Esco2 Regulates cx43 Expression During Skeletal Regeneration in the Zebrafish Fin

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Introduction

The skeleton supports soft tissues, provides for muscle attachment and protects internal organs from risk of injury. Additional functions include calcium and phosphorus storage, blood cell production and immune response. Given this diverse array of functionality, mutations that affect skeletal functions exhibit pleiotropic defects that include osteoporosis, osteoarthritis, and hematopoietic and immunity deficiencies. Aberrations in skeletal development, especially long bone growth and morphogenesis, are also components of several multi-spectrum developmental abnormalities such as Roberts syndrome (RBS) and Cornelia de Lange syndrome (CdLS). In addition to severe long-bone growth defects and missing digits, both RBS and CdLS patients may exhibit craniofacial abnormalities, cleft palate, syndactyly, organ defects, and severe mental retardation (Liu and Krantz, 2009; Mannini et al., 2010).

Recent genetic mapping studies reveal that mutations in cohesin pathways are responsible for RBS and CdLS disorders, as well as a host of related diseases collectively termed cohesinopathies (Krantz et al., 2004; Tonkin et al., 2004; Schule et al., 2005; Vega et al., 2005; Musio et al., 2006; Deardorff et al., 2007, 2012a,b; Gordillo et al., 2008; Van der Lelij et al., 2010 Yuan et al., 2015). Elucidating the molecular basis of these disorders span topics of both chromosome segregation and transcriptional regulation (Rudra and Skibbens, 2013). For instance, RBS arises from mutations in ESCO2. High fidelity chromosome segregation requires that sister chromatids be identified from DNA synthesis (S-phase) to anaphase onset during mitosis (M-phase). Identity is achieved by cohesin complexes that tether together sister chromatids. ESCO2 is an acetyltransferase that converts chromatin-bound cohesins to a tether-competent state (Skibbens et al., 1999; Toth et al., 1999; Ivanov et al., 2002; Zhang et al., 2008; Unal et al., 2008; Ben-Shahar et al., 2008). Thus, it is not surprising that cells isolated from RBS patients exhibit mitotic failure, elevated levels of apoptosis, reduced proliferation and genotoxic hypersensitivities (Horsfield et al., 2012; Mehta et al., 2013). On the other hand, CdLS arises from mutations in the cohesin genes SMC1A, SMC3, and RAD21, the cohesin deposition factor encoded by NIPBL, and the de-acetylase encoded by HDAC8 that targets SMC3 (Krantz et al., 2004; Toth et al., 2004; Musio et al., 2006; Deardorff et al., 2007, 2012a,b). Intriguingly, CdLS patient cells typically exhibit normal mitosis and retain a euploid genomic state, revealing that CdLS instead arises mainly through transcriptional dysregulation (Liu and Krantz, 2009; Dorsett and Article is online at: http://onlinelibrary.wiley.com/doi/10.1002/dvdy.24354/abstract © 2015 Wiley Periodicals, Inc.)
Merkenschlager, 2013). A transcriptional basis of CdLS is supported by findings that cohesins are critical for (1) transcription termination, (2) enhancer-promoter registration, (3) CTCF-insulator recruitment and (4) POLII transitioning from a paused to elongating state (Dorsett and Merkenschlager, 2013). Despite the similarities to CdLS, a transcriptional basis for RBS that involves ESCO2 remains undefined.

The zebrafish caudal fin contains 16 to 17 segmented bony fin rays with new growth occurring by the distal addition of bony segments and associated fin ray joints (Goss and Stagg, 1957; Haas, 1962). Because of its simple structure, rapid regeneration following amputation, and the ability to knockdown gene expression through gene-specific morpholinos, the zebrafish fin is emerging as an excellent model system from which to elucidate tissue and bone growth pathways. For instance, mutations in connexin43 (cx43) cause the short fin (sof123) phenotype, which is characterized by defects in bony fin ray growth and joint formation (Iovine et al., 2005; Hoptak-Solga et al., 2008). Missense mutations in human CX43 cause oculodentodigital dysplasia (ODDD), a genetic disorder that effects both craniofacial and distal skeleton limb development (Paznekas et al., 2003). Thus, the regenerating fin provides clinically relevant insights into highly conserved pathways critical for human skeletal development.

ESCO2-knockdown studies in zebrafish and medaka embryos produce severe developmental defects, in part recapitulating RBS phenotypes (Monnich et al., 2011; Morita et al., 2012). Interpreting the effects of esco2-knockdown in these studies, however, is complicated by large-scale cell death and subsequent indirect or downstream effects. Here, we report on the role of esco2 during fin regeneration, in the absence of complications produced by whole embryo lethality. The regenerating fin is an ideal system to study the effects of reduced esco2 on the skeleton in that it avoids the possible confounding effects of reduced esco2 during development as previously reported. In the current study, results reveal that esco2 expression is up-regulated in regenerating fins, particularly in the blastema, which is a specialized compartment that contains the majority of proliferative cells. Moreover, esco2-knockdown results in defects in both fin regeneration and bony segment length. Importantly, the results reveal that esco2-knockdown reduces cx43 expression and diminishes cx43 signaling pathways but does not globally reduce other gene expression pathways. In combination, these results suggest the possibility that Esco2 may act as a specific transcriptional regulator with targets that include cx43.

Results

esco2 mRNA is Up-regulated in the Blastema of Regenerating Fins

Despite the fact that ESCO2 is critical for proper human development (Schule et al., 2005; Vega et al., 2005), little information exists regarding its expression or the molecular basis through which mutations in ESCO2 result in skeletal disorders. Here, we exploit the adult zebrafish regenerating fin to address both of these fundamental issues in the absence of confounding effects due to embryonic death. To ascertain the temporal regulation of esco2 expression in regenerating fins, fins were amputated at the 50% level and regenerating tissue harvested at 1, 3, 5, and 8 days postamputation (dpa). The expression of esco2 was then assessed by whole-mount in situ hybridization (Fig. 1). At 1 dpa, esco2 was not detectable. esco2 was readily apparent at both 3 and 5 dpa with levels starting to diminish at this later time point. By 8 dpa, esco2 expression was significantly reduced. High esco2 expression at 3 dpa is consistent with previous studies that map this period as the peak rate of regeneration in the zebrafish fin (Lee et al., 2005; Hoptak-Solga et al., 2008) and suggest that the localization of esco2 expression might similarly correlate with the highly proliferative blastemal compartment. To test this possibility, 5 dpa fins stained for esco2 expression by in situ hybridization were cryosectioned. The results reveal that esco2 expression is specifically up-regulated in the blastemal compartment (Fig. 2). To establish that the probe had access to all esco2-positive tissue, 5 dpa fins were cryosectioned before hybridization (Smith et al., 2008). Results from this regimen confirm that esco2 is expressed specifically within the blastema (Fig. 2). In summary, esco2 expression is temporally regulated and occurs specifically in the blastemal compartment of the zebrafish regenerating fin.

esco2 is a Critical Regulator of Fin Regeneration and Specifically of Bone Growth

esco2 is an essential gene (Monnich et al., 2011; Whelan et al., 2012; Morita et al., 2012), requiring knockdown strategies to ascertain function in the adult regenerating fin. Therefore, we depleted Esco2 using morpholino (MO)-mediated knockdown methodologies (Hoptak-Solga et al., 2008; Sims et al., 2009) using one of the two validated MOs for Esco2 (Monnich et al., 2011). As a control, we used either a custom mismatch morpholino (5MM) containing five mismatches compared with the esco2 targeting MO or the “standard control” MO from Gene Tools that does not recognize target genes in zebrafish. Uptake is accomplished by first injecting the MO into the blastema of the regenerating fin, followed by electroporation across the fin. All MOs are modified with fluorescein, permitting validation of cellular uptake. Only fins positive for MOs at 1 day postelectroporation (dpe) are kept for further analysis. Procedural details for all knockdown experiments are outlined in Figure 3.

Regenerate length and segment length were evaluated at 4 dpe/7 dpa. All fin regenerate and segment length measurements were obtained from the 3rd fin ray and results compared between injected (esco2-MM or esco2-MO) and uninjected portions of the same fin, a strategy previously documented as providing for both internal controls and standardized analyses (Iovine and Johnson, 2000; Hoptak-Solga et al., 2008). Uninjected control fins regenerated in a robust manner. esco2-MM injected regenerating fins exhibited identical growth to the uninjected control (Fig. 4). In contrast, regenerating fins injected with the esco2-MO exhibited a significant decrease in regenerate length compared with uninjected controls within the same fins (Fig. 4), documenting that esco2 is critical for fin regeneration.

RBS patients exhibit significant bone growth deficiencies, especially in the arms and legs (Horsfield et al., 2012; Mehta et al., 2013). Thus, it became important to quantify the extent that esco2 depletion may specifically impact bone segment growth in regenerating fins. To address this question, segment length was measured in uninjected, esco2-MM injected, and esco2-MO injected fins. The results show that segment length in uninjected fish was nearly identical to that of esco2-MM injected fish (Fig. 4). However, segment length was significantly reduced in esco2-MO injected fish compared with the uninjected side of the same fish fins (Fig. 4). Thus, esco2 is critical for bone growth.
in regenerating fins, consistent with its role in skeletal development in humans.

To confirm that the esco2-MO was effective in reducing Esco2 protein levels, we generated an antibody against Esco2 and verified its specificity using bacterial lysates expressing GST-Esco2. The results show that the anti-Esco2 antibody recognizes GST-Esco2, which migrates at the predicted size of 94 kDa. The anti-GST antibody also recognizes the GST-Esco2 band at the predicted size of 94 kDa and a 26 kDa band in the GST alone lane (Fig. 5). The anti-Esco2 antibody does not recognize GST alone. To confirm the specificity of the anti-Esco2 antibody, a peptide competition assay was performed. Esco2-directed antibody was preincubated with the peptide that the antibody was generated against. The resulting competed antibody produced a greatly reduced signal, compared with noncompeted antibody, when used to detect GST-Esco2, confirming antibody specificity (Fig. 5). Upon validating the specificity of the Esco2-directed antibody, we tested the effectiveness of the esco2-MO to knockdown Esco2 levels in vivo. At 3 dpa, fins were injected with either esco2-MM or esco2-MO and harvested the next day (1 dpe/4 dpa) to prepare fin lysate (see also Fig. 3). The lysates were used to test for Esco2 reduction by western blot. Quantification of the resulting western blots show that the esco2-MO reduces Esco2 protein levels by approximately 70% while robust levels of Esco2 persist in MM injected fins (Fig. 5). The results of the Western blot analyses document the efficacy of the esco2-MO to significantly reduce Esco2 protein levels.

**Role of esco2 in Cell Proliferation and Programmed Cell Death**

Reduced tissue and bone segment growth in regenerating fins could be due to decreased cell proliferation, increased programmed cell death (PCD), or both. To address possible changes in the level of cell proliferation, we evaluated both 5-bromo-2'-deoxyuridine (BrdU) as a marker for S-phase (Iovine et al., 2005)
and histone-3-phosphate (H3P) as a marker for M phase (Wei et al., 1999). Morpholino-injected fish (where half of the fin was injected with either esco2-MO or esco2-MM, see also Fig. 3) were allowed to swim in water supplemented with BrdU at 1 dpe/4 dpa and fins harvested immediately. To quantify BrdU staining through which BrdU incorporation could be directly compared between the two fin halves, we compared the ratio of the BrdU-positive domains in the regenerating tip with the total regenerate length. As expected, esco2-MM injected and uninjected fins exhibited nearly identical ratios of BrdU labeled cells to regenerate length. In contrast, esco2-MO injected and uninjected sides produced a significant decrease in the ratio of BrdU labeled cells to regenerate length (Fig. 6), demonstrating fewer dividing progenitor cells at the time of labeling. Next we evaluated H3P-positive cells at 1 dpe/4 dpa (see also Fig. 3). To compare H3P-positive cells between esco2-MM and MO injections in fin halves of the same fins, we counted the total number of H3P-positive cells in the 250 μm area that defines the proliferative blastema, normalizing for the area (Nechiporuk et al., 2002). Regenerating fins in which one fin half was uninjected and the other half injected with esco2-MM exhibited nearly identical numbers of H3P-positive cells within this defined area (Fig. 6). In contrast, the esco2 MO-injected side exhibited a statistically significant reduced level of H3P-staining cells compared with the uninjected sides of the fins (Fig. 6). Thus, cell proliferation appears to play a critical role in skeletal regrowth defects that occur in regenerating fins depleted of Esco2.

Esco2 and Cx43 Appear to Function in a Common Pathway

The pattern of esco2 expression and localization during fin regeneration, coupled with impact of esco2 depletion on bone segment regrowth, are strikingly similar to those previously reported for cx43 mutations that cause the short fin (sof b123) phenotype (Iovine et al., 2005; Hoptak-Solga et al., 2008). Could Esco2 and Cx43 function in a common pathway to influence bone segment growth? To address this possibility, we first tested whether Esco2 function is downstream of Cx43. If true, then esco2 expression might be reduced in sof b123 mutant fins. Whole-mount in situ hybridization was performed to monitor esco2 message levels in wild-type (WT) and sof b123 regenerating fins. The results show that esco2 expression levels are nearly identical in regenerating WT and sof b123 fins (Fig. 8), suggesting that esco2 is not downstream of cx43. An alternate possibility is that cx43 is downstream of esco2. Whole-mount in situ hybridization was performed on esco2-knockdown fins (1 dpe/4 dpa, see also Fig. 3) to determine if cx43 expression is reduced in the half of the fin injected with esco2-MO (Fig. 9). Indeed, cx43 was reduced in fin rays injected with esco2-MO and not reduced in either MM control or uninjected controls (Fig. 9). To independently test for cx43 dependency on esco2, we performed quantitative real-time polymerase chain reaction (qRT-PCR) at 1 dpe/4 dpa (see also Fig. 3). The results from three independent esco2-knockdown samples show that cx43 is significantly down-regulated in esco2-knockdown regenerating fins (Table 1; Fig. 10). Because reduced cell proliferation is not sufficient to cause reduced cx43 expression (Govindan and Iovine, 2014; Bhadra and Iovine, 2015), the observed reduction of cx43 expression is not likely the result of reduced cell proliferation in esco2-knockdown fins. In combination, these findings support a model where Cx43 acts downstream of, and may be regulated by, Esco2.

To further examine the possibility that Esco2 and Cx43 function in a common pathway, we next evaluated expression of both semaphorin3d (sema3d) and hyaluronan and proteoglycan link protein 1a (hapln1a), two genes recently found to function downstream of cx43 (Ton and Iovine, 2013; Govindan and
ESCO2 MEDIATES CX43 FUNCTION

If cx43 truly acts downstream of Esco2 and cx43 reduction in part mediates the bone growth defects observed upon Esco2 knockdown, then we reasoned that Cx43 overexpression might attenuate the regenerate and bone segment growth defects in regenerating fins. To test this prediction, we next attempted to rescue esco2-dependent skeletal growth defects by overexpression of Cx43. We used the Tg(hsp70:miR-133sp mdc4a) line that harbors an EGFP cDNA followed by three miR-133 binding sites (Ebert et al., 2007; Loya et al., 2009). Prior studies found that miR-133 knockdown by means of this “sponge” transgene increases cx43 levels during zebrafish heart regeneration (Yin et al., 2012). We confirmed up-regulation of both cx43 mRNA and Cx43 protein in regenerating fins of this transgenic line treated for heat shock (Fig. 11). Moreover, to rule out the possibility that increased Cx43 leads to an increase in Esco2, we further confirmed that the levels of esco2 mRNA and Esco2 protein are not up-regulated in this transgenic line treated for heat shock (Fig. 11).

Either transgenic-positive (Tg+) or transgenic-negative (control siblings, Tg-) fins were treated for esco2-knockdown (Fig. 12). Four hours postelectroporation, Tg+ and Tg- fish were both heat shocked (HS+) at 37°C for 1 hr. These fish are denoted as Tg+HS+ and Tg-HS+, respectively. Alternatively, a second group of esco2-knockdown Tg+ fish was not heat shocked and thus denoted as Tg+HS-. To demonstrate that heat shock alone does not rescue esco2-dependent phenotypes, we performed esco2-knockdown in Tg-HS+. Results from three independent trials show that esco2-knockdown in Tg-HS+ exhibited the predicted growth defects for regenerate fin and bone segment lengths (i.e., the percent similarity between the esco2-knockdown side and the untreated side is low) (Fig. 12). In contrast, esco2-knockdown followed by induction of the transgene (causing cx43 over-expression) in Tg-HS+, exhibited increased regenerate length and segment length, demonstrating Cx43-dependent rescue of the esco2-knockdown phenotypes (i.e., the percent similarity between the esco2-knockdown side and the untreated side is significantly increased when cx43 is overexpressed). We performed the same experiment using esco2-MM construct as a negative control. As expected, we did not observe skeletal phenotypes in these fins (data not shown). To demonstrate that...
the rescue of phenotype is heat shock dependent and, therefore, requires overexpression of cx43. In fish that carry the transgene but in which we do not induce its expression by means of the heat pulse (Fig. 12), the combination of these experiments provide compelling evidence that Esco2 and Cx43 function in a common pathway, and that esco2 effects are partially mediated by reduced cx43 expression.

Discussion

Cohesinopathies are a growing collection of severe and phenotypically pleiotropic developmental maladies, but at present the molecular mechanisms through which cells and developing organisms respond to cohesion mutations remains unclear (Horsfield et al., 2012; Mehta et al., 2013; Dorsett and Merkenschlager, 2013; Barbero, 2013). For instance, CDSLS appears to arise through transcriptional dysregulation that occurs in response to heterozygous or X-linked mutations in NIPBL, SMC1, SMC3, RAD21, or HDAC8—phenotypes also mimicked in PDS5 knockout mice (Krantz et al., 2004; Tonkin et al., 2004; Gillis et al., 2004; Musio et al., 2006; Deardorff et al., 2007, 2012a, b; Zhang et al., 2009). In contrast, the sister cohesinopathy RBS, which arises through homozygous mutation of ESCO2 (Schule et al., 2005; Vega et al., 2005; Gordinello et al., 2008), is thought to arise instead through reduced progenitor cell proliferation and increased mitotic failure and apoptosis (Horsfield et al., 2012; Whelan et al., 2012; Mehta et al., 2013). Our current studies suggest that Esco2 is a critical transcriptional regulator, leading us to speculate that RBS is most likely a transcriptional dysregulation malady. Support for this model is four-fold. First, esco2-knockdown reduces transcription of specific cell signaling pathways such as cr43 (mutations in which disrupt proper development [Paznekas et al., 2003; Iovine et al., 2005; Musa et al., 2009], but not other developmentally-relevant signaling pathways such as shh (Table 1; Fig. 10). Second, recent evidence reveals that Esco2 regulates Notch signaling through binding/sequestration of the intracellular Notch domain (Leem et al., 2011). Notch is required for neuronal differentiation, skeletal development, and hematopoietic lineages, consistent with pleiotropic phenotypes that result in both esco2 and notch mutations (Zanotti and Canalis, 2013). Third, other lines of evidence suggest that ESCO proteins may recruit or link chromatin modifiers to DNA (Choi et al., 2010; Kim et al., 2008). Fourth, our results reveal no significant increase in the incidence of apoptotic cells following esco2-knockdown in the blastemal compartment of regenerating fins. At present, we cannot exclude the possibility that a small increase in apoptotic cells is masked by a low but consistent background level required for remodeling in the regenerating fin, but the fact that elevated levels were not discernible in MO-injected fins compared with MM-injected and uninjected matched-fin controls suggests instead that the effects on fin and bone segment regeneration can occur independent of apoptosis. These findings contrast studies involving esco2-knockdown in zebrafish and medaka embryos and mice in which reproducible elevations in apoptotic cells were reported (Monnich et al., 2011; Morita et al., 2012; Whelan et al., 2012). However, the effects in those studies are modest. ESCO2-knockdown in mice neuroepithelium, for instance, produced severe microcephaly but succeeded in inducing only a two-fold increase in the number of apoptotic cells (Whelan et al., 2012). In zebrafish embryos, esco2 depletion resulted in Caspase 8 activation that was temporally limited (Monnich et al., 2011). Esco2 depletion also resulted in a four-fold increase in the number of apoptotic cells in zebrafish embryos and global induction of apoptosis in medaka embryos, but such increases that occur in inviable or failing embryos are plausibly downstream events indirectly coupled to esco2 effects (Monnich et al., 2011; Morita et al., 2012). Thus, it remains possible that apoptosis is not the major clinically relevant etiologic...
effect of esco2 mutation in RBS patients, especially because apoptosis is not a feature of CdLS cells (Tonkin et al., 2004; Castro-

novo et al., 2009; Revenkova et al., 2009). We hypothesize that the transcriptional dysregulation that arises from ESCO2 mutation may be the underlying etiologic basis for the developmental abnormalities of RBS—upon which are overlaid cohesion defects, mitotic failure and apoptosis.

Currently, mutation of ESCO2 represents the sole etiologic agent of the severe developmental disorder Roberts syndrome (RBS) in humans (Vega et al., 2005). We posit that a second revelation from our current study is that Esco2 may be an upstream regulator of Cx43 function in zebrafish regenerating fins. For instance, esco2 depletion not only reduces cr43 expression, but also expression of sema3d and hapln1a, two genes in the bone growth signaling pathways that rely upon Cx43. Conversely, genes that appear independent of Cx43, such as shh, spry4, and mps1 were not reduced in esco2-knockdown regenerating fins. Notably, the spatial and temporal expressions of esco2 and cr43 are nearly identical within the regenerating fin (lovine et al., 2005; current study). Moreover, the demonstration that overexpression of cr43 rescues esco2-dependent skeletal phenotypes strongly suggests that Esco2 and Cx43 function in a common pathway. Inhibition of miR-133 could influence the expression of genes in addition to cr43; thus, we cannot formally rule out the possibility that rescue of esco2-phenotypes occurs through a more complex pathway. If our model is correct that Esco2-dependent expression of Cx43 is an important factor in human development, then cr43 mutation should similarly manifest developmentally abnormalities. In fact, mutations of human CX43 cause ODDD, a malady that includes craniofacial dysmorphia, distal limb skeletal growth defects, and abnormal eye and teeth development (Paznekas et al., 2003; Musa et al., 2009). We note prior studies in which mutation/depletion of either esco2, nipbl, or rad21 were found to impact a plethora of developmentally relevant genes, including CX43/GJA1, observations that helped to inform the current study (Kawauchi et al., 2009; Monnich et al., 2011). Our findings suggest that Esco2 function may be coupled to a Cx43-dependent signaling pathway previously shown to directly promote proper development. Based on these results, we suggest that ODDD could be a mild form of cohesinopathies.

Conservatively, the current study is consistent with a model that Esco2 regulates the expression, either directly or indirectly, of cr43 and its downstream targets (i.e., sema3d and hapln1a), thereby influencing growth and skeletal patterning (Fig. 13). Future experiments to test this model include measuring cr43
expression level in RBS patient cells and directly assessing esco2 localization/regulation of the cx43 promoter. Alternatively, the impact of Esco2 on the skeleton may occur through its roles in DNA damage repair, DNA replication fork progression, chromosome compaction or ribosome maturation/assembly (Skibbens et al., 1999, 2010, 2013; Unal et al., 2004; Strom et al., 2004; Gard et al., 2009; Terret et al., 2009; Bose et al., 2012; Gerton, 2012). We also note differences in H3P labeling between esco2-knockdown in embryos and fins (Monnich et al., 2011, and the current study). As stated previously, whole embryos respond to esco2-knockdown through tissue, organ and whole embryo failure, which likely produce multiple downstream consequences superimposed on direct effects. It is possible, however, that the outcome of esco2 knockdown may be different during development compared with regeneration. For example, esco2 knockdown in embryos (i.e., during deployment of developmental programs) may cause increased aneuploidy (and, therefore, more cell death and mitotic arrest) and transcriptional deregulation (developmental defects), while during regeneration esco2 knockdown preferentially influences gene expression that leads predominantly to reduced cell proliferation and developmental defects.

**Experimental Procedures**

**Statement on the Ethical Treatment of Animals**

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols used for this manuscript were approved by Lehigh’s Institutional Animal Care and Use Committee (IACUC) (Protocol identification # 128, approved 11/16/2014). Lehigh University’s Animal Welfare Assurance Number is A-3877-01. All experiments were performed to minimize pain and discomfort.

**Housing and Husbandry**

Zebrafish (Danio rerio) were housed in a re-circulating system built by Aquatic Habitats (now Pentair). Both 3-L tanks (up to 12 fish/tank) and 10-L tanks (up to 30 fish/tank) were used. The fish room had a 14:10 light:dark cycle. Room temperature was tightly regulated and varied from 27 to 29°C (Westerfield, 1993). Water quality was monitored automatically and dosed to maintain conductivity (400–600 μS) and pH (6.95–7.30). Nitrogen levels were maintained by a biofilter. A 10% water change occurred daily. Recirculating water was filtered sequentially through pad filters, bag filters, and a carbon canister before circulating over ultraviolet lights for sterilization. Fish were fed three times daily, once with brine shrimp (hatched from INVE artemia cysts) and twice with flake food (Aquatox AX5) supplemented with 7.5% micro pellets (Hikari), 7.5% Golden Pearl (300–500 micron, Brine Shrimp direct), and 5% Cyclo-Peeze (Argent).

**Zebrafish Strains and Surgical Procedures**

The zebrafish strains used in this study were wild-type (C32), sof b123 (Iovine and Johnson, 2000), and Tg(hsp70:miR-133sp pd48) (Monnich et al., 2011, and the current study). As stated previously, whole embryos respond to esco2-knockdown through tissue, organ and whole embryo failure, which likely produce multiple downstream consequences superimposed on direct effects. It is possible, however, that the outcome of esco2 knockdown may be different during development compared with regeneration. For example, esco2 knockdown in embryos (i.e., during deployment of developmental programs)
Caudal fin amputations, fin regeneration, and harvesting were done as previously described (Sims et al., 2009; Ton and Iovine, 2013; Govindan and Iovine, 2014). Briefly, fish were first anaesthetized in 0.1% tricaine solution and their caudal fin rays amputated to the 50% level using a sterile razor blade and visualized using a dissecting scope. Fin regeneration proceeded until the desired time period depending on the type of experiment. At the required time point, the regenerated fins were harvested and fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) overnight at 4°C. The fins were then dehydrated in 100% methanol and stored at 20°C until further use.

**Gene Knockdown by Morpholino Injection and Electroporation**

All morpholinos (MOs) used in the study were fluorescein-tagged and purchased from Gene Tools, LLC. The MOs were reconstituted in sterile water to a final concentration of 1 mM. The sequences

![Fig. 9. In situ hybridization on morpholino mediated esco2-knockdown fins shows reduced levels of cx43 expression. The MO/MM was injected in 3 dpa fins and fins were harvested at 1 dpe/4 dpa. The amputation plane is identified by black lines unless it is out of the field of view (i.e., as occurs in most of the higher magnification images). The fin in panel A appears curved because there was less growth on the esco2-knockdown side compared with the uninjected side. A: (Top) Representative image of a fin with esco2-knockdown side (esco2 MO) showing decreased staining of cx43, compared with the uninjected side (UN). (Bottom left) Higher magnification of the knockdown side (MO) of the same fin (fin rays from top image marked by *) showing reduced cx43 expression. (Bottom right) Higher magnification of the uninjected side (UN) of the same fin (fin rays from top image marked by +) showing robust cx43 expression. Scale bar = 100 μm.](image)

### TABLE 1. Quantitative RT-PCR Confirms Changes in Gene Expression

<table>
<thead>
<tr>
<th>Gene</th>
<th>Average C&lt;sub&gt;T&lt;/sub&gt; (esco2 KD)</th>
<th>Average C&lt;sub&gt;T&lt;/sub&gt; (keratin)</th>
<th>ΔC&lt;sub&gt;T&lt;/sub&gt; esco2</th>
<th>ΔC&lt;sub&gt;T&lt;/sub&gt; MM</th>
<th>ΔC&lt;sub&gt;T&lt;/sub&gt; esco2/MM</th>
<th>Fold difference relative to MM&lt;sup&gt;c&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>cx43</td>
<td>23.31 ± 0.11</td>
<td>17.14 ± 0.15</td>
<td>6.16 ± 0.19</td>
<td>5.21 ± 0.14</td>
<td>0.95 ± 0.24</td>
<td>0.51 (0.43–0.61)</td>
</tr>
<tr>
<td>sema3d</td>
<td>24.25 ± 0.15</td>
<td>17.25 ± 0.07</td>
<td>7.0 ± 0.16</td>
<td>6.05 ± 0.12</td>
<td>0.94 ± 0.21</td>
<td>0.52 (0.44–0.6)</td>
</tr>
<tr>
<td>hapln1a</td>
<td>24.43 ± 0.05</td>
<td>18.09 ± 0.04</td>
<td>6.34 ± 0.07</td>
<td>5.57 ± 0.42</td>
<td>0.85 ± 0.42</td>
<td>0.58 (0.43–0.78)</td>
</tr>
<tr>
<td>mps1</td>
<td>22.27 ± 0.08</td>
<td>17.72 ± 0.08</td>
<td>4.55 ± 0.12</td>
<td>4.47 ± 0.34</td>
<td>–0.08 ± 0.36</td>
<td>0.94 (0.73–1.21)</td>
</tr>
<tr>
<td>shh</td>
<td>22.7 ± 0.1</td>
<td>19.51 ± 0.05</td>
<td>3.24 ± 0.11</td>
<td>3.53 ± 0.14</td>
<td>–0.28 ± 0.18</td>
<td>1.21 (1.07–1.38)</td>
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<tr>
<td>spry4</td>
<td>23.3 ± 0.08</td>
<td>19.35 ± 0.1</td>
<td>3.91 ± 0.13</td>
<td>4.45 ± 0.31</td>
<td>–0.54 ± 0.34</td>
<td>1.45 (1.14–1.85)</td>
</tr>
</tbody>
</table>

<sup>a</sup>The ΔC<sub>T</sub> value is determined by subtracting the average Keratin C<sub>T</sub> value from the average Gene C<sub>T</sub> value. The standard deviation of the difference is calculated from the standard deviations of the gene and Keratin values using the Comparative Method.

<sup>b</sup>The calculation of ΔΔC<sub>T</sub> involves subtraction by the ΔC<sub>T</sub> calibrator value. This is subtraction of an arbitrary constant, so the standard deviation of ΔΔC<sub>T</sub> is the same as the standard deviation of the ΔC<sub>T</sub> value.

<sup>c</sup>The range given for gene relative to MM is determined by evaluating the expression: 2<sup>–ΔΔC<sub>T</sub></sup> with ΔΔC<sub>T</sub>±s and ΔΔC<sub>T</sub>–s, where s = the standard deviation of the ΔΔC<sub>T</sub> value.
for MOs used in the study are as follows: MO targeted against esco2 (esco2-MO: 5'-CTCTTTCGGATAACATCTTCAATC-3', from Monnich et al., 2011), esco2 -5-base mismatch control MO (esco2-MM: 5'-CTCTTTCGGATAACATCTTCAATC-3', and Gene Tools standard control MO (5'-CCTCTAACCCTGTTACAATT-TATA-3'). Microinjection and electroporation procedures were carried out as described previously (Thummel et al., 2006; Hoptak-Solga et al., 2008; Sims et al., 2009). Briefly, caudal fins were amputated at 50% level. At 3 days postamputation (3 dpa), fish were anesthetized and MOs injected using a Narishige IM 300 Microinjector. Approximately 50 nl of MO was injected per ray into either the dorsal or ventral side of the regenerating fin tissue (approximately first five to six bony fin rays), keeping the other side uninjected. The uninjected side served as the internal control. Immediately after injection, both sides of the fin were electroporated using a CUY21 Square Wave electroporator (Protech International Inc). To minimize nonspecific effects of the electroporation procedure, both sides of the fin were electroporated. The following parameters were used during electroporation: ten 50-ms pulses of 15 V with a 1-s pause between pulses. These fish were returned back to the system water for regeneration to proceed. After 24 hr (i.e., 1 day postelectroporation (1 dpe), which is equivalent to 4 dpa, the injected side of the fins were evaluated by fluorescence using a Nikon Eclipse 80i Microscope (Diagnostic Instruments) to confirm MO uptake. The MO injected fins were evaluated for regenerate length, segment length, cell proliferation, cell death, in situ hybridization, protein levels by Western blots and RNA levels by qRT-PCR. For fins used for

**Fig. 10.** cx43 and cx43-dependent target genes are reduced following esco2-knockdown. The fold difference values from qRT-PCR are shown. A fold difference of 1 indicates no change with respect to standard MO treated fins. Three independent esco2-knockdown (KD) samples were prepared. Each sample was tested in duplicate (trials 1–3) for cx43, sema3d, hapln1a, shh, spry4, and mps1, and compared with the internal reference gene. The individual trials are represented by circles and the averages are represented by solid circles.

**Fig. 11.** Up-regulation of both cx43 mRNA and Cx43 protein in regenerating fins of the transgenic line treated for heat shock. A: Expression of cx43 mRNA in transgenic hsp70:mR-133sp−p++;positive with heat shock (Tg+HS+) and hsp70:mR-133sp−p--;negative with heat shock (Tg-HS+) fins by whole-mount ISH. The Tg+HS+ fins show a higher expression of cx43 mRNA compared with Tg-HS+ fins. The brackets mark the zone of cx43 expression in each fin ray and the horizontal line represents the amputation plane. Scale bar = 50 μm. B: Western blot reveals a 50% increase of Cx43 protein levels (normalized to tubulin) in fin lysates of Tg+HS+ compared with Tg-HS+. Tubulin was used as a loading control. The single band marked was used for relative band intensity analysis using the gel analysis tool (ImageJ software). C: Expression of esco2 mRNA in transgenic hsp70:mR-133sp−p++;positive with heat shock (Tg+HS+) and hsp70:mR-133sp−p--;negative with heat shock (Tg-HS+) fins by whole-mount ISH. Tg+HS+ and Tg-HS+ fins show similar expression level of esco2 mRNA. The brackets mark the zone of esco2 expression in each fin ray and the horizontal line represents the amputation plane. Scale bar = 50 μm. D: Western blot reveals the Esco2 protein expression (normalized to tubulin) is nearly similar (90%) in fin lysates of Tg+HS+ and Tg-HS+. Tubulin was used as a loading control. The single band marked was used for relative band intensity analysis using the gel analysis tool (ImageJ software).
lysate preparation or for qRT-PCR, all fins were injected and elec-
trooporated before harvesting.

Regenerate Length and Segment Length Measurement

At 4 dpe/7 dpa, MO injected (esco2-MO or esco2-MM) fins were
calcein stained before measuring regenerate length and segment
length (Du et al., 2001; Sims et al., 2009). Briefly, fish from all three
groups were amputated at 50% level and all were allowed to regenerate for 3 days. At 3 dpa, morpholino was injected to one half
of the regenerating fin tissue, immediately followed by electroporation on both sides. After an interval of 4 hr fish receiving
heat shock were shifted to 37°C for 1 hr. Induction of the transgene
expression upon heat shock was confirmed after 24 hr by screening
for GFP-positive fins in the Tg+HS+ group. The control groups (Tg+HS- and Tg±HS-) were negative for GFP expression. For ISH experiments, the
fins were harvested at 1 dpe/4 dpa. For measurement of regenerate length and segment length, fins were calcine stained at 4 dpe/7 dpa. For each
experiment, at least 6–8 fish were used per trial and at least 3 independent trials were performed.

RNA Probe Preparation and In Situ Hybridization on

Whole-Mount and Cryosectioned Fins

RNA probes were made using linear PCR product as template,
where the T7 RNA polymerase binding site was included in the

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Fig. 12. Overexpression of cx43 rescues esco2 knockdown phenotypes. A: Figure depicting the timeline of fin amputation, morpholino injection
and electroporation, heat shock, and data analysis. The 3 groups included in this experiment were Tg+HS- (transgenic hsp70:miR-133sp posit-
itive, heat shock), Tg+HS+ (transgenic hsp70:miR-133sp double-negative, heat shock) and Tg±HS- (transgenic hsp70:miR-133sp single-positive, not
heat shocked). Briefly, fins from all three groups were amputated at 50% level and all were allowed to regenerate for 3 days. At 3 dpa, morpholino was
injected to one half of the regenerating fin tissue, immediately followed by electroporation on both sides. After an interval of 4 hr fish receiving
heat shock were shifted to 37°C for 1 hr. Induction of the transgene expression upon heat shock was confirmed after 24 hr by screening for
GFP-positive fins in the Tg+HS+ group. The control groups (Tg+HS- and Tg±HS-) were negative for GFP expression. For ISH experiments, the
fins were harvested at 1 dpe/4 dpa. For measurement of regenerate length and segment length, fins were calcine stained at 4 dpe/7 dpa. For each
experiment, at least 6–8 fish were used per trial and at least 3 independent trials were performed. B: Graph reveals significant (*) rescue of esco2-
dependent segment length defects in heat shock miR-133sp double-positive esco2-knockdown fins (Tg+HS+) compared both with heat shock
transgene-negative (Tg+HS-) and with miR-133sp double-positive with no heat shock (Tg±HS-). Measurements from the injected side and the unin-
jected side of the same fin were used to calculate percent similarity and the average was calculated for each group. Note that a percent similarity
of 100% indicates no difference between the knockdown side and the control sides of the fin. Student’s t-test was used for determining statistical
significance where P < 0.05. Standard error is represented by error bars. C) Graph reveals significant (*) rescue of esco2-dependent regenerate
length defects in heat shocked miR-133sp double-positive esco2-knockdown fins (Tg+HS+) compared both with heat shocked transgene-negative
(Tg+HS-) and with miR-133sp double-positive in the absence of heat shock (Tg±HS-). Measurements from the injected side and the uninjected side
of the same fin were used to calculate percent similarity and the average was calculated for each group. Note that a percent similarity of 100%
indicates no difference between the knockdown side and the control sides of the fin. Student’s t-test was used for determining statistical signifi-
cance where P < 0.05. Standard error is represented by error bars.

Fig. 13. New model depicts the connection between Esco2 and Cx43 skeletal patterning pathway during fin regeneration. Proposed pathway
for Esco2 regulation of cx43 expression levels and genes downstream
of cx43. Esco2 may have additional targets.
reverse primer. The cdx3 template was made as described (Iovine et al., 2005). The esco2 product was generated using gene-specific primers (forward primer: 5’AGCAGGGACTCTTACAAG CA3’ and reverse primer: 5’TATAGCCTACTATAGGGGGGAT CATCTGGAAGAACG3’). RNA probes were labeled with digoxigenin (DIG) following manufacturer instructions (Roche). In situ hybridization (ISH) was performed on WT fins of different time points (1, 3, 5, and 8 dpa) and 5 dpa sof M1223 fins. Briefly, fins were amputated at 50% level and harvested at the appropriate time point. For ISH on knockdown fins, MO (esco2-MO or esco2-MM) was injected and electroporated on WT-3 dpa fins and harvested after 24 hr (1 dpe/4 dpa). For ISH on whole-mount fins, the standard protocol was followed (Ton and Iovine, 2013; Govindan and Iovine, 2014). For ISH on transgenic hsp70:miR-133sp pda4- positive and -negative fins, 3 dpa fish were heat shocked at 37degC for 1 hr and harvested after 24 hr (1 dpe/4 dpa). For all ISH experiments, approximately six to eight fins were used per trial and three independent trials were performed. A Nikon Eclipse 80i Microscope equipped with a SPOT-RTKE digital camera (Diagnostic Instruments) and SPOT software (Diagnostic Instruments) was used to acquire images.

ISH on sections was done as described with the following modifications (Smith et al., 2008). WT-5 dpa fins were first rehydrated sequentially by methanol-PBS washes, cryosectioned, and stored at -20degC. Slides were defrosted for approximately 1 hr before hybridization and section locations marked with a hydrophobic barrier pen (ImmEdge Pen; PAP pen, VWR Laboratories). RNA probe was mixed with hybridization buffer: 1 x salt solution (NaCl, Tris HCl, Tris Base, Na2HPO4, and 0.5 M EDTA), 50% deionized formamide (Sigma), 10% dextran sulfate, 1 mg/ml tRNA, and 1 x Denhart’s (Fisher) and denatured by incubating at 70degC for 5 min. The denatured probe mix was added to the sections and hybridized overnight at 65degC. Slides were washed at 65degC with a 1 x SSC, 50% formamide and 0.1% Tween-20 solution, rinsed with MABT (100 mM Maleic acid, 150 mM NaCl, and 0.1% Tween-20) and incubated in a blocking solution (MABT, goat serum, and 10% milk) for 2 hr. Anti- DIG antibody (1:5,000) was diluted in MABT and the slides were incubated overnight at 4degC. Slides were washed 4 x in MABT, 2 x in alkaline phosphatase staining buffer (100 mM Tris, pH 9.5, 50 mM MgCl2, 100 mM NaCl, and 0.1% Tween20), then incubated overnight at 37degC in 10% polyvinyl alcohol staining solution and NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) stock solution (Roche). The reaction was stopped by washing extensively with PBST. Sections were mounted in 100% glycerol and images acquired using a Nikon Eclipse 80i Microscope equipped with a SPOT-RTKE digital camera (Diagnostic Instruments) and SPOT software (Diagnostic Instruments).

**Immunoblotting and Esco2 Antibody**

*Escherichia coli* lysates from cells expressing either GST or GST-Eesco2 fusion protein (protein expression was induced using 0.3 mM IPTG for 4 hr) were prepared as described (Gerhart et al., 2012). Briefly, cells from 1 ml of culture were pelleted and lysed using 50 µg/ml lysozyme in lysis buffer (100 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM EDTA, pH 8, complete protease inhibitor cocktail, Roche). To this mixture, 2.2 N NaOH and 8% BME were added. Protein precipitation was carried out using 55% TCA followed by wash with 0.5% TCA. The protein pellets were resuspended in 2 x sodium dodecyl sulfate buffer. GST or GST-Eesco2 were detected by Western blot using anti-GST antibody (Santa Cruz, 1:5,000) or affinity-purified anti-Eesco2 (1:1,000). Affinity purified polyclonal Anti-Eesco2 was generated in rabbit against the N-terminal peptide LSRRKKGSPDAESC (Genscript) and used at a concentration of 1:1,000 for the noncompeted Western blot. For the anti-Eesco2 antibody specificity assay, identical gels were loaded with decreasing volumes of the GST-Eesco2 protein samples. For the competed blot, the anti-Eesco2 antibody was preincubated with the Esco2 peptide (100 µM). The blots were incubated with primary antibody overnight at 4degC.

Fin lysates were prepared as previously described (Hoptak-Solga et al., 2008; Gerhart et al., 2012; Govindan and Iovine, 2014). Briefly, approximately 9–10 MO-injected (esco2 MO or esco2 MM) 1 dpe/4 dpa regenerating fins were pooled, and then suspended in incubation buffer (136.8 mM NaCl, 5.36 mM KCl, 0.34 mM Na2HPO4, 0.35 mM KH2PO4, 0.8 mM MgSO4, 2.7 mM CaCl2, 20 mM HEPES with pH adjusted to 7.5) supplemented with protease inhibitor (Thermo scientific, Halt™ Protease and Phosphatase Inhibitor Cocktail, 100X). The harvested fin tissue was homogenized by a tissue homogenizer (Bio-Gen, PRO 200) at high speed (3 x) for 5 sec with 10-sec cooling intervals. Homogenized samples were centrifuged at 200 g for 10 min at 4degC and supernatant protein levels normalized according to Bradford assays. Note that for preparation of proteins lyase from heat shocked Tg(hsp70:miR-133sp pda4)-positive and Tg(hsp70:miR-133sp pda4)-negative fish, 37degC heat shock was performed for 1 hr at 3 dpa and fins harvested at 1 dpe/4 dpa as previously described.

GST, Esco2, Cx43, or tubulin was detected using anti-GST (1:5,000) (Santa Cruz), anti-Eesco2 (1:1,000), anti-Cx43 (1:1,000, Hoptak-Solga et al., 2008) or anti-a-tubulin (1:1,000) (Sigma), followed by exposure to peroxidase-conjugated goat anti-rabbit IgG (GST, Esco2 and Cx43) or goat anti-mouse IgG (tubulin) (Pierce Rockford, IL) at a concentration of 1:20,000. Signal detection was performed using ECL chemiluminescent reagent (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce Rockford, IL) and X-ray film.

Image J software was used to measure the band intensities and the percent change was calculated. Relative pixel densities of gel bands were measured using a gel analysis tool in ImageJ software as described (Bhadra and Iovine, 2015). The density of each band was obtained as the area under the curve using the gel analysis tool. For relative density calculation, the density of the Esco2, Cx43, or tubulin bands for the experimental sample was first normalized against the density of the Esco2, Cx43, or tubulin bands from the control sample. Relative pixel density was calculated as the ratio of Esco2 and tubulin or Cx43 and tubulin, where tubulin is the loading control.

**Cell Proliferation and Cell Death Assays**

For detection of proliferating cells in S-phase, bromodeoxyuridine (5-bromo-2′-deoxyuridine, BrdU) labeling was performed with few modifications (Nechiporuk and Keating, 2002, Iovine et al., 2005). Briefly, 3 dpa MO injected (esco2-MO or esco2-MM) fish were allowed to swim for 5 min in 50 µg/ml of BrdU (Roche) mixed in system water at 1 dpe/4 dpa and harvested on the same day. The BrdU-labeled fins were fixed in 4% PFA overnight at 4degC and then dehydrated by keeping in 100% methanol overnight. Before use, the fins were rehydrated gradually in a series of methanol solutions containing 0.1% Triton X-100 in PBS (PBTrx).
Next, fins were treated for 30 min in a solution containing 2N HCl in PBTx. Following that, the fins were blocked for 2 hr (0.25% bovine serum albumin in PBTx). The primary antibody against BrdU (Roche) is a mouse monoclonal and used at a 1:50 dilution and incubated overnight at 4degC. Extensive washes (4 hr) in the PBTx solution were performed the next day and fins incubated overnight at 4degC in 1:200 dilution of anti-mouse antibody conjugated to Alexa-546 (Invitrogen). The next day extensive washes (4 hr) were performed and the fins mounted in 100% glycerol and visualized under a Nikon Eclipse 80i Microscope equipped with a SPOT-RTKE digital camera (Diagnostic Instruments) and SPOT software (Diagnostic Instruments). For measuring BrdU labeled cells the Image Pro software was used. A ratio of distance migrated by BrdU positive cells from the regenerating tip in μm (a) and the regenerating length in μm (b) was calculated. BrdU labeling was then obtained by measuring a/b ratio of the uninjected and injected (esco2-MO and esco2-MM) side of the fin.

For both histone-3-phosphate (H3P) and TUNEL assays, 3 dpa MO-injected (esco2-MO or esco2-MM) fins were harvested at 1 dpe/4 dpa and fixed in 4% PFA overnight at 4degC. These fins were then dehydrated in 100% methanol overnight before use. To detect mitotic cells, H3P staining and the number of H3P positive cells per unit area were carried out as described previously (Ton and Iovine, 2013). The primary and secondary antibodies used for H3P assay are as follows: rabbit anti-histone-3-phosphate (1:200) (anti-H3P, Millipore) and anti-rabbit Alexa 546 (1:200) (Invitrogen). H3P-positive cells were counted without software. Excised fins were then dehydrated in 100% methanol overnight before use. To detect mitotic cells, H3P staining and the number of H3P positive cells per unit area were carried out as described previously (Ton and Iovine, 2013). The primary and secondary antibodies used for H3P assay are as follows: rabbit anti-histone-3-phosphate (1:200) (anti-H3P, Millipore) and anti-rabbit Alexa 546 (1:200) (Invitrogen). H3P-positive cells were counted without software from within the distal-most 250 μm of the 3rd fin ray as previously established (Iovine et al., 2005; Hoptak-Solga et al., 2008).

TUNEL assay (ApopTag Kit, Chemicon) was performed as described in the manufacturer’s instructions with the following modifications. The fins were rehydrated by successive washes in methanol/PBST, treated with proteinase K at a concentration of 5 μg/ml for 45 min at room temperature, and then re-fixed in 4% PFA in PBS for 20 min. After extensive PBST washes, fins were incubated in ethanol: acetic acid (2:1; v:v) at −20degC for 10 min. Following extensive PBST washes, fins were incubated in equilibrium buffer (from ApopTag Kit) for 1 hr at room temperature, and then incubated overnight in 37degC water bath in TdT solution. The enzymatic reaction was stopped by extensive washes in stop/ wash buffer for 3 hr in 37degC water bath, briefly rinsed in PBST and blocked with blocking solution (from ApopTag Kit) for 1 hr. The fins were incubated overnight at 37deg in Rhodamine antibody solution (from ApopTag Kit). Fins were washed extensively in PBST and then mounted in 100% glycerol. Image acquisition was performed using a Nikon Eclipse 80i microscope equipped with a SPOT-RTKE digital camera (Diagnostic Instruments) and SPOT software (Diagnostic Instruments). Cell death was analyzed by counting the number of TUNEL-positive cells without software from within the distal-most 250 μm of the 3rd fin ray, similar to the H3P-positive cell counting analysis. For all the experiments at least 6–8 fins were used per trial and at least 3 independent trials were performed. Student’s t-test (P < 0.05) was used for statistical analysis.

qRT-PCR Analysis

The qRT-PCR analysis was completed on total mRNA extracted from 1 dpe/4 dpa harvested fins (3 dpa esco2-MO and standard control-MO injected). Total RNA extraction was carried out by following the standard protocol (Sims et al., 2009). Briefly, Trizol reagent (Gibco) was used to extract mRNA from minimum of 10 fins. For making cDNA, 1 μg of total RNA was reverse transcribed with SuperScript III reverse transcriptase (Invitrogen) using oligo (dT) primers. The following primers (2.5 μM) for keratin, cx43 (Sims et al., 2009), sema3d (Ton and Iovine, 2013), hapln1a (Govindan and Iovine, 2014), and mps1 (Bhadra and Iovine, 2015) were used for qRT-PCR analysis. The primers for shh and spry4 were designed using Primer express software (shh: forward primer: 5’-GGCTCATGACACAGATGCA-3’, reverse primer: 5’-CATTACAGAGATGGCCAGCA-3’ and spry4: forward primer: 5’-CGCAAAGCTTCTGTATCTGA-3’, reverse primer: 5’-GCCACTTGCAATCGAAAGCA-3’). Data from three independent esco2-knockdown RNA samples were used, with qRT-PCR for each gene performed in duplicate, for comparison between experimental treatments. RNA and subsequent cDNA synthesized from standard control MO injected fins served as the control. The standard control MO does not target any zebrafish genes. Analyses of the samples were done using Rotor–Gene 6000 series software (Corbett Research) and the average cycle number (Ct) determined for each amplicon. Keratin was used as a housekeeping gene, and the delta Ct (ΔCt) values represent expression levels normalized to keratin values. ΔΔCt values represent the relative level of gene expression and the fold difference was determined using the 2−ΔΔCt method as described (Ton and Iovine, 2013). Standard deviation was calculated using the comparative method described in User Bulletin 2 # ABI PRISM 7700 Sequence Detection System (http://www3.appliedbiosystems.com/cms/groups/ mch_support/documents/generaldocuments/cms_040980.pdf).

Heat Shock Induction of cx43 Expression

Tg(hsp70:miR-133sp pd48) are denoted as transgene-positive (Tg+) and their siblings denoted as transgene-negative (Tg−) were used in the heat shock experiment (Yin et al., 2012). For all the experiments at least six to eight fish were used per trial and at least 3 independent trials were performed. esco2-knockdown was performed on 3 dpa Tg+ and Tg− fish as described above. After 4 hr, both groups were heat shocked at 37 C for 1 hr and returned to the system water for recovery. These groups were then denoted by Tg+HS+ and Tg−HS+, respectively. To confirm that rescue depended on cx43, we also examined phenotypes in the transgenic line without heat shock (Tg+HS−). Induction of the transgene expression upon heat shock was confirmed after 24 hr by screening for GFP-positive fins in the Tg+HS+ group. The control groups (Tg+HS− and Tg−HS+) were negative for GFP expression after heat shock. For measurement of regenerate length and segment length fins were harvested at 4 dpe/7 dpa and calcine stained as previously described (Du et al., 2001; Sims et al., 2009). The measurement and data analysis were done as described below. Image acquisition was carried out by using the Nikon Eclipse 80i microscope equipped with a SPOT-RTKE digital camera (Diagnostic Instruments) and SPOT software (Diagnostic Instruments). Image Pro software was used for regenerate and segment length measurements.

To evaluate the regenerate length and segment length of Tg(hsp70:miR-133sp pd48) fins, the esco2-MO injected side of each fin was compared with its un-injected side by % similarity method as described (Bhadra and Iovine, 2015). Briefly, the length of the injected side and un.injected sides were measured in μm and denoted as A and B respectively. The % similarity for
each fin was calculated by using the formula: \( \frac{[(A/B) \times 100]}{C2} \). Values close to 100% indicate that the esco2-MO has no effect on the phenotype whereas a value less than 100% indicate that the MO has an effect on the observed phenotype. The mean of % similarity for the esco2-knockdown experimental group (Tg(HS+) and the corresponding esco2-knockdown control groups (Tg(HS+ and Tg(HS-) were estimated and compared, and the statistical significance between the groups was determined using two tailed unpaired student’s t-test \( (P < 0.05) \). Segment length analysis was performed on calcein stained fins. Briefly, for segment length, the distance between the first two newly formed joints following amputation was measured (in the 3rd fin ray from either the dorsal or ventral end) because that was previously established as a standard (Iovine and Johnson, 2000). To evaluate the phenotypic effect of segment length, the % similarity method was used as described above.

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References


