Coordinate control of cell cycle regulatory genes in zebrafish development tested by cyclin D1 knockdown with morpholino phosphorodiamidates and hydroxyprolyl-phosphono peptide nucleic acids

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ABSTRACT

During early zebrafish (Danio rerio) development zygotic transcription does not begin until the mid-blastula transition (MBT) ~3 h after fertilization. MBT demarcates transition from synchronous short cell cycles of S and M phases exclusively to full cycles encompassing G1 and G2 phases. Transcriptional profiling and RT–PCR analyses during these phases enabled us to determine that this shift corresponds to decreased transcript levels of S/M phase cell cycle control genes (e.g. ccna2, ccnb1, ccnb2 and ccne) and increased transcript levels of ccnd1, encoding cyclin D1, and orthologs of p21 (p21-like) and retinoblastoma (Rb-like). To investigate the regulation of this process further, the translation of ccnd1 mRNA, a G1/S checkpoint control element, was impaired by microinjection of ccnd1-specific morpholino phosphorodiamidate (MO) 20mer or hydroxyprolyl-phosphono peptide nucleic acid (HypNA-pPNA) 16mer antisense oligonucleotides. The resulting downregulation of cyclin D1 protein resulted in microophthalmia and microcephaly, but not lethality. The phenotypes were not seen with 3-mismatch MO 20mers or 1-mismatch HypNA-pPNA 16mers, and were rescued by an exogenous ccnd1 mRNA construct with five mismatches. Collectively, these results indicate that transcription of key molecular determinants of asynchronous cell cycle control in zebrafish embryos commences at MBT and that the reduction of cyclin D1 expression compromises zebrafish eye and head development.

INTRODUCTION

Control of cell cycle progression is central to maintaining homeostasis in multicellular organisms. Loss of cell cycle control may lead to imbalances in proliferation and cell death that contribute to various disease states including neoplasia. Current knowledge of cell cycle regulation has largely been instructed by studies on cells and tissues in the adult organism and the extensive use of knockout mice (1).
These studies have illuminated the complex interplay of cyclins, cyclin-dependent kinases (cdks) and regulators thereof, which control progression through the G1, S, G2 and M phases of the cell cycle. Furthermore, they highlighted the existence of cell cycle checkpoints, i.e. molecular switches controlling cell cycle progression. In malignant tumor cells, molecular determinants of checkpoint control are frequently inactivated permitting unchecked cell cycle progression and relaxed genome surveillance (2). Cell cycle checkpoints are also absent during very early stages of amphibian development as initially described in fertilized Xenopus laevis eggs (3). In Xenopus, synchronous, rapid cycling and the absence of the G1 and G2 phases of the cell cycle characterize the first rounds of DNA replication and cell division. The molecular underpinnings of this phenomenon are poorly understood.

We used the zebrafish (Danio rerio) as a simple model to study cell cycle regulation during vertebrate embryogenesis. The zebrafish has in recent years evolved as a novel and facile in vivo model to study human disease since many key genes are highly conserved between the two vertebrate species; these include cyclins, cdks and inhibitors of cdks. Importantly, zebrafish and Xenopus embryos share the absence of G1 and G2 cell cycle phases during very early development, followed by the establishment of asynchronous cell cycles within a short time frame (~3 h) after fertilization, coincident with the mid-blastula transition (MBT) (4).

The onset of cell cycle regulation in zebrafish embryos occurs when zygotic transcription commences, raising the issue whether and how these two phenomena are linked. Here, we describe global transcription profiles in zebrafish embryos at distinct stages of embryonal development before and after establishment of cell cycle checkpoints. We observed that key cell cycle regulators involved in S to M phase transition, specifically ccnb1, encoding cyclin B1, ccnb2, encoding cyclin B2, and ccne, encoding cyclin E, were expressed at high levels in pre-MBT embryos followed by a rapid decline in post-MBT, congruent with the expected pattern of these cell cycle regulators during early zebrafish development. These observations led us to evaluate the expression profiles of other cell cycle regulatory genes, including those involved in G1–S transition specifically ccnd1, encoding cyclin D1, and Rb, with initial low expression levels, followed by increased expression correlation with the onset of cell cycle asynchrony. Of these three, ccnd1 mRNA expression was most markedly upregulated after MBT, in accordance with an earlier study (5). This prompted us to investigate the consequences of interfering with ccnd1/cyclin D1 expression in developing zebrafish embryos.

Morpholino phosphorodiicamide (MO) oligonucleotides are used frequently for sequence-based knockdown studies in zebrafish (6). Recently, we and others have described the hybridization strength and activity of antisense oligonucleotides with alternating trans-4-hydroxy-L-proline peptide nucleic acid (PNA) and phosphono PNA residues (7,8). The hydroxyproplyl-phosphono PNA (HypNA-pPNA) oligomers are more specific than conventional MOs (9,10). The highly soluble HypNA-pPNA analog exhibited higher affinity towards DNA and RNA, and more stringent mismatch discrimination than MOs. Hence, we compared both MOs and HypNA-pPNAs for their ability to knock down ccnd1 mRNA translation to cyclin D1 protein based on the published ccnd1 cDNA sequence (4). We report that the reduction of cyclin D1 expression was associated with impaired development of the eye and the head region in zebrafish embryos, the two anatomical sites with the highest cyclin D1 expression levels during early zebrafish development.

MATERIALS AND METHODS

Embryo harvesting and maintenance

Zebrafish husbandry, embryo collection, decolorization and embryo maintenance were performed according to the Standard Operating Procedures as described elsewhere (11) and with approval by the Institutional Animal Care and Use Committee at Thomas Jefferson University. Zebrafish were maintained in the Kimmel Cancer Center Zebrafish Facility at 28.5°C on a 14 h light/10 h dark cycle. Selected embryos with >24 h post-fertilization (hpf) were placed in embryo medium with 0.2 mM 1-phenyl-2-thio urea (Sigma, St Louis, MO) to prevent pigment formation.

Transcription profiles

Embryos were obtained from natural crosses of wild-type zebrafish at various times during development and staged as described elsewhere (12). Total RNA was isolated from groups of 100 staged embryos corresponding to 1.5, 3, 6 and 24 h post-fertilization (hpf) at 28.5°C using TriReagent (Sigma, St Louis, MO) according to the manufacturer’s protocol. Gene expression in the zebrafish embryos was determined using biotin-labeled and in vitro-transcribed anti-sense RNA (aRNA) generated from the total RNA template. The labeled aRNA was hybridized to a microarray chip with 17 000 65mer sense oligonucleotides representing 16 399 zebrafish genes (Compugen/Sigma-Genosys, Kimmel Cancer Center Microarray Facility), with three replicate chips for each developmental time point. Each chip was scanned and quantified using a ScanArray Express laser scanner (Packard Bioscience). Raw gene expression data were normalized to housekeeping gene controls on each chip. The data were exported to MS Excel for further analysis. After local background correction, each chip was normalized globally by dividing each array by its respective median signal. The different developmental time points were compared with Student’s t-test. Significantly different genes were identified at \( P < 0.01 \) (3\( \sigma \)) and ratio >10 cutoffs. These genes were grouped using the Cluster method of Eisen et al. (13), and clusters were visualized by using TreeView. For hierarchical clustering, data were log transformed, median centered and normalized. Clusters were identified by average linkage.

RT–PCR analysis

The expression profiles of selected cell cycle control transcripts were confirmed by RT–PCR analysis of the original RNA samples using the following target-specific primer sets: cyclin A2 (ccna2, GenBank accession no. AF234784) forward 5'-GCCACGGGTATAAAAGCAAC-3' and reverse 5'-GGCCTCTCTCCAAACTCC-3'; cyclin B1 (ccnb1, GenBank accession no. AB040435) forward
5'-GAGTCACACGAAATAAAACCAC-3' and reverse 5'-AGG-AAGCTTGACAGACACAC-3'; cyclin B2 (ccnb2, GenBank accession no. AW242010) forward 5'-AGGTTGAGTTGGACGAACAC-5' and reverse 5'-GAAAGAGGCTGTGTC-3'; cyclin D1 (ccnd1, GenBank accession no. X87581) forward 5'-ACACGACCTGTTGATAG-3' and reverse 5'-GGCCGATCCCCACTTCA-3'; cyclin E (ccne, GenBank accession no. X83594) forward 5'-GGAC-TGCAGAAGACATC-3' and reverse 5'-CGGTICCTGCA-TCTCATCAG-3'; p21 ortholog (p21-like, GenBank accession no. B887574) forward 5'-CCGTAGACCGAG-GAGC-3' and reverse 5'-GTCTGGCCACTTCTTTCTCTC-3'; Rb ortholog (Rb-like 1, GenBank accession no. AW281574) forward 5'-CTTCAGGCCACCAAAGTGT-3' and reverse 5'-GCAGCTTCTCTCTACTG-3'; and β-actin (GenBank accession no. AP057040) forward 5'-GTCTGGCCACTTCTTTCTCTC-3' and reverse 5'-GGTGTTGAAGGTCTCGA-3'.

The trimer complementary to the AUG initiation codon is underlined and italicized, and the mismatched nucleotides in the control sequences are shown in lower case.

3.5% octyl-glucopyranoside, pH 6.4. Samples were diluted 1:1 (v/v) in 2× Tris–glycine SDS sample buffer (Invitrogen, Carlsbad, CA) and boiled for 5 min. The total lysates were resolved by 12% SDS–PAGE and transferred onto nitrocellulose membranes. For the detection of cyclin D1, the membranes were blocked with 1% powdered milk, then probed with a 1:1000 dilution of polyclonal rabbit anti-cyclin D1 antibodies (Neomarkers, Fremont, CA).

<table>
<thead>
<tr>
<th>type</th>
<th>antisense</th>
<th>mismatch</th>
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<tbody>
<tr>
<td>HypNA-pPNA</td>
<td>N-GTGCTCCAATCTTCA-C</td>
<td>N-GTGCTCCAATCTTCA-C</td>
</tr>
<tr>
<td></td>
<td>Tm: 74.1 ± 0.2°C</td>
<td>Tm: 68.4 ± 0.1°C</td>
</tr>
<tr>
<td>MO</td>
<td>6'-ACTGGTGCTTCAATCTTCA-3'</td>
<td>6'-ACTGGTGCTTCAATCTTCA-3'</td>
</tr>
<tr>
<td></td>
<td>Tm: 78.9 ± 0.3°C</td>
<td>Tm: 52.9 ± 0.1°C</td>
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Figure 1. ccnd1 antisense sequences. The ccnd1-specific MO and HypNA-pPNA antisense and corresponding mismatch control sequences are displayed with their respective Tm's. The trimer complementary to the AUG initiation codon is underlined and italicized, and the mismatched nucleotides in the control sequences are shown in lower case.
The bound antibodies were visualized using a horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) and the SuperSignal West Femto Maximum Sensitivity substrate (Pierce, Rockford, IL). Samples were analyzed similarly for the expression of GAPDH as a control for sample loading using a mouse anti-rabbit polyclonal antibody (BDI, Flanders, NJ).

Zebrafish embryo retinal histology

Control uninjected, ccnd1 MO-injected and ccnd1 HypNA-pPNA-injected 5-day-old larvae were fixed, embedded in agarose arrays, sectioned (4 μm) and stained as described elsewhere (15). Slide images were acquired using a Qimaging Retiga EXi and a CRI RGB tunable filter on a Zeiss Axiophot using a ×20 0.75NA Plan-Apo objective, and processed using Adobe Photoshop and Illustrator CS.

RESULTS

Transcription profile analysis of zebrafish gene expression during embryogenesis

The first 10 embryonic cell cycles in zebrafish proceed in a synchronous fashion and in the absence of RNA synthesis. During this early phase, cells rapidly cycle through DNA synthesis (S phase) and mitosis (M phase) without transition through either G₁ or G₂ (12). After 10 divisions and ~3 h post-fertilization (hpf) embryos enter MBT characterized by the start of zygotic transcription and the gradual lengthening of the cell cycle. The coincident start of zygotic transcription and onset of G₁ phase at MBT raises the question of which early zygotic transcripts direct the establishment of cell cycle control post-MBT (5). To gain a representative view of gene expression patterns during this important transition, we analyzed transcription profiles before and after MBT. To this end, we used a 17 000-oligonucleotide microarray platform containing most of the cell cycle regulatory genes known in zebrafish to date. The analysis was performed in triplicates on RNA preparations collected in three different experiments performed independently of each other. Hierarchical cluster analysis of the results of these experiments revealed massive changes in gene expression reflecting the shift from maternal transcripts pre-MBT to zygotic transcription post-MBT.

Normalized mean mRNA levels from each time point investigated by microarray analysis were directly compared with the expression level at all other time points for a total of six possible comparisons. Level of significance is based on P < 0.01 by Student's t-test. Expressed genes with three or more pairwise comparisons demonstrating the indicated fold difference (>10, >5 or >3).

Table 1. Change in global gene expression patterns in early zebrafish development

<table>
<thead>
<tr>
<th>Change in mRNA levels</th>
<th>Significant difference by pairwise comparison&lt;sup&gt;a&lt;/sup&gt;</th>
<th>&gt;3 Comparisons&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Single comparison&lt;sup&gt;c&lt;/sup&gt;</th>
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<tr>
<td>&gt;10-Fold</td>
<td>293&lt;sup&gt;d&lt;/sup&gt;</td>
<td>945</td>
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<tr>
<td>&gt;5-Fold</td>
<td>1024</td>
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<tr>
<td>&gt;3-Fold</td>
<td>2464</td>
<td>5208</td>
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</table>

<sup>a</sup>Normalized mean mRNA levels from each time point investigated by microarray analysis were directly compared with the expression level at all other time points.

<sup>b</sup>Expressed genes with three or more pairwise comparisons demonstrating the indicated fold difference (>10, >5 or >3).

<sup>c</sup>Expressed genes with only one pairwise comparison demonstrating the indicated fold difference (>10, >5 or >3).

<sup>d</sup>This clustered dataset is shown in Figure 3.

Stage-dependent expression of cell cycle regulatory genes in zebrafish development

Next, we focused our analysis of the microarray results to transcripts encoding cell cycle regulators and genes involved in checkpoint control. This analysis revealed that transcripts for S/M phase cyclin genes, ccna2, ccnb1, ccnb2 and cccn, were expressed at high levels of pre-MBT and declined thereafter (Figure 4). In contrast, genes of major importance to G₁/S phase transition, including orthologs of the Rb, p21 and ccnd1, were expressed at very low levels initially and increased markedly between 3 and 6 hpf (Figure 4), with the greatest increase...
We confirmed the expression patterns of these genes by RT–PCR analysis. Collectively, these results underscore that several key molecular determinants of G1/S transition, including cyclin D1, are expressed as a result of zygotic transcription consistent with the delayed establishment of cell cycle checkpoints after MBT.

MO and HypNA-pPNA-mediated knockdown of cyclin D1 in zebrafish embryos

Whole mount in situ hybridization of ccnd1 mRNA demonstrated ubiquitous expression during gastrulation, and was prominently expressed in the retina and brain from the 18-somite stage to 24 hpf (16). After 48 hpf, expression remained strong in the retina and tectum of the brain (18).

The distinctive expression pattern of ccnd1 mRNA in zebrafish embryos prompted us to investigate the functional contribution of cyclin D1 protein expression to embryonal development in zebrafish larvae. To this end, we used antisense oligonucleotides complementary to the ccnd1 mRNA encoding cyclin D1 in order to block translation of the targeted message; thereby, reducing the level of the target protein. To test the efficacy of MOs and HypNA-pPNAs in reducing cyclin D1 expression in zebrafish embryos, target sequences were selected as shown in Figure 1. The MO 20mer was only 5°C more stable than the HypNA-pPNA 16mer. A single mismatch in the latter lowered Tm by 6°C. In the MO 20mer, three mismatches lowered Tm by 26°C.

The effects of MO and HypNA-pPNA injections on cyclin D1 mRNA expression were assessed by western blot analyses (Figure 5) showing that both treatments were equally effective in downregulating target protein expression. This analysis was performed at 24 hpf, at which time substantial expression of cyclin D1 was expected based on the transcription profiles shown in Figure 4.

Figure 4. Cell cycle regulatory gene expression during zebrafish embryogenesis. Expression profiles of selected genes involved in cell cycle regulation (ccna2, ccnb1, ccnb2, ccne, ccnd1, p21-like and Rb-like 1) identified by zebrafish-specific microarray analysis of embryos at 1.5, 3, 6 and 24 h post-fertilization (hpf) are clustered according to similar time-dependent expression patterns with upregulated expression in red, downregulated genes in green and unchanged expression in black. The RT–PCR analyses of the corresponding mRNA levels are shown at right to test the results of the microarray analysis. Amplification of β-actin transcripts was performed as a loading control for each RT–PCR experiment with representative expression levels shown.

Figure 5. Western blot analysis of protein expression in antisense-treated zebrafish embryos. Western blot analysis of cyclin D1 protein in zebrafish embryo total protein extracts prepared at 24 hpf as a function of vehicle or antisense microinjection shortly after fertilization: Lane 1, Phenol red control embryo extract; lane 2, MO-treated embryo extract; lane 3, HypNA-pPNA-treated embryo extract. GAPDH expression was determined as a loading control for each sample.
(Figure 6). Cyclin D1 knockdown by MO and HypNA-pPNA injection was associated with comparable phenotypic changes confined to the head and eye regions. Knockdown of ccnd1 did not markedly affect body size, but did decrease eye and head size. Closer examination revealed that these changes consisted largely of growth inhibition as evidenced by the reduced circumference of the eye and the reduced size of the head region without overt evidence for necrosis or gross malformation. A quantitative analysis of the effects of ccnd1-targeted HypNA-pPNA and MO treatments based on measuring the diameters of the eyes clearly confirmed the visual impression of microophthalmia (Figure 7). Histological analysis of ccnd1 HypNA-pPNA-treated or MO-treated fish larvae further confirmed reduced gross size of the eyes throughout the observation period. Representative examples of control, MO-treated and HypNA-pPNA-treated larval eyes at day 5 of development are shown in Figure 8. The most striking antisense effects were a sharp reduction in the number of retinal cells and in the thickness of the inner plexiform layer (white arrowheads, Figure 8). This difference was not due to programmed cell death as only a few apoptotic cells were observed between days 2 and 5 of larval development in all experimental conditions (data not shown).

Importantly, the developmental consequences of both MO and HypNA-pPNA cyclin D1 knockdown were sequence-specific. Even a one-base mismatch control, in the case of HypNA-pPNA injections, was sufficient to prevent the phenotypic changes produced by ccnd1-targeted HypNA-pPNA. In contrast, in the case of MOs, three-base mismatch controls were necessary to prevent inhibitory effects. To further assess the specificity of the effects observed, we performed rescue

Figure 6. Effect of ccnd1 knockdown on morphology of zebrafish embryos. Photomicrographs demonstrate the morphology of zebrafish embryos following knockdown of cyclin D1 at 24 h after microinjection with 0.1–1 mM ccnd1 antisense MO or HypNA-pPNA: (A) Uninjected control; (B) MO-treated embryo; (C) HypNA-pPNA-treated embryo; (D) Phenol Red control; (E) MO 3-base mismatch control; and (F) HypNA-pPNA 1-base mismatch control; all images were taken at ×100 magnification. The embryos injected with either antisense oligonucleotide displayed microophthalmia and microcephaly consistent with the restricted expression of cyclin D1 in 24 hpf zebrafish embryos. No gross morphologic defect was observed in the mismatch control-injected embryos. Labels: h, head; e, eye; y, yolk sac; b, body.

Figure 7. Sense mRNA rescue of ccnd1 antisense-treated zebrafish embryo morphology. Zebrafish embryos were microinjected with 0.5 mM ccnd1 antisense MO or HypNA-pPNA as described in Materials and Methods. Sense ccnd1 mRNA at 1 g/l was co-microinjected with the either MO or HypNA-pPNA into selected groups of embryos (designated ‘rescue’), and morphology recorded at 24 h after injection: (A) Phenol red-treated control; (B) MO-treated embryo; (C) HypNA-pPNA-treated embryo; (D) MO plus mRNA co-injected embryo; (E) HypNA-pPNA plus mRNA co-injected embryo; all images were taken at ×100 magnification. All embryos were treated with PTU following microinjection to inhibit pigment formation; therefore, the circumference of the eyes are delineated for better visualization. There is visual evidence of rescue of the ccnd1 antisense-induced microophthalmia and microcephaly in the ccnd1 mRNA co-injected embryos. The incidence of microophthalmia in MO- (F) or HypNA-pPNA-treated (G) embryos, with or without sense mRNA rescue, was calculated and expressed as percent (±SD) of 100 embryos per sample.
experiments by co-injection of ccnd1 mRNA and either HypNA-pPNA or MO. In either case, almost complete rescue was observed, confirming that the effects of either antisense construct were sequence-specific (Figure 7). In summary, these results revealed similar phenotypic effects produced by ccnd1 antisense oligonucleotides with quite divergent structures.

DISCUSSION

This study identifies molecular mechanisms controlling the establishment of cell cycle control during early zebrafish development. The delayed onset of G1/S transition control in this vertebrate model system allowed us to monitor expression of cell cycle regulators that contribute to the establishment of G1/S transition. The transcription profile analysis enabled simultaneous assessment of temporal changes in the expression patterns of multiple cell cycle regulators, cyclins, cdks and cdk inhibitors in early zebrafish development. Specifically, we observed high expression levels of cyclin genes ccna2, ccnb1, ccnb2 and ccne before the onset of zygotic transcription. This pattern was expected as these cyclins play prominent roles in S and M phases of the cell cycle, which are prevalent in the first hours of embryonal development (12).

A previous study demonstrated that zygotic transcription is necessary for the gradual slowing of cell cycle progression after MBT (5). In agreement with this earlier study we describe here that the expression of various molecular determinants of G1/S checkpoint control, the putative Rb and p21 gene orthologs, and ccnd1, commences after MBT. It should be noted that, as yet, zebrafish orthologs of ccnd2 and ccnd3 are not fully validated. Previous work demonstrated that, in zebrafish, ccnd1 expression occurred after MBT at the onset of G1 phase (4). Our results are consistent with this earlier report, and raised the question of what role cyclin D1 plays in zebrafish embryogenesis.

We found that downregulation of cyclin D1 by use of two antisense oligonucleotides with different backbones resulted in a phenotype restricted to the organ sites characterized by highest expression of cyclin D1 during gastrulation, i.e. the developing eye and the head region. Elimination of knockdown effects by a single mismatch in the HypNA-pPNA 16mer correlated with our previous observations of non-activity with one and two mismatches in 18mers, versus the need for four mismatches in an MO 25mer to abrogate activity (9).

The main effect of cyclin D1 downregulation on eye development was a proportional decrease in the size of the organ owing to reduced cellularity, but not overt cell death. This result is remarkably similar to results described in cyclin D1-deficient mice generated by gene targeting in embryonic stem cells (17,18). As in zebrafish (16), ccnd1 mRNA expression is very high in the developing retina and brain of mice. Furthermore, cyclin D1 deficiency in mice led to impaired development of all layers of the retina in a fashion very similar to the results described in this study. Collectively, these results show a remarkable degree of functional conservation of cyclin D1 function across species and open the door to the study of other cell cycle regulators in zebrafish.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.
ACKNOWLEDGEMENTS

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Conflict of interest statement. None declared.

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