Zebrafish Dkk3a Protein Regulates the Activity of myf5 Promoter through Interaction with Membrane Receptor Integrin α6b

Received for publication, June 27, 2012, and in revised form, September 11, 2012 Published, JBC Papers in Press, September 28, 2012, DOI 10.1074/jbc.M112.395012

Chuan-Yang Fu, Ying-Fang Su, Ming-Hsuan Lee, Geen-Dong Chang, and Huai-Jen Tsai
From the Institutes of Molecular and Cellular Biology and Biochemical Sciences, National Taiwan University, Number 1, Section 4, Roosevelt Road, Taipei 106, Taiwan

**Background:** Dkk3a regulates the promoter activity of myf5 through an unknown receptor. **Results:** Igα6b is a high affinity Dkk3a receptor through its β-propeller domains. **Conclusion:** Dkk3a regulates the promoter activity of myf5 through interaction with receptor Igα6b. **Significance:** This study discovered a novel mechanism for Dkk3a to regulate the promoter activity of myf5 during myogenesis.

Myogenic regulatory factor Myf5 plays important roles in muscle development. In zebrafish myf5, a microRNA (miR), termed miR-3906 or miR-ln300, was reported to silence dickkopf-3-related gene (dkk3r or dkk3a), resulting in repression of myf5 promoter activity. However, the membrane receptor that interacts with ligand Dkk3a to control myf5 expression through signal transduction remains unknown. To address this question, we applied immunoprecipitation and LC-MS/MS to screen putative membrane receptors of Dkk3a, and Integrin α6b (Igα6b) was finally identified. To further confirm this, we used cell surface binding assays, which showed that Dkk3a and Igα6b were co-expressed at the cell membrane of HEK-293T cells. Cross-linking immunoprecipitation data also showed high affinity of Igα6b for Dkk3a. We further proved that the β-propeller repeat domains of Igα6b are key segments bound by Dkk3a. Moreover, when dkk3a and Igα6b mRNAs were co-injected into embryos, luciferase activity was up-regulated 4-fold greater than that of control embryos. In contrast, the luciferase activities of dkk3a knockout embryos co-injected with Igα6b mRNA and Igα6b knockout embryos co-injected with dkk3a mRNA were decreased in a manner similar to that in control embryos, respectively. Knockdown of Igα6b resulted in abnormal somite shape, fewer somitic cells, weaker or absent myf5 expression, and reduced the protein level of phosphorylated p38α in somites. These defective phenotypes of trunk muscular development were similar to those of dkk3a knockout embryos. We demonstrated that the secreted ligand Dkk3a binds to the membrane receptor Igα6b, which increases the protein level of phosphorylated p38α and activates myf5 promoter activity of zebrafish embryos during myogenesis.

The Dickkopf (Dkk) family consists of dkk1–4, and soggy (sgy), also named dkk3-related gene (dkk3l) (1). Dkks contain two conserved cysteine-rich domains: an N-terminal cysteine-rich domain unique to Dkks and a C-terminal cysteine-rich domain related to the collapse fold. All Dkks share 37–50% protein identity, but they contain a variable linker region to separate the N- and C-terminal conserved cysteine-rich regions (1). In addition to a secretory form of Dkk, which contains a signal sequence, an intracellular form of Dkk was also reported (2).

Dkk1, Dkk2, and Dkk4 inhibit Wnt signaling through binding to LRP5/6 by the transmembrane protein Kremen, resulting in internalization of LRP5/6, which prevents Wnt and Frizzled receptor from forming an active complex with LRP5/6 (3, 4). Dkk2 can also activate the Wnt pathway in certain situations, depending on the cell type, the presence of Wnt ligands, and levels of LRP5/6 (5–7). Dkk3 is the most divergent member of the Dkk family in terms of DNA structure, protein function, and evolutionary impact (8). For example, it has been reported that Dkk3 does not physically interact with LRP5/6 or Kremen (3, 9). Unlike the other Dkk members, Dkk3 does not regulate Wnt signaling in various activity assays, including Wnt-dependent secondary axis induction in Xenopus embryos and Wnt1/Fz8 signaling in cultured cells (1, 5, 6). On the other hand, some studies have demonstrated that Dkk3 does regulate Wnt signaling, including weak inhibition of Wnt7A signaling in PC12 cells (10) as well as inhibition of Wnt activity in the osteosarcoma Saos-2 cell line (11). Thus, whether Wnt signaling is regulated by Dkk3 is still controversial.

The dkk3 gene is identical to the reduced expression in immortalized cells gene (REIC) (12), and REIC/Dkk3 has been shown to act as a tumor suppressor or antioncogene because the expression of dkk3 is decreased in a variety of cancer cell lines (13–16). Thus, human Dkk3 provides a new strategy against some human malignant tumors. For example, the
forced expression of REIC/Dkk inhibited cell growth in HeLa and liver cancer cell lines (17). Furthermore, overexpression of dkk3 suppresses the growth of cells and the invasive capacity of cancer cell lines (11, 15, 18, 19). Apart from the study of Dkk3 function in cell lines, dkk3 is reported to be expressed in many organs during mouse embryonic development, including neural epithelium, limb bud, bone, and heart, particularly in regions of epithelial-mesenchymal transformation (20). The dkk3 gene is also widely expressed in adult tissues with the highest levels found in the heart and brain (1). In zebrafish, two dkk3 genes, dkk3a and dkk3r (NCBI Reference Sequence NM_001159454.1) and dkk3b (NCBI Reference Sequence NM_001089523.1), have been reported (21). The dkk3a is strongly expressed in various neuronal structures of the head, whereas dkk3b is expressed mainly in the endoderm cells of the pancreas and brachial arches. Nevertheless, the function of Dkk3 during embryonic development is extremely limited.

Myf5, a myogenic regulatory factor, plays important roles in the specification and differentiation of muscle cells during myogenesis. Recently, we found a novel microRNA, named mir-1300 (miR-3906), which binds to the 3′-untranslated region (UTR) of the mRNA of dkk3a (22). Knockdown of dkk3a with dkk3a morpholino (MO) resulted in the down-regulation of myf5 expression, suggesting that Dkk3a is involved in the upstream positive regulation of myf5. Furthermore, Hsu et al. (23) discovered that secreted Dkk3a can activate the phosphorylation of p38a and that phosphorylated p38a (p-p38a) causes stabilization of Smad4 through sumoylation. Stabilization of Smad4 enables formation of a Smad2/3a/4 complex, which then enters the nucleus to activate the myf5 promoter.

Although β-transducin repeat-containing proteins (24) and dynein light chain Tctex-1 (25) have been identified to associate with intracellular Dkk3 in human ovary, the membrane receptor that interacts with extracellular ligand Dkk3a to control zebrafish myf5 expression through p38a signal transduction is totally unknown. In this study, we found that Integrin α6b (Itgα6b) is a membrane receptor of secretory Dkk3a and that the interaction of Dkk3a and Itgα6b drives the downstream signal transduction to regulate myf5 promoter activity in somites during the development of zebrafish embryos.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—Full-length cDNAs of zebrafish dkk3a and itgα6b were cloned from embryos at 16 hours postfertilization (hpf) by polymerase chain reaction (PCR). The open reading frames (ORF) of dkk3a and itgα6b were inserted into pCS2+ to generate pCS2-dkk3a and pCS2-itgα6b, respectively. Plasmids pCS2-dkk3a-FLAG and pCS2-itgα6b-MYC were engineered by ligation of FLAG and MYC epitopes fused after the ORFs of dkk3a and itgα6b, respectively. The serial deletions of itgα6b were obtained by PCR using pCS2-itgα6b as a template. The baculovirus recombinant transfer vectors pVL1392-dkk3a-FLAG, pVL1392-EGFP-FLAG, pVL1393-itgα6b-MYC, pVL1393-itgα6bΔ1-MYC, pVL1393-itgα6bΔ2-MYC, and pVL1393-itgα6bΔ3-MYC were constructed from the transfer vector pVL1393 (BD Biosciences) in which DNA fragments of dkk3a-FLAG, EGFP-FLAG, itgα6b-MYC, itgα6bΔ1-MYC, itgα6b-Δ2-MYC, and itgα6bΔ3-MYC were inserted, respectively. Plasmid pCMV-Itgα6b-EGFP was constructed by inserting itgα6b ORF into plasmid pCMV-EGFP (Clontech). Plasmid pCS2-itgα6b-MO-target-EGFP was constructed by inserting a target sequence for itgα6b MO, which is located at +52/+86 within the 5′-UTR of itgα6b mRNA, into pCS2-EGFP. Plasmid pCS2-NSL-EGFP was inserted as a nuclear localization signal (NLS) sequence into pCS2-EGFP. Plasmids pCS2-itgα6bΔ1 lacking five repeat domains, pCS2-itgα6bΔ2 lacking the first seven repeat domains, and pCS2-itgα6bΔ3 lacking the last four repeat domains were constructed to study the activity of binding domains and interaction between Itgα6b and Dkk3a.

In the rescue experiment, we used PCR to mutate the target nucleotide sequences of dkk3a MO (ATGTTTCTGCTCGATTACGTCTC) and itgα6b MO (ATGGAAATCTTACAGCACTTTAACC) into dkk3aMO (wobble) (ATTGTTCTCTCTGGGTATGCTT) and itgα6bMO (wobble) (ATGGAGTCCTATAGACCTTGAC), respectively, (the mutated nucleotides are underlined) without changing their amino acid sequences. The dkk3aMO (wobble) and itgα6bMO (wobble) were inserted into pCS2+ and pCS2+-FLAG to generate pCS2-dkk3aMO (wobble), pCS2-itgα6bMO (wobble), and pCS2-dkk3a (wobble)-FLAG.

Baculovirus Protein Expression System—Plasmids pVL1392-dkk3a-FLAG, pVL1392-EGFP-FLAG, pVL1393-itgα6b-MYC, pVL1393-itgα6bΔ1-MYC, pVL1393-itgα6bΔ2-MYC, and pVL1393-itgα6bΔ3-MYC were used to express recombinant proteins in the insect cell line Sf21 using a baculovirus protein expression system according to procedures described in the BaculoGold™ transfection kit (BD Biosciences).

Immunoprecipitation—Recombinant baculovirus containing plasmids pVL1392-dkk3a-FLAG and pVL1392-EGFP-FLAG were individually transfected into Sf21 cells. The recombinant proteins Dkk3a-FLAG and EGFP-FLAG were purified by anti-FLAG beads (Sigma). Immunoprecipitation of Dkk3a-FLAG and EGFP-FLAG with extracts isolated from zebrafish embryos at 16 hpf followed the protocols described in the Pierce Crosslink Immunoprecipitation kit (Thermo). The resultant immunoprecipitates were analyzed by SDS-PAGE followed by silver staining and in-gel digestion.

In-gel Digestion and LC-MS/MS Analysis—The immunoprecipitates shown on SDS-PAGE were cut into many gel pieces (26) using Mascot Distiller (Matrix Science, UK). The resultant MGF file was searched using the Mascot search engine (v2.2, Matrix Science) with the following conditions. 1) The protein database was set as Swiss-Prot. 2) Taxonomy was set as Danio rerio (zebrafish). 3) One trypsin missed oxidation (Met) and deamidation (Asn and Gln) were chosen. 4) The peptide mass tolerance was set to ±0.5 Da, and the fragment mass tolerance was set to ±0.5 Da. 5) Carbamidomethyl (Cys) was chosen as a fixed modification. 6) Oxidation (Met) and deamidation (Asn and Gln) were chosen as variable modifications.

Cell Surface Binding Assay—The conditional media used to culture Sf21 cells infected with recombinant baculovirus containing pVL1392-dkk3a-FLAG to produce extracellular Dkk3a-FLAG were concentrated about 40-fold using Amicon Ultra (Millipore). The concentration of secreted Dkk3a-FLAG was
determined by Western blotting using anti-FLAG antiserum. For cell surface binding experiments, HEK-293T cells were separately transfected with pCMV-itg\(\alpha 6b\)-EGFP and pCMV (served as control) using Lipofectamine 2000 (Invitrogen). After transfection for 48 h in a 6-well plate, cells were incubated with Dkk3a-FLAG proteins for 30 min at 4 °C. We discarded the conditional media, washed cells with PBS, and fixed them with 4% paraformaldehyde. Then cells were stained with anti-FLAG-Alexa Fluor 555 (Cell Signaling Technology) and observed under confocal microscopy (Zeiss LSM 780).

**Cross-linking Immunoprecipitation**—The recombinant protein Dkk3a-FLAG in concentrated media and the insect cell line separately infected with Itg\(\alpha 6b\)-MYC, Itg\(\alpha 6b\)1-MYC, Itg\(\alpha 6b\)2-MYC, and Itg\(\alpha 6b\)3-MYC were mixed with bis(sulfo)succinimidyl]suberate to perform cross-linking immunoprecipitation according to the procedures described for bis(sulfo)succinimidyl]suberate cross-linkers (Thermo). Cell lysates were then subjected to immunoprecipitation with anti-MYC beads (Sigma). The immunoprecipitates were prepared for Western blot analysis.

**In Vitro Transcription, Whole-mount in Situ Hybridization (WISH), Fluorescence Microscopy Observation, Western Blot Analysis, and Luciferase Activity Detection**—The synthesis of capped mRNAs, labeling of probes for WISH, observation of embryos under fluorescence microscopy, extraction of total proteins from embryos, Western blotting, and quantitative measurement of luciferase activity followed the methods described by Hsu et al. (23).

**Knockdown Experiments**—The antisense MOs designed specifically to knock down itg\(\alpha 6b\) (itg\(\alpha 6b\) MO) and dkk3a (dkk3a MO) were CGGTTAAAGTCCTGTAAGATTCCAT and GAGGCTGAATCCGAGCAGAAACATG, respectively. The itg\(\alpha 6b\) negative control MO (itg\(\alpha 6b\) control MO) and dkk3a

---

**FIGURE 1.** The recombinant protein Dkk3a fused with FLAG reporter was produced by the baculovirus expression system in insect cells. The recombinant Dkk3a fused with FLAG reporter (Dkk3a-FLAG) was produced by insect cells through a baculovirus expression system and was purified by immunoprecipitation with antiserum against FLAG peptide (anti-FLAG). The precipitated proteins were analyzed by SDS-PAGE and stained with Coomassie Blue. The bands shown on SDS-PAGE, including 25-kDa EGFP-FLAG (marked with *), 40-kDa non-glycosylated Dkk3a-FLAG, 48-kDa glycosylated Dkk3a-FLAG (marked with **), 55-kDa antibody heavy chain (marked with △), and 22-kDa antibody light chain (marked with ▽), were identified. M, protein markers.

**FIGURE 2.** The protein profiles of Dkk3a-FLAG immunoprecipitated with zebrafish membrane proteins. The recombinant Dkk3a-FLAG produced by a baculovirus expression system in insect cells was immunoprecipitated with membrane proteins extracted from zebrafish embryos. The precipitated proteins were stained with silver stain. Lane 1, protein markers; lane 2, protein profiles when Dkk3a-FLAG was added with anti-FLAG beads; lane 3, protein profiles when zebrafish membrane proteins were immunoprecipitated with anti-FLAG beads; lane 4, protein profiles when Dkk3a-FLAG was purified by anti-FLAG beads and then immunoprecipitated with zebrafish membrane proteins. Compared with lanes 2 and 3, many extra protein bands are shown in lane 4 that were used to further identify their amino acids by LC-MS/MS. The bands excised for LC-MS/MS are marked with brackets.
TABLE 1
A profile of putative genes and their encoded proteins obtained by LC-MS/MS analysis

After the Dkk3a-FLAG fusion protein was purified by anti-FLAG beads, it was immunoprecipitated with zebrafish membrane proteins.

| Location       | Gene ID   | Common name                                      |
|----------------|-----------|--------------------------------------------------|
| Cell membrane  | gi77682215| Voltage-gated sodium channel type IV α subunit   |
|                | gi117606157| Interleukin 1 receptor accessory protein-like   |
|                | gi63101376| Gtbp1 protein                                    |
|                | gi111955346| Potassium voltage-gated channel, subfamily H, member 2-like |
| Mitochondrion  | gi61806466| Integrin-66                                     |
|                | gi192447383| Serotonin receptor 1B (5-HT1B)                   |
|                | gi47550717| Adenine nucleotide translocator 1               |
|                | gi45709332| Solute carrier family 25, member 3              |
|                | gi62955065| ATP-binding cassette, subfamily B, member 8       |
|                | gi71892474| ATPase, Ca2+-transporting, cardiac muscle, slow twitch 2B |
|                | gi117606266| ATPase, Ca2+-transporting, cardiac muscle, fast twitch 1-like |
|                | gi16033712| Solute carrier family 45, member 2              |
|                | gi47777298| Inner membrane protein, mitochondrial            |
|                | gi116325975| ATP synthase, H+-transporting, mitochondrial F complex, α subunit 1, cardiac muscle |
| Endoplasmic reticulum | gi41152375| Mitochondrial ATP synthase γ subunit           |
|                | gi44890302| Ribophorin II                                   |
|                | gi41393119| Valosin-containing protein                       |
|                | gi37362194| Chaperonin-containing TCP1, subunit 8           |
|                | gi40807135| Cct4 protein                                    |
|                | gi40548312| ADP-ribosylation factor 5                       |
|                | gi4502203| ADP-ribosylation factor 3                       |
|                | gi50540124| Sec61 β subunit                                 |
| Cytoskeleton   | gi38174455| Myhβ protein                                    |
|                | gi57222229| Talin 1                                         |
|                | gi188588249| Actin, α, cardiac muscle 1b                     |
|                | gi37681963| Tubulin, β, 2                                   |
|                | gi47087047| ARP1 actin-related protein 1 homolog B           |
|                | gi29179446| Rpn1 protein                                    |
| Other          | gi226283315| Heat shock protein 90-kDa α, class B member 1    |
|                | gi113681112| Heat shock protein 90-kDa α                      |
|                | gi80751129| Ubiquitin A 52-residue ribosomal protein fusion 1 |
|                | gi18858871| Heat shock cognate 70-kDa protein               |
|                | gi189530294| Predicted: similar to type I thrombospondin domain-containing 7A-like |

negative control MO (dkk3a control MO) were designed as CGGTAAAATCATGTTAAATTCAT AT and GACGCT-CAAATCGAACCACCAGATG, respectively (the mutated-mismatched nucleotides are underlined). All MOs were prepared at a stock concentration of 1 mM and diluted to the desired concentration for microinjection into each embryo.

RESULTS


dkk3a Is a High Affinity Dkk3a Receptor—To search for the membrane receptor bound by the secreted Dkk3a, we first produced recombinant Dkk3a fused with the FLAG reporter using the baculovirus expression system in insect cells (Fig. 1), and the protein function of dkk3a was not altered by adding a FLAG reporter at the C terminus (supplemental Fig. S1). This recombinant Dkk3a-FLAG was immunoprecipitated with membrane proteins extracted from zebrafish embryos at 16 hpf. The precipitated proteins were analyzed by SDS-PAGE with silver staining (Fig. 2). We further applied LC-MS/MS to reveal the putative proteins bound by Dkk3a-FLAG, including cell membrane proteins, mitochondrial proteins, endoplasmic reticulum proteins, and cytoskeleton proteins (Table 1). Among them, we focused on the membrane proteins and then screened them by an in vivo luciferase assay of myf5 promoter. Because Itgα6b was capable of up-regulating myf5 promoter activity (see Fig. 4), we proposed that Itgα6b might be a likely membrane receptor bound by Dkk3a. To further confirm this, we used a cell surface binding assay, which demonstrated that Dkk3a and Itgα6b were co-expressed at the cell membrane of HEK-293T cells (Fig. 3). Data from the cell surface cross-linking experiment combined with cross-linking immunoprecipitation also demonstrated the high affinity of Itgα6b for Dkk3a (see Fig. 5C). Based on this evidence, we suggested that Itgα6b is a high affinity Dkk3a receptor.

Binding between Ligand Dkk3a and Membrane Receptor Itgα6b Up-regulates the Promoter Activity of myf5—To further confirm the impact of binding between ligand Dkk3a and membrane receptor Itgα6b on myf5 expression in zebrafish embryos, we injected several constructs. The luciferase activity of embryos injected with control plasmid pZmyf5 6.3R in which the reporter was driven by the upstream 6.3 kb of myf5 served as 100%. Compared with control embryos, the luciferase activity of embryos injected with excessive dkk3a mRNA and itgα6b mRNA increased 223 and 217%, respectively (Fig. 4). Interestingly, when dkk3a and itgα6b mRNAs were co-injected into embryos, the luciferase activity was up-regulated as high as 396%, which was 4-fold greater than that of control embryos (Fig. 4), suggesting that binding between ligand Dkk3a and membrane receptor Itgα6b results in synergistic enhancement of myf5 promoter activity. In contrast, the luciferase activity of embryos injected with dkk3a MO alone, itgα6b MO alone, dkk3a MO plus itgα6b MO, dkk3a MO plus itgα6b mRNA, or itgα6b MO plus dkk3a mRNA was decreased to 49–69% of control embryos (Fig. 4), suggesting that the binding between ligand Dkk3a and its receptor, Itgα6b, results in up-regulation of myf5 promoter activity.

The β-Propeller Repeat Domains of Itgα6b Are Key Segments Bound by Dkk3a to Regulate myf5 Promoter Activity—To determine which segment of Itgα6b acts as a key motif in regulating myf5 promoter activity, we analyzed the domain structure of Itgα6b using the NCBI database. The data showed a total of five β-propeller repeat domains at the N-terminal region of Itgα6b (Fig. 5A). Because the β-propeller repeat domain has been reported as a ligand-binding region of the integrin α subunit for signal transduction (27), we designed three constructs, namely Itgα6bΔ1 lacking five repeat domains, Itgα6bΔ2 lacking the first repeat domains, and Itgα6bΔ3 lacking the last four repeat domains (Fig. 5A), and performed an in vivo myf5 promoter luciferase assay. Compared with the embryos injected with pZmyf5 6.3R alone, the luciferase activity driven by myf5 promoter in the embryos injected with excessive itgα6b mRNA was increased up to 227% (Fig. 5B). In contrast, the luciferase activity of embryos injected with deletion clones (itgα6bΔ1 mRNA, itgα6bΔ2 mRNA, and itgα6bΔ3 mRNA) remained unchanged (Fig. 5B), suggesting that the β-propeller repeat domains of Itgα6b are required for the regulation of myf5 promoter activity by Itgα6b. The cell surface cross-linking experiment combined with co-immunoprecipitation also detected the binding between the fusion proteins Itgα6b-MYC and Dkk3a-FLAG.
On the other hand, the fusion proteins Itgα6b/H92516b/H90041-MYC, Itgα6b/H92516b/H90042-MYC, and Itgα6b/H92516b/H90043-MYC could not bind with Dkk3a-FLAG (Fig. 5C), indicating that β-propeller repeat domains of Itgα6b are key segments interacting with Dkk3a to regulate myf5 promoter activity.

Knockdown of Itgα6b and Dkk3a Results in Similar Defects in Trunk Muscular Development—To observe the defects in muscle development induced by knockdown of Itgα6b and dkk3a, we injected itgα6b MO alone, dkk3a MO alone, and itgα6b MO plus dkk3a MO separately into one-celled fertilized eggs. Com-
Dkk3a Regulates myf5 through Interaction with Itgα6b

FIGURE 5. The β-propeller repeat domains of Itgα6b are key segments that interact with Dkk3a to control the promoter activity of myf5. A, schematic drawing of three different deletions of the Itgα6b domain. The mRNA encoding for each deletion construct was co-microinjected into the one-celled stage of embryos with pZmyf5 6.3R in which the luciferase reporter was driven by the upstream 6.3 kb segment of zebrafish myf5 promoter to carry out the transient luciferase assay. The luciferase activity driven by injected construct(s) was measured in three independent experiments. The average value of the embryos injected with only pZmyf5 6.3R served as 1 (100%). Compared with the embryos injected with pZmyf5 6.3R, the luciferase activity was increased in the embryos injected with pZmyf5 6.3R and itgα6b mRNA. In contrast, the luciferase activity was decreased in the embryos injected with mRNAs encoding for three different deletions of β-propeller repeat domains of itgα6b. The data were presented as the average ± standard deviation from measurements collected from three independent experiments and two measurements for each experiment. *** indicates a significant difference at p < 0.001. C, fusion proteins Itgα6b-MYC, Itgα6bΔ1-MYC, Itgα6bΔ2-MYC, and Itgα6bΔ3-MYC produced by Sf21 insect cells were incubated in medium containing recombinant protein Dkk3a-FLAG followed by cross-linking immunoprecipitation (IP) using anti-MYC. Afterward, proteins were extracted and analyzed by Western immunoblot (IB) using anti-MYC to detect Itgα6b-MYC, Itgα6bΔ1-MYC, Itgα6bΔ2-MYC, and Itgα6bΔ3-MYC (IB: MYC) and anti-FLAG to detect Dkk3a-FLAG (IB: FLAG). No band was observed if wild-type Sf21 (WT) was incubated with Dkk3a-FLAG (lane 1). However, two positive bands were detected when Itgα6b-MYC was incubated with Dkk3a-FLAG (lane 2); a band representing an interaction complex between Itgα6b-MYC and Dkk3a-FLAG had the highest molecular mass and is marked with *, and a band representing recombinant Itgα6b-MYC displayed strong intensity. When Itgα6bΔ1-MYC (lane 3), Itgα6bΔ2-MYC (lane 4), or Itgα6bΔ3-MYC (lane 5) was separately incubated with Dkk3a-FLAG, no band representing an interaction complex was observed except for a band representing the recombinant protein per se.
Dkk3a Regulates myf5 through Interaction with Itgα6b

Knockdown of Itga6b and dkk3a causes similar defective phenotypes in somites of zebrafish embryos. WT embryos and embryos injected with 12 ng of itga6b MO alone, 2 ng of dkk3a MO alone, and 12 ng of itga6b MO plus 2 ng of dkk3a MO were observed at 16 hpf under light microscopy (A, B, C, and D). Compared with WT (A), the itga6b MO-injected embryos exhibited such abnormal phenotypes as incomplete muscle development and irregular shape of somatic cells with no clear boundary (B). These phenotypes also occurred in embryos injected with dkk3a MO alone (C) and with itga6b MO plus dkk3a MO (D). Embryos were co-injected with NLS-EGFP and Lyn-Tomato to label nuclei in Fig. S2 and S3. Compared with WT (A), the itga6b MO-injected embryos (B, 1–3), dkk3a MO-injected embryos (C, 1–3), and itga6b MO- plus dkk3a MO-injected embryos (D, 1–3) exhibited a decreased number of cells in somites. E, cell number within somites of each group is quantified as indicated. Data are presented as the average of five embryos for three independent times. ** and *** indicate the significant differences of values at p < 0.01 and p < 0.001 levels, respectively. Scale bar: A–D, 10 μm; A’–D’, 25 μm.

Knockdown of Itga6b and Dkk3a Results in the Down-regulation of myf5 Expression—To investigate and characterize myf5 expression induced by knockdown of itga6b and dkk3a in the somites of embryos, we injected itga6b MO alone, dkk3a MO alone, and itga6b MO plus dkk3a MO separately into one-celled fertilized eggs. These embryos were then subjected to WISH to examine the expression of myf5 at 16 hpf. The non-injected embryos (WT) served as the control group, which showed myf5 expression in presomitic mesoderm (indicated by a bracket) and five somites from −II, −I, to 0 and from 1 to 2 (Fig. 7 A). We noted a weak expression of myf5 in the mature somites (I and II) (Fig. 7 A, black arrowheads) but a strong expression of myf5 in the newly forming somites (0, −I, and −II), presenting a V-shape (Fig. 7 A, red arrowheads). However, in the itga6b MO-injected, dkk3a MO-injected, and itga6b MO- plus dkk3a MO-injected embryos, myf5 was expressed normally in presomitic mesoderm, but it was significantly down-regulated in the newly forming somites (0, −I, and −II), presenting a horizontal shape, and absent in the mature somites.

pared with the non-injected wild-type embryos at 16 hpf (Fig. 6A), all injected embryos exhibited similar abnormal phenotypes, such as incomplete muscle development and irregular shape of somitic cells with no clear boundary (Fig. 6, B, C, and D, and Table 2). However, the number of somites in all defective embryos was unaffected. Furthermore, we injected NLS-EGFP and Lyn-Tomato mRNA, which were used to label cell nuclei and cell membrane, respectively, into wild-type embryos or embryos injected as noted above. Compared with the non-injected wild-type embryos at 16 hpf (Fig. 7), we noted a weak expression of myf5 in the mature somites (I and II) (Fig. 7A), black arrowheads) but a strong expression of myf5 in the newly forming somites (0, −I, and −II), presenting a V-shape (Fig. 7A, red arrowheads). However, in the itga6b MO-injected, dkk3a MO-injected, and itga6b MO- plus dkk3a MO-injected embryos, myf5 was expressed normally in presomitic mesoderm, but it was significantly down-regulated in the newly forming somites (0, −I, and −II), presenting a horizontal shape, and absent in the mature somites.
Dkk3a Regulates myf5 through Interaction with Itgα6b

TABLE 2
Morphological phenotypes and myf5 expression of zebrafish embryos derived from fertilized eggs injected with different materials

| Injected materials | Concentration | Morphological phenotypes | myf5 expression |
|--------------------|---------------|---------------------------|----------------|
|                    |               | Wild type-like | Abnormal | Normal | Defect |
| itgα6b control MO  | 12 ng         | 93% (198/214) | 7% (16/214) | 90% (141/156) | 10% (15/156) |
| itgα6b MO          | 12 ng         | 37% (71/193)  | 63% (122/193) | 30% (40/133) | 70% (93/133) |
| dkk3a control MO  | 2 ng          | 89% (171/193) | 11% (22/193) | 86% (111/129) | 14% (18/129) |
| dkk3a MO           | 2 ng          | 24% (46/190)  | 76% (144/190) | 22% (33/149) | 78% (116/149) |

|                   |               | Wild type-like | Abnormal | Normal | Defect |
| itgα6b control MO | 12 ng         | 85% (182/214) | 15% (33/214) | 83% (114/129) | 17% (24/138) |
| dkk3a control MO  | 2 ng          | 83% (149/180) | 21% (24/116) | 79% (92/116) |
| itgα6b MO         | 12 ng         | 17% (31/180)  | 83% (149/180) | 21% (24/116) | 79% (92/116) |

FIGURE 7: Knockdown of itgα6b and dkk3a resulted in abnormal expression of myogenesis genes in zebrafish embryos. We performed WISH to detect the expression of two myogenesis genes, myf5 and myod, in embryos at 16 hpf. In the non-injected control embryos (WT), the mature somites (1 and 2) displayed a weak expression of myf5 (A, black arrowheads), but the newly forming somites (0, 1, and 2) displayed a strong expression of myf5 (A, red arrowheads). Compared with WT (A), embryos injected with 12 ng of itgα6b MO alone (B), 2 ng of dkk3a MO alone (C), and 12 ng of itgα6b MO plus 2 ng of dkk3a MO (D) showed normal myf5 expression in presomitic mesoderm (indicated by a bracket) but showed significant down-regulation of myf5 in the newly forming somites (0, 1, to 2), whereas myf5 expression was absent in mature somites (1 to 2). We also used WISH to detect the expression patterns of myod in the WT, itgα6b MO-injected, dkk3a MO-injected, and itgα6b MO- plus dkk3a MO-co-injected embryos and found that myod was expressed normally; it was present in the mature somites (from 0 to 6) but absent in the newly forming somites (E, F, G, and H), suggesting that the decrease or absence of myf5 expression in somites did not result from a lack of somites.

(1 and 2) (Fig. 7, B, C, and D, and Table 2). These data indicated that ligand Dkk3a regulates myf5 expression through receptor Itgα6b in zebrafish. Interestingly, we noticed that the defective phenotypes of myf5 expression and its occurrence percentage caused by co-injection of itgα6b MO plus dkk3a MO were similar to those of embryos injected with either itgα6b MO alone or dkk3a MO alone, suggesting that no negative synergistic effect occurred under these conditions. In addition, we also used WISH to detect the expression patterns of myod in the WT, itgα6b MO-injected, dkk3a MO-injected, and itgα6b MO- plus dkk3a MO-injected embryos. Results showed that myod was expressed normally in the mature somites (from 0 to 6) but absent in the newly forming somites in all injected embryos (Fig. 7, E, F, G, and H), suggesting that the decrease or absence of myf5 expression in somites did not result from a lack of somites.

Knockdown of Itgα6b and Dkk3a Results in the Decrease of the Protein Level of p-p38a—To study whether the phosphorylation of p38a is affected by knockdown of either receptor Itgα6b or ligand Dkk3a, we extracted total proteins of the 16-hpf embryos derived from one-celled fertilized eggs injected with either itgα6b MO or dkk3a MO. The total proteins were analyzed by Western blot. Compared with the wild-type embryos, the amounts of p38a protein in the itgα6b MO- and dkk3a MO-injected embryos were relatively unchanged; i.e. the p38a protein levels relative to α-tubulin levels among wild-type embryos, itgα6b MO-injected embryos, and dkk3a MO-injected embryos were 1:0.92:0.99, respectively (Fig. 8A). However, the protein level of p-p38a from embryos injected with either itgα6b MO or dkk3a MO was greatly reduced. In this case, the p-p38a levels relative to α-tubulin levels among wild-type embryos, itgα6b MO-injected embryos, and dkk3a MO-injected embryos were 1:0.32:0.23, respectively (Fig. 8A). On the other hand, we found that the amounts of p38a protein extracted from wild-type embryos, from embryos injected with dkk3a mRNA alone, and from embryos injected with dkk3a mRNA plus itgα6b MO were quite close. Under these conditions, the p38a levels relative to α-tubulin levels among wild-type embryos, dkk3a mRNA-injected embryos, and dkk3a mRNA- plus itgα6b MO-injected embryos were 1:0.97:0.99, respectively (Fig. 8B). However, although the protein level of p-p38a was increased in the dkk3a mRNA-injected embryos, it was reduced in the embryos injected with dkk3a mRNA plus itgα6b MO (Fig. 8B). Results showed that protein levels of p-p38a relative to α-tubulin levels among wild-type embryos, dkk3a mRNA-injected embryos, and dkk3a mRNA- plus itgα6b MO-injected embryos were 1:1.73:0.23, respectively (Fig. 8B). Similarly, the protein level of p-p38a was increased in the itgα6b mRNA-injected embryos but reduced in the embryos injected with itgα6b mRNA plus dkk3a MO (Fig. 8C). Specifically, the protein levels of p-p38a relative to α-tubulin levels among wild-type embryos, itgα6b mRNA-injected embryos, and itgα6b mRNA- plus dkk3a MO-injected embryos were 1:1.62:0.10, respectively (Fig. 8C). This line of evidence indicated that the
**FIGURE 8.** The protein level of p-p38a is enhanced by interaction between **Dkk3a** and **Itgα6b**. Total proteins (20 μg) were extracted from 16-hpf WT embryos and from 16-hpf embryos injected with the indicated materials. Western blot analysis was performed using a specific antibody against p38a, p-p38a, or α-tubulin, which served as an internal control. **A**, the Western signals of p38a and p-p38a levels from WT embryos, 12-ng *itgα6b* MO-injected embryos, and 2-ng *dkk3a* MO-injected embryos are shown. The p38a and p-p38a levels relative to α-tubulin levels are also indicated. **B**, the Western signals of p38a and p-p38a levels from WT embryos, 200-pg *dkk3a* mRNA-injected embryos, and 200-pg *dkk3a* mRNA- plus 12-ng *itgα6b* MO-injected embryos are shown. The p38a and p-p38a protein levels relative to α-tubulin levels are indicated. C, the Western signals of p38a and p-p38a levels from WT embryos, 200-pg *itgα6b* mRNA-injected embryos, 200-pg *itgα6b* mRNA- plus 2-ng *dkk3a* MO-injected embryos are shown. The p38a and p-p38a protein levels relative to α-tubulin levels are also indicated.

**Dkk3a Regulates myf5 through Interaction with Itgα6b**

ligand Dkk3a activates the phosphorylation of p38a through binding the receptor Itgα6b, which in turn up-regulates *myf5* expression in somites during muscle development.

The **Defective Expression of myf5 Caused by Either itgα6b MO or dkk3a MO Can Be Rescued by Introducing Either p38a mRNA or smad4 mRNA**—We asked whether *myf5* defects caused by *itgα6b* MO could be rescued by overexpression of *itgα6b* mRNA. In the *itgα6b* MO-injected embryos, 70% exhibited defects of *myf5* expression, but 30% (*n* = 133) did not (supplemental Fig. S4). However, when *itgα6b* MO was co-injected with *itgα6b*(wobble) mRNA, the percentage of embryos that exhibited no defect of *myf5* expression increased to 72% (*n* = 95) (supplemental Fig. S4), suggesting that the defect induced by *itgα6b* MO was specific. Interestingly, when *itgα6b* MO was we co-injected with *p38a* mRNA and *smad4* mRNA, the percentage of embryos that exhibited no defect of *myf5* expression increased to 63 (*n* = 104) and 64% (*n* = 98), respectively (supplemental Fig. S4). Similarly, when *dkk3a* was knocked down, we observed 74% of embryos (*n* = 141) with a defect in *myf5* expression (supplemental Fig. S4). However, the percentages of defects in the embryos injected with MO and excessive mRNAs of *dkk3a*(wobble), *p38a*, and *smad4* were reduced to 37 (*n* = 96), 42 (*n* = 98), and 41% (*n* = 103), respectively (supplemental Fig. S4). This line of evidence suggested that Dkk3a interacts with Itgα6b to regulate *myf5* expression through the downstream p38a and Smad4 pathways.

**Conclusion**—Based on the collective evidence shown in this study, we hypothesized a model to depict the impact of binding between ligand Dkk3a and receptor Itgα6b on the activation of zebrafish *myf5* promoter in somites during embryogenesis (Fig. 9). In summary, we demonstrated that secreted Dkk3a binds to the membrane receptor Itgα6b, which results in enhancement of the phosphorylation of p38a, which in turn increases the protein level of p-p38a and activates the *myf5* promoter. This finding further demonstrates that Dkk3a regulates the promoter activity of zebrafish *myf5* through the p38a signaling pathway.

**DISCUSSION**

**Dkk3a and Itgα6b Are Co-expressed in Zebrafish Embryonic Somites during Developmental Stages**—Integrins are important membrane receptors that are located in the extracellular matrix of animal cells. Integrins play important roles in cell adhesion, migration, gastrulation, morphogenesis, growth, proliferation, differentiation, apoptosis, and signaling transduction during development. The cytosolic integrins interact with actin filaments through binding cellular cytoskeleton proteins, such as vinculin, talin, β-actin, paxillin, and tensin (28), and activate many cytosolic signal transduction pathways (29). In this study, we demonstrated that zebrafish Dkk3a regulates muscle development through its interaction with the α form of integrins, in this case Itgα6b. In fact, the expression pattern and function of Itgα6b are largely unknown except for mouse *itgα6b*, which is expressed in the early developmental stages and is continuously presented in lateral plated mesoderm, heart, head, and forelimbs at embryonic day E9.5 (30). In somites, expression of mouse *itgα6b* is decreased in dermomyotome and sclerotome but elevated in myotome. In early epaxial myotome, the expres-
Dkk3a Regulates myf5 through Interaction with Itgα6b

This study demonstrated that secreted Dkk3a binds to the membrane receptor Itgα6b and activates the phosphorylation of p38a, consequently increasing the protein level of phosphorylated p38a. In the downstream pathway reported by Hsu et al. (22), Dkk3a causes the sumoylation (SUMO) of Smad4 through activation of phosphorylated p38a. Stabilized Smad4 then enables the formation of a Smad2/3a/4 complex, which then enters the nucleus to activate the myf5 promoter.

FIGURE 9. Schematic model of Dkk3a regulation of zebrafish myf5 promoter activity through binding receptor Itgα6b. This study demonstrated that secreted Dkk3a binds to the membrane receptor Itgα6b and activates the phosphorylation of p38a, consequently increasing the protein level of phosphorylated p38a. In the downstream pathway reported by Hsu et al. (22), Dkk3a causes the sumoylation (SUMO) of Smad4 through activation of phosphorylated p38a. Stabilized Smad4 then enables the formation of a Smad2/3a/4 complex, which then enters the nucleus to activate the myf5 promoter.
knockdown of myf5 as reported by Chen and Tsai (45) because knockdown of dkk3a results in the decrease of myf5 expression in somites. This line of evidence indicates that Dkk3a is involved in the positive regulation of myf5 expression. In this study, we found that knockdown of itgα6b results in embryos at 16 hpf having defective somites similar to those induced by knockdown of dkk3a. Additionally, myf5 expression in somites was decreased. On the other hand, when either excessive dkk3a mRNA or itgα6b mRNA was injected into embryos, myf5 promoter activity was activated. Furthermore, when dkk3a mRNA and itgα6b mRNA were combined and co-injected into embryos, myf5 promoter activity was greatly up-regulated, suggesting that dkk3a and itgα6b produce a synergism that strongly up-regulates myf5 expression. Recently, Wilshutz et al. (46) demonstrated that knockdown of Itgα6b causes down-regulation of myf5 and inhibits muscle cell proliferation in porcine primary muscle stem cells, indicating that Itgα6b is very important for muscle cell proliferation. Taken together, we proposed that ligand Dkk3a activates myf5 promoter through interaction with membrane receptor Itgα6b in somites during muscle development.

A Plausible Regulatory Model Showing How Ligand Dkk3a Interacts with Membrane Receptor Itgα6b to Control myf5 Expression in Zebrafish Embryos—Based on the evidence shown in this study and the report of Hsu et al. (23), we have formulated the following hypothesis. First, when secreted ligand Dkk3a binds to the membrane receptor Itgα6b, phosphorylation of p38a is activated, consequently increasing the protein level of p-p38a. Second, such increase in p38a phosphorylation and protein level causes Smad4 sumoylation to occur at the protein level of p-p38a. Second, such increase in p38a phosphorylation of p38a is activated, consequently increasing the activity of p-p38a. Third, such increase in p38a phosphorylation of p38a is activated, consequently increasing the activity of p-p38a. Additionally, p-p38a is activated, consequently increasing the activity of p-p38a. Second, such increase in p38a phosphorylation of p38a is activated, consequently increasing the activity of p-p38a. Additionally, p-p38a is activated, consequently increasing the activity of p-p38a. Further, such increase in p38a phosphorylation of p38a is activated, consequently increasing the activity of p-p38a. Additionally, p-p38a is activated, consequently increasing the activity of p-p38a. Therefore, such increase in p38a phosphorylation of p38a is activated, consequently increasing the activity of p-p38a. Additionally, p-p38a is activated, consequently increasing the activity of p-p38a. Second, such increase in p38a phosphorylation of p38a is activated, consequently increasing the activity of p-p38a. Additionally, p-p38a is activated, consequently increasing the activity of p-p38a. Thus, it is highly likely that Itgα6b may process inside-out signaling through binding of cytosolic talin 1, resulting in an increase in its affinity to outer membrane ligand Dkk3a. On the other hand, in the inside-out signaling pathway, integrins might be changed to increase affinity with outer membrane ligands if cytosolic proteins, such as talin or kindlins, bind integrins through the β tail. Based on the mass spectrometry data of Dkk3a-associated putative proteins as shown in Table 1, we found that such an interaction can be observed in zebrafish embryos at early stages.

Thus, it is highly likely that Itgα6b may process inside-out signaling through binding of cytosolic talin 1, resulting in an increase in its affinity to outer membrane ligand Dkk3a. On the other hand, in the inside-out signaling pathway, integrins might be changed to increase affinity with outer membrane ligands if cytosolic proteins, such as talin or kindlins, bind integrins through the β tail. Based on the mass spectrometry data of Dkk3a-associated putative proteins as shown in Table 1, we found that such an interaction can be observed in zebrafish embryos at early stages.

Furthermore, when dkk3a mRNA and itgα6b mRNA were combined and co-injected into embryos, myf5 promoter activity was greatly up-regulated, suggesting that dkk3a and itgα6b produce a synergism that strongly up-regulates myf5 expression. Recently, Wilshutz et al. (46) demonstrated that knockdown of Itgα6b causes down-regulation of myf5 and inhibits muscle cell proliferation in porcine primary muscle stem cells, indicating that Itgα6b is very important for muscle cell proliferation. Taken together, we proposed that ligand Dkk3a activates myf5 promoter through interaction with membrane receptor Itgα6b in somites during muscle development.

Dkk3a Regulates myf5 through Interaction with Itgα6b

REFERENCES

1. Krupnik, V. E., Sharp, J. D., Jiang, C., Robison, K., Chickering, T. W., Amaravadi, L., Brown, D. E., Guyot, D., Mays, G., Leiby, K., Chang, B., Duong, T., Goodearl, A. D., Gearing, D. P., Sokol, S. Y., and McCarthy, S. A. (1999) Functional and structural diversity of the human Dickkopf gene family. Gene. 238, 301–313
2. Nakamura, R. E., Hunter, D. D., Yi, H., Brunken, W. J., and Hackam, S. S. (2007) Identification of two novel activities of the Wnt signaling regulator Dickkopf 3 and characterization of its expression in the mouse retina. BMC Cell Biol. 8, 52
3. Mao, B., Wu, W., Davidson, G., Marhold, J., Li, M., Mechler, B. M., Delius, H., Hoppe, D., Stannek, P., Walter, C., Glinka, A., and Niehrs, C. (2002) Kremen proteins are Dickkopf receptors that regulate Wnt/β-catenin signaling. Nature 417, 664–667
4. Semenov, M. V., Tamai, K., Brett, B. K., Kühl, M., Sokol, S., and He, X. (2001) Head inducer Dickkopf-1 is a ligand for Wnt coreceptor LRP6. Curr. Biol. 11, 951–961
5. Brett, B. K. and Sokol, S. Y. (2002) Regulation of Wnt/β-catenin signaling by distinct domains of Dickkopf proteins. Mol. Cell. Biol. 22, 6100–6110
6. Wu, W., Glinka, A., Delius, H., and Niehrs, C. (2000) Mutual antagonism between dickkopf1 and dickkopf2 regulates Wnt/β-catenin signalling. Curr. Biol. 10, 1611–1614
7. Li, L., Mao, J., Sun, L., Liu, W., and Wu, D. (2002) Second cysteine-rich domain of Dickkopf-2 activates canonical Wnt signaling pathway via LRP-6 independently of dishevelled. J. Biol. Chem. 277, 5977–5981
8. Niehrs, C. (2006) Function and biological roles of the Dickkopf family of Wnt modulators. Oncogene 25, 7469–7481
9. Mao, B. and Niehrs, C. (2003) Kremen2 modulates Dickkopf2 activity during Wnt/LRP6 signaling. Gene 302, 179–183
10. Caricaseo, A., Ferraro, T., Iacovelli, L., Barletta, E., Caruso, A., Melchiorri, D., Terrapstatt, G. C., and Nicoletti, F. (2003) Functional characterization of WNT7A signaling in PC12 cells: interaction with a FZD5-LRP6 receptor complex and modulation by Dickkopf proteins. J. Biol. Chem. 278, 37024–37031
11. Hoang, B. H., Kubo, T., Healey, I. H. Yang, R., Nathan, S. S., Kolb, E. A., Mazza, B., Meyers, P. A., and Gorlick, R. (2004) Dickkopf 3 inhibits invasion and motility of Saos-2 osteosarcoma cells by modulating the Wnt-β-catenin pathway. Cancer Res. 64, 2734–2739
12. Tsuji, T., Nozaki, I., Miyaizaki, M., Sakaguchi, M., Pu, H., Hamazaki, Y., Iijima, O., and Namba, M. (2001) Antiproliferative activity of REIC/Dkk-3 and its significant down-regulation in non-small-cell lung carcinomas. Biochem. Biophys. Res. Commun. 289, 257–263
13. Nozaki, I., Tsuji, T., Iijima, O., Ohmura, Y., Andou, A., Miyaizaki, M., Shimizu, N., and Namba, M. (2001) Reduced expression of REIC/Dkk-3 gene in non-small-cell lung cancer. Int. J. Oncol. 19, 117–121
14. Kurose, K., Sakaguchi, M., Nasu, Y., Ebara, S., Kaku, H., Kariyama, R., Arao, Y., Miyaizaki, M., Tsushima, T., Namba, M., Kumor, H., and Huh, N. H. (2004) Decreased expression of REIC/Dkk-3 in human renal clear cell carcinoma. J. Urol. 171, 1314–1318
15. Lodygin, D., Epanchintsev, A., Menssen, A., Diebold, J., and Hermeking, H. (2005) Functional epigenomics identifies genes frequently silenced in prostate cancer. Cancer Res. 65, 4218–4227
16. Kuphal, S., Lodermeier, S., Bataille, F., Schuierer, M., Hoang, B. H., and Bosserhoff, A. K. (2006) Expression of Dickkopf genes is strongly reduced in malignant melanoma. Oncogene 25, 5027–5036
17. Kawasaki, K., Watanabe, M., Brown, D. E., Guyot, D., Mays, G., Leiby, K., Chang, B., Duong, T., Goodearl, A. D., Gearing, D. P., Sokol, S. Y., and McCarthy, S. A. (1999) Functional and structural diversity of the human Dickkopf gene family. Gene. 238, 301–313
18. Abarzua, F., Sakaguchi, M., Takashi, M., Nasu, Y., Kurose, K., Ebara, S., Miyazaki, M., Namba, M., Kumor, H., and Huh, N. H. (2005) Adenovirus-mediated overexpression of REIC/Dkk-3 selectively induces apoptosis in human prostate cancer cells through activation of c-Jun-NH2-kinase.
Dkk3a Regulates myf5 through Interaction with Itgα6b

Cancer Res. 65, 9617–9622.

19. Kawano, O.; Sasaki, H.; Endo, K.; Suzuki, E.; Haneda, H.; Yukiue, H.; Kubayashi, Y.; Yano, M.; and Fujii, Y. (2006) PIK3CA mutation status in Japanese lung cancer patients. Lung Cancer 54, 209–215.

20. Monaghan, A. P.; Kioschis, P.; Wu, W.; Zuniga, A.; Bock, D.; Poustka, A.; Delius, H.; and Niehrs, C. (1999) Dickkopf genes are co-ordinately expressed in mesodermal lineages. Mech. Dev. 87, 45–56.

21. Untergasser, G.; Martowicz, A.; Hermann, M.; Töchterle, S.; and Meyer, D. (2011) Distinct expression patterns of dickkopf genes during late embryonic development of Danio rerio. Gene Exp. Pattern 11, 491–500.

22. Hsu, R. J.; Lin, C. Y.; Hoi, H. S.; Zheng, S. K.; Lin, C. C.; and Tsai, H. J. (2010) Novel intronic microRNA represses zebrafish myf5 promoter activity through silencing dickkopf-3 gene. Nucleic Acids Res. 38, 4384–4393.

23. Hsu, R. J.; Lin, C. C.; Su, Y. F.; and Tsai, H. J. (2011) dickkopf-3-related gene regulates the expression of zebrafish myf5 gene through phosphorylated p38α-dependent Smad4 activity. J. Biol. Chem. 286, 6855–6864.

24. Lee, E. J.; Jo, M.; Rho, S. B.; Park, K.; Yoo, Y. N.; Park, J.; Chae, M.; Zhang, W.; and Lee, J. H. (2009) Dkk3, downregulated in cervical cancer, functions as a negative regulator of β-catenin. Int. J. Cancer 124, 287–297.

25. Ochiai, K.; Watanabe, M.; Ueki, H.; Huang, P.; Fujii, Y.; Nasu, Y.; Noguchi, H.; Hirata, T.; Sakaguchi, M.; Huh, N. H.; Kashikawa, Y.; Kaku, H.; and Kumon, H. (2011) Tumor suppressor REIC/Dkk-3 interacts with the dynein light chain, Tctex-1. Biochem. Biophys. Res. Commun. 412, 391–395.

26. Chiang, Y. H.; Wu, Y. J.; Lu, Y. T.; Chen, K. H.; Lin, T. C.; Chen, Y. K.; Li, D. T.; Shi, F. K.; Chen, C. C.; and Hsu, J. L. (2011) Simple and specific dual-wavelength excitable dye staining for glycoprotein detection in polyacrylamide gels and its application in glycoproteomics. J. Biomed. Biotechnol. 2011, 780108.

27. Springer, T. A. (1997) Folding of the N-terminal, ligand-binding region of integrin α-subunits into a β-propeller domain. Proc. Natl. Acad. Sci. U.S.A. 94, 65–72.

28. Chong, S. W.; Korzh, V.; and Jiang, Y. J. (2009) Myogenesis and molecules—insights from zebrafish Danio rerio. J. Fish Biol. 74, 1693–1755.

29. Hynes, R. O. (1987) Integrins: a family of cell surface receptors. Cell 48, 549–554.

30. Bajanca, F.; Luz, M.; Duxson, M. J.; and Thorsteinsdottir, S. (2004) Integmins in the mouse myotome: developmental changes and differences between the epaxial and hypaxial lineage. Dev. Dyn. 231, 402–415.

31. Otey, C. A. (1996) pp125FAK in the focal adhesion. Int. Rev. Cytol. 167, 161–183.

32. Lewis, J. M.; Baskaran, R.; Taagepera, S.; Schwartz, M. A.; and Wang, J. Y. (1996) Integrin regulation of c-Abl tyrosine kinase activity and cytoplasmic-nuclear transport. Proc. Natl. Acad. Sci. U.S.A. 93, 15174–15179.

33. Clark, E. A.; and Bruggue, J. S. (1993) Redistribution of activated pp60c-src to integrin-dependent cytoskeletal complexes in thrombin-stimulated platelets. Mol. Cell. Biol. 13, 1863–1871.

34. Cobb, B. S.; Schaller, M. D.; Leu, T. H.; and Parsons, J. T. (1994) Stable association of pp60src and pp59fyn with the focal adhesion-associated protein tyrosine kinase, pp125FAK. Mol. Cell. Biol. 14, 147–155.

35. Chen, Q.; Kinch, M. S.; Lin, T. H.; Burridge, K.; and Juliano, R. L. (1994) Integrin-mediated cell adhesion activates mitogen-activated protein kinases. J. Biol. Chem. 269, 26602–26605.

36. Zhu, X.; and Assoian, R. K. (1995) Integrin-dependent activation of MAP kinase: a link to shape-dependent cell proliferation. Mol. Biol. Cell 6, 273–282.

37. Chung, J. S.; and Jacobson, B. S. (1993) Requirement for diacylglycerol and protein kinase C in HeLa cell-substratum adhesion and their feedback amplification of arachidonic acid production for optimum cell spreading. Mol. Biol. Cell 4, 271–281.

38. Vuori, K.; and Ruoslahti, E. (1993) Activation of protein kinase C precedes α5β1 integrin-mediated cell spreading on fibronectin. J. Biol. Chem. 268, 21459–21462.

39. McNamee, H. P.; Inghir, D. E.; and Schwartz, M. A. (1993) Adhesion to fibronectin stimulates inositol lipid synthesis and enhances PDGF-induced inositol lipid breakdown. J. Cell Biol. 121, 673–678.

40. Laramche, N.; Tapon, N.; Stowers, L.; Burbelo, P. D.; Aspenström, P.; Bridges, T.; Chant, J.; and Hall, A. (1996) Rac and Cdc42 induce actin polymerization and G1 cell cycle progression independently of p65PAK and the JNK/SAPK MAP kinase cascade. Cell 87, 519–529.

41. Segat, D.; Cornai, R.; Di Marco, E.; Strangio, A.; Cancelleda, R.; Franzoi, A. T.; and Tacchetti, C. (2002) Integrins αα6β1 and αα8β1 promote different stages of chondrogenic cell differentiation. J. Biol. Chem. 277, 31612–31621.

42. Buckingham, M.; Bajard, L.; Chang, T.; Daubas, P.; Haddouel, M.; Meilhac, S.; Montarras, D.; Rocancourt, D.; and Relaix, F. (2003) The formation of skeletal muscle: from somite to limb. J. Anat. 202, 59–68.

43. Rudnicki, M. A.; Schneegolsberg, P. N.; Stead, R. H.; Braun, T.; Arnold, H. H.; and Jaenisch, R. (1993) MyoD or Myf-5 is required for the formation of skeletal muscle. Cell 75, 1351–1359.

44. Tajbakhsh, S. (2005) Skeletal muscle stem and progenitor cells: reconciling genetics and lineage. Exp. Cell Res. 306, 364–372.

45. Chen, Y. H.; and Tsai, H. J. (2002) Treatment with Myf5-morpholino results in somite patterning and brain formation defects in zebrafish. Differentiation 70, 447–456.

46. Wilschut, K. J.; van Tol, H. T.; Arkesteijn, G. J.; Haagsman, H. P.; and Roelen, B. A. (2011) α6 integrin is important for myogenic stem cell differentiation. Stem Cell Res. 7, 112–123.

47. Shattil, S. J.; Kim, C.; and Ginsberg, M. H. (2010) The final steps of integrin activation: the end game. Nat. Rev. Mol. Cell Biol. 11, 288–300.

48. Annes, J. P.; Munger, J. S.; and Rikfink, D. B. (2003) Making sense of latent TGFβ activation. J. Cell Sci. 116, 217–224.