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Insulin-like growth factor receptor 1b is required for zebrafish primordial germ cell migration and survival

Peter J. Schlueter[1,3], **Xianpeng Sang**[2], **Cunming Duan**[1], and **Antony W. Wood**[2,4]

1 *Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, MI 48109*

2 *Vincent Center for Reproductive Biology, Vincent Obstetrics and Gynecology Service, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114, Tel: 617-726-0654, Fax: 617-724-9935*

Abstract

Insulin-like growth factor (IGF) signaling is a critical regulator of somatic growth during fetal and adult development, primarily through its stimulatory effects on cell proliferation and survival. IGF signaling is also required for development of the reproductive system, although its precise role in this regard remains unclear. We have hypothesized that IGF signaling is required for embryonic germline development, which requires the specification and proliferation of primordial germ cells (PGCs) in an extragonadal location, followed by directed migration to the genital ridges. We tested this hypothesis using loss-of-function studies in the zebrafish embryo, which possesses two functional copies of the Type-1 IGF receptor gene (*igf1ra*, *igf1rb*). Knockdown of IGF1Rb by morpholino oligonucleotides (MO) results in mismigration and elimination of primordial germ cells (PGCs), resulting in fewer PGCs colonizing the genital ridges. In contrast, knockdown of IGF1Ra has no effect on PGC migration or number despite inducing widespread somatic cell apoptosis. Ablation of both receptors, using combined MO injections or overexpression of a dominant-negative IGF1R, yields embryos with a PGC-deficient phenotype similar to IGF1Rb knockdown. TUNEL analyses revealed that mismigrated PGCs in IGF1Rb-deficient embryos are eliminated by apoptosis; overexpression of an antiapoptotic gene (Bcl2l) rescues ectopic PGCs from apoptosis but fails to rescue migration defects. Lastly, we show that suppression of IGF signaling leads to quantitative changes in the expression of genes encoding CXCL-family chemokine ligands and receptors involved in PGC migration. Collectively, these data suggest a novel role for IGF signaling in early germline development, potentially via cross-talk with chemokine signaling pathways.

Introduction

Insulin-like growth factor (IGF) signaling plays a central role in the regulation of animal growth and development (Barbieri et al., 2003;LeRoith et al., 2001;Wood et al., 2005a). In mammals, IGF-1 and IGF-2 ligands exert their effects by activating the Type-1 IGF receptor (IGF1R), triggering a variety of cellular responses including proliferation, migration, and cell survival (Butler et al., 1998;Yano et al., 1999). During postnatal growth, IGF signaling serves as the primary endocrine mediator of growth hormone (GH) signaling (LeRoith et al., 2001), but appears to function independently of GH during prenatal development (Wollmann, 2000).

[4]Author to whom correspondence should be addressed (awwood@partners.org)
[3]Current Address: Cardiovascular Research Center, Massachusetts General Hospital, 149-13th Street, Charlestown MA 02129

The importance of IGF signaling to animal growth was clearly demonstrated by targeted deletion of genes encoding IGF ligands and receptors in mice (Baker et al., 1993;DeChiara et al., 1990;DeChiara et al., 1991;Liu et al., 1993). Mice lacking either IGF ligand survive to adulthood, but exhibit proportional growth retardation during fetal and postnatal life, while deletion of both ligands or IGF1R leads to severe fetal dwarfism and perinatal mortality. Importantly, these effects are consistent with growth defects in humans with mutations in IGF-related genes, confirming a conserved role for IGF signaling in regulating vertebrate growth (Abuzzahab et al., 2003;Nakae et al., 2001).

There is also evidence that IGF signaling is specifically required for both development and function of the reproductive system. Both IGF-1 and the IGF1R are abundantly expressed in reproductive tissues of adult vertebrates (Nuttinck et al., 2004;Ohtsuki et al., 2005;Perrot et al., 2000), and IGF-1 nullizygous mice exhibit significantly reduced fertility as adults (Baker et al., 1996;Liu et al., 1993). The precise cause of the fertility defects remains unclear; an analysis of testicular germ cell numbers in IGF-1 nullizygous mouse revealed a disproportionate reduction in the number of germ cells (relative to both organ and body size; Baker et al., 1996), suggesting specific defects in germ cell development in the absence of normal IGF signaling. To our knowledge however, a detailed analysis of germ cell development in IGF-deficient animals has not been performed.

In vitro studies indicate that IGF-1 can inhibit apoptosis of fetal mouse germ cells (Morita et al., 1999) and chicken primordial germ cells (Park and Han, 2000), but these findings have not been extended to an examination of IGF signaling and germline development *in vivo*. However, insulin-like signaling was shown to play a direct role *in vivo* in promoting germline stem cell renewal in *Drosophila* (LaFever and Drummond-Barbosa, 2005), suggesting that regulation of germline development by insulin-like signaling may be conserved throughout animal evolution.

The zebrafish is an excellent model for *in vivo* studies of vertebrate germline development. The transparency of zebrafish embryos permits direct visualization of PGC development, a coordinated sequence of events involving cell fate specification, proliferation, and directed migration. While a number of conserved signaling pathways required for PGC development (e.g., Cxcl-family chemokine signaling) have been identified in zebrafish (Ara et al., 2003;Doitsidou et al., 2002;Knaut et al., 2003;Molyneaux et al., 2003;Schier, 2003;Stebler et al., 2004), our understanding of this process remains largely incomplete. The well-described effects of IGF signaling on both cell proliferation and migration led us to hypothesize that IGF signaling regulates one or more facets of PGC development. In this study we used the zebrafish to test this hypothesis, using two strategies to suppress IGF signaling during the PGC migration period. We examined PGC development in zebrafish embryos after single or combined knockdown of IGF1Ra and IGF1Rb, using morpholino antisense oligonucleotides or a dominant-negative IGF1R. Our results indicate that in zebrafish embryos, IGF signaling through IGF1Rb is necessary to ensure correct migration of PGCs to the genital ridges, and that defective migration results in subsequent elimination of mismigrated PGCs by apoptosis. We also present evidence that the effects of IGF signaling on PGC development may be manifest through modifications of CXCL-family chemokine signaling.

Materials and Methods

Animals

Adult wild-type zebrafish were reared and maintained using standard methods. Embryos were generated from natural crosses, staged according to Kimmel et al. (1995), and reared as previously described (Wood et al., 2005b). All experiments were conducted in accordance with

guidelines as established by the Subcommittee on Research Animal Care at Massachusetts General Hospital.

Chemicals and reagents

Standard chemicals and reagents were purchased from Fisher (Pittsburgh, PA) unless otherwise specified. RNA polymerases and DNase (RNase-free) were purchased from Promega (Madison, WI), and restriction endonucleases were purchased from New England BioLabs (Beverly, MA). Platinum® Taq DNA polymerase, Superscript II reverse transcriptase and oligonucleotide primers (for conventional and quantitative RT-PCR) were purchased from Invitrogen Life Technologies, Inc. (Carlsbad, CA). FailSafe™ PCR reaction buffers were purchased from EpiCentre (Madison, WI), and the *In Situ* Cell Death Detection Kit (TMR Red) was purchased from Roche Applied Science (New Jersey, NJ). Gene-specific morpholino-modified oligonucleotides (MO) were purchased from Gene Tools, LLC (Philomath, OR). Polyclonal antiserum raised against zebrafish Vasa protein was a generous gift from Holger Knaut and Christiane Nüsslein-Volhard (Max Planck Institute for Developmental Biology, Tübingen, Germany). Alexa Fluor® 488 secondary antibodies were purchased from Molecular Probes (Eugene, OR).

In situ hybridization

Partial cDNA sequences corresponding to divergent regions of *igf1ra* and *igf1rb* were generated by RT-PCR using gene-specific primers as previously described (Wood et al., 2005b), and subcloned into the PCRII-TOPO™ vector. The gene-specific oligonucleotide primer sequences are as follows:

igf1ra (forward): 5'-GCCATCTTTCCTGGAGATCA-3'

igf1ra (reverse): 5'-AGACAAAGGGAGGAGGGAAA-3'

igf1rb (forward): 5'-CCCTCTAGAACCGTCTTCCA-3’

igf1rb (reverse): 5'-GATCCTGTCTGGCGGAAATA-3'.

Accuracies of amplified sequences were confirmed by automated sequencing using commercial primers. Template DNA for riboprobe synthesis was generated by restriction enzyme digestion, and digoxigenin-labeled ribonucleotide probes were generated by *in vitro* transcription. Whole-mount *in situ* hybridizations were performed as previously described (Wood et al., 2005b). Images were captured with a digital camera (MicroFire, Optronics®, Goleta, CA) mounted to a Nikon Eclipse TE-2000SE microscope (Melville, NY).

Messenger RNA synthesis

Capped synthetic messenger RNA was generated by *in vitro* transcription (mMESSAGE mMACHINE®, Ambion, Austin, TX) according to supplier’s instructions. Template plasmid DNA for zebrafish *bcl2l* mRNA was generously provided by Monte Westerfield (University of Oregon, Eugene, OR). The dominant-negative IGF1R:GFP fusion protein construct (dnIGF1R:GFP) has been previously described (Schlueter et al., 2007).

Embryo microinjections

Stock MO solutions were diluted in Danieau buffer and injected into fertilized embryos (~1 nl/embryo) at the one-cell stage, as previously described (Wood et al., 2005b). To ensure efficient knockdown, two non-overlapping MO sequences were used against the translational start site of each *igf1r* subtype; the specificity and efficacy of each of these MO sequences have been confirmed using multiple approaches (Schlueter et al., 2006). The MO against each receptor subtype were injected together (2.5 ng total MO per embryo); it was determined in pilot studies that these quantities of MO (i.e., 1.25 ng *igf1ra*-MO1 + 1.25 ng *igf1ra*-MO2)

yielded reproducible effects (mild, proportional growth restriction) for each receptor subtype, whereas equivalent quantities of a control-MO cocktail (Control MO-a + Control MO-b) yielded embryos indistinguishable from non-injected embryos. For phenotypic analyses, embryos were raised as described above, fixed at desired stages of development in 4% buffered paraformaldehyde, dehydrated in methanol, and stored at –20ºC until analysis. For quantitative RT-PCR (qRT-PCR) analyses, injected embryos were snap-frozen in liquid N_2 in preparation for RNA extraction.

For synthetic mRNA injections, stock solutions were diluted in RNase-free Danieau buffer, and either injected alone (dnIGF1R:GFP, Blc2l, GFP), or co-injected (Bcl2l) with gene-specific MO. Synthetic mRNA was microinjected at nominal concentrations of 750 pg/embryo (dnIGF1R:GFP) or 100 pg/embryo (Bcl2l); for controls, mRNA encoding GFP was injected at 750 pg/embryo.

Whole-mount immunostaining

Fluorescent immunostaining for PGCs was performed in whole embryos as previously described (Westerfield, 1995), using polyclonal antiserum against zebrafish Vasa protein, diluted 1:8000 in blocking buffer (PBS, containing 0.1% Tween 20, 1% DMSO, 2% goat serum). The secondary antibody (Alexa Fluor™ 488 goat anti-rabbit IgG) was diluted 1:1000 in blocking buffer. Primordial germ cells (Vasa-positive cells) were localized and enumerated by examining whole embryos under a fluorescent microscope. Negative controls were incubated in the absence of primary antibody to confirm the germ cell-specificity of the primary antibody.

Western immunoblot analysis

Western immunoblot analysis was used to confirm expression of the dnIGF1R:GFP fusion protein, using prim-5 stage zebrafish embryos. Yolk proteins were removed by puncturing the yolk sac and washing the embryos in PBS. Residual carcasses were then homogenized in SDS sample buffer (1% SDS, 0.1 M Tris-HCl, 10% glycerol, 0.02% bromophenol blue, 2% 2-mercaptoethanol), and the homogenates were electrophoretically fractionated in 10% SDS-PAGE gels under denaturing conditions. After transfer to Immobilon-P PVDF membranes, the blots were incubated for 4 h in blocking buffer (Tris-buffered saline containing 0.1% Tween-20 and 5% non-fat dry milk; TBS-TM), and then incubated overnight at 4ºC in TBS-TM containing rabbit antiserum against GFP (Torrey Pines Biolabs, Houston, TX). Primary antibody was detected using HRP-conjugated donkey anti-rabbit IgG (1:5000; Jackson ImmunoResearch Labs, West Grove, PA), and labeled proteins were visualized by the ECL detection system.

Whole-mount *in situ* cell death (TUNEL) analysis

Fixed embryos were rehydrated and permeabilized as described (Schlueter et al., 2006), and incubated for 1 h at 37ºC in TUNEL assay reagents. Negative controls were incubated in the absence of terminal transferase enzyme. Stained embryos were resuspended in glycerol:PBS (70:30) and examined by fluorescence microscopy. To specifically identify TUNEL-positive PGCs, we performed subsequent immunocytochemical staining (as above) before analysis by fluorescence microscopy.

Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was employed to determine if knockdown of IGF1Ra or IGF1Rb affected mRNA levels for genes encoding known germ cell guidance molecules. Efforts focused on mRNA encoding the chemokine ligand Cxcl12a (NP_840092) and its cognate receptor Cxcr4b (AAF17561), a conserved ligand-receptor pair whose functions are

essential for directional PGC migration in vertebrate embryos (Doitsidou et al., 2002;Knaut et al., 2003;Molyneaux et al., 2003;Schier, 2003). Ornithine decarboxylase 1 (*odc1*; NM_131801) mRNA was used as the internal reference standard (Draper et al., 2001). The following primers were used (lower case letters indicate hairpin primer extensions; [FAM] indicates position of fluorescent label):

cxcl12a (forward): 5'-cgccCGTAGTAGTCGCTCTGATGG[FAM]G-3'

cxcl12a (reverse): 5'-TGGGACTGTGTTGACTGTGGAA-3'

cxcr4b (forward): 5'-cggcCTGGTTGCCTTACTGTGC[FAM]G

cxcr4b (reverse): 5'-CCATTTCTCCAGACCCTGTTCC-3'

odc1 (forward): 5'-gacaGCGGTGAACCTCCTTGGCTG[FAM]C-3'

odc1 (reverse): 5'-CGGTGCAAGCCGTCATAGTG-3'.

Total RNA was isolated from control-MO-injected and MO-injected embryos (Prim-5 stage) using Trizol reagent, and residual genomic DNA was removed by digestion with RNase-free DNase. Each sample consisted of mRNA isolated from a pool of ten embryos, to ensure sufficient quantities of mRNA for analysis. First-strand cDNA was generated by reverse-transcription as described (Wood et al., 2005b), using 500 ng of DNA-free RNA. Real-time PCR amplifications were performed in a Cepheid SmartCycler II (Fisher Scientific) for 45 cycles under the following conditions: 95ºC for 150 s, 95ºC for 10 s; 60ºC for 10 s; 72ºC for 10 s. The identity of PCR products amplified with FAM-labeled primers was confirmed by electrophoretic and melting point analysis; quantification of relative mRNA levels for each target gene in treatment and control groups was determined as previously described (Pfaffl, 2001).

Data analysis

Values are presented as means ± standard error of the mean (SEM). Mean PGC numbers in treatment and control groups were compared statistically using one-way analysis of variance (ANOVA), followed by the Tukey's *post-hoc* test for multiple comparisons. Target gene (*cxcl12a*, *cxcr4b*) mRNA levels are presented as relative (percentage) values normalized to *odc* (100%); mean relative changes in mRNA levels were statistically compared among treatment and control groups by paired Student's *t*-test. In all statistical tests, mean values were considered significantly different when $P < 0.05$.

Results

Both *igf1ra* and *igf1rb* are ubiquitously expressed in zebrafish embryos

Although it was previously reported that mRNAs encoding both IGF1Ra and IGF1Rb are ubiquitously expressed in zebrafish embryos, as determined by whole-mount *in situ* hybridization (Maures et al., 2002), we sought to re-examine mRNA expression in greater detail, specifically in PGCs and the genital ridges (Supplemental Fig. 1). As previously reported, both genes exhibit ubiquitous mRNA expression in zebrafish embryos (Supplemental Fig. 1A–D). To examine the mRNA expression of *igf1ra* and *igf1rb* specifically within the PGCs and the genital ridges, we used fluorescence immunohistochemistry (Supplemental Fig. 1E–H) with an antibody raised against zebrafish Vasa protein (Knaut et al., 2000) to visualize PGCs in whole embryos directly following *in situ* hybridization labeling of mRNA for either IGF1Ra or IGF1Rb.

The double-labeling approach confirmed that both PGCs and the genital ridge regions express mRNA for both IGF1Ra and IGF1Rb (Supplemental Fig. 1G–H), but we were unable to detect

any relative differences in expression of the receptor subtypes in either PGCs or the genital ridges.

Morpholino knockdown of *igf1rb* reduces total PGC number

To test the hypothesis that PGC development requires signaling through IGF1R, we first counted the total number of PGCs in equivalent-stage embryos after separate or combined knockdown of IGF1Ra and IGF1Rb with gene-specific morpholino antisense oligonucleotides (MO). The prim-5 stage of development was chosen for analysis because in normal untreated (wild-type) embryos, virtually all PGCs have completed their migration to the genital ridges by this stage. The efficacy and specificity of the MO sequences have recently been confirmed in zebrafish using multiple approaches (Schlueter et al., 2006). We identified and quantified PGCs by fluorescence immunostaining of Vasa protein (Fig. 1) as described above.

Control-MO-injected embryos (Fig. 1A) developed similarly to non-injected (wild-type) embryos, and at the prim-5 stage no significant differences were detected in the mean number of PGCs among Control-MO-injected and wild-type embryos (Table 1). Knockdown of IGF1Ra by antisense MO injection resulted in slightly delayed development, as described previously (Schlueter et al., 2006), but had no detectable effects on the mean number of PGCs, relative to control-MO-injected or wild-type embryos, when compared at the prim-5 stage (Fig. 1B, Table 1). Knockdown of IGF1Rb by MO injection similarly induced slightly delayed development, but also induced a significant reduction in the total number of visible PGCs at the prim-5 stage (Fig. 1C, Table 1). Simultaneous MO-mediated knockdown of both IGF1Ra and IGF1Rb, using combined *igf1ra*-MO + *igf1rb*-MO injections, yielded embryos with PGC numbers similar to those observed in IGF1Rb-deficient embryos (Fig. 1D, Table 1), suggesting no additive effects of double receptor knockdown beyond those observed after IGF1Rb knockdown.

Overexpression of dominant-negative IGF1R reduces PGC number

To confirm the MO knockdown results using an alternative approach to suppress IGF signaling, we injected embryos with synthetic mRNA encoding a dominant-negative IGF1R:GFP fusion protein (dnIGF1R:GFP; Schlueter et al., 2007). Overexpression of dnIGF1R:GFP yielded GFP-positive embryos (Fig. 2A) with a developmental delay phenotype similar to that observed in *igf1r*-MO-injected embryos, confirming functionality of the dnIGF1R:GFP fusion protein. Overexpression of dnIGF1R:GFP (Fig. 2C) also resulted in a significant reduction in mean PGC number in embryos at the prim-5 stage, relative to control and wild-type embryos (Table 1). The mean number of visible PGCs in embryos overexpressing dnIGF1R:GFP was statistically similar to the mean values observed in both *igf1rb*-MO-injected embryos and *igf1ra*-MO + *igf1rb*-MO-injected embryos (ANOVA, Tukey's, $P > 0.05$; Table 1). Injection of mRNA encoding GFP alone had no significant effects on mean PGC number relative to wild-type or Control-MO-injected embryos.

Suppression of IGF signaling does not disrupt early PGC proliferation

IGF signaling stimulates mitotic proliferation in a diversity of cell types (Onagbesan et al., 1994;Otteson et al., 2002;Pozios et al., 2001), and zebrafish PGCs undergo 2–3 mitotic divisions prior to initiating migration towards the genital ridges (Braat et al., 1999). To determine whether the PGC deficiency in *igf1rb*-MO-injected embryos is due to defects in PGC proliferation, we enumerated PGCs in *igf1r*-MO-injected embryos at an earlier stage of development (18S). As shown (Table 1), the mean number of PGCs in 18S *igf1r*-MO-injected embryos (*igf1ra*-MO, *igf1rb*-MO, *igf1ra*-MO + *igf1rb*-MO), and embryos overexpressing dnIGF1R:GFP, were not significantly different from PGC numbers in 18S control-MO-injected embryos, that were likewise similar to PGC numbers in control-MO-injected embryos at the prim-5 stage. These results suggest that PGCs initially proliferated normally in all

treatment groups, but subsequently declined in number between 18 and 24 hpf, specifically after targeted knockdown of IGF1Rb. Notably, this time interval corresponds directly to the terminal stages of PGC migration to the genital ridges (Weidinger et al., 1999).

Knockdown of IGF1Rb disrupts PGC migration

In the course of enumerating PGCs in 18S embryos, we noticed that more Vasa-positive cells in *igf1rb*-MO-injected embryos were found in "ectopic" positions (i.e., distant from the genital ridges) relative to other treatment groups. Because cell migration is a universal feature of PGC development in metazoans (Molyneaux and Wylie, 2004), we hypothesized that the PGC deficiency in *igf1rb*-MO-injected embryos may be a secondary consequence of defective PGC migration. We therefore counted the number of ectopic PGCs in 20-somite (20S) stage MO-injected embryos; we specifically chose this stage of development to facilitate discrimination between mismigrated PGCs and normally migrating PGCs that had not yet reached the genital ridges. We defined ectopic PGCs as Vasa-positive cells found in cranial, dorsal, and caudal regions of the embryo proper, and in regions of the yolk cell distal to the genital ridges (Fig. 3A–C). Vasa-positive cells found along the yolk cell extension, or in regions of the yolk cell directly adjacent to the genital ridges were considered to be normal PGCs in the terminal stages of migration toward the genital ridges. Consistent with our hypothesis, we found a significantly increased mean number of ectopic PGCs in 20S *igf1rb*-MO-injected embryos (6.2 ± 0.8; n=12) relative to 20S control MO-injected embryos (0.7 ± 0.2; n=27, ANOVA, $P < 0.001$; Fig. 3D). Notably, ectopic PGCs in *igf1rb*-MO-injected embryos appeared to be distributed in a random fashion, suggesting their displacement by an active, but unguided, mechanism. The mean number of ectopic PGCs in 20S *igf1ra*-MO-injected embryos (0.5 ± 0.2; n=10) was not significantly different from 20S control MO-injected embryos ($P = 0.6$).

Ectopic PGCs are selectively eliminated by apoptosis

Signaling through the IGF1R is a potent anti-apoptotic signal in many cell types, including germ cells (Byrne et al., 2002;Delaney et al., 1999;Loir, 1999). We therefore reasoned that the decline in mean PGC number in *igf1rb*-MO-injected embryos may be due to the elimination of PGCs by apoptosis. We therefore examined *igf1ra*-MO- and *igf1rb*-MO-injected embryos by TUNEL analysis for evidence of apoptotic DNA fragmentation.

We first examined whole embryos, and consistent with the known anti-apoptotic functions of IGF signaling, TUNEL analyses revealed increased DNA fragmentation throughout MO-injected embryos after knockdown of either receptor subtype, in both 18S (Fig. 4A–C) and prim-5 (Fig. 4D–F) embryos. TUNEL-positive cells in both *igf1ra*-MO- and *igf1rb*-MO-injected embryos were most abundant in anterior neural tissues and dorsal regions of the trunk, with greater intensities observed in 18S versus prim-5 stage embryos. Overall, DNA fragmentation was most intense in *igf1ra*-MO-injected embryos (Fig. 4B, E); although DNA fragmentation was visibly increased in *igf1rb*-MO-injected embryos (Fig. 4C, F) relative to control MO-injected embryos, the intensity of DNA fragmentation in *igf1rb*-MO-injected embryos was noticeably less than that in *igf1ra*-MO-injected embryos. When considered in the context of the PGC-deficient phenotype, the difference in relative intensities of DNA fragmentation among *igf1ra*-MO- and *igf1rb*-MO-injected embryos suggests that IGF1Rb may play a more selective role in promoting PGC survival, whereas IGF1Ra is predominantly involved in somatic cell survival.

To examine this in more detail, we used combined immunostaining (anti-Vasa, Fig. 5A, D) and TUNEL (Fig. 5B, E) to look specifically for TUNEL-positive PGCs in MO-injected embryos. We again examined 20S-stage embryos, to facilitate detection of PGCs before their degradation and elimination, which appears to occur by the prim-5 stage (based on mean PGC numbers, Table 1). As shown in Fig. 5A–C, TUNEL-positive PGCs were readily identifiable

in *igf1rb*-MO-injected embryos; notably however, TUNEL-positive PGCs were restricted exclusively to ectopic positions. We did not detect TUNEL-positive PGCs in the genital ridges of any embryo treatment group (e.g., Fig. 5D–F). This observation supports the hypothesis that ectopic PGCs are selectively eliminated by apoptosis, whereas PGCs that correctly migrate to the genital ridges are resistant to death by apoptosis. Thus, more total PGCs are eliminated in *igf1rb*-MO-injected embryos (relative to other treatment groups), because they contain comparatively more ectopic PGCs, ultimately leading to fewer total PGCs at the prim-5 stage (Table 1).

Overexpression of Bcl2l rescues ectopic PGCs in IGF1Rb-deficient embryos

We next analyzed rescue of PGCs by overexpression of an anti-apoptotic Bcl2 protein family member (Bcl2l; Chen et al., 2001;Her et al., 2006), to confirm further that PGCs are eliminated by apoptosis. Co-injection of synthetic mRNA encoding zebrafish Bcl2l significantly rescued mean total PGC numbers in *igf1rb*-MO-injected embryos, when examined at the prim-5 stage (Table 1), supporting our interpretation that the PGC deficiency in these embryos is due to the elimination of PGCs by apoptosis. The mean number of PGCs in embryos co-injected with *bcl2l* mRNA and *igf1rb*-MO was statistically similar to the mean number of PGCs in both control MO-injected and wild-type embryos, and significantly greater than the mean number of PGCs in embryos after injection with *igf1rb*-MO, *igf1ra*-MO + *igf1rb*-MO, and overexpression of dnIGF1R:GFP. These data confirm a statistically significant rescue of PGC numbers by Bcl2l overexpression.

To determine whether Bcl2l overexpression rescues ectopic PGCs specifically, we quantified the number of ectopic PGCs in both 20S and prim-5 embryos, with or without Bcl2l overexpression. As reported above (Fig. 3D), a significantly greater mean number of ectopic PGCs was detected in 20S *igf1rb*-MO-injected embryos (6.2 ± 0.8, n = 11), relative to 20S control-MO-injected embryos (0.6 ± 0.3, n = 18). Similarly, we detected a significantly increased mean number of ectopic PGCs in 20S *igf1rb*-MO/*bcl2l* mRNA co-injected embryos (6.7 ± 0.9; n = 12; ANOVA, $P < 0.001$), relative to control-MO-injected embryos (Fig. 3D). Injection of *bcl2l* mRNA alone had no effects on the mean number of ectopic PGCs in 20S embryos (1.0 ± 0.3; n = 15; ANOVA, $P > 0.05$) relative to control MO-injected embryos (Fig. 3D) indicating that Bcl2l overexpression does not affect PGC migration.

At the prim-5 stage, we detected significantly fewer ectopic PGCs in *igf1rb*-MO-injected embryos (2.1 ± 0.38; n = 10; ANOVA, $P < 0.001$), relative to 20S *igf1rb*-MO-injected embryos (Fig. 3D), supporting our hypothesis that the decline in total PGC number in *igf1rb*-MO-injected embryos is due to the elimination of ectopic PGCs by apoptosis. Accordingly, we detected a significantly greater mean number of ectopic PGCs in prim-5 *igf1rb*-MO/*bcl2l* mRNA co-injected embryos (4.7 ± 0.6; n = 10), relative to both prim-5 *igf1rb*-MO-injected (ANOVA, $P < 0.05$) and prim-5 control MO-injected embryos (ANOVA, $P < 0.001$). These data confirm that overexpression of Bcl2l promotes the survival of ectopic PGCs that would otherwise be eliminated by apoptosis. Combined TUNEL analysis and Vasa immunostaining of *igf1rb*-MO/*bcl2l* mRNA co-injected embryos confirmed a reduction in the overall number of TUNEL-positive cells, and an absence of DNA fragmentation in ectopic PGCs (Fig. 6).

Increased expression of *cxcr12a* in IGF1Rb-deficient embryos

In efforts to elucidate the mechanism by which loss of IGF1Rb leads to PGC mismigration, we sought to determine whether knockdown of IGF1Rb leads to detectable changes in the expression of genes encoding a chemokine ligand/receptor pair (Cxcl12a/Cxcr4b) that plays a critical role in guiding germ cell migration in vertebrates (Knaut et al., 2003). Embryos in all treatment groups were examined during the PGC migratory period (20S). Targeted knockdown of IGF1Rb had no detectable effect on *cxcr4b* mRNA levels (111.3 ± 19.5, n=4, P=0.48; Fig.

7) as determined by qRT-PCR analysis, but resulted in a significant increase in *cxcl12a* mRNA levels (152.3 ± 13.9; n=4, $P<0.01$). Conversely, knockdown of IGF1Ra had no detectable effect on relative *cxcl12a* mRNA levels (84.8 ± 5.0%; n=4, $P=0.11$), though did lead to a detectable reduction in *cxcr4b* mRNA levels (34.8 ± 2.6%; n=4, $P=0.001$).

Discussion

In this study we employed an *in vivo* loss-of-function approach to investigate the role of IGF signaling in primordial germ cell development, using the embryonic zebrafish as a model. Our findings suggest novel functions for IGF signaling in PGC development, and provide further support for recent suggestions of divergent evolution of IGF1R signaling following duplication of the ancestral *igf1r* gene (Schlueter et al., 2006). Specifically, we have shown that suppression of IGF signaling through one (IGF1Rb) of the two duplicate IGF receptors in zebrafish results in defective migration and apoptosis of PGCs, resulting in fewer numbers of PGCs colonizing the genital ridges. Suppressing expression of the other receptor duplicate (IGF1Ra) results in widespread somatic cell death, but has no observable effects on PGC development.

While IGF signaling is a known survival factor for multiple cell types, we believe the PGC apoptosis observed after suppression of IGF1Rb is a secondary consequence of misdirected PGC migration. This interpretation is based upon the observations that PGC apoptosis does not occur until the terminal stages of PGC migration, and among this cell population, appeared to be restricted to ectopic (mismigrated) cells. Furthermore in 18S embryos, significant somatic cell apoptosis could be observed after suppression of both IGF1Ra and IGF1Rb, whereas normal numbers of PGCs were observed in embryos of both groups at this stage. This suggests that in IGF1Rb-deficient embryos, normal numbers of PGCs are initially specified but subsequently exhibit a differential susceptibility to apoptosis, corresponding to their stage of development and/or position in the embryo. Only upon mismigration were PGCs observed to degenerate, which is not wholly unexpected; the elimination of mismigrated PGCs has been well-documented in other vertebrates (Anderson et al., 1999;Molyneaux and Wylie, 2004), possibly as a mechanism to avert the development of extragonadal germ cell tumors (Donovan and de Miguel, 2003).

There are three developmental defects that could lead to PGC migratory defects, and one or more of these could explain the mismigration phenotype observed in IGF1Rb-deficient embryos: (1) An intrinsic loss of cell motility, leaving PGCs unable to respond to extrinsic migratory cues; (2) an intrinsic failure to correctly interpret extrinsic cues; and (3) defective development or death of IGF-dependent somatic cells that provide guidance cues to migratory PGCs. We believe the first possibility can be ruled out, based upon the random distribution of ectopic PGCs in IGF1Rb-deficient embryos. For example, we detected ectopic PGCs in cranial regions of some IGF1Rb-deficient embryos, but in caudal or ventral regions of others, none of which corresponds to the normal pathways followed by migratory PGCs (Weidinger et al., 1999;Weidinger et al., 2002). This random distribution of PGCs in IGF1Rb-deficient embryos suggests that these cells retain intrinsic migratory behaviors, but fail to either receive or correctly interpret extrinsic migratory cues. While this may rule out intrinsic cell-motility defects, it remains unclear whether PGCs cell-autonomously require IGF signaling through IGF1Rb to correctly interpret extrinsic directional cues, or whether the observed defects stem from defective development of IGF-dependent somatic cells upon which PGCs rely for directional cues. Resolving this issue is complicated by the spatially and temporally ubiquitous patterns of expression of both IGF1Ra and IGF1Rb during zebrafish embryogenesis (Maures et al., 2002). On-going studies seek to develop methods to cell-autonomously suppress IGF1R function specifically within PGCs, while maintaining normal receptor function in somatic cells.

Our findings do contribute to recent progress in identifying signaling pathways regulating vertebrate PGC development. For example, signaling by the chemokine CXCL12 through the G-protein-coupled receptor CXCR4 is a highly conserved mechanism regulating directional migration of PGCs in mice, chick, and zebrafish (Ara et al., 2003;Doitsidou et al., 2002;Knaut et al., 2003;Molyneaux et al., 2003;Schier, 2003;Stebler et al., 2004), while stem cell factor, leukemia-inhibitory factor, and IGF-1 have all been shown to function as survival factors for PGCs *in vitro* (Morita et al., 1999;Park and Han, 2000;Pesce et al., 1993). Our study provides novel *in vivo* evidence that IGF signaling is also required to promote correct PGC migration, in a manner that is distinct from its documented effects on PGC survival, and more general effects on cell proliferation. Our findings also provide a potential explanation for the germ cell-deficient phenotype observed in mice after targeted ablation of IGF-1 (Baker et al., 1996).

The mechanism by which IGF promotes germ cell migration remains unclear, and warrants further investigation. A potential mechanism was suggested in a recent study with migratory breast cancer cells, which provided evidence for intracellular cross-talk between IGF1R and CXCR4 (Akekawatchai et al., 2005). Although demonstrating cross-talk at the protein level is a difficult prospect *in vivo*, we chose to examine mRNA levels of *cxcl12a* and *cxcr4b* after targeted knockdown of IGF1Ra and IGF1Rb. In IGF1Rb-deficient embryos, we failed to detect any significant changes in *cxr4b* mRNA levels, but observed a significant increase in *cxcl12a* mRNA levels. Conversely in IGF1Ra-deficient embryos, there were no significant changes in *cxcl12a* mRNA levels, though we did observe a significant reduction in *cxcr4b* mRNA levels. The significance of these changes in gene expression are not presently clear; while disruptions to endogenous *cxcl12a* expression has been shown to result in PGC mismigration (Knaut et al., 2003), the observed increase in *cxcl12a* expression in IGF1Rb-deficient embryos could alternatively be explained as a compensatory response by the embryo to mismigration and/or elimination of PGCs. Likewise, the decline in *cxcr4b* expression in IGF1Ra-deficient embryos could be a secondary effect associated with the demise of *cxcr4b*-expressing somatic cells, as cell death was particularly intense in IGF1Ra-deficient embryos (Chong et al., 2001).

Overall, our data are consistent with numerous published studies implicating a central role for phosphatidylinositol 3-kinase (PI3K)-Akt signaling during cell migration. Both IGF1R and CXCR4 are known activators of the PI3K-Akt pathway, and in zebrafish embryos, pharmacological suppression of PI3K signaling results in PGC migratory defects (Dumstrei et al., 2004). The specific signal transduction pathways utilized by zebrafish IGF1Ra and IGF1Rb have not been investigated in detail, although it was shown that MO-mediated suppression of both IGF1Ra and IGF1Rb in zebrafish embryos results in reduced Akt phosphorylation (using whole embryos lysates) (Schlueter et al., 2006). Thus, while both receptors appear to activate the PI3K-Akt pathway in zebrafish embryos, cell-specific responses have yet to be investigated in detail. It is possible that functional evolution of the receptor duplicates has occurred at the level of intracellular signal transduction in a cell-specific manner.

The sequence identity between the zebrafish receptor duplicates is relatively high (~70%), and both are highly similar to IGF1R orthologs in other vertebrates (Wood et al., 2005a). However, differences within selected regions of the mature zebrafish IGF1R peptides could account for the observed functional differences. For example, the IGF1Ra peptide contains a contiguous sequence of 15 amino acids in the cytoplasmic domain (directly adjacent to the tyrosine kinase domain) that is not present in IGF1Rb (Maures et al., 2002). While this region does not encode any known functional domains, its presence could presumably influence the activation of downstream signaling pathways, leading to differential cellular responses.

In summary we have shown that IGF signaling, through one of two duplicate IGF receptors, is required for successful migration of PGCs to the genital ridges during embryogenesis. As a

consequence of suppressed IGF1Rb signaling, misdirected PGCs are eliminated by apoptosis, resulting in reduced numbers of PGCs colonizing the genital ridges. These findings provide new information about the embryonic functions of IGF signaling in vertebrates, and contribute to a growing body of evidence of the importance of insulin-like signaling for germline development in metazoans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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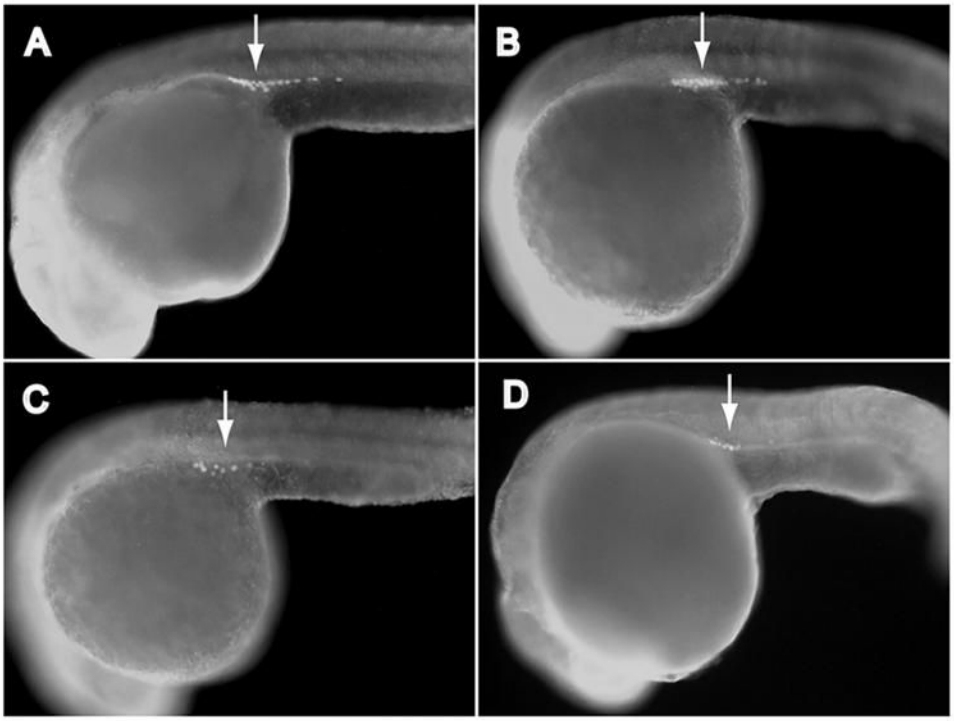

Figure 1.

Igf1rb is required to establish normal primordial germ cell (PGC) numbers at the prim-5 stage. Immunohistochemical identification of PGCs (Vasa-positive cells, arrows) in prim-5 stage zebrafish embryos, after ablation of Type-1 IGF receptors (*igf1ra, igf1rb*): (A) control (non-targeting) morpholinos; (B) *igf1ra*-MO; (C) *igf1rb*-MO; (D) *igf1ra*-MO + *igf1rb*-MO. Magnification 200x. Mean PGC numbers of treatment groups are summarized in Table 1.

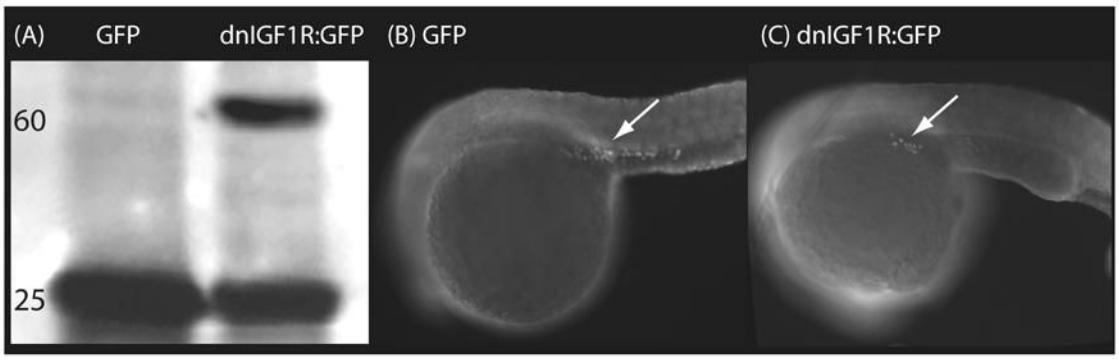

Figure 2.

(A) Western immunoblot analysis of zebrafish lysates demonstrating overexpression of dominant-negative IGF1R:GFP fusion protein (dnIGF1R:GFP). First lane, lysates from zebrafish embryos injected with synthetic mRNA encoding GFP only; second lane, lysates from zebrafish embryos injected with synthetic mRNA encoding dnIGF1R:GFP. Upper band in dnIGF1R:GFP corresponds to the predicted molecular weight (~60 kDa) of dnIGF1R:GFP after denaturation in reducing conditions. (B) Vasa immunostaining of PGCs (arrows) in zebrafish embryo overexpressing GFP only (control); (C) Vasa immunostaining of PGCs in zebrafish embryo overexpressing dnIGF1R:GFP fusion protein. Magnification (B–C), 200x. Mean PGC numbers in B and C are summarized in Table 1.

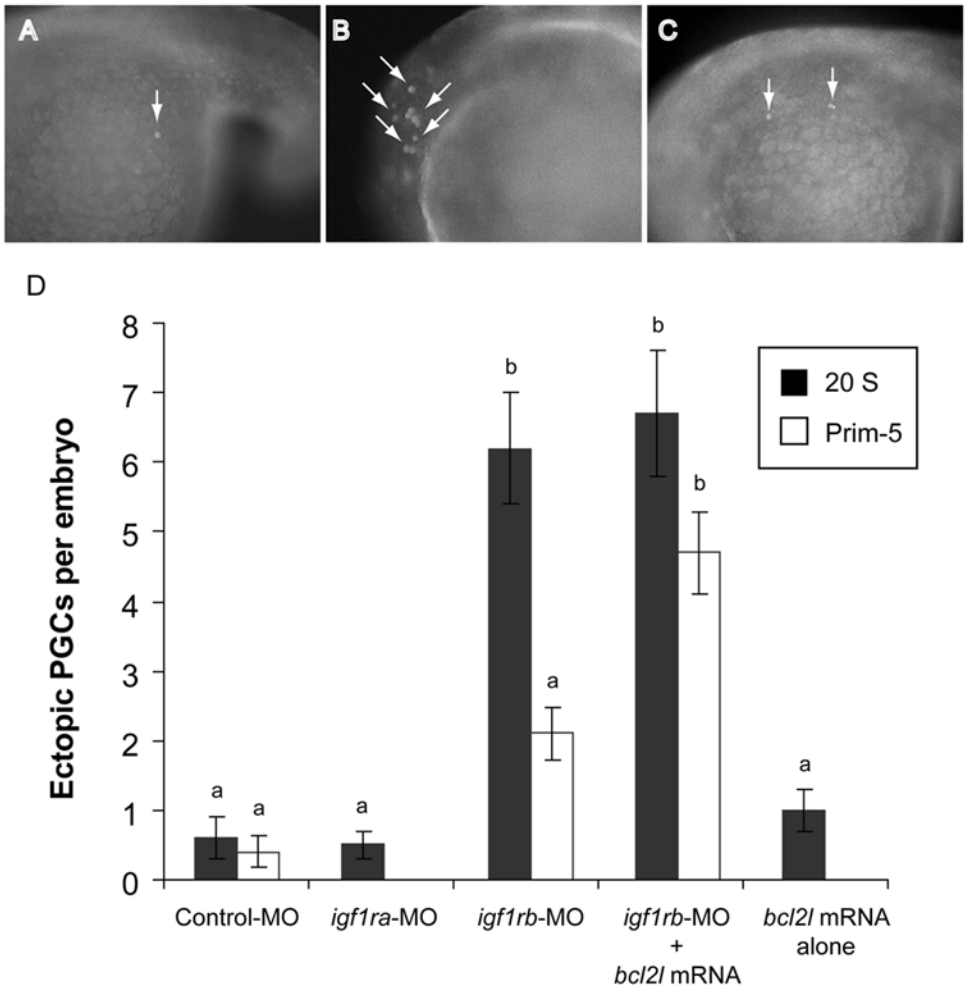

Figure 3.

IGF1Rb is required for normal PGC migration. Ectopic PGCs (arrows) were detected rarely in Control-MO-injected embryos (A), whereas they were frequently observed, in both somatic tissues (B) and throughout the yolk cell (C) of *igf1rb*-MO-injected embryos. (D) Mean numbers of ectopic PGCs in zebrafish embryos after morpholino injections, and/or over-expression of an anti-apoptotic Bcl2-like protein (Bcl2l). Data represent means ± SEM, with sample size indicated in parentheses. Superscript letters denote significant differences between groups (ANOVA, Tukey's, $P < 0.05$). Magnification (A–C), 200x.

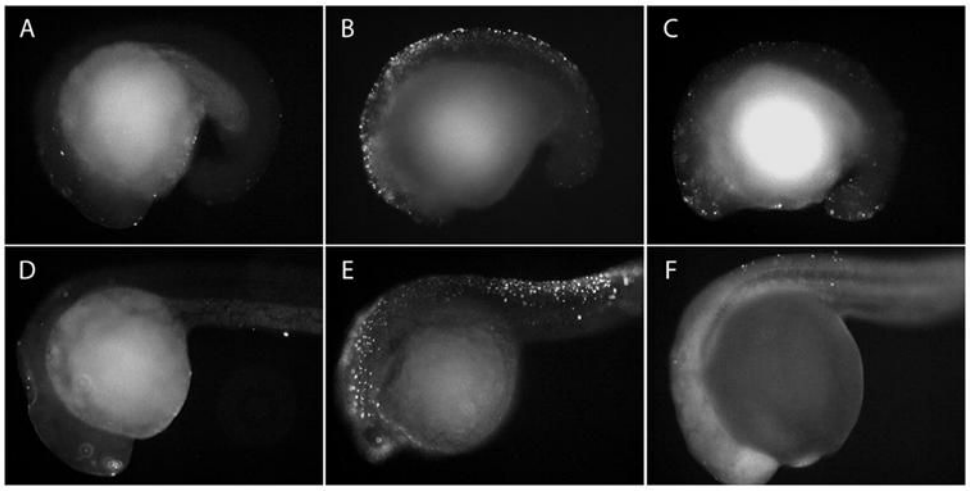

Figure 4.

Targeted knockdown of either IGF1R subtype results in increased apoptosis. *In situ* cell death (TUNEL) analyses of zebrafish embryos after injection with (A, D) control morpholinos; (B, E) IGF1Ra antisense morpholinos (*igf1ra*-MO); (C, F) IGF1Rb antisense morpholinos (*igf1rb*-MO). Embryos in upper panels are 18-somite stage embryos; embryos in lower panels are prim-5 stage embryos. Relative to control-MO-injected embryos (A, D), TUNEL-positive cells are more abundant in both *igf1ra*-MO- and *igf1rb*-MO-injected embryos, at both stages of development. Magnification 100x.

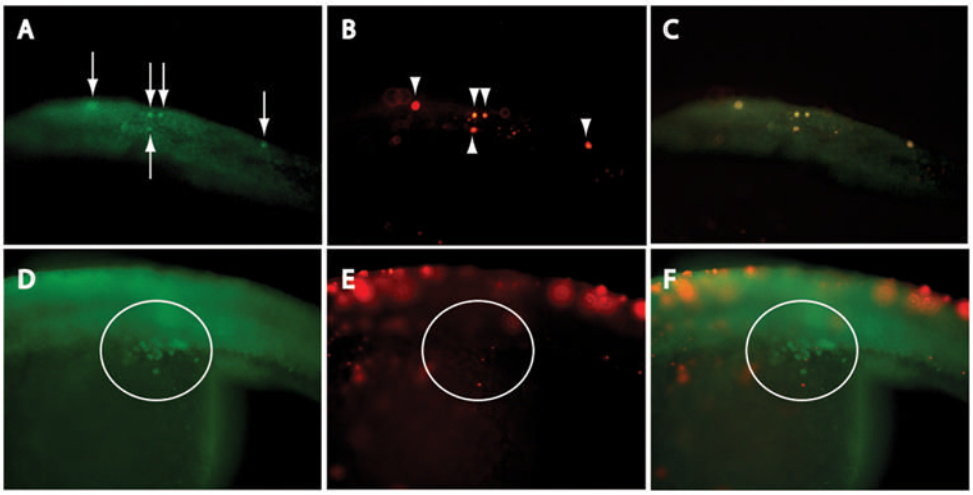

Figure 5.
Ectopic PGCs undergo apoptosis. (A) Vasa immunostaining, and (B) TUNEL analysis of ectopic PGCs in dorsal trunk of *igf1rb*-MO-injected embryo; (C; merged image) colocalization of Vasa-positive (arrows) and TUNEL-positive (arrowheads) cells confirms DNA fragmentation in ectopic PGCs. (D) Vasa-positive, but (E) TUNEL-negative PGCs in genital ridge (circled) of *igf1rb*-MO-injected embryo; images merged in (F). An absence of DNA fragmentation in genital ridge PGCs confirms survival of the subpopulation of PGCs that successfully migrate to the genital ridge. Anterior is to the left in all images; magnification 200x.

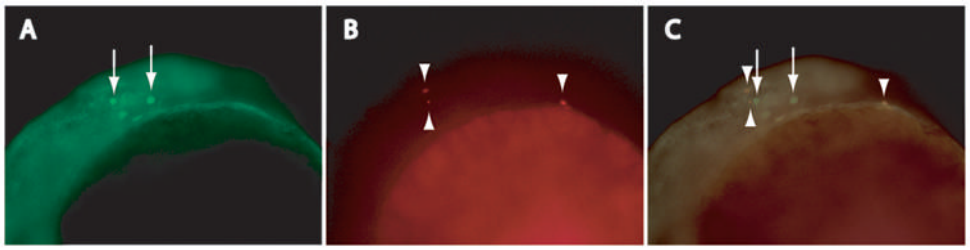

Figure 6.

Ectopic PGCs are rescued from apoptosis by overexpression of Bcl2l. (A) Vasa positive cells (arrows), and (B) rare TUNEL-positive cells (arrowheads) in dorsal trunk of *igf1rb*-MO/*bcl2l* mRNA co-injected embryo; (C; merged image) lack of colocalized Vasa and TUNEL signals (arrows, arrowheads) confirms absence of DNA fragmentation in ectopic PGCs (compare with Fig. 6A–C). Magnification 200x.

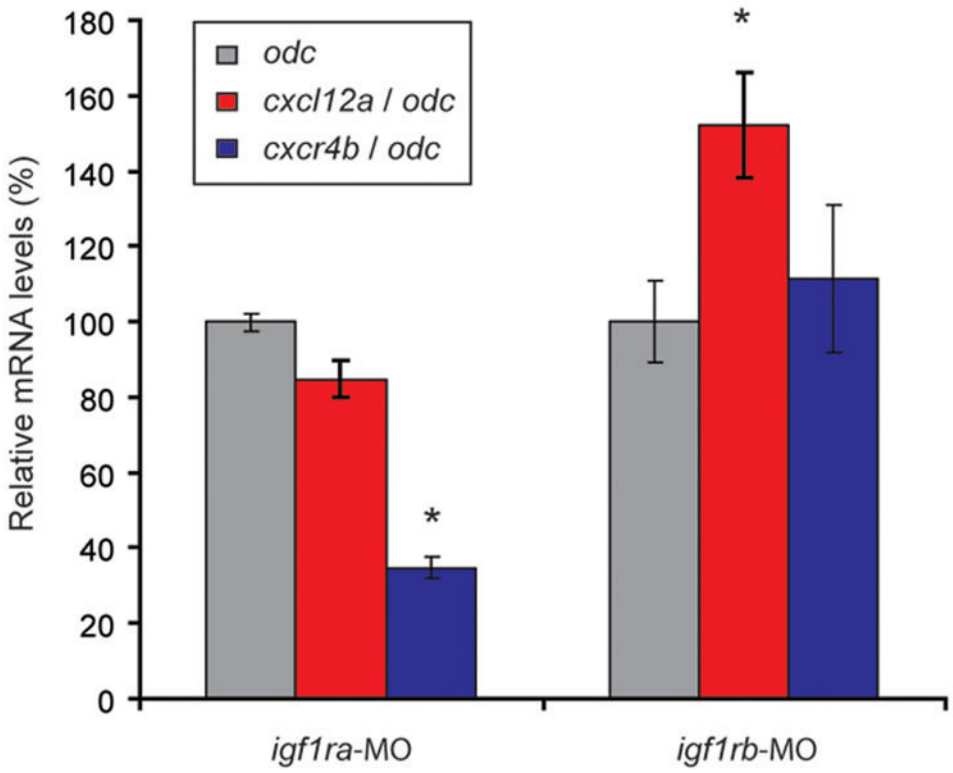

Figure 7.
Relative mRNA levels of *cxcl12a* and *cxcr4b* in zebrafish embryos injected with morpholino oligonucleotides targeting IGF1Ra (*igf1ra*-MO) and IGF1Rb (*igf1rb*-MO). Values are normalized relative to *odc* mRNA levels (100%), and presented as means ± SEM (n=4). *denotes statistical difference from Control-MO-injected embryos (paired Students *t*-test, $P < 0.05$).

Table 1

Mean number of PGCs in zebrafish embryos after targeted knockdown of Type-1 IGF receptors using morpholino oligonucleotides (MO), or a dominant-negative IGF1R:GFP fusion protein (dnIGF1R:GFP). Sample size indicated in parentheses. N.D., not determined.

	Stage of Development	
Treatment Group	**Prim-5**	**18-somite (18S)**
Wild-type (non-injected)	32.6 ± 0.9 (10)[a]	N.D.
Control MO-a/MO-b	33.9 ± 1.1 (21)[a]	34.6 ± 3.4 (10)
igf1ra-MO	33.1 ± 1.8 (9)[a]	30.6 ± 0.5 (8)
igf1rb-MO	18.2 ± 2.2 (18)[b]	32.9 ± 3.2 (10)
igf1ra-MO + *igf1rb*-MO	19.3 ± 2.8 (8)[b]	32.7 ± 1.82 (15)
igf1rb-MO + *bcl2l*[*]	28.8 ± 1.5 (20)[a]	N.D.
dnIGF1R:GFP	16.0 ± 3.1 (9)[b]	34.4 ± 2.6 (8)
GFP	35.9 ± 1.7 (20)[a]	N.D.

Superscript letters denote significant differences among same-stage embryos (ANOVA, Tukey's, $P < 0.05$). No significant differences were detected among means of 18S embryo groups.

[*] mRNA encoding zebrafish Bcl2l.