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博 士 学 位 论 文

## Dlk2 在骨骼肌发育中的作用研究

### Study on Roles of Dlk2 in Skeletal Muscle Development

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厦门大学博硕士学位论文摘要库

## 摘要

Dlk2 (Delta-like 2 homolog) 属 EGF 样跨膜蛋白, 与同家族成员 Dlk1 (Delta-like 1 homolog) 高度同源。现有研究发现 Dlk1 在包括脂肪生成和肌肉生成在内的多个发育过程中发挥重要作用。但是目前对 Dlk2 的研究还主要限于脂肪生成过程, 迄今为止尚未见 Dlk2 在骨骼肌生成方面的报道。

本研究首次考察了 Dlk2 在骨骼肌发育中的作用。首先, 通过检测不同发育时期的鸡骨骼肌组织中 *cDLK2* 的表达水平, 我们发现其在胚胎骨骼肌发育早期较高, 而随发育的进行逐渐降低并在出壳后维持较低水平, 说明 Dlk2 可能在胚胎骨骼肌早期发育中发挥作用。接着, 我们利用小鼠成肌细胞系 C2C12 模型发现, *Dlk2* 在成肌细胞诱导分化前表达水平较高, 而在诱导分化后大幅下降, 进一步说明 Dlk2 涉及到成肌分化过程中。敲低 Dlk2 后不影响 C2C12 早期成肌分化, 但显著削弱了肌管的融合, 说明中晚期成肌分化受到抑制。过表达 Dlk2 对 C2C12 的早期成肌分化基因的表达也没有显著影响, 但同样抑制中晚期分化。这种矛盾暗示了 Dlk2 可能在调控骨骼肌生成方面具有双重作用。进一步分析 *Dlk2* 在诱导分化前成肌细胞中的表达, 我们发现成肌细胞的密度可以负调控 *Dlk2* 表达, 鉴于成肌细胞密度与其定向肌细胞分化潜能密切相关, 我们推测 Dlk2 可能参与维持成肌细胞的分化潜能。

为研究 Dlk2 调控成肌细胞分化的分子机制, 首先我们在 C2C12 细胞中考察了 Dlk2 与 Notch 信号通路之间的关系, 发现虽然过表达 *Dlk2* 引起 Notch 通路下游响应基因 *Hey1* 的表达水平显著上调, 但这并不是由 Notch1 信号的激活造成的。考察另一个影响 *Hey1* 的信号通路——BMP 通路, 我们发现 *Hey1* 的上调也不是经由 BMP-Smad1/5/8 信号实现的。因此我们推测 Dlk2 可能不通过 Notch 或 BMP 信号通路调控骨骼肌生成。另一方面, 过表达 Dlk2 导致 IGF 通路基因 *Igf2* 表达水平显著下调, 而 *Igfbp2* 和 *Igfbp5* 表达水平显著上调。进一步研究发现 Dlk2 可以与 IGFs 受体 IGF-IR 直接结合并抑制 IGF-Akt 信号活性, 施加外源 IGF-II 蛋白可以恢复过表达 Dlk2 C2C12 细胞的分化能力, 说明 Dlk2 能够经由 IGF-Akt 通路调控骨骼肌生成。

接下来我们利用小鼠肌肉损伤修复模型检测了骨骼肌再生过程中 *Dlk2* 的表达水平变化,发现在此过程中 *Dlk2* 的表达明显上调,并且其表达模式与 *Pax7* 高度相似,表明 *Dlk2* 参与了骨骼肌再生的早期阶段。最后我们成功利用 CRISPR/Cas9 技术获得了 *zDlk2* 敲除的斑马鱼,通过对 F<sub>1</sub> 代突变体胚胎的初步表型分析,发现与野生型相比 *zDlk2* 敲除斑马鱼胚胎发育明显迟滞。

综上所述,*Dlk2* 参与胚胎骨骼肌的早期发育和成体后骨骼肌再生的早期阶段,并与维持成肌细胞定向分化潜能密切相关,它在成肌细胞分化过程中可能发挥双重调控作用,而其调控作用至少部分是经由 IGF-Akt 通路实现的。

**关键词:** *Dlk2*; 基因表达; 骨骼肌发育; C2C12 细胞模型; 斑马鱼; CRISPR/Cas9

**ABSTRACT**

The protein Dlk2 (Delta-like 2 homolog) belongs to the EGF-like family of membrane proteins. Accumulating evidence suggests that Dlk1, highly homologous to Dlk2, is involved in several differentiation processes, including adipogenesis and myogenesis. However, limited previous studies on Dlk2 are almost directed to adipogenesis, and its function in myogenesis has not been reported.

The present study concerned, for the first time, the roles of Dlk2 in skeletal muscle development. First, the expression of *Dlk2* mRNA in chicken skeletal muscle showed a high level at the early embryonic stage and a gradual decrease during development, finally followed by a stable but low level after hatch. This result provides a possibility that Dlk2 may play a role in embryonic skeletal myogenesis. Then using mouse C2C12 myoblasts as model, we found that *Dlk2* highly expressed in myoblasts before differentiation induction but was greatly downregulated after that, which further demonstrated Dlk2 was involved in myogenesis. Knockdown of Dlk2 did not affect the expression of the early myogenic regulatory genes, but significantly impaired the fusion of myotubes, indicating that the mid-late myogenesis was suppressed. Surprisingly, overexpression of Dlk2 showed, however, the similar results that the mid-late but not the early myogenesis was inhibited. These seemingly contradictory results suggest the possible dual roles Dlk2 plays in myogenesis. By further analysis, we found a negative correlation between Dlk2 expression and the density of myoblasts. Moreover, in view of the close relationship between the density and the myogenic potential of myoblasts, it is implied that Dlk2 may be involved in maintaining the myogenic potential of myoblasts.

To investigate the molecular mechanism by which Dlk2 regulates myogenesis, the correlation between Notch signaling, the antagonist target of Dlk2 in adipogenesis, and Dlk2 was concerned first. Although Dlk2 overexpression remarkably upregulated the expression of *Hey1*, a Notch1 downstream gene, that proved not to be induced by the activation of Notch1 signaling itself. Taking BMP pathway into account, which is

thought to be another inducer of *Hey1*, we found BMP-Smad1/5/8 signaling not responsible for the upregulation of *Hey1*, either. Therefore, we speculate that *Dlk2* may not regulate myogenesis via Notch or BMP signaling. Otherwise, *Dlk2* overexpression had a significant effect on IGF signaling. The expression of *Igf2* was dramatically downregulated in *Dlk2*-overexpressed C2C12 cells, while the expression of *Igfbp2* and *Igfbp5* was markedly upregulated. Further investigation illustrated that *Dlk2* protein could interact with IGF receptor IGF-IR directly and suppress IGF-Akt signaling, which impaired myogenesis subsequently. Adding IGF-II restored the myogenic differentiation of the *Dlk2*-overexpressed C2C12. These findings demonstrate that *Dlk2* can regulate myogenesis via IGF-Akt pathway.

Next we examined the expression of *Dlk2* during skeletal muscle regeneration with the help of mouse TA muscle injury and repair model. Interestingly, *Dlk2* was considerably upregulated during muscle regeneration, what's more, following the highly similar expression pattern with *Pax7*. This evidence suggests *Dlk2* participates in the early stage of skeletal muscle regeneration. Finally, using the CRISPR/Cas9 system, we have successfully harvested *zDlk2* mutant zebrafishes. By preliminary observation on F<sub>1</sub> mutants, we found the development of the mutant embryos was evidently retarded comparing with wild type, which stated *Dlk2* was of importance to embryonic development.

In conclusion, our research shows that *Dlk2* is deep involved in the embryonic development and adult regeneration of skeletal muscle. It seems to play dual roles in skeletal myogenesis and to be important to maintain the myogenic differentiation potential of myoblasts. Furthermore, *Dlk2* functions in myogenesis at least partly via IGF-Akt signaling.

**Keywords:** *Dlk2*; Gene expression; Skeletal muscle development; C2C12 cell model; Zebrafish; CRISPR /Cas9

# 1 前言

## 1.1 骨骼肌发育概述

### 1.1.1 胚胎骨骼肌发育

脊椎动物的骨骼肌 (skeletal muscle) 是一个复杂的组织系统, 它起源于胚胎形成 (embryogenesis) 时期的轴旁中胚层 (paraxial mesoderm)<sup>[1]</sup>。在胚胎早期发育过程中, 轴旁中胚层在神经管 (neural tube) 两侧沿胚胎前-后轴向 (anterior-posterior axis) 形成左右对称的分节单位——体节 (somites)<sup>[2]</sup>。羊膜动物 (amniote) 的体节进一步生成生骨节 (sclerotome) 和生皮肌节 (dermomyotome), 生骨节位于体节腹侧, 将来形成软骨和骨; 生皮肌节位于体节背侧, 将来形成真皮 (dermis)、躯干和四肢骨骼肌、内皮细胞 (endothelial cells) 以及平滑肌 (smooth muscle)<sup>[3]</sup>。与躯干和四肢的骨骼肌不同, 头部骨骼肌来源于胚胎头部和脊索前的轴旁中胚层<sup>[3]</sup> (图 1-1A)。生皮肌节内的部分细胞表达标志性转录因子 Pax3 和 Pax7 (paired box 3 and 7) 和低水平的碱性螺旋-环-螺旋转录因子 [basic helix-loop-helix (bHLH) transcription factors] Myf5 (myogenic factor 5), 它们决定了这些细胞的成肌命运<sup>[4]</sup>。生皮肌节周围的 4 个上皮边沿 (epithelial lips) 将发育为生肌节 (myotome), 生肌节是一个包含定型 (committed) 肌细胞的原始结构, 这些细胞高水平表达 Myf5 和另一个 bHLH 转录因子 MyoD (myogenic differentiation 1, 也称 Myod1)<sup>[4]</sup>。

肌肉生成 (myogenesis) 的过程首先开始于初级生肌节 (primary myotome) 的形成<sup>[2]</sup>。肌源性祖细胞 (myogenic progenitor cells, MPCs) 自生皮肌节的背内侧沿 (dorsomedial lip, DML) 和腹外侧沿 (ventrolateral lip, VLL) 分别向腹面和背面迁移, 在生皮肌节和生骨节之间形成轴上 (epaxial) 和轴下 (hypaxial) 生肌区, 组成初级生肌节<sup>[2, 4, 5]</sup> (图 1-1B)。来自周围组织的信号分子将促进或抑制生肌节中基因的表达, 比如来自神经管和侧板中胚层 (lateral plate mesoderm) 的 BMP4 (bone morphogenetic protein 4) 会对 Myf5 和 MyoD 等基因的表达产生抑制作用, 而来自脊索、神经管和表面外胚层 (surface ectoderm) 的 Shh (Sonic Hedgehog) 和 Wnts (Wnt1、Wnt3、Wnt7a 和 Wnt11) 则会激活生肌节内基因的表

达<sup>[3]</sup> (图 1-1B)。轴上生肌节最终将形成背部肌肉，而轴下生肌节最终将形成体壁 (body wall) 和四肢肌肉<sup>[6]</sup>。

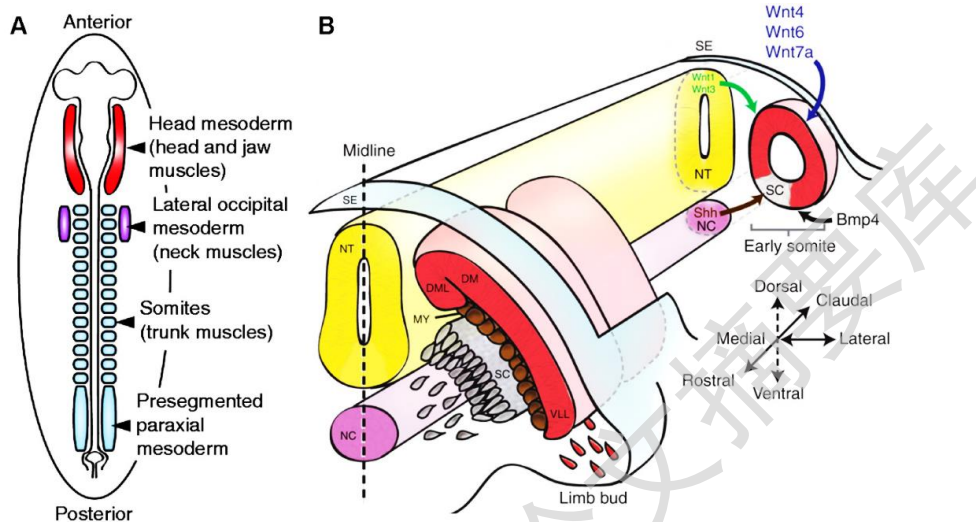


图 1-1 脊椎动物胚胎骨骼肌发育

(A 图引自 Mok, *Reproduction*, 2011<sup>[6]</sup>, B 图引自 Bentzinger, *Cold Spring Harb Perspect Biol*, 2012<sup>[4]</sup>)

(A) 13 体节羊膜动物胚胎示意图，相当于 Carnegie stage 11 人胚 (~24d)，E8.5 鼠胚，HH11 鸡胚 (40-45h)。  
(B) 胚胎早期肌肉发育示意图。NT= neural tube, NC= notochord, SE= surface ectoderm, SC=sclerotome, DM= dermomyotome, DML=dorsomedial lip, VLL=ventrolateral lip.

### Fig. 1.1 Embryonic myogenesis of vertebrate

肌原性祖细胞 (MPCs) 是单核、纺锤形细胞，在成肌调节因子 (myogenic regulatory factors) 的作用下激活增殖，然后分化为胚胎成肌细胞 (embryonic myoblasts)。这些成肌细胞在经历一定的增殖阶段后将退出细胞周期，继而分化为肌细胞 (myocytes)，再经排列 (alignment) 和重组 (rearrangement)，细胞之间相互融合延伸形成多核的肌管 (myotubes)。随着分化的进行，肌管中的细胞核由中心位置逐渐移向边缘，多个肌管将组成初级肌纤维 (primary myofibers)<sup>[4]</sup> (图 1-2)。在随后的胚胎发育过程中，胎儿成肌细胞 (fetal myoblasts) 分化形成次级肌纤维 (secondary myofibers)，次级肌纤维依附于初级肌纤维上发育。同时期不同个体的初级肌纤维在数量上没有显著差异，但粗细却有明显不同，而正是初级肌纤维的粗细决定了依附其上的次级肌纤维的数量，成体肌肉组织主要由



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