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miR-27 regulates chondrogenesis by suppressing focal adhesion kinase during pharyngeal arch development

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ABSTRACT

Cranial neural crest cells are a multipotent cell population that generate all the elements of the pharyngeal cartilage with differentiation into chondrocytes tightly regulated by temporal intracellular and extracellular cues. Here, we demonstrate a novel role for *miR-27*, a highly enriched microRNA in the pharyngeal arches, as a positive regulator of chondrogenesis. Knock down of *miR-27* led to nearly complete loss of pharyngeal cartilage by attenuating proliferation and blocking differentiation of pre-chondrogenic cells. Focal adhesion kinase (FAK) is a key regulator in integrin-mediated extracellular matrix (ECM) adhesion and has been proposed to function as a negative regulator of chondrogenesis. We show that FAK is downregulated in the pharyngeal arches during chondrogenesis and is a direct target of *miR-27*. Suppressing the accumulation of FAK in *miR-27* morphants partially rescued the severe pharyngeal cartilage defects observed upon knock down of *miR-27*. These data support a crucial role for *miR-27* in promoting chondrogenic differentiation in the pharyngeal arches through regulation of FAK.

1. Introduction

Craniofacial abnormalities are among the most common human birth defects, cleft lip and palate being among the five most common congenital malformations (Gorlin et al., 1990). Although an increasing number of genetic mutations have been implicated with these malformations, there is limited information about the etiology of congenital craniofacial disorders. In zebrafish, many features that control craniofacial development and pharyngeal skeletal elements are conserved with that of higher vertebrates (Yelick and Schilling, 2002). Most skeletal structures in the skull and the entire pharyngeal skeleton are derived from a unique population of cells, cranial neural crest (CNC) cells (Couly et al., 1993; Lumsden et al., 1991; Schilling and Kimmel, 1994). CNC cells migrate from the dorsal neural tube in three streams to populate the pharyngeal arches. Post-migratory CNC cells go through mesenchymal condensation during which pre-chondrogenic cells (PCCs) aggregate and increase their cell-cell contacts. Coincident with dynamic changes in the extracellular matrix (ECM), PCCs differentiate into chondrocytes surrounded by a type-II collagen and aggrecan rich matrix (Hall and Miyake, 2000; Kozhemyakina et al., 2015).

Vertebrate CNCs are a migratory, multipotent cell population, able to differentiate into cartilage, bone, teeth forming cells, and non-ectomesenchyme derivatives, such as neurons, pigment cells and glia (Baroffio et al., 1991). Chondrogenic differentiation of CNC cells is regulated by various signaling pathways including Tgf-β, Bmp, and Fgf pathways, as well as changes in cell shape (Kozhemyakina et al., 2015). As cell-cell interactions increase during mesenchymal condensation, PCCs become more rounded. Recent studies have shown that restricting cell spreading on synthetic substrates, or by maintaining high-cell density to prevent cell spreading, promotes chondrogenic differentiation of mesenchymal stem cells (Gao et al., 2010; McBride and Knothe Tate, 2008). Interestingly, mechanical forces or changes in the ECM that perturb cell shape lead to the formation of integrin-mediated focal adhesions, which in turn prevents chondrogenesis (Eyckmans et al., 2011; Tang et al., 2013; Yim and Sheetz, 2012). Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase and an essential component of focal adhesions (Parsons, 2003). Apart from its well-established roles in cell adhesion and migration, FAK is also involved in regulating mesenchymal stem cell fates in response to cell shape changes and integrin-β1 activation (Mitra et al., 2005; Pala et al., 2008; Takahashi et al., 2003; Tang et al., 2013). Exactly how FAK is regulated in the pharyngeal arches during chondrogenesis is not known.

miRNAs are small noncoding RNAs that regulate the expression of target mRNAs at the post-transcriptional level. miRNAs bind to the 3′UTR of their targets with imperfect base pairing and induce dead-

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enylation, translational repression, and degradation of the target mRNA (Huntzinger and Izaurralde, 2011; Krol et al., 2010). Tissue-specific expression of miRNAs allows them to regulate multiple developmental processes in diverse organisms (Flynt et al., 2007; Kloosterman and Plasterk, 2006; Li et al., 2011; Wei et al., 2013; Wienholds, 2005). Previous studies reported that miRNAs are required for skeletal development using mice with conditional deletion of Dicer, an RNaseIII-like enzyme required for miRNA biogenesis, in either NC cells or early chondrocytes in the craniofacial cartilage or growth plate (Kobayashi et al., 2008; Zehir et al., 2010). Global deficiency of all miRNAs in NC cells resulted in the loss of the majority of NC-derived craniofacial cartilage and bone (Zehir et al., 2010). These studies show that miRNA expression is crucial for skeletal development but only a small subset of miRNAs have been characterized as to their targets and control of whole organism cartilage and bone development (*miR-92a*, *miR-140* and *miR-452*) (Eberhart et al., 2008; Nakamura et al., 2011; Ning et al., 2013; Sheehy et al., 2010).

In this study, we demonstrate a novel role for *miR-27*, a highly conserved miRNA family, during craniofacial cartilage development. Knock down of *miR-27* inhibited pharyngeal arch morphogenesis and

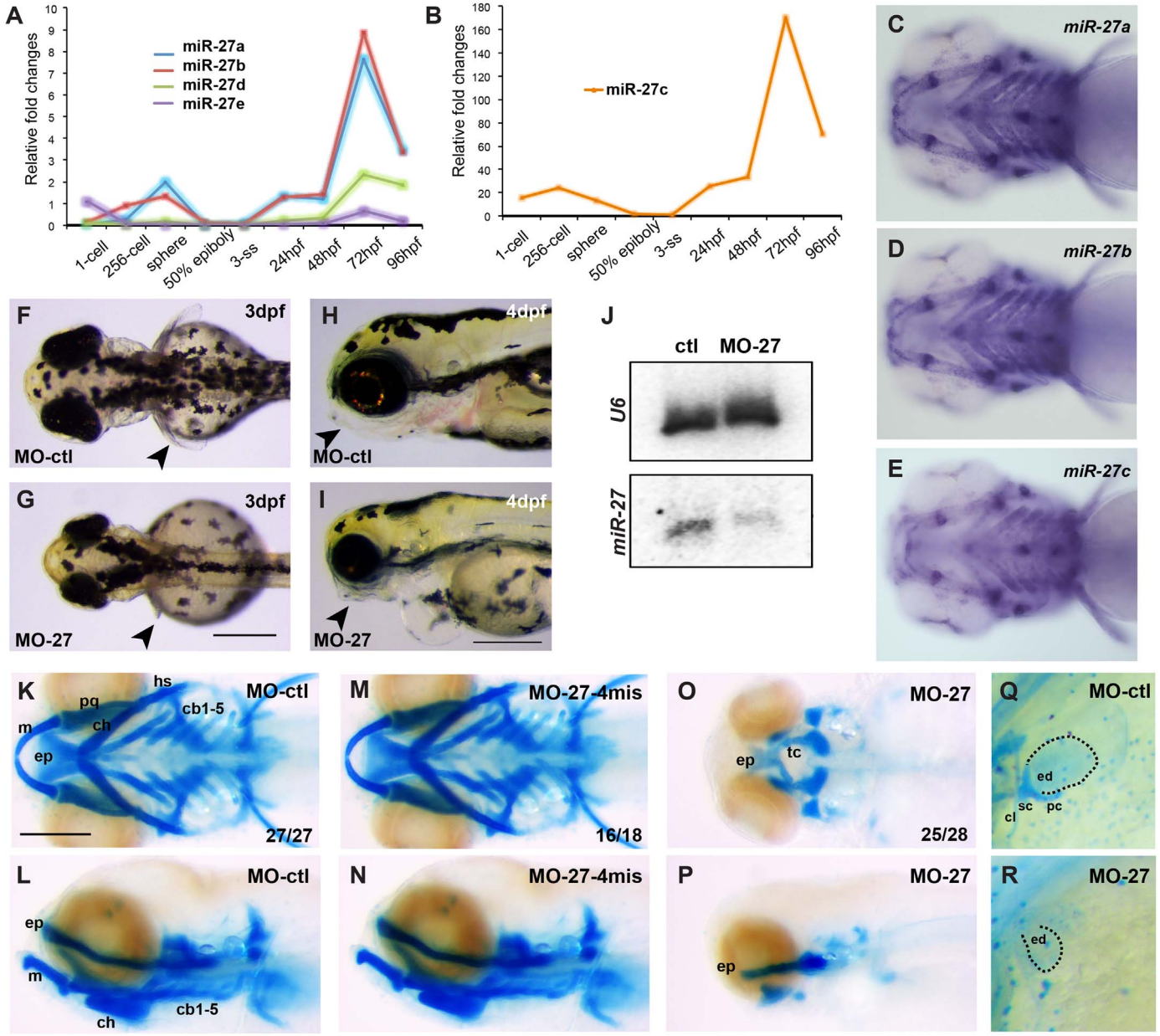

Fig. 1. Knock down of *miR-27* leads to craniofacial and pectoral fin defects. (A,B) qRT-PCR for *miR-27a-e* at the indicated developmental stages normalized to U6 snRNA. Fold changes were calculated using ΔΔC(t) method comparing all *miR-27* levels to *miR-27c* levels at the 3 somite stage (ss). Due to comparably higher levels, *miR-27c* expression profile is shown separately. (C-E) Expression of *miR-27a, miR-27b* and *miR-27c* in 4dpf embryos detected by whole-mount *in situ* hybridization by locked nucleic acid (LNA) probes. All are ventral views of the head. (F,G) Dorsal view of 3 dpf live embryos injected with either 5 ng standard control morpholino (MO-ctl) or MO-27 at the single-cell stage. Pectoral fins are indicated with arrowheads. (H,I) Morphology of the head in 4 dpf embryos injected with either MO-ctl or MO-27. Lateral views, jaws are indicated with arrowheads. Scale bar, 300 μm. (J) *miR-27* and *U6* levels in uninjected control and *miR-27* morpholino (MO-27) injected embryos at 48hpf detected by Northern blot. (K-P) Head cartilages stained with Alcian blue in 4dpf embryos injected with (K,L) standard control morpholino (MO-ctl), (M,N) 4-mismatch *miR-27* morpholino (MO27-4mis) and (O,P) MO-27. Top panels, ventral views; bottom panels, lateral views. The indicated ratio represents the number of embryos with the represented phenotype/total number of observed embryos. Cartilage labels: ep, ethmoid plate; tc, trabeculae cranii; m, Meckel's cartilage; pq, palatoquadrate; ch, ceratohyal; hs, hyosymplectic; cb, ceratobranchial. Anterior side of the embryos is to the left. (Q,R) Staining of pectoral fin skeleton in 4dpf embryos by Alcian blue. The right side pectoral fin is shown with anterior to the top. The cleithrum (cl) and scapulocoracoid (sc) cartilages and postcoracoid process (pc) of pectoral fins are missing and the endoskeletal disc cartilage (ed) is smaller in *miR-27* morphants compared to the controls. Scale bar, 200 μm.

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