed in 4% paraformaldehyde and embedded in paraffin according to standard protocols. Adjacent 6-μm sections were used for comparative analysis. Sections were deparaffinized and rehydrated, and some of them were stained with hematoxylin and eosin. Sections were incubated with a 1:300 dilution of purified anti-Duxbl polyclonal antibodies or with 1:500 dilution of mouse monoclonal anti-MyoD antibody clone 5.8A (IMGENEX). After washing, sections were incubated with a 1:200 dilution of biotinylated goat anti-rabbit IgG or biotinylated goat anti-mouse IgG followed by incubation with horse radish peroxidase-streptavidin complexes. Positive signals were visualized by incubation with diaminobenzidine (DAB), a kit from Molecular Probes, and sections were then lightly counterstained with methyl green (Sigma-Aldrich). Negative controls consisted of identical reactions with normal rabbit immunoglobulin G as the primary antibodies.

Cell Cultures

C2C12 cells were kept in DMEM supplemented with 10% fetal bovine serum. Differentiation in C2C12 cells was induced by replacing the medium with differentiation medium (2% horse serum in DMEM). HeLa cells were kept in alpha-MEM supplemented with 10% fetal calf serum, 1% nonessential amino acids, and 1% sodium pyruvate.

Subcellular Localizations of Duxbl and Duxbl-s Proteins

The coding regions of *Duxbl* and *Duxbl-s* were inserted into pcDNA3.1/V5-His (Life technologies) and pFLAG-CMV2 (Sigma-Aldrich) to produce the Duxbl-V5 and FLAG-Duxbl-s expression vectors, respectively. The expression vectors were transfected into HeLa cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After transfection, cells were immunostained with a 1:500 dilution of purified anti-Duxbl polyclonal antibodies and then a 1:200 dilution of fluorescein isothiocyanate (FITC) -conjugated goat anti-rabbit immunoglobulin. After immunostaining, cell nuclei were counterstained with 4′-6-diamidino-2-phenylindole (DAPI; Sig-ma-Aldrich). The fluorescent signals were obtained by fluorescence microscopy (Axioplan, Zeiss).

Immunocytochemistry

Expressions of Duxbl and MyoD proteins in C2C12 cells were examined by immunocytochemistry. C2C12 myoblasts were grown in 4-well chamber slides and were allowed to differentiate into myotubes. The cells were then fixed in 4% paraformaldehyde followed by permeable with 0.1% Triton. Cells were blocked with 1% bovine serum albumin in phosphate buffered saline (PBS). C2C12 cells were immunostained with rabbit anti-Duxbl (1:500) polyclonal antibodies and mouse monoclonal anti-MyoD (1:500) antibody. Cells were washed with PBS, incubated with FITC-conjugated chicken anti-mouse IgG antibodies and Texas red-conjugated goat anti-rabbit IgG antibodies at a 1:300 dilution, and then counterstained for nuclei with DAPI. The fluorescent signals were obtained by fluorescence microscopy.

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