THE INHIBITOR OF DNA BINDING PROTEINS IN CELLULAR PROLIFERATION AND DIFFERENTIATION: REGULATION BY THE RETINOIC ACID SIGNALING PATHWAY

by

CAREN M. VILLANO

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and approved by

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ABSTRACT OF THE DISSERTATION

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By Caren M. Villano

Dissertation Director:
Lori A. White, Ph.D.

The Id (Inhibitor of differentiation or DNA binding) helix-loop-helix proteins mediate cellular differentiation and proliferation in a variety of cell types through regulation of gene expression. The goal of the experiments in this thesis is to determine the effect of the retinoic acid signaling pathway on Id expression using cell culture and whole animal models. Retinoids, vitamin A analogues, are powerful regulators of cell growth and differentiation and are widely used in the prevention and treatment of a variety of cancers in humans. We found that exposure of normal human keratinocytes to all-trans retinoic acid (RA) results in increased expression of Id1 and Id3, which is mediated by increased transcription involving cis- acting elements in the distal portion of the promoter. To examine the effect of the Id proteins in development, we used the zebrafish (*Danio rerio*) model. Morpholino knockdown of Id1 in the developing zebrafish embryo results in pericardial, yolk sac and/or brain edema, as well as an undulating notochord. Loss of Id1 in early zebrafish embryogenesis results in defects in larval development, such as decreased body size, lack of swim bladder inflation, and craniofacial defects. We conclude that Id1 is critical for early and late zebrafish

development. Our findings also demonstrate that RA exposure decreases expression of Id1 specifically in the heart, and RA deficiency results in increased Id1 expression. Id1 knockdown results in increased expression of the cardiac-specific transcription factors gata5 and nkx2.5 which are also targets of the RA signaling pathway. To further examine the role of Id1 in differentiation and proliferation, we examined Id expression during caudal fin regeneration. Our data demonstrate that Id1 expression is induced in the blastema during caudal fin regeneration, and that exposure to RA during regeneration decreases Id1 expression. Taken together, the data presented in this thesis demonstrate that Id1 is a target for RA signaling in both human skin cells and in zebrafish. Further, these data suggest that Id1 may be an important intermediate in the RA signaling pathway, by altering expression of genes involved in proliferation and differentiation.

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CHAPTER 1:

GENERAL INTRODUCTION

The inhibitor of DNA binding (Id) proteins are members of the basic helix-loop-helix (bHLH) family of transcription factors. bHLH proteins are involved in regulating a variety of developmental processes including cellular differentiation, lineage commitment, and sex determination through binding to a hexanucleotide sequence, CANNTG, termed the E-box (Massari and Murre, 2000). There are seven distinct classes of bHLH proteins. Class I proteins, also known as the E proteins, include E12, E47, and Daughterless, and can form homo- or heterodimers (Cronmiller et al., 1988; Murre et al., 1989). Class II members include myogenic and hematopoietic regulatory factors such as myoD, myogenin, scl/tal-1 and lyl-1 and these cannot form homodimers, but heterodimerize preferentially with class I bHLH proteins (Murre et al., 1989). The proteins in class III contain a leucine zipper adjacent to the HLH region and include members of the Myc family, transcripton factor E3 (TFE3) and sterol regulatory element binding protein 1 (SREBP-1) (Massari and Murre, 2000). Binding partners of the Myc proteins, including Max, Mad and Mxi make up the members of the class IV bHLH proteins (Bernards, 1995). Class V members are negative regulators of class I and II, and include the Ids and the *Drosophila* protein extramachrochaetae (emc) (Ellis et al., 1990). Class VI includes proteins related to the *Drosophila* proteins hairy and enhancer of split (Davis and Turner, 2001). Finally, class VII members contain the bHLH-PAS (Per-Arnt-Sim) domain and include the aryl hydrocarbon receptor (AhR), AhR nuclear translocator (Arnt), and hypoxia factor 1α (hif1α) (Crews, 1998).

The Id proteins are distinct from the other HLH proteins in that they contain a HLH region that mediates dimerization, but lack the adjacent basic region, which is required for DNA binding. Ids heterodimerize with class I and class II HLH transcription factors and form an inactive complex that is unable to bind to DNA effectively. The Id proteins have been shown to bind with high affinity to the E proteins; however, low, variable binding affinities were shown for myogenic and hematopoietic factors (Langlands et al., 1997). The HLH region was found to be necessary and sufficient for Ids' dominant negative function (Pesce and Benezra, 1993). In addition, intracellular localization is important for Ids' dominant negative function. Nuclear localization and export signals allow translocation between the cytoplasm and the nucleus (Kurooka and Yokota, 2005; Makita et al., 2006). The Ids also disrupt subnuclear chromatin compartmentalization of bHLH proteins by sequestering pools of transiently diffusing transcription factors (O'Toole et al., 2003).

Four mammalian Id proteins (-1, -2, -3, and -4), the products of distinct genes, have been identified (Norton, 2000). Their function is tightly regulated on the level of transcription and protein stability. The expression of Id1 and Id2 is transiently induced in early passage human fibroblasts, and repressed later during senescence (Hara et al., 1994). Transcription of Id1 is regulated through elements on its promoter including Egr-1 (early growth response 1), YY-1 (ying yang 1), and CREB (cAMP responsive element binding protein) binding sites (Tournay and Benezra, 1996; Singh et al., 2002). Transcriptional regulators of Ids include bone morphogenic proteins (BMPs), transforming growth factor β (TGFβ), and insulin-like growth factor (IGF) (reviewed in (Perk et al., 2005)). Id1, Id2, and Id3 are rapidly degraded by the ubiquitin-proteasome

pathway, with half lives of approximately 60 minutes (Bounpheng et al., 1999). However, when bound to other bHLH proteins, this half life increases significantly, demonstrating increased stability (Bounpheng et al., 1999). Interactions with E proteins, such as the growth inhibitory E2A, regulates cell cycle entry at the G1 phase, demonstrating that Ids function to increase cell proliferation while inhibiting differentiation (Barone et al., 1994; Peverali et al., 1994). Id proteins also play a critical role in the regulation of cell cycle progression by promoting G1/S transitions, through direct interactions with non-HLH transcription factors such as the E26 (ETS) family of transcription factors, pRb tumor suppressor proteins and the centrisome (Norton, 2000; Zebedee and Hara, 2001; Hasskarl and Munger, 2002). The ternary complex factor (TCF) subfamily of ETS transcription factors interacts with Ids to regulate immediate-early genes, such as c-fos and egr-1, in response to mitogenic stimulation (Yates et al., 1999). pRb prevents cell proliferation by binding to and inactivating transcription factors, such as E2F (Zebedee and Hara, 2001). Phosphorylation of pRb by cyclin-dependent kinases (CDKs) releases it from its repressive binding and allows transcription of genes required for S phase. The tumor suppressor protein $p16^{INK4A}$ inhibits CDKs that phosphorylate pRb, thus blocking cellular proliferation (Shapiro et al., 2000). Id2, but not Id1 or Id3, binds to pRb and inhibits growth arrest (Lasorella et al., 1996). Additionally, regulation of cellular senescence by Id proteins is coupled to changes in $p16^{INK4A}$ expression. Primary fibroblasts from Id1-null mice undergo premature senescence, which is associated with increased expression of $p16^{INK4A}$ (Alani et al., 2001). Further, senescence in human fibroblasts is associated with reduction of Id1 levels as well as $p16^{INK4A}$ activation by ETS transcription factors (Ohtani et al., 2001).

Ids in epithelial tissues

Ectopic expression of the Id proteins inhibits differentiation of many cell types in culture, suggesting that the Id proteins are regulators of cellular differentiation (Norton et al., 1998). Specifically these proteins are implicated in regulating the homeostasis of epithelial cells (reviewed in Coppe et al., 2003). The Id proteins are expressed in proliferating keratinocytes *in vitro* as well as in intact epidermis (Langlands et al., 2000). Primary human keratinocytes express Ids during active proliferation with decreased expression upon calcium-induced differentiation (Schaefer et al., 2001). Additionally, established keratinocyte cell lines (HaCaT, SCC9) exhibit dysregulated Id expression (Langlands et al., 2000). Overexpression of Id1 leads to changes in differentiation and proliferation in keratinocytes. However, some discrepancies exist regarding the exact mechanisms that regulate these changes. One study demonstrated that ectopic Id1 immortalized normal human keratinocytes (NHKs) through activation of telomerase activity and inactivation of the tumor suppressor retinoblastoma (pRb) protein (Alani et al., 1999). However, data from other laboratories demonstrated that Id1 did not cause immortalization, but instead delayed replicative sensence in NHKs and human oral keratinocytes through the inactivation of $p16^{INK4A}$ (Nickoloff et al., 2000). Mammary epithelial cell growth and differentiation is also affected by Id levels (Desprez et al., 1995). Ectopic expression of Id1 in murine mammary epithelial cells inhibits differentiation, stimulates cell growth, and enhances glucocorticoid stimulation of tight junction sealing (Desprez et al., 1998; Woo et al., 2000). The Id proteins are also expressed in a variety of other protective and glandular epithelia including cells of the lung, esophagus, small intestines, liver, pancreas, thyroid, kidney, prostate, testis, cervix,

and ovaries (reviewed in Coppe et al., 2003). In most of these cell types, increased Id expression was associated with normal proliferation, with decreased or low expression during differentiation.

Ids and cancer

The ability of Ids to extend the life span of epithelial cells in culture suggests that they may be important regulators in tumorigenesis. In fact, Id proteins are often overexpressed in tumor cells, stimulating proliferation and tumor angiogenesis (Benezra, 2001). Elevated Id levels have been observed in carcinomas of the breast, prostate, ovary, endometrium, cervix, colon, pancreas, liver, and thyroid. Id levels are also high in a variety of squamous cell carcinomas including those of the esophagus, oral cavity, and nasopharynx, as well as melanomas, neural tumors, gastric adenomas, and leukemia. In some cases, Ids have been used as prognosis factors, with high expression levels being associated with severity and poor prognosis. In the rat model of hormone-induced prostate cancer, high levels of Id1 correlate with increased malignancy (Ouyang et al., 2001). Overexpression of Ids also induces the expression and secretion of matrix metalloproteinases (MMP2 and MMP7) enzymes that play a signficiant role in tumor invasion and metastasis due to their ability to degrade extracellular matrix proteins (Desprez et al., 1998; Coppe et al., 2004; Ouyang et al., 2001; Lyden et al., 1999; Brinckerhoff et al., 2000). Ids also transcriptionally activate expression of vascular endothelial growth factor (VEGF), which promotes angiogenesis, and may be responsible for tumor neovascularization (Ling et al., 2005). The role that Id proteins are shown to play in cancer progression makes them a rational target for cancer therapeutic strategies (Fong et al., 2004).

Ids and development

During development, the Ids are involved in tight regulation of cell proliferation and differentiation required for proper body plan (Yokota, 2001). Expression of Id1, -2 and -3 overlaps significantly during murine development, while Id4 expression patterns appear unique (Jen et al., 1996). Generally, Ids are expressed in many organs and tissues including the developing nervous system, limbs, branchial arches, and the heart (Evans and O'Brien, 1993). Id1-null mice show no obvious abnormalities during development and are viable for at least two years (Yan et al., 1997). Id2 knockout mice exhibit defects in natural killer cell differentiation, lack lymph nodes, and fail to produce lactating mammary glands during pregnancy (Yokota et al., 1999; Mori et al., 2000). Additionally, Id3 knockout mice do not show any obvious phenotypes during development, but do have compromised immunity (Pan et al., 1999). The importance of the Id proteins in development is supported by the findings that Id1/Id3 double knockout mice are nonviable and exhibit reduced body size, cranial hemorrhaging, and small brain size *in utero* (Lyden et al., 1999). Double and triple Id knockouts show severe cardiac defects leading to embryonic lethality at E13.5 (Fraidenraich et al., 2004). Also, overexpression of Id1 during mouse development inhibits neuronal development (Yokota, 2001). Further investigation into the regulation of Id expression will allow for a more complete understanding of their role in developmental processes.

Retinoic Acid

Retinoic acid (RA) is a natural product (lipid soluble hormone) derived from the metabolism of vitamin A. Vitamin A is an essential nutrient obtained from food either as preformed vitamin A (retinyl ester, retinol and small amount of RA) from animal products (eggs, liver, milk) or as pro-vitamin A (carotenoids) from fruits and vegetables (Sporn et al., 1994; Fisher and Voorhees, 1996). Vitamin A and its natural and synthetic derivatives are also referred to as retinoids. Dietary derived all-trans RA (atRA) is the main signaling retinoid in the body and is vital for biological functions such as embryogenesis, growth and differentiation, as well as for vision and reproduction (Dragnev et al., 2000). Levels of atRA in the tissue are tightly regulated through its biosynthesis, metabolism and storage in the liver.

Storage and transport

Vitamin A is obtained from the diet, either as preformed vitamin A (retinyl ester, retinol and small amount of RA) or as provitamin A (carotenoids). In the lumen of the small intestine or in the intestinal mucosa, dietary retinyl esters are hydrolyzed to retinol, through the action of retinyl ester hydrolases (REHs). Provitamin A (mainly in the form of β-carotene) absorbed by the mucosal cells is converted to retinaldehyde through the actions of carotene-15,15'-dioxygenase, and this form is further reduced to retinol by retinaldehyde reductase. Within the enterocyte, retinol, independent of its dietary origin, is resterified by the enzyme lecithin:retinol acyltransferase (LRAT), and retinyl esters are packaged into chylomicrons, together with other dietary lipids. Once packaged into chylomicrons, the bulk of dietary retinoid is taken up by the liver, while the remaining

25% is taken up by extrahepatic tissues (Goodman, 1962; Goodman et al., 1965) In the liver, chylomicron retinyl esters are once again hydrolyzed to retinol, which can either be secreted by the hepatocyte bound to retinol-binding protein (RBP) or it can be transferred to the hepatic stellate cells for storage (Vogel et al., 1999). At this time the mechanism of transfer of retinol from the hepatocytes to the stellate cells for storage is still not completely elucidated. However, when dietary vitamin A is abundant, ~80-90% of the stored retinyl esters are in the stellate cells. Both hepatocyte and stellate cells produce significant amounts of REH and LRAT, as well as the cellular retinol binding protein type I (CRBPI). CRBPI is a chaperone protein necessary to solubilize retinol in the aqueous environment of the cell (Vogel et al., 1999). To maintain solubility in an aqueous environment, retinoids are bound to retinoid specific binding proteins. The cellular retinol binding proteins (CRBPI and CRBPII) and the cellular retinoic acid binding proteins (CRABPI and CRABPII) are entirely intracellular; whereas the RBP and the intercellular retinol binding proteins (IRBP) are extracellular. These essential proteins are ubiquitously expressed and highly conserved among species. The retinoic binding proteins help regulate RA concentrations by protecting retinol and RA from oxidation and isomerization and acting as substrates for metabolism (Napoli, 1997).

Retinoic Acid Catabolism

Tight regulation of RA concentrations maintains retinoid homeostasis in the body. The enzyme lecithin:retinol acyltransferase (LRAT) is considered the primary enzyme for esterification of retinoids, LRAT null mice maintain some ability to convert retinol to retinyl esters, supporting the notion that another enzyme, namely acyl-CoA:retinol acyltransferase, is involved in this process (O'Byrne et al., 2005). A variety of CYP450s

are known to have metabolizing activity towards RAs, including members of the CYP1A, 2, 3A and CYP26 families (Honkakoski and Negishi, 2000). CYP26 mediates the oxidation of atRA to polar metabolites that are less biologically active.

Retinoic Acid Signaling

The majority of RA's biological activity is mediated through regulation of gene expression. A comprehensive table of genes regulated by RA signaling reveals over 300 genes upregulated, 100 genes downregulated, and 100 more variably regulated (Balmer and Blomhoff, 2002). RA binds to two types of nuclear receptors, the retinoic acid receptors (RARs), which bind both 9-cis and all-trans forms of RA, and retinoid X receptors (RXRs), which bind 9-cis RA (reviewed in (Mangelsdorf et al., 1995; Chambon, 1996)). The RARs and RXRs each contain a well conserved DNA binding domain, a well conserved ligand binding domain, and three or four domains that are not as well conserved (Renaud and Moras, 2000). Both RAR and RXR have three subtypes (α, β, and γ), and gene knockout experiments suggest that the RAR subtypes may be functionally redundant (Mark et al., 1999). Furthermore, RAR and RXR subtypes form multiple isotypes through the use of alternate promoters, alternative splicing, and alternative initiation of translation (Chambon, 1996). Although these isoforms appear to have tissue-specific expression, it is unclear whether or not they differentially regulate gene transcription. RAR and RXR homo- and hetero-dimers stimulate transcription of target genes through binding to retinoic acid response elements (RAREs) consisting of direct repeats of 5'-AGGTCA-3' separated by 1-5 spaces (termed DR-1 or DR-5, respectively) (Glass et al., 1997). Although RARs and RXRs can form homodimers, heterodimerization of RARs with RXRs increases the affinity of these receptors for the

RARE. In addition to directly altering gene expression, atRA also can reduce the expression of certain genes through interference with other transcription factors, most notably AP-1 (Schule et al., 1991). In an un-induced state, the RAR/RXR heterodimers are in complex with the RARE and are bound to the nuclear co-repressors SMRT (silencing mediator of retinoid and thyroid receptors) or N-CoR (nuclear receptors co-repressor) (Li et al., 1997; Yoh and Privalsky, 2001). These co-repressors function by recruiting complexes containing histone deacetylases to the promoter. Upon binding of ligand, the RAR/RXR heterodimers are released from this complex to interact with co-activator complexes (SRC1/TIF2/RAC3 and CBP/p300) that mediate transactivation (Chen and Li, 1998).

Steric interconversion of atRA occurs *in vitro* and *in vivo* (Zile et al., 1967; Sundaresan and Bhat, 1982; Vane et al., 1982; Kojima et al., 1994; Marchetti et al., 1997), and the conversion between atRA and 9- and 13-cisRAs alters the available binding forms. Although atRA is considered the primary signaling retinoid in the body, data indicate that other RA isoforms also contribute to signaling. 9-cis RA has also been accepted as a potent signaling retinoid, binding effectively to the RXRs. No binding activity is associated with the 13-cis RA isomer, and it is believed the biological effects of 13-cis RA were mediated through isomerization to 9-cis RA. However, data now suggest that, although the 13-cis isomer is unable to activate transcription through the retinoid receptors, it may function through inhibition of enzymes involved in steroid metabolism or through other membrane receptors (Blaner, 2001).

Endogenous levels of atRA in the body are maintained through a balance between atRA biosynthesis, metabolism, and storage. A variety of metabolizing enzymes,

including CYPs, UGTs and ALDHs, are potentially involved in the synthesis and catabolism of RA (Fig. 3). Under normal physiological conditions the cytosolic medium-chain alcohol dehydrogenases (ADHs) and the microsomal short chain alcohol dehydrogenases (SCADs) are responsible for the oxidation of ROH to RAL. Alternatively, retinol dehydrogenase (RDH) can catalyze the oxidation of 9-cisROH, but not atROH (Gamble et al., 1999). The irreversible oxidation of retinal to atRA is catalyzed by members of the aldehyde dehydrogenase (ALDH) family (Duester, 2000), including retinal dehydrogenase type 2 (RALDH2).

RESEARCH OBJECTIVES

The overall goal of this thesis is to examine the ability of all-trans retinoic (atRA) acid to regulate the expression of the inhibitor of DNA binding 1 (Id1) protein and to examine the effect of the Id proteins on cellular differentiation and proliferation.

The specific aims of are to:

1. Determine the ability of atRA to regulate Id1 expression in normal human keratinocytes.
2. Examine the role of Id1 in zebrafish embryonic development.
3. Determine the effects of atRA exposure on Id1 expression in the developing zebrafish embryo.
4. Examine the role of Id1 in zebrafish tailfin regeneration and the effects of atRA on Id1 expression during regeneration.

Specific Aim 1:

The normal human keratinocyte cell culture model system allows for the in depth investigation of the transcriptional activation of Id1 by atRA, which could not be easily accomplished in a whole animal model. The Id proteins are expressed in proliferating keratinocytes *in vitro* as well as in intact epidermis (Langlands et al., 2000). We chose normal human keratinocytes for experiments in Specific Aim 1, because these cells express Ids during active proliferation and have decreased Id expression upon calcium-induced differentiation. Additionally, established keratinocyte cell lines (HaCaT, SCC9) exhibit dysregulated Id expression (Langlands et al., 2000). *Given the effect of the Id proteins on cellular differentiation and proliferation, we hypothesized that the Id proteins may be functional intermediates for the effects of retinoic acid in NHKs*. Our data show that atRA-exposure of keratinocytes results in a significant increase in both Id-1 mRNA

and protein and this activation is mediated through multiple *cis*-acting elements in the distal region of the promoter (Villano and White, 2006).

Specific Aim 2:

Retinoic acid is a potent teratogen and is known to affect the development of many organ systems in the zebrafish embryo, including the central nervous system, heart, eye, fin, and pancreas. *Based on our previous findings in keratinocytes, we hypothesize that Id1 may be an important mediator in atRA- induced developmental defects in the zebrafish.* In the developing embryo, cellular proliferation and differentiation are orchestrated by tight regulation of signaling pathways to achieve a normal body plan. The basic helix-loop-helix (bHLH) transcription factors promote cellular differentiation and are balanced by the the Id proteins which promote proliferation. This delicate balance is essential for proper development (reviewed in (Yokota, 2001)). To examine the interaction of the atRA signaling pathway and the Id proteins during development, we chose the zebrafish (*Danio rerio*) model. The advantages of this model system include the availability of genetic and molecular techniques, extensive information on stages of development and genetics, as well as transparency of the embryo, and physiological similarity to mammals.

Specific Aim 3:

The toxicity and mortality associated with atRA-exposure in the embryo makes detailed investigation into atRA effects on the Id proteins in embryonic development challenging. The caudal fin regeneration model offers the same level of complexity (i.e.

multiple cell types, post-embryonic development) without the secondary effects that result from physiological imbalances during development. Epimorphic regeneration requires both cellular differentiation and proliferation to replicate the pattern of lost tissue. Although retinoic acid is required for normal regenerative processes, excess retinoic acid can have both teratogenic and morphogenetic effects (reviewed in (Maden, 2000)). In the zebrafish caudal fin, exogenous atRA inhibits regeneration (Ferretti and Geraudie, 1995). To date, there is little information available on the role of Id proteins in regeneration. *We hypothesize that Id1 is involved in normal and atRA-inhibited zebrafish caudal fin regeneration.*

CHAPTER 2:

MATERIALS AND METHODS

Human Keratinocyte Culture

Normal human keratinocytes (NHKs) were purchased from Cascade Biological. NHKs were incubated at 37^{o}C, in a humidified, 5% CO_2 incubator in Media 154 supplemented with Human Keratinocyte Growth Supplement (HKGS) and PSA (Cascade Biological). For experiments, confluent NHK cultures were washed three times in Ca^{2+}/Mg^{2+} free PBS, and cultured in serum and additive-free media containing vehicle control (Me_2SO) or the indicated treatment. Treatments were performed at the following concentrations: all-trans retinoic acid (atRA; 10^{-6} M; Sigma), cycloheximide (CHX; 25μg/ml; Sigma), 5,6-dichlorobenzimidazole riboside (DRB; 75μM; Sigma), actinomycin D (ActD; 5μg/ml; Sigma).

RNA Isolation

For qRT-PCR analysis in NHKs and zebrafish embryos total RNA was isolated using Trizol Reagent (Invitrogen) per manufacturer's instructions. For Northern analysis, poly(A^+) RNA was isolated from confluent NHK cultures as previously described (Sadek and Allen-Hoffmann, 1994). For zebrafish heart isolations and caudal fin tissue, total RNA was extracted from the using the Qiagen RNeasy Mini Kit.

Northern Analysis

Poly(A^+) RNA (3 μg) was electrophoresed in a 1.2% agarose gel containing formaldehyde and transferred onto a Immobilon NY^+ membrane (Millipore). The blot was UV cross-linked, and hybridized to ^{32}P random-primer labeled probes. The cDNA probes used were specific to human ID-1 cDNA. Blots were also hybridized with

glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a loading control. Blots were exposed on phosphorimaging screens, and scanned using a Phosphorimager (Molecular Dynamics). Band intensities were analyzed using the ImageQuant 5.2 software (Molecular Dynamics).

Quantitative RT- PCR

RNA samples isolated with TRIzol were DNase treated (DNA-free kit, Ambion) to remove residual genomic DNA, and reverse transcription was performed on 1μg of total RNA to produce cDNA using the iScript cDNA Synthesis Kit (BioRad) according to manufacturer's instructions. For RNA isolated with the Qiagen RNeasy Mini Kit, elimination of genomic DNA is completed by use of a spin column provided in the kit. Primer sets for quantitative PCR are shown in Table 1 for human and Table 2 for zebrafish. For fluorescent detection of PCR products, reactions containing template and specific primers reactions were amplified using BioRad iQ SYBR Green Supermix on a BioRad iCycler equipped with an iCycler iQ Detection System. Reactions were performed in triplicate. Data were quantified using standard curves generated for each primer set, and normalized to either GAPDH (human) or 28S (zebrafish) to control for total RNA concentration. Melting curves were generated for each sample after amplification for determination of primer dimers and product amplification was verified using acrylamide gel electrophoresis.

Transient Transfection and Luciferase Assays

The full length Id-1 promoter (2.2kb) in the pGL3 vector (Promega) was a gift from Dr. P.-Y. Desprez (Singh et al., 2002). To prepare the Δ1 promoter construct, PCR was performed on the full length promoter using the following primers: Δ1Sac F: 5'-

ATCTaa *gAGCTc* C CGCGCCGTG-3'and Δ1HindIII R: 5'-TC*AaGcTT*CTTG GCGACTGGCT-3'. Inserted restriction sites are in italics and changed bases are in lower case. The product was digested with SacI and HindIII, ligated in the multiple cloning site of the pGL3 vector and transformed into ECOS-Blue competent cells (ISC BioExpress). Colony PCR was performed to screen for positive clones. The Δ2 and Δ3 promoter constructs were prepared similarly, using the forward primers Δ2Sac: 5'gagCCg*GAGcTC*TTTTCATTATAAGGC-3' and Δ3Sac: 5'- at*gagctc*CCGTGGC GTGTTTATAAAAGAC -3' and Δ1HindIII reverse primer. NHKs were transfected with the pGL3 constructs using Keratinocyte TransIT (Mirus) (2 μg DNA: 6 μl TransIT). NHKs were co-transfected with the renilla luciferase vector phRL-TK (Promega) at a ratio of 1:100 phRL-TK: pGL3 Id1 constructs to control for variations in transfection efficiency. NHKs were plated at a density of 1.5 X 10^5 cells/well on a 6 well plate. Twenty-four hours post plating, cells were transfected, and the following day the cells were treated with vehicle (Me_2SO) or atRA. Cellular lysates were harvested 6 h following treatment using the Dual Luciferase Kit (Promega) and luciferase activities determined using a Dynex Multiplate Luminometer. Protein concentrations of the lysates were determined using a modified Lowry assay (Pierce). Luciferase activities were normalized to transfection efficiency using the phRL values, and to protein content. Treatments and transfections were performed in groups of six.

Preparation of Nuclear Extracts

Nuclear extract harvest and mobility shift assays were performed as previously described (White and Brinckerhoff, 1995). Nuclear proteins were isolated from Me_2SO and atRA treated adherent cultures of normal human keratinocytes. Cell monolayers

were washed twice in ice-cold PBS. Cells were harvested by scraping, and pelleted at 1850 x g for 10 min. The packed cell volume was estimated, the cells resuspended in Buffer A (10mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10mM KCl, 0.5mM DTT, 0.5mM PMSF), and allowed to swell on ice for 10 min. Cells were lysed by passing through a 25 gauge needle, and the nuclei were pelleted by centrifugation at 3300 x g for 10 min. Packed nuclear volume was estimated, the nuclei resuspended in 1 volume of Buffer C (20 mM Hepes, pH 7.9, 25% glycerol, 1.5M $MgCl_2$, 0.42M KCl, 0.2mM EDTA, 0.5mM DTT, 0.5mM PMSF), and rocked at 4^{o}C for 30 min. The nuclear proteins were harvested by centrifugation of the nuclei at 14,000 x g for 30 min. at 4^{o}C, and stored at –70^{o}C. Protein concentrations were determined using a modified Lowry Assay (BioRad).

Electrophoretic Mobility Shift Assays (EMSA)

Probes for EMSA were ^{32}P-labeled by primer extension. Oligonucleotides corresponding to regions of the human ID-1 5' region are shown in Fig. 6 and Fig. 7. Binding reactions were performed in nuclear extract Buffer C, and contained 10μg nuclear protein, 5μg dIdC, and 10 μg BSA. Binding reactions were electrophoresed on a 7% acrylamide gel in 0.5 X TBE at 125 V. Gels were dried, exposed on a phosphorimager screen, and scanned using a Storm 860 Phosphorimager (Molecular Dynamics). Antibody specific to CREB-1 (Santa Cruz Biotechnology: c-21x) was used in supershift analysis.

Western Analysis

Whole cell protein was isolated from the cell lines using RIPA buffer, and 10 μg separated on a NuPAGE Bis-Tris acrylamide gel (4-12%) (Invitrogen) under reducing conditions. Separated proteins were transferred onto Hybond-ECL membrane

(Amersham) and successful transfer was confirmed by Ponceau-S staining of the membrane. Western blots were probed using antibody specific to Id1 (Santa Cruz Biotechnology: c20) and detected using an ECL Plus Kit (Amersham). Signal was detected by exposure to Kodak X-ray film. Blots were stripped and re-probed using an antibody specific to human β-actin (Sigma) to determine accurate loading.

Zebrafish Strains and Husbandry

The AB and fli1: EGFP strains of zebrafish (*Danio rerio*), obtained from the Zebrafish International Resource Center (ZIRC), were used for experiments described. Zebrafish were maintained and bred in an Aquatic Habitats recirculation system according to a husbandry protocol approved by the Rutgers University Animal Care and Facilities Committee.

***In Vitro* Transcription/Translation**

Verification of Id1 morpholino knockdown was performed using the High Yield Coupled *in vitro* transcription/translation kit (Promega). The zebrafish Id1 cDNA (cb108) was obtained from Zebrafish Information Network (ZFIN) and subcloned into the Promega pTNT vector using EcoRI and KpnI sites. Reactions were run using 2 μg of circular plasmid cDNA and 10 μCu of ^{35}S Methionine (GE Healthcare) for incorporation. 10 μM of Id1 morpholino was added to the reaction to block translation. A control reaction which included 10 μM control morpholino was also run. One microliter of the finished reaction was run on a 10% Bis-Tris polyacrylamide gel (Invitrogen), which was subsequently dried down and exposed overnight to a phosphorimager screen. Screens were scanned on a Storm PhosphorImager (Molecular Dyamics).

Microinjection of Antisense Morpholino Oligonucleotides

A zebrafish antisense morpholino (Id1-MO) designed to block initiation of Id1 mRNA translation was obtained from Gene Tools, LLC (Philomath, Oregon). The sequence of the Id1-MO was 5'- CGCAGGTAGGTCC CACAACTTTCAT-3' and the sequence of the Gene Tools standard control morpholino (control-MO*)* was 5'- CCTCTTACCTCAGTTACAATTTATA-3'. For confirmation of morpholino specificity, a second Id1 morpholino with the following sequence was used: 5'- TGCGAGTAAGTCAAAGAGGAATAGC-3'. The sequence of the raldh2-MO was the same as used in Stafford et al. (2006): 5'- GCA GTTCAAC TTCACTGGAGGTCAT-3'. Morpholinos are fluorescein-tagged for visualization and monitoring of injection success. Prior to injections, 1 mM stocks of each morpholino were diluted to concentrations ranging from 10 μM – 100 μM in 1 X Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM HEPES, pH 7.6) (Nasevicius and Ekker, 2000). Embyros were injected with 4 nL of control-MO or Id1-MO at the 1-2 cell stage as described in Nasevicius and Ekker (2000) using a General Valve Corporation Picospritzer II. Approximately 2 h following the injection, embryos were visualized using an Olympus IX51 inverted microscope equipped with an EXFO X-Cite 120 fluorescence illumination system. Embryos that were found to be unfertilized, damaged, and/or possess uneven distributions of the morpholino were discarded. Embryos that were successfully injected with the morpholino, as judged both by even distribution throughout the cell mass (at 2 hours post fertilization, embryos are at the 64-cell stage) and a strong fluorescent signal, were maintained at 28°C in embryo medium. Embryos

were assessed every 24 hours and any changes in normal morphology were photographed using a Scion CFW-1310C color digital camera on the Olympus IX51 inverted microscope.

Alcian Blue Staining

Embryos were fixed overnight at 4°C in 4% paraformaldehyde (Electron Microscopy Sciences) and then transferred to 70% ethanol for storage. Prior to staining, embryos were bleached in 30% hydrogen peroxide for approximately 2 h. Staining was performed overnight at room temperature using 0.1% Alcian Blue 8GX (Sigma-Aldrich) that had been filtered through a 2 μm syringe filter. Following staining, embryos were placed in acidified ethanol for 2-4 h to allow for clearing of non-specific staining. Finally embryos were washed in increasing concentrations of glycerol (15 min each of 20%, 50%, 80%, and 100%) and visualized as described previously. Photographs were taken on an Olympus SZ4060 zoom stereo microscope equipped with a Scion CFW-1310C color digital camera.

Collection & Treatment of Zebrafish Embryos

Embryos were collected at 3 hours post fertilization from breeding stocks of zebrafish. Treatments consisted of exposure for 24, 48, or 72 hours to 10, 5, and 1nM *all-trans* retinoic acid (atRA; Sigma-Aldrich) in embryo medium (Westerfield, 2000). A 1μM stock solution of atRA was made in ethanol (EtOH) prior to being diluted in embryo medium. In all treatments EtOH was kept at 0.1% final concentration. All experiments were carried out in the dark, due to the light sensitivity of atRA.

In Situ Hybridization

An Id1 EST (cb108) was purchased from Zebrafish International Resource Center (Eugene, OR) and used to make RNA probe. The cb108 construct was linearized with NotI, and T7 polymerase was used to create antisense digoxigenin (DIG)-labeled RNA probes (DIG RNA Labeling Kit - SP6/T7, Roche) from the linearized DNA. Embryos to be used for *in situ* hybridization were grown in 0.003% (0.033 mg/mL in embryo medium) phenylthiourea to inhibit formation of pigmentation. Embryos were manually dechorionated and fixed overnight at 4°C in BT-fix (4% sucrose, 4% paraformaldehyde (Electron Microscopy Sciences), 0.1 M sodium phosphate, 0.15 mM calcium chloride, pH 7.3) (Westerfield, 2000). For adult caudal fin studies, fish were anesthetized with MS222 (ethyl 3-aminobenzoate methanesulfonate; Sigma) and caudal fin regenerates were collected at 2 and 4 days post amputation. For each timepoint, ten adult zebrafish were used. Regenerated fin tissue was fixed overnight at 4°C in 4% paraformaldehyde/1XPBS. The *in situ* hybridization protocol was modified from that described by (Oxtoby and Jowett, 1993). Following staining, embryos were cleared of non-specific staining by being transferred into methanol for 10 minutes, isopropanol for 10 minutes, and then placed in 1,2,3,4-tetrahydronaphthalene (Sigma-Aldrich) for visualization.

Zebrafish Embryo Heart Isolations

Hearts were isolated according to the method described in Burns and MacRae (2006) except fli1:EGFP zebrafish were used. Briefly, approximately 300 embryos were collected at 48 hours post fertilization, anesthetized with MS222, and rinsed in embryo disruption medium (Gibco Leibovitz's L-15 medium (Invitrogen) + 10% fetal bovine serum). Embryos were fragmented using a 19 gauge needle with a 5mL syringe.

Fragmented embryos were passed through a 105 μm nylon mesh (Small Parts, Inc.). The flow-through was applied to a 40 μm nylon mesh (Small Parts, Inc.) and the retained material was washed off and retained for RNA isolation.

Fin amputations and retinoic acid treatments of adult zebrafish

Retinoic acid treatments were done according to (White et al., 1994). Briefly, fish were anesthetized in MS222, caudal fins were amputated proximal to the first ray bifurcation and fish were allowed to recover for one day. At one day post amputation, fish were placed in water containing either DMSO or 10^{-6} M atRA (Sigma) at 33°C. At this concentration, the regenerative process is inhibited and regenerated fins are narrower and show fusion of rays (Geraudie et al., 1995). Concentrations higher than this were shown to affect survival while lower concentrations had no effect on regeneration. Since retinoic acid is light sensitive, both treated and solvent control animals will be kept in the dark. At 2 and 4 days post amputation, fish were anesthetized and fin regenerates were collected.

BrdU incorporation

At 3 days post amputation, fish were anesthetized as previously described and injected with 25mg/g body weight bromodeoxyuridine (Roche Diagnostics) in sterile saline. After recovering, animals were placed back in treatement for six hours. After six hours, regenerated tail fins were amputated and fixed in 4% paraformaldehyde/1X PBS at 4C overnight. Fixed tails were stored in methanol at -20C until used for immunohistochemistry. Fins were rehydrated in a methanol:PBS series, washed in 1X PBS + 0.25% Trition X-100 (PBSTx) and incubated for 30 minutes in 2N HCl/PBSTx. Next, fins were inclubated in a 1:100 dilution of anti-BrdU antibody (Roche Diagnostics) in PBSTx overnight at 4C. The following day, fins were washed 5 x 10 minutes in

PBSTx and incubated at room temperature for 4 hours in a fluoresceine-tagged anti-mouse IgG (Southern Biotech). Fins were washed twice in PBSTx and viewed on an Olympus IX51 inverted microscope equipped with an EXFO X-Cite 120 fluorescence illumination system.

Statistical Analysis

Statistical analysis was performed using the SigmaStat v.1.0 software package (Jandel Scientific). The probability level for statistical significance was $p < 0.05$. Changes in mRNA levels were evaluated using an unpaired Student's t-test.

TABLE 1: Primer sets used for quantitative PCR - Human.

	Primers	**Product**	**Reference**
GAPDH	F: 5'-CGCCAGCCGAGCCACAT-3'	295 bp	(Murphy et al., 2004)
	R: 5'-TCGCCCCACT TGATTTTG-3'		
Id1	F: 5'- CTCTACGACATGAACGGCTG T -3'	78 bp	(Lee et al., 2003)
	R: 5'- TGCTCACCTTGCGGTTCTG -3'		
	hnRNA R: 5'- TGATCTAGTGGTCGGATCTGGATC-3'		
Id2	F: 5'- TCAGCCTGCATCAC AGAGA -3'	92 bp	
	R: 5'- CTGCAAGGACAGGATGCTGATA -3'		
Id3	F: 5'- TCAGCTTAGCCAGGTGGAAATC -3'	76 bp	
	R: 5'- TGGCTCGGCCAGGACTAC -3'		
Id4	F: 5'- CCGAGCCAGGAGCACTAGAG -3'	116 bp	
	R: 5'- CTTGGAATGACGAATGAAAACG -3'		
P16ink4a	F: 5'- GCGGAAGGTCCCTCAGACA -3'	73 bp	(Lee et al., 2003)
	R: 5'- TCTAAGTTTCCCGAGGTTTCTCA -3'		
BMP2	F: 5'- GGAGAAGGAGGAGGCAAG -3'	100 bp	(Emmanuele et al., 2003)
	R: 5'- GACACGTCCATTGAAAGAGC -3'		
BMP4	F: 5'-TTCCGGACTACATGCGGGATCTTT-3'	395 bp	
	R: 5'-TGTCACATTGTGGTGGACCAGTCT-3'		

TABLE 2: Primer sets used for quantitative PCR - Zebrafish.

28S	F: 5'- CCTCACGATCCTTCTGGCTT -3' R: 5'- AATTCTGCTTCACAATGATA -3'	151 bp	
Id1	F: 5'- GTTTCTCCAAGACATGAA CAG C -3' R: 5'- TGCTGGCTTTCTTGTTGGTCGGTA -3'	76 bp	
Gata5	F: 5' - TCTGCGTTTCTCCTCCACAGTGTT -3' R: 5' - CAGAAACGCCTGCAAAGCACATCA -3'	103 bp	
Nkx2.5	F: 5' - ACCTACAACACCTACCCTGCGTTT -3' R: 5' - ACTGTGAAGGTTGGATGCTGGACA -3'	92 bp	

CHAPTER 3:

EXPRESSION OF THE HELIX-LOOP-HELIX PROTEIN INHIBITOR OF DNA BINDING-1 (ID-1) IS ACTIVATED BY ALL-TRANS RETINOIC ACID IN NORMAL HUMAN KERATINOCYTES

INTRODUCTION

Helix-loop-helix (HLH) proteins are important transcriptional regulators of a variety of developmental processes such as lineage commitment, cell differentiation and sex determination (Massari and Murre, 2000). The HLH region mediates homo- and heterodimerization that is required for DNA binding, and conventional HLH transcription factors have a highly basic region immediately adjacent to this region that mediates binding to E-box sequences. The Id (Inhibitor of DNA binding) class of HLH proteins is distinct from the other classes in that these proteins lack the basic DNA binding domain, and form inactive heterodimers with other HLH proteins (Norton, 2000). Ids are believed to effect cellular processes such as cell proliferation and differentiation through inhibiting the functions of other HLH proteins. The current hypothesis is that the actions of bHLH homo- and heterodimer transcription factors and the Id inhibitor proteins coordinate regulation of gene expression during differentiation.

Four mammalian Id proteins (-1, -2, -3, and -4), the products of distinct genes, have been identified (Norton, 2000), and ectopic expression of the Id proteins inhibits differentiation of many cell types in culture (Norton et al., 1998) suggesting that the Id proteins are regulators of cellular differentiation. Furthermore, Id proteins have a critical role in the regulation of cell cycle progression by promoting G1/S transitions, through interactions with the retinoblastoma protein and the centrisome (Norton, 2000; Zebedee

and Hara, 2001; Hasskarl and Munger, 2002). Id proteins play a critical role in epidermal homeostasis and keratinocyte differentiation and recent reports suggest they are also involved in promoting angiogenesis, tumor formation and metastasis (de Candia et al., 2003; Fong et al., 2003; Ruzinova and Benezra, 2003; Sikder et al., 2003). For example, ectopic expression of Id1 in the intestinal epithelia of mice results in increased adenoma formation, demonstrating a role for Id1 in regulation of intestinal epithelia replication (Wice and Gordon, 1998). In squamous cell carcinoma (SCC) the majority of the malignant/poorly differentiated SCC expressed Id1 and Id3 while Id1 and –3 were reduced in the well-differentiated SCC (Langlands et al., 2000). Further, forced overexpression of Id1, -2 and –3 in normal human keratinocytes extends the normal *in vitro* lifespan of keratinocytes in culture(Alani et al., 1999; Nickoloff et al., 2000). These data suggest that Ids are important for maintaining the proliferation/ differentiation balance in the epidermis, and that disruption of Id expression may be a critical factor in the development of skin cancer.

Id gene expression is altered by cytokines, growth factors, and hormones, including members of the transforming growth factor-β family (TGF-β, BMP-2/4/7), platelet derived growth factor (PDGF), epidermal growth factor (EGF), neural growth factor (NGF), and estrogen (Yokota and Mori, 2002). Recent data indicate that Id expression is also influenced by exposure to retinoic acid (Ma et al., 2003; Nigten et al., 2005; Zhang and Rosdahl, 2005). Dietary derived all-trans RA (atRA) is the main signaling retinoid in the body and is vital for biologic functions such as embryogenesis, growth and differentiation, vision and reproduction (Dragnev et al., 2000). Furthermore, RA is necessary for the maintenance of epithelial differentiation, and demonstrates an

inhibitory action on skin carcinogenesis (Fisher et al., 1995; Fisher and Voorhees, 1996). The majority of RA's biological activity is mediated by alteration of gene expression. The binding of specific nuclear receptors, the retinoic acid receptors (RARs) and retinoid X receptors (RXRs) transcriptionally activates gene expression (reviewed in(Mangelsdorf et al., 1995; Chambon, 1996)).

As retinoids are important mediators of epidermal differentiation, our investigations focused on the effects of retinoic acid on Id expression in normal human keratinocytes. Our data show that expression of Id1 and Id3 is increased by exposure to atRA, and that atRA-induced expression of Id1 is regulated at the level of transcription. Several elements in the human Id1 promoter appear to be involved in atRA induced expression, including a YY1 element, an Egr1 site, as well as a CREB binding site.

RESULTS

All-trans retinoic acid stimulated expression of ID1 mRNA and protein.

To investigate whether Id gene expression in keratinocytes is altered by all-trans retinoic acid (atRA) exposure, we cultured normal human keratinocytes (NHKs) in medium containing atRA (10^{-6}M) or vehicle control (Me_2SO). RNA was isolated 6 and 24 h following atRA exposure and used for Northern and quantitative RT-PCR analysis. Figure1A shows that atRA exposure of NHKs results in an increase in Id1 mRNA, which was confirmed by Northern analysis (data not shown). Id1 was expressed at higher levels than the other Id family members (Fig. 1A and 1B). A significant increase in Id3 was also observed following 6 h atRA exposure; however, this increase was gone by 24 h of treatment (Fig. 1B). A small, but statistically significant increase is seen in Id2

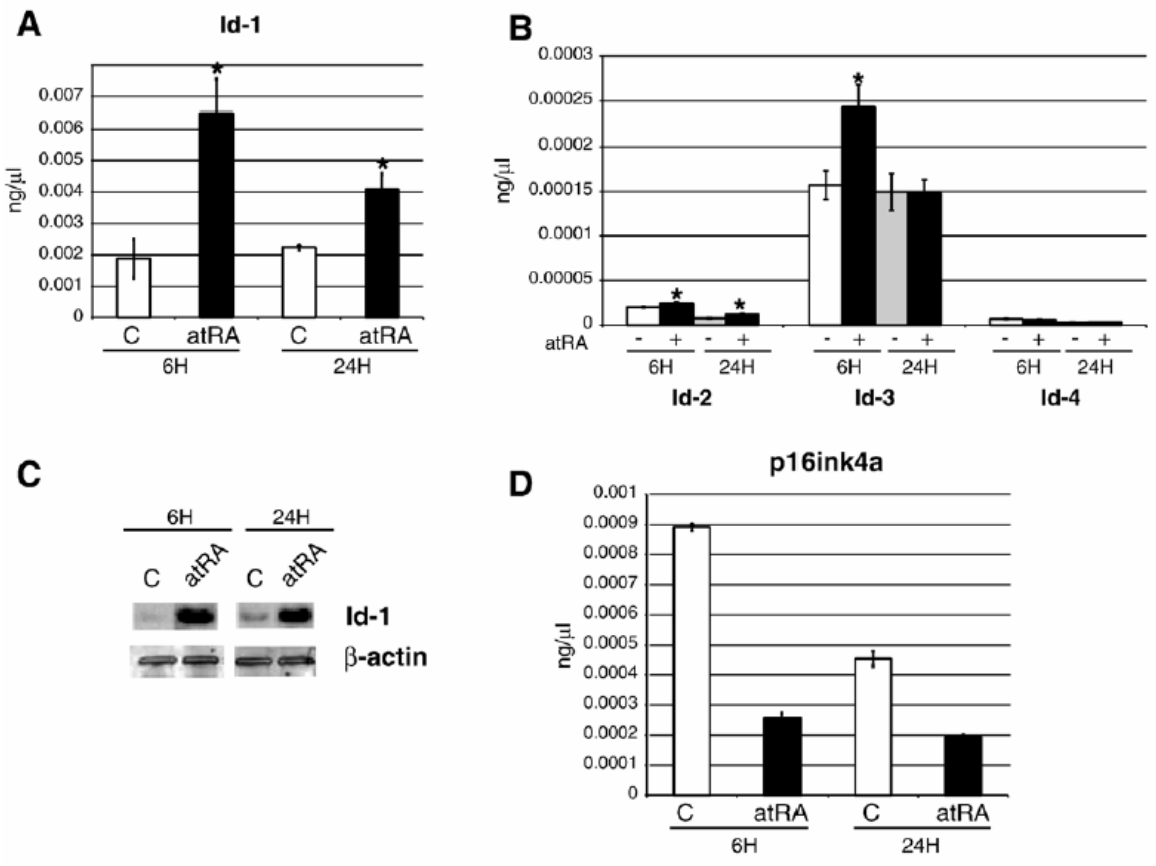


Figure 1. Expression of ID1 is induced by atRA in normal human keratinocytes. Total RNA was isolated from keratinocytes cultured in media containing atRA (10^{-6}M) or vehicle (Me_2SO) for 6 and 24h and was used as template for quantitative RT-PCR using primers specific for human Id1 (A), Id2, Id3, Id4 (B), and GAPDH as shown in Table 1. PCR products were detected using SYBR green as a fluorogenic marker and an iCYCLERQ (BioRad). Data were quantified using a standard curve generated using specific PCR products at concentrations ranging from100 ng to 1 pg . Data were normalized to GAPDH to control for total RNA concentration. Data were plotted as concentration of template after normalization to total RNA concentration by GAPDH. PCR reactions were performed in triplicate and data are representative of three separate experiments. Error bars denote standard deviation. (C) Whole cell protein extracts were isolated from the keratinocyte cultures for use in western analysis. Ten µg of protein was separated on a NuPAGE Bis-Tris acrylamide gel (4-12%) (Invitrogen) under reducing conditions. Separated proteins were transferred onto Hybond-ECL membrane (Amersham). Id1 protein was detected using a polyclonal antibody (c-20; Santa Cruz Biotechnology) or an antibody to β-actin (Sigma) and visualized using chemiluminescence. Blots were stained with Ponceau S prior to western analysis to visualize total protein loading and quality of transfer. (D) Expression of $p16^{ink4a}$ following atRA exposure was determined using quantitative RT-PCR as described in (A). Primers specific to $p16^{ink4a}$ are shown in Table 1. Error bars denote standard deviation. Statistical significance, determined by students t-test, is denoted by (*) ($p<0.05$).

expression. No increase was seen in expression of Id4. To confirm that the atRA induction of Id1 mRNA results in an increase in Id1 protein, total protein was isolated from NHKs that had been cultured in media containing atRA or Me_2SO for 6 and 24h. Western analysis was performed using a polyclonal antibody to Id1 (c-20, Santa Cruz Biotechnology) (Fig. 1C). Data demonstrate that the atRA induced increase in Id1 mRNA results in an increase in Id1 protein. Both atRA treatment and Id1 overexpression result in repression of the cell cycle control gene p16ink4a in a variety of cell types (Alani et al., 2001; Alisi et al., 2003). Our data confirm that atRA treatment results in a significant down regulation of p16ink4a in NHK (Fig. 1D).

ID1 mRNA stability is not altered by atRA exposure in NHKs.

To determine whether the atRA induced increase in Id1 expression results from changes in mRNA stability, we co-treated the cells with the transcriptional inhibitor actinomycin D. NHKs were cultured in media containing atRA or vehicle control for 6 h. At this time the cultures were washed and serum-free media containing actinomycin D (5μg/ml; Sigma) was added to the cultures. mRNA was isolated at 3, 6 and 18 h post actinomycin D treatment (Fig. 2A). Id1 mRNA stability in vehicle-treated cultures was transient, confirming data from other laboratories; no Id1 message was observed by 3h post actinomycin D exposure. No change in Id1 mRNA stability was observed in NHKs that had been exposed to atRA for 6h. These data indicate that atRA induction of Id1 mRNA does not involve an increase in Id1 mRNA stability.

To determine whether atRA-induced Id1 mRNA is a result of increased transcription, we examined the expression of Id1 heteronuclear RNA (hnRNA). Levels

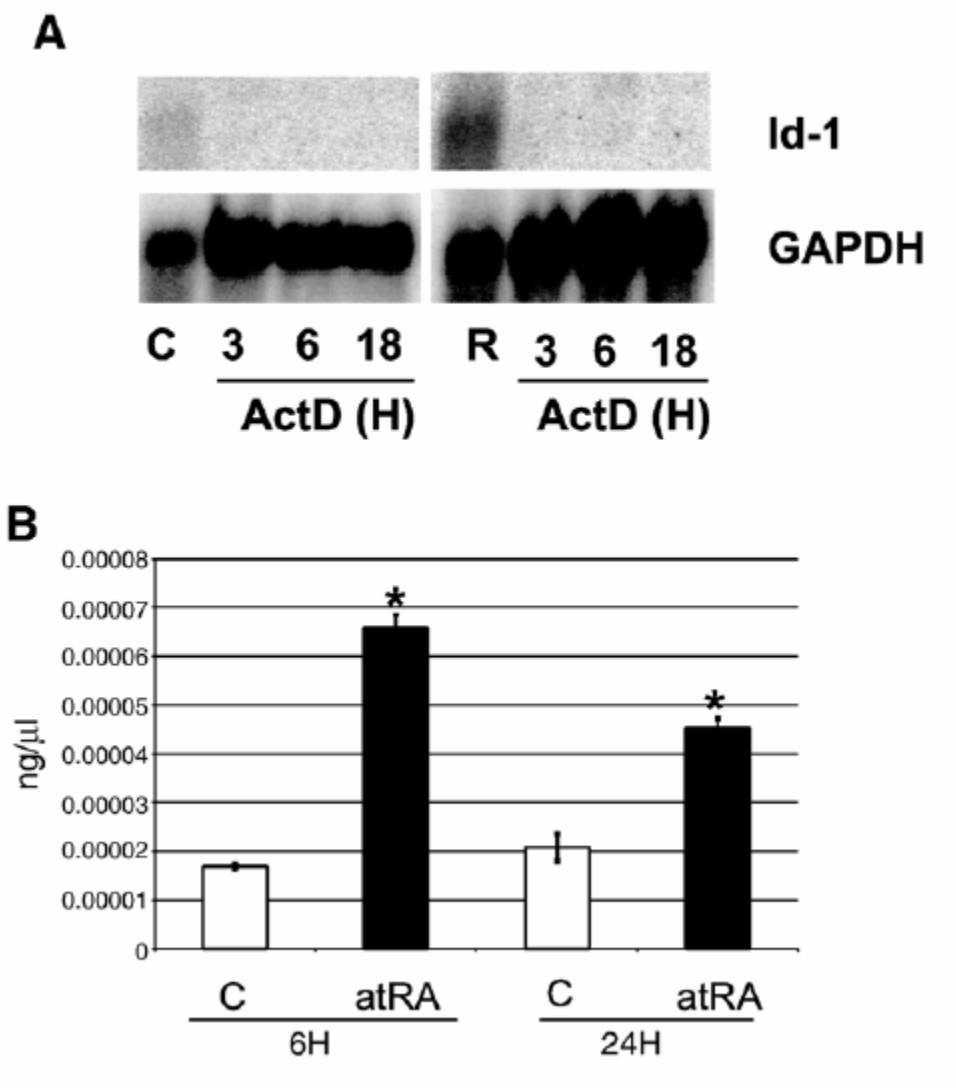


Figure 2. Stability of the Id1 mRNA is not altered following exposure to atRA. (A) NHKs were cultured in media containing atRA (10^{-6}M) or vehicle (Me_2SO) for 6 h. At this time, the treatment media was removed, the cultures washed in PBS, and media containing actinomycin D (5µg/ml) was added to the cultures. Total RNA was isolated and used in quantitative RT-PCR reactions as described above. (B) Quantification of Id1 heteronuclear RNA (hnRNA) was determined using quantitative PCR and primers specific to unspliced Id1 sequence (Table 1). Statistical significance, determined by students t-test, is denoted by (*) ($p<0.05$).

of hnRNA are indicative of transcription when effects of stability have been eliminated (Delany, 2000). No changes in Id1 mRNA stability were observed using either actinomycin D as shown in Fig. 2A, or DRB (data not shown). However, a significant increase in Id1 hnRNA was observed following atRA exposure of NHKs (Fig. 2B). These data, taken together, suggest that atRA-induced expression of Id1 is a result of increased transcription.

atRA induced expression of Id1 is independent of de novo synthesis of a mediator.

To determine whether atRA-induced expression of Id1 involves the *de novo* synthesis of a protein mediator, NHKs were co-treated with atRA and the protein synthesis inhibitor cycloheximide (CHX). Although both basal and atRA-induced levels were lowered in cultures exposed to CHX, atRA-induced Id1 mRNA was still observed (Fig. 3). This indicates that atRA-stimulation of Id1 does not require the synthesis of an intermediary protein.

atRA induced transcriptional activity of the Id1 promoter in NHKs.

Serum and growth factor induced expression of Id1 is mediated through several elements in the promoter, including several SMAD binding sites in the distal promoter, as well as a cluster of elements in the proximal promoter (Fig. 4A**)** (Korchynskyi and ten Dijke, 2002; Lopez-Rovira et al., 2002; Singh et al., 2002)**.** To identify the atRA responsive region of the Id1 promoter, we generated a deletional series of Id1 promoter sequences linked to firefly luciferase, ranging from 2.2 kb to 140 bp of the Id1 promoter (Fig. 4A). Constitutive promoter activity varied between the deletional series, as seen in other cell types (Korchynskyi and ten Dijke, 2002; Lopez-Rovira et al., 2002; Singh et al., 2002), a result of loss of positive and negative cis-acting elements along the sequence.

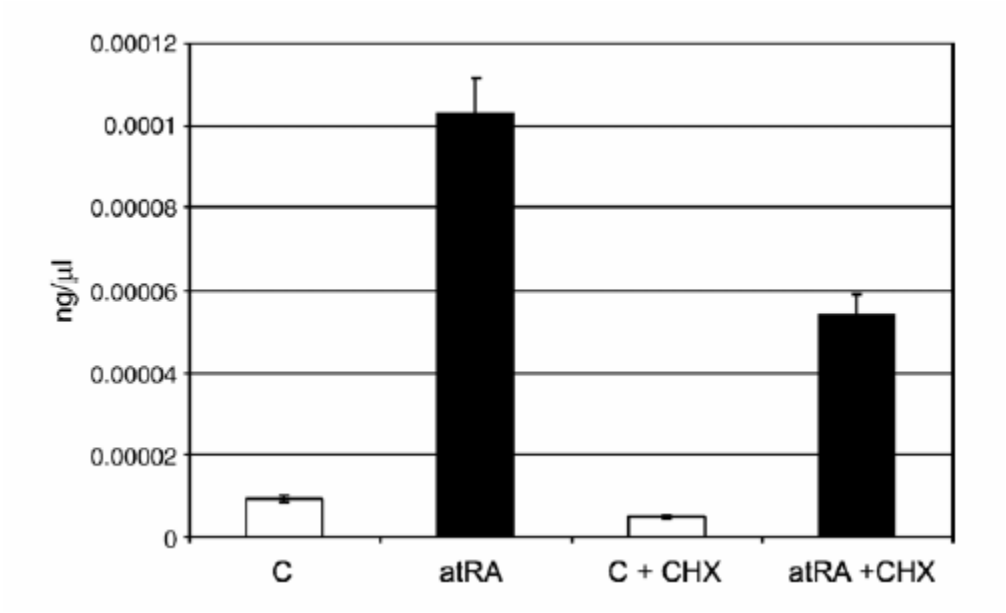


Figure 3. at-RA induced expression of Id1 is independent of *de novo* protein synthesis. Total RNA was isolated from NHKs cultured in media containing atRA (10^{-6}M), cycloheximide (CHX;75 µM), atRA + CHX or vehicle (Me_2SO) for 6 h and was used as template for quantitative RT-PCR using primers specific for human Id1, Id3 and GAPDH. Quantitative RT-PCR was performed as described in Fig. 1. PCR reactions were performed in triplicate and data are representative of three separate experiments. Error bars denote standard deviation.

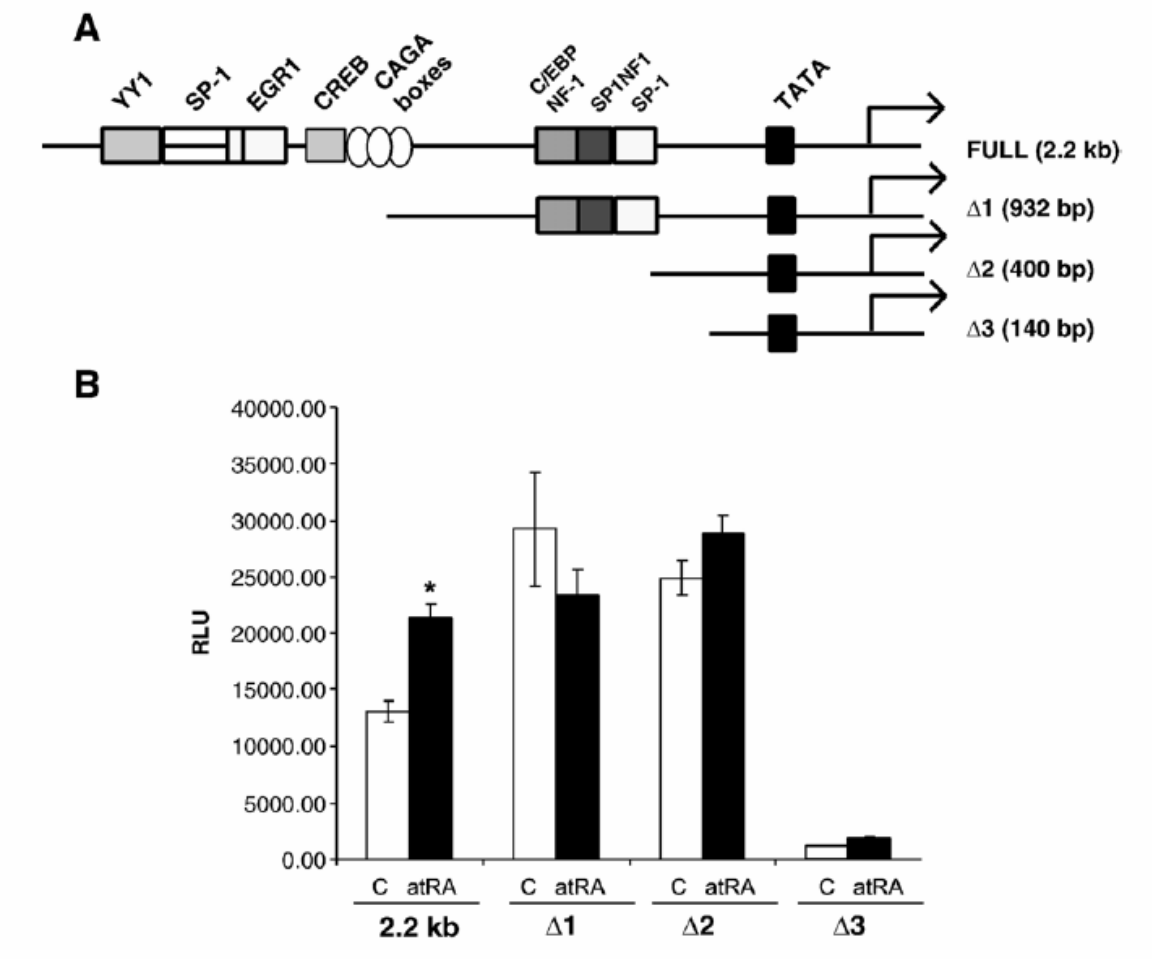


Figure 4. atRA induces Id1 promoter activity. (A) Constructs containing 2200 to 140 bp of the human ID1 promoter linked to the luciferase gene (pGL3; Promega) were used in transient transfection assays. (B) The ID1/luciferase plasmids were transfected into normal human keratinocytes. Twenty-four hours following transfection, cultures were treated in serum-free media containing vehicle (Me_2SO) or atRA (10^{-6}M). Cell lysates were harvested 6h after treatment and luciferase assays performed using the Promega Dual Luciferase Kit and detected on a Dynex luminometer plate reader. Data are presented as RLU (relative light units). All treatments and transfections were performed in groups of six, and normalized to total protein. Data shown are representative of three separate transfections. Statistical significance was determined by the paired Students t-test (*) ($p<0.05$).

These luciferase constructs were transiently transfected into NHK, and examined for responsiveness to atRA. The largest construct (2.2kb), demonstrated a significant response to atRA. However, loss of 1.2 kb from 2.2 to 932 bp, resulted in loss of atRA induction. This indicates that sequences in the distal region of the promoter are necessary for atRA-induced Id1 expression. This area contains many known sites, including several CAGA boxes (SMAD binding sites), a binding site for CREB, and a cluster containing a YY1 site, a SP-1 element and a Egr1 site.

Involvement of BMP2/4 in atRA induced Id1 expression

In other cell types, Id1 expression is mediated through the bone morphogenic proteins (BMPs), through activation of the SMAD sites in the distal promoter (Hollnagel et al., 1999; Clement et al., 2000; Lopez-Rovira et al., 2002; Valdimarsdottir et al., 2002). Furthermore, data indicates that atRA induces the BMP pathway and SMAD binding (Rogers et al., 1992; Hatakeyama et al., 1996; Dupe et al., 1999; Weston et al., 2000). To investigate the potential involvement of BMPs in atRA-induced Id1 expression in NHKs, we exposed NHKs to atRA or to recombinant BMP2. RNA was isolated at 3, 6, and 24 h post treatment. Id1 expression was induced by both agents, however the time course indicates that BMPs are not likely to be an intermediary for atRA signaling. atRA-induced Id1 expression was observed as early as 3 h and was maintained through 24 h (Fig. 5A). BMP2 induced Id1 expression was not evident until 6h, and appeared to be gone by 24 h. Furthermore, no observable increase in expression of either BMP2 or BMP4 was seen in NHKs following atRA exposure (Fig. 5B). Indeed, a decrease was seen in both BMPs 24 h post exposure. Western analysis confirmed the findings shown

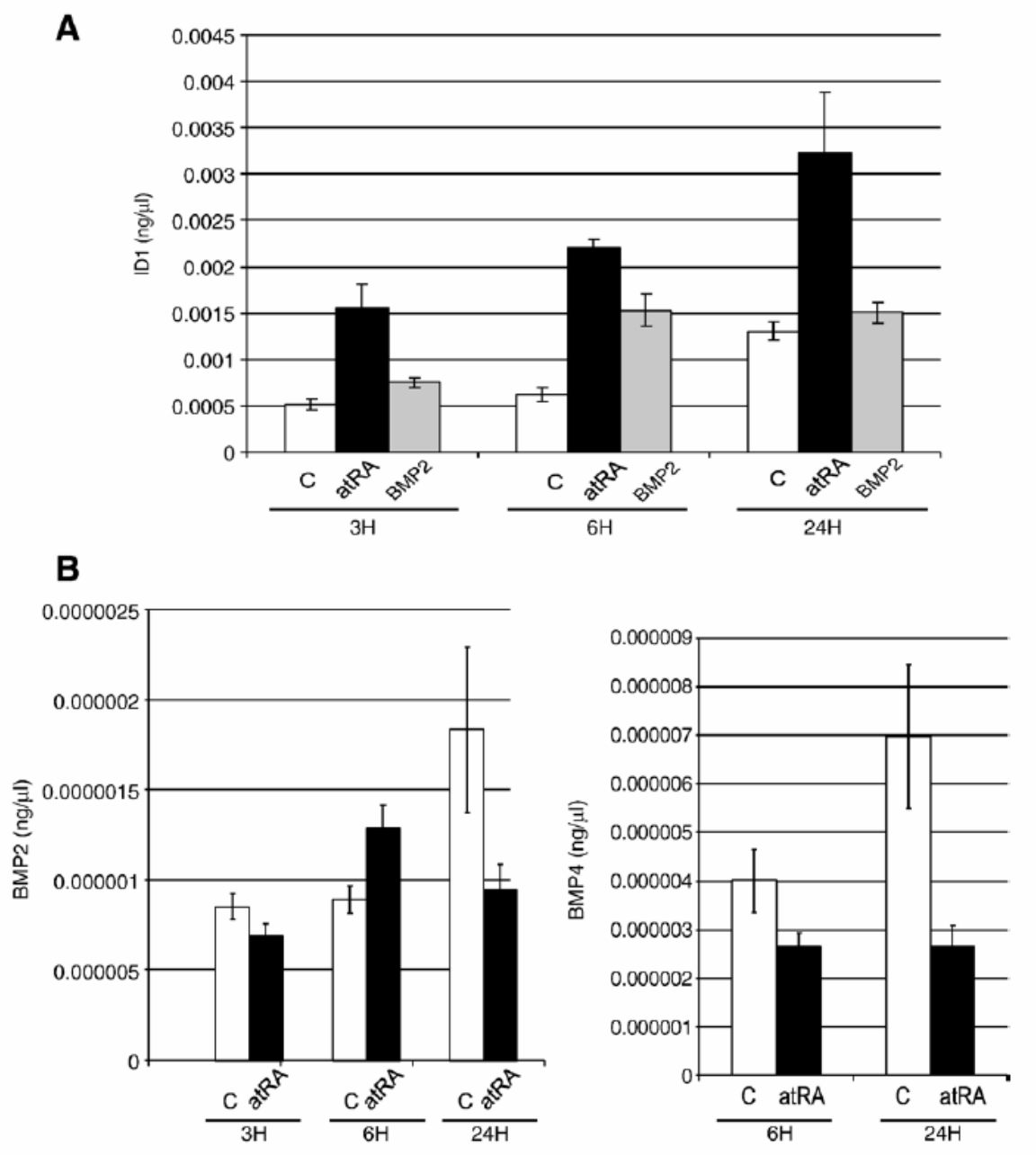


Figure 5. BMP involvement in atRA-induced Id1 expression. (A) NHKs were exposed to atRA or recombinant BMP2 (Research Diagnostics, Inc.) for 3, 6 or 24 h. Total RNA was isolated and used to generate cDNA template for quantitative PCR using primers shown in Table 1. (B) Expression of BMP2 and BMP4 was examined in RNA isolated from NHKs treated with at RA for 3, 6 or 24 h. Primers used to amplify BMP2 and BMP4 are shown in Table 1.

for the RNA (data not shown). These data, taken together, indicate that the BMPs are not likely to be involved in atRA-induction of Id expression.

Retinoic acid induces binding to several sites in the distal region of the Id1 promoter.

To determine potential elements mediating atRA-induced Id1 expression, electrophoretic mobility shift assays (EMSA) were performed using segments of the promoter with identified elements. The first region (-959bp to –919 bp) depicted in Fig. 6, contains a YY1 element, an SP-1 site, and an Egr1 sequence. In addition, a GC-rich region on the 3' portion of this region has been shown to have some activity in Id1 expression(Korchynskyi and ten Dijke, 2002). Nulcear extracts prepared from cultures that were exposed to vehicle or atRA were used to bind to the region, and to several oligonucleotides in which specific elements had been altered to eliminate binding (Fig. 6A). Binding reactions containing the wild-type oligonucleotide demonstrated specific binding, which was increased upon treatment with atRA (Fig. 6B). Alterations in the SP-1 site and the GC-rich region do not appear to alter atRA-induced binding to the oligonucleotide. The two-bp change in the Egr1 element slightly reduces atRA-induced binding. However, the removal of the YY1 element results in a significant reduction in atRA induced binding. This data demonstrate that atRA does induce changes in binding of factors to this region of the Id1 promoter, and suggest that the YY1 element and perhaps the Egr1 site are contributing to this activity.

A second region of interest spans from –899 to –826 bp of the Id1 distal promoter, and contains a CREB binding site and three SMAD binding sites (Fig. 7A). Binding

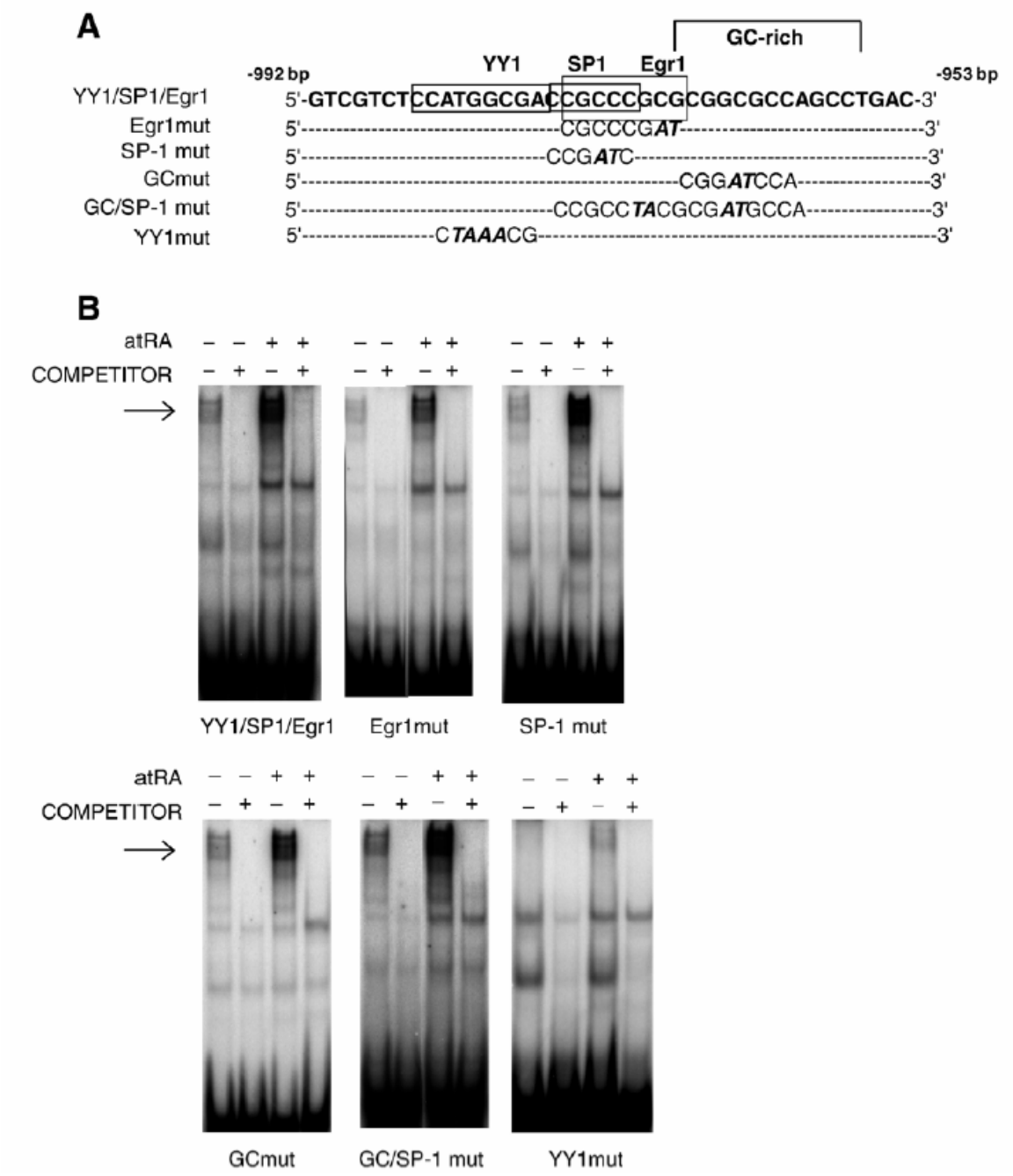


Figure 6. Retinoic Acid treatment increases binding to oligonucleotide containing YY1, SP-1 and Egr1 elements. (A) Sequences of oligonucleotides used in eletrophoretic mobility shift assays (EMSA). (B) Nuclear extracts were isolated from keratinocyte cultures following 2h exposure to atRA (10^{-6}M) or vehicle Me_2SO. P^{32}-labeled probes were used in binding reactions with or without 100-fold excess of self oligonucleotide or 100-fold excess of non-specific oligonucleotide. Specific complexes are indicated by the arrows.

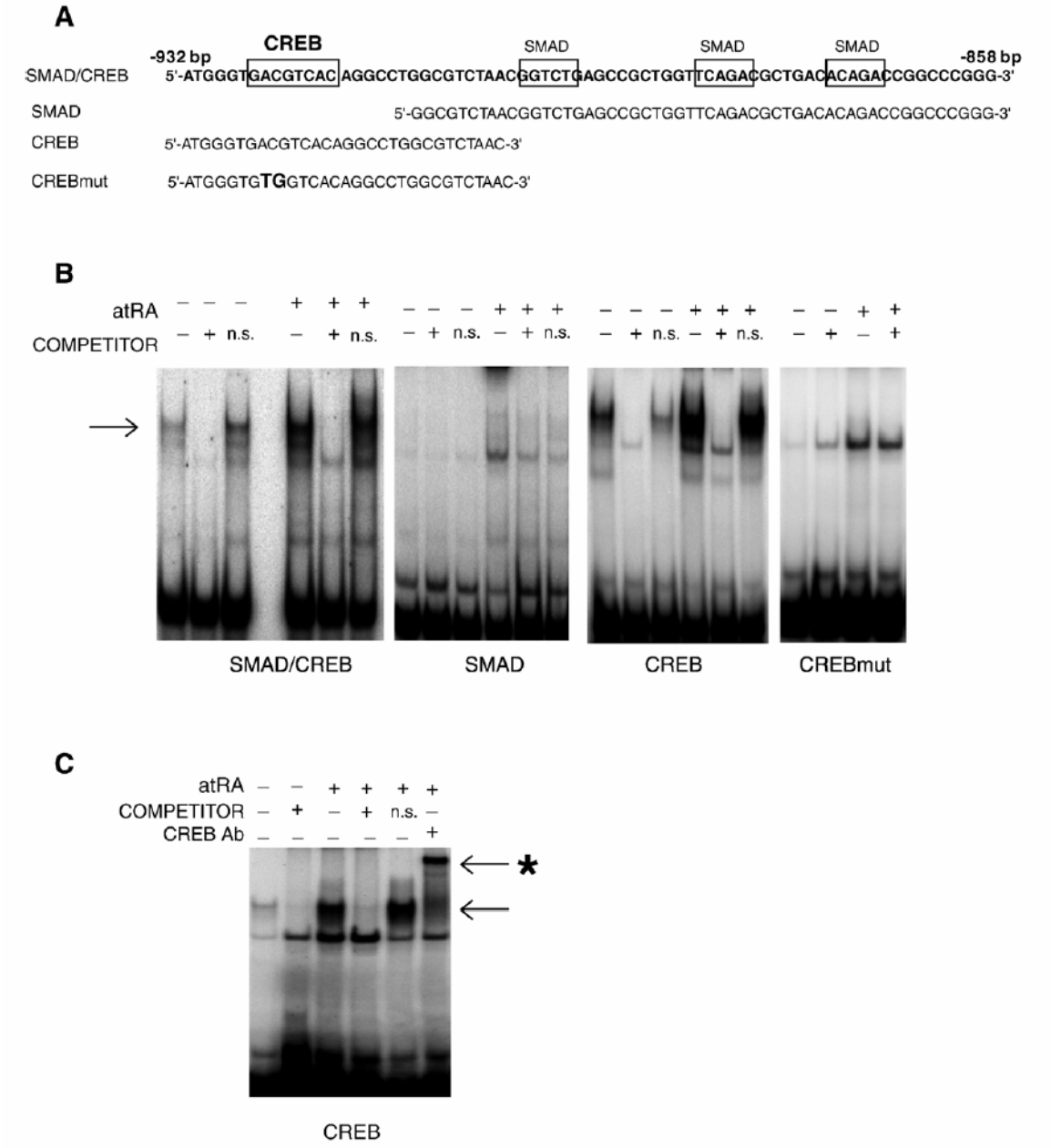


Figure 7. atRA exposure increases CREB binding to the an element at –894 bp in the Id1 promoter. (A) Sequences of oligonucleotides used in EMSA. (B) Nuclear extracts were isolated from keratinocyte cultures following 2h exposure to atRA (10^{-6}M) or vehicle Me_2SO. P^{32}-labeled probes were used in binding reactions with or without 100-fold excess of self oligonucleotide or 100-fold excess of non-specific (n.s.) oligonucleotide. Specific complexes are indicated by the arrows. (C) Antibody specific to CREB was incubated with nuclear extracts prior to binding to probe. Supershifted complex is denoted by the arrow (*).

reactions using probes that span the entire region demonstrate specific binding that is increased in extracts from cultures treated with atRA (Fig. 7B). Oligonucleotide probes containing only the three SMAD sites do not demonstrate any increase in binding from atRA treatment. However, probes containing the CREB element demonstrate an increase in binding following atRA exposure. Mutation of the CREB site results in a loss of the binding from extracts isolated from atRA treated cultures. To confirm CREB binding to this site, CREB specific antibodies were added to the binding reactions. Addition of the CREB specific antibody results in a higher molecular weight complex, indicating that the CREB protein is involved in binding to this site (Fig. 7C). These data, along with the data in Fig. 6, demonstrate that binding to several elements in this responsive region is increased following atRA treatment, and suggests that atRA induced Id1 expression may be mediated through multiple elements in the promoter.

DISCUSSION

The goal of the experiments presented in this paper was to demonstrate that the Id proteins are targets of the atRA signaling pathway in NHKs. This pathway is involved in the regulation of embryogenesis, reproduction, proliferation and differentiation, as well as of epithelial differentiation (Fisher et al., 1995; Fisher and Voorhees, 1996). Recent reports demonstrate that atRA treatment of other cell types induces Id expression (Ma et al., 2003; Zhang and Rosdahl, 2005). Id1, -2 and -3 are all expressed in the basal keratinocytes in normal human epidermis, though some differences are observed in their cell-type and subcellular localization. Importantly, data from other laboratories indicate

that the Id proteins have profound effects on keratinocyte proliferation, both in animal models and in culture.

Our data show that basal expression of Id1 in NHKs is higher than the other three Id family members, with Id-3 being the second highest. In addition, expression of both Id1 and -3 is increased following atRA exposure. Basal levels of Id-2 and -4 were low but detectable; however, no increase following atRA exposure was observed. atRA-induced Id1 and -3 expression are observed by 3h following exposure and this increase does not require *de novo* synthesis of a mediator. Taken together, these data suggest that Id1 and Id3 are direct targets of the RA signaling pathway.

atRA-induced expression of Id1 in NHKs appears to be primarily transcriptional. Treatment with the transcriptional inhibitor actinomycinD demonstrates no change in Id1 mRNA stability following atRA exposure. Further, increased levels of Id1 heteronuclear RNA (hnRNA) are seen following atRA treatment. Taken together, these data suggest that atRA induced Id1 mRNA is mediated by transcriptional activation. Transient transfection of a luciferase construct containing 2.2 kb of the human Id1 promoter demonstrates that atRA is able to induce activity of the Id1 promoter, supporting the hnRNA data.

A variety of cis-acting elements involved in cytokine and growth factor induced expression have been identified in the Id1 promoter. One of particular interest is the SMAD binding sites that mediate expression of Id1 by the bone morphogenic proteins (BMPs). Other reports have demonstrated that atRA induced expression of BMPs and SMAD binding, suggesting the SMAD binding sites in the Id1 promoter were likely targets for atRA induction (Rogers et al., 1992; Hatakeyama et al., 1996; Li et al., 2003).

However, our data do not support this in keratinocytes. First, no induction of BMP2 or 4 mRNA or protein was observed following atRA exposure of NHKs. Furthermore, although recombinant BMP2 was able to induce Id1 expression, the timing differed from that observed with atRA: atRA-induced Id1 expression is observed by 3h post treatment, whereas BMP2 induced Id1 expression is not observed until 6 h post treatment. Although it is possible that the difference in timing is reflective of the concentration or delivery of the BMP2 protein, taken together these data suggest that BMP induced binding to the SMAD elements is not the mechanism of atRA-induced Id1 expression.

atRA-induced Id1 promoter activity was lost using a 932 bp construct that lacks a region containing several cis-acting elements, including several SMAD elements, a CREB binding site, and a region with overlapping Egr1/SP-1/YY1 elements. Mobility shift analysis using oligonucleotides spanning this region demonstrates that atRA exposure induces protein binding to the Egr1/SP-1/YY1 region and to the CREB binding site. No increase in binding was observed using the oligonucleotide containing the SMAD binding sites, supporting our hypothesis that the BMP/SMAD pathway is not involved. To determine which element(s) in the Egr1/SP-1/YY1 oligonucleotide is contributing to atRA-induced binding, alterations were made to eliminate the contribution of each site. These data suggest that both the Egr1 and YY1 sites are contributing to the binding in this region. The Egr1 (early growth response gene) is a zinc finger transcription factor essential for growth proliferation and differentiation (Thiel and Cibelli, 2002) , and is induced by retinoic acid exposure in a variety of cell types including those of human skin (Larsen et al., 1994). The YY1 (Yin Yang 1) elements can mediate either activation or inhibition of transcription depending on promoter context.

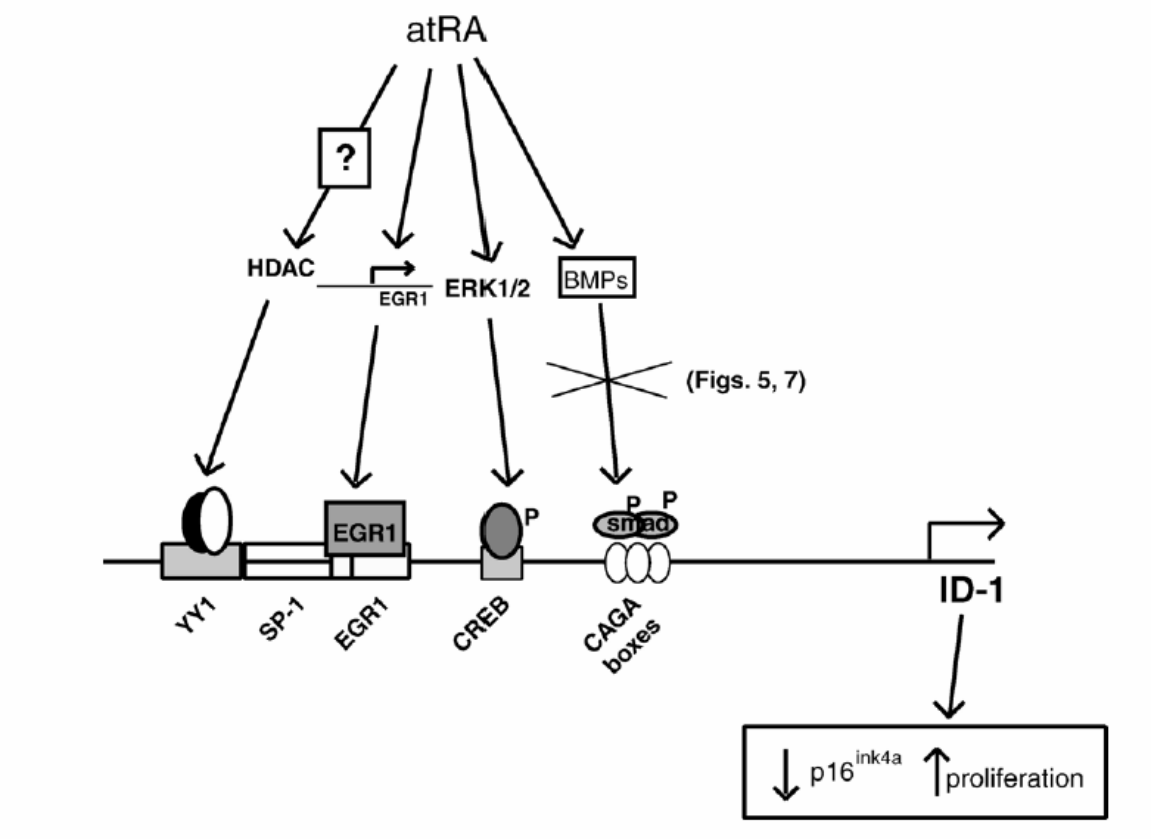


Figure 8. atRA induced Id1 expression involves several cis-acting elements in the 5' regulatory region. Our data indicate that binding to several cis-acting elements may influence atRA-induced Id1 expression. Specific binding activity to the YY1 element is increased in extracts from NHKs treated with atRA (Fig. 6). YY1 is a constitutively expressed transcription factor which has been shown to be both a positive and a negative regulator of gene expression (Shi et al., 1997). No link between YY1 and atRA has been shown. Binding activity to the Egr1 site was also increased following atRA exposure (Fig. 6). Egr1 is a zinc finger transcription factor and its expression is transcriptionally induced by retinoic acid exposure in a variety of cell types including those of human skin (Larsen et al., 1994). Binding to the CREB site was also higher in extracts isolated from atRA treated NHKs (Fig. 7). CREB protein is regulated by phosphorylation at serine residue (ser133), and atRA exposure has been shown to induce rapid phosphorylation of CREB in neuronal cell types (Canon et al., 2004). atRA also induces expression of the BMPs which regulate transcription through SMAD elements. However, our data do not support a role for this pathway in atRA regulation of Id1 (Fig. 5, 7).

YY1 is a constitutively expressed member of the GLI-krüppel family of zinc-finger transcription factors which is involved in the regulation of a diverse array of cellular and transcriptional machinery (Guo et al., 2001; Riquet et al., 2001; Patel and Dressler, 2004; Xu et al., 2004; Alvarez-Salas et al., 2005).

Our data also indicate the CREB binding element is involved in atRA induced Id1 expression. CREB, cAMP response-element binding protein, mediates changes in gene expression induced by the second messenger cyclic adenosine 3',5'-monophosphate (cAMP) (Johannessen et al., 2004). CREB protein is regulated by phosphorylation at serine residue (ser133), and atRA exposure has been shown to induce rapid phosphorylation of CREB in neuronal cell types (Canon et al., 2004). Our data show that increased specific binding to an oligonucleotide containing the CREB site is seen in reactions using extracts from atRA treated keratinocytes, and mutation of the CREB binding site eliminates binding to this oligonucleotide. Furthermore, incubation of extracts with antibody specific to CREB-1 results in a distinct supershifted complex, indicating that CREB proteins are binding to this site. These data suggest that atRA induced Id1 expression is mediated through the interactions of multiple elements in the distal part of the promoter. However, the relative contributions of these elements are unclear. Future experiments will focus on the mechanism of atRA induced Id expression, including the contributions of these elements and the proteins that are binding to them.

Mounting evidence indicates that the Id proteins are critical mediators of cell differentiation in a variety of cell types, including keratinocytes where overexpression leads to delayed senescence in culture. Therefore, it is important to understand how the expression of these proteins is regulated in these cells. Our data provide an important link

between the Id proteins and the retinoic acid signaling pathway. Retinoids are important endogenous mediators of epidermal differentiation, and are extensively used in pharmaceuticals to treat skin disorders, such as psoriasis, in addition to treating non-melanocytic skin cancers. Given the function of Ids, and their link to cell cycle regulation, angiogenesis and tumor promotion, these proteins may play an important role in mediating the effects of atRA and related compounds.

CHAPTER 4:

INHIBITOR OF DNA BINDING-1 IS REQUIRED FOR ZEBRAFISH (*DANIO RERIO*) DEVELOPMENT

INTRODUCTION

The inhibitor of DNA binding (Id) proteins are important regulators of tissue development in the embryo, functioning as dominant negative inhibitors of the basic helix-loop-helix (bHLH) transcription factors (Massari and Murre, 2000). They are capable of binding class I and class II bHLH transcription factors including the E proteins E12 and E47, and MyoD (Benezra et al., 1990; Sun et al., 1991; Loveys et al., 1996). Id proteins also regulate cell cycle progression by promoting G1/S transitions through interactions with the ETS (E26) family of transcription factors (reviewed in (Yordy and Muise-Helmericks, 2000), Rb tumor suppressor proteins and the centrisome (Norton, 2000; Zebedee and Hara, 2001; Hasskarl and Munger, 2002). For example, in normal human keratinocytes, overexpression of Id1 results in immortalization or delayed senescence (Alani et al., 1999; Nickoloff et al., 2000). This constitutive expression of Id1 was associated with increased telomerase activity and inactivation of the $p16^{INK4A}$/retinoblastoma protein (Rb) signaling pathway. Id proteins also interact with members of the ternary complex factor (TCF) subfamily of ETS-domain proteins, such as Elk1 and Sap1, and are part of the immediate-early response following serum induction (Yates et al., 1999).

Due to their impact on cellular proliferation, research on the Id proteins has focused mainly on their role in embryonic development and cancer progression. Id proteins have been detected in a variety of tumor types, including breast cancer,

melanoma, squamous cell carcinoma, and prostate cancer and are used as a prognosis factor, where increased Id1 expression correlates with more aggressive phenotypes (reviewed in (Lasorella et al., 2001). Further evidence implicating the role of Ids in cancer progression is that $Id1^{+/-}$/ $Id3^{-/-}$ mice are unable to support the growth of tumor xenografts due to failure to vascularize the tumor mass (Lyden et al., 1999). The expression of Id1, -2 and -3 overlaps significantly during murine development, while Id4 expression patterns are unique (Jen et al., 1996). Id1-null mice show no obvious abnormalities during development and are viable for at least two years (Yan et al., 1997). Id2 knockout mice exhibit defects in natural killer cell differentiation, lack lymph nodes, and fail to produce lactating mammary glands during pregnancy (Yokota et al., 1999; Mori et al., 2000). Additionally, Id3 knockout mice do not show any obvious phenotypes during development, but do have compromised immunity (Pan et al., 1999). The importance of the Id proteins in development is supported by the findings that Id1/Id3 double knockout mice are nonviable and exhibit reduced body size, cranial hemorrhaging, and small brain size (Lyden et al., 1999). Double and triple Id knockouts show severe cardiac defects leading to embryonic lethality at E13.5 (Fraidenraich et al., 2004). Furthermore, overexpression of Id1 during mouse development inhibits neuronal development (Yokota, 2001). Taken together, these data indicate that Ids are an integral part of cellular signaling and organ development during embryogenesis.

In this chapter, we investigated the effects of the loss of Id1 expression on zebrafish embryogenesis. The zebrafish model system is one of the most widely used animal models for developmental research because of its fecundity and its genetic and physiological similarity to mammals (reviewed in (Grunwald and Eisen, 2002). The

utility of the zebrafish is enhanced by the complete sequencing of the zebrafish genome, and the availability of full-length cDNAs and DNA microarrays for expression analysis. Three homologs to the mammalian Ids have been identified in the zebrafish, including zId1, zId2, and zId3, the homologs to human Id1, Id2 and Id3 (Sawai and Campos-Ortega, 1997; Dickmeis et al., 2002; Chong et al., 2005). The Id1 homolog, previously referred to as Id6, is the main focus of this chapter.

RESULTS

Expression of zebrafish Id1 during normal embryo development.

Zebrafish Id1 begins zygotic expression at 4 hours post fertilization (hpf) and maintains steady expression through 36 hpf (Sawai and Campos-Ortega, 1997). To further characterize the expression of Id1 during normal zebrafish embryogenesis, RNA was collected from embryos at four different time points, 4, 10, 24, and 48 hpf, and used as template for quantitative RT-PCR (Fig. 9). At 4 hpf, low levels of expression are observed, which corresponds to the beginning of zygotic expression, as previously reported (Sawai and Campos-Ortega, 1997). Expression levels increase approximately 25-fold by 10 hpf, corresponding to the end of the gastrula stage and the beginning of the segmentation period where the embryo has reached 100% epiboly and a tail bud has formed. By 24 hpf, the beginning of the pharyngula stage, levels decrease by half and this level of expression is maintained through 48hpf, the long-pec stage. From this data, we conclude that zebrafish Id1 expression occurs throughout zebrafish development. This observation suggests that Id1 expression is necessary for normal embryogenesis and that knockdown of Id1 will likely affect this process.

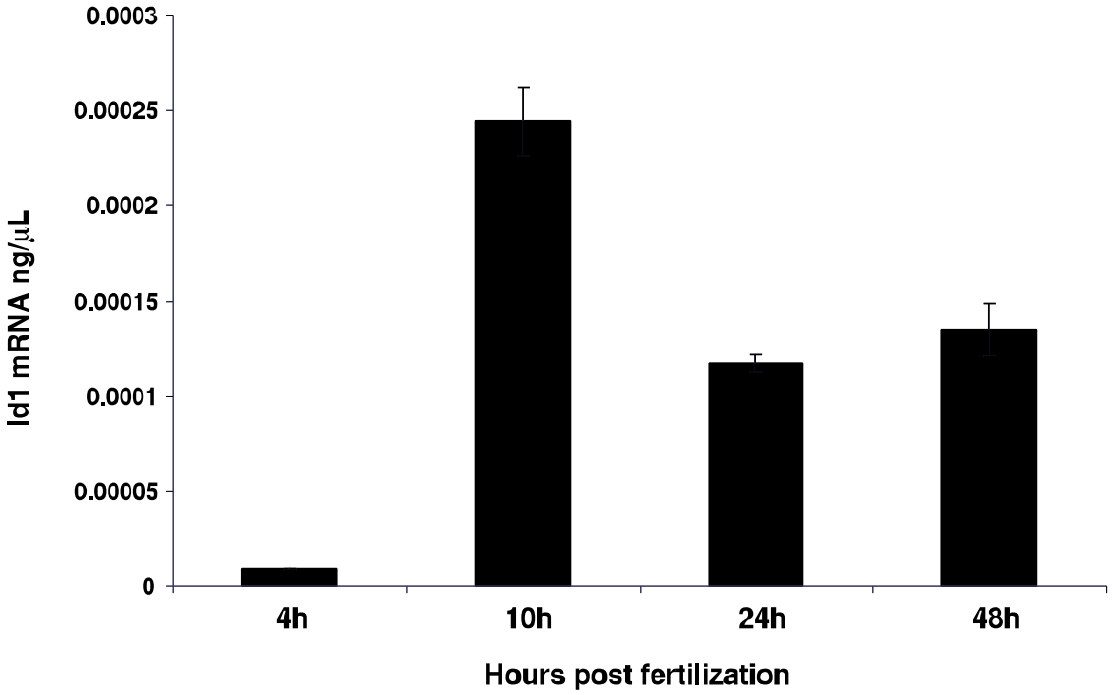


Figure 9. Zebrafish Id1 expression occurs throughout zebrafish development. Total RNA was isolated from developing zebrafish at 4, 10, 24, and 48 hpf and used as template for quantitative RT-PCR using primers specific for zebrafish Id1 and 28S rRNA. Data were quantified by utilizing a standard curve generated using specific PCR products at concentrations ranging from 100pg to 0.1pg. Data were normalized to 28S rRNA. PCR reactions were performed in triplicate. Data are representative of three separate experiments.

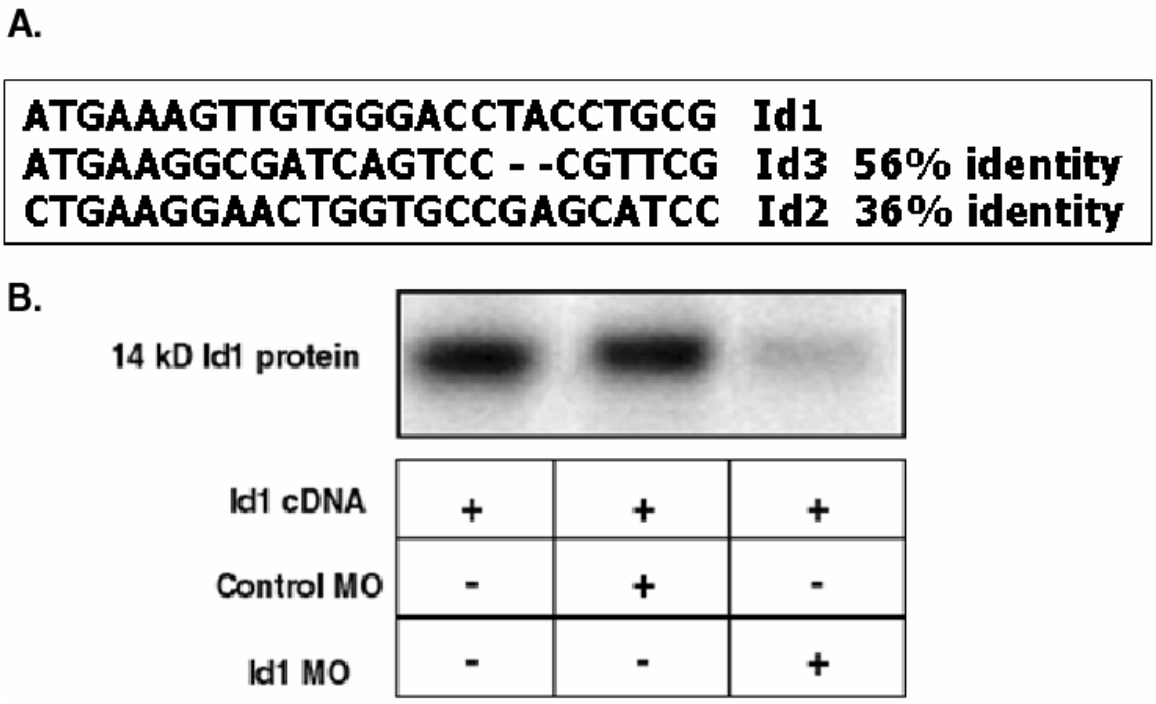


Figure 10. Verification of Id1 MO specificity and effect on *in vitro* translation.
(A) Sequence alignment of zebrafish Id1, 2, and 3 in the region which the morpholino binds to shows that there is low homology between the three genes. **B)** To demonstrate that the Id1-MO is able to block translation of the Id1 protein, the Id1 (cb108) cDNA was transcribed and translated using the High Yield Coupled *in vitro* transcription/translation kit (Promega). The reaction was performed with 2 μg of cDNA alone (Lane 1), cDNA plus 10 μM control-MO (Lane 2), and cDNA plus 10 μM Id1-MO (Lane 3). All reactions were run in the presence of [^{35}S] methionine. Samples were electrophoresed on a 10% Bis-Tris polyacrylamide gel, and dried gels were exposed overnight on a phosphorimaging screen for detection on a Storm PhosphorImager (Molecular Dynamics).

Verification of Id1 morpholino knockdown.

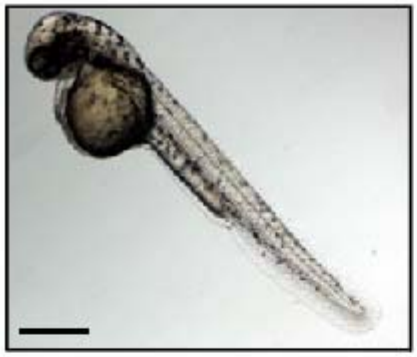
In the zebrafish, sequence specific antisense morpholino oligonucleotides (MO) are used to block protein translation (Nasevicius and Ekker, 2000). For these experiments, we used a sequence-specific morpholino antisense oligo (GeneTools, LLC), complementary to the 5' UTR of the zebrafish Id1 mRNA. To show that the Id1 morpholino is specific for Id1 and will not bind to Id2 or 3, we aligned the three sequences using the William Pearson's web based LALIGN program to examine the homology in the region that the Id1 morpholino was designed to. Id1 and Id3 show 56% homology, while Id1 and Id2 show only 36% sequence homology (Figure 10A). A 4 or 5 basepair mismatch morpholino is often used to control for specificity of a morpholino (Corey and Abrams, 2001), demonstrating that this difference in sequence is enough to inhibit binding of a target sequence. Our sequence homology results suggest that there are sufficient differences between Id1 and Id2 or Id3 to allow our morpholino to be specific to Id1. Next, to verify that the Id1 morpholino (Id1-MO) was blocking translation of Id1, *in vitro* transcription and translation were performed using the Id1 cDNA (cb108; (Thisse, 2001) obtained from ZFIN as template. Id1 cDNA alone produces a 14kDa protein (Fig. 10B, Lane 1). The addition of Id1-MO results in a significant decrease in Id1 protein (Fig. 10B, Lane 3). However, the addition of control morpholino shows no effect on protein production (Fig. 10B, Lane 2). Taken together, these data demonstrate that the Id1-MO is able to effectively knockdown Id1 protein translation and that the control MO has no effect on translation.

Id1 is required for early embryo development.

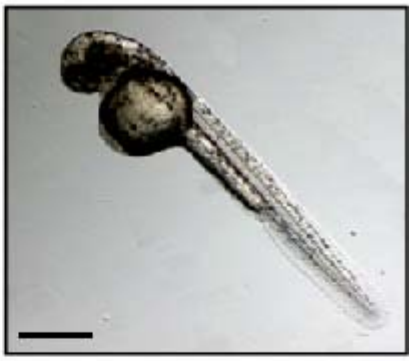
Id1 knockout mice show no abnormal phenotype and are viable (Fraidenraich et al., 2004). However, loss of Id1, Id2, or Id3 in any combination results in severe cardiac abnormalities including ventricular septal defects, thinning of the myocardial wall, and failure of atrium-ventricle separation and is lethal by E13.5 (Lyden et al., 1999; Fraidenraich et al., 2004). To demonstrate the importance of Id1 in normal zebrafish embryogenesis, Id1-MO was used to knockdown protein expression by blocking the translational start site. The Id1-MO was injected at 10, 50,100, and 150 μM in order to establish an effective morpholino concentration. Embryos injected with 10 μM MO (Fig. 11B) exhibit no gross morphological changes and appear identical to those injected with control-MO (Fig. 11A). When the concentration of morpholino is increased to 50 μM, embryos exhibit an abnormal phenotype at 48 hpf consisting of pericardial and yolk sac edema (Fig. 11C), decreased blood flow, and improperly looped hearts (Fig. 12A and B). Only five percent of these embryos die by 24 hpf (Fig. 11F). Additionally, there are some incidences of backflow in the beating heart, suggesting improper valve formation. Twenty percent of embryos injected with 100 μM Id1-MO are dead by 24 hpf (Fig 11F), and of the surviving embryos, approximately 51% exhibit additional phenotypes by 48 hpf including cranial edema and an undulating notochord (Fig. 11D). At higher magnification, the notochord appears intact and fully developed, suggesting that this defect may be caused by improper development of the surrounding muscle or of the

Figure 11. Zebrafish Id1 is required for normal embryo development. Microinjection of Id1-MO into 1- to 2-cell embryos was used to knockdown Id1 protein expression. Representative photographs taken at 48 hpf of embryos injected with either **A.** 100μM control-MO, **B.** 10 μM Id1-MO, **C.** 50 μM Id1-MO, or **D.** 100 μM Id1-MO. **E.** Higher magnification of 100 μM Id1-MO injected embryo showing notochord undulation. **F.** Percent mortality at 24 hpf was recorded for control MO and Id1-MO at 50, 100, and 150 uM concentrations to help determine the effective dose with the lowest occurrence of mortality. Scale bars = 500 μm, except in **E.** scale bar =100 μm.

A.



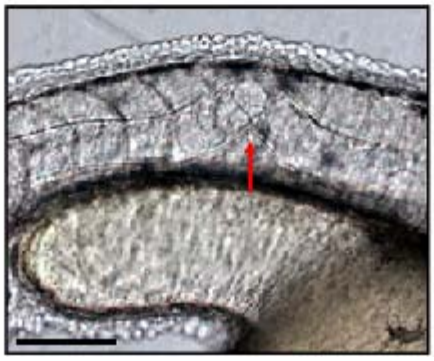
B.



C.

D.

E.



F.

Morpholino	% Mortality by 24 hpf
100μM C	0
50μM Id1	5
100μM Id1	20
150μM Id1	61

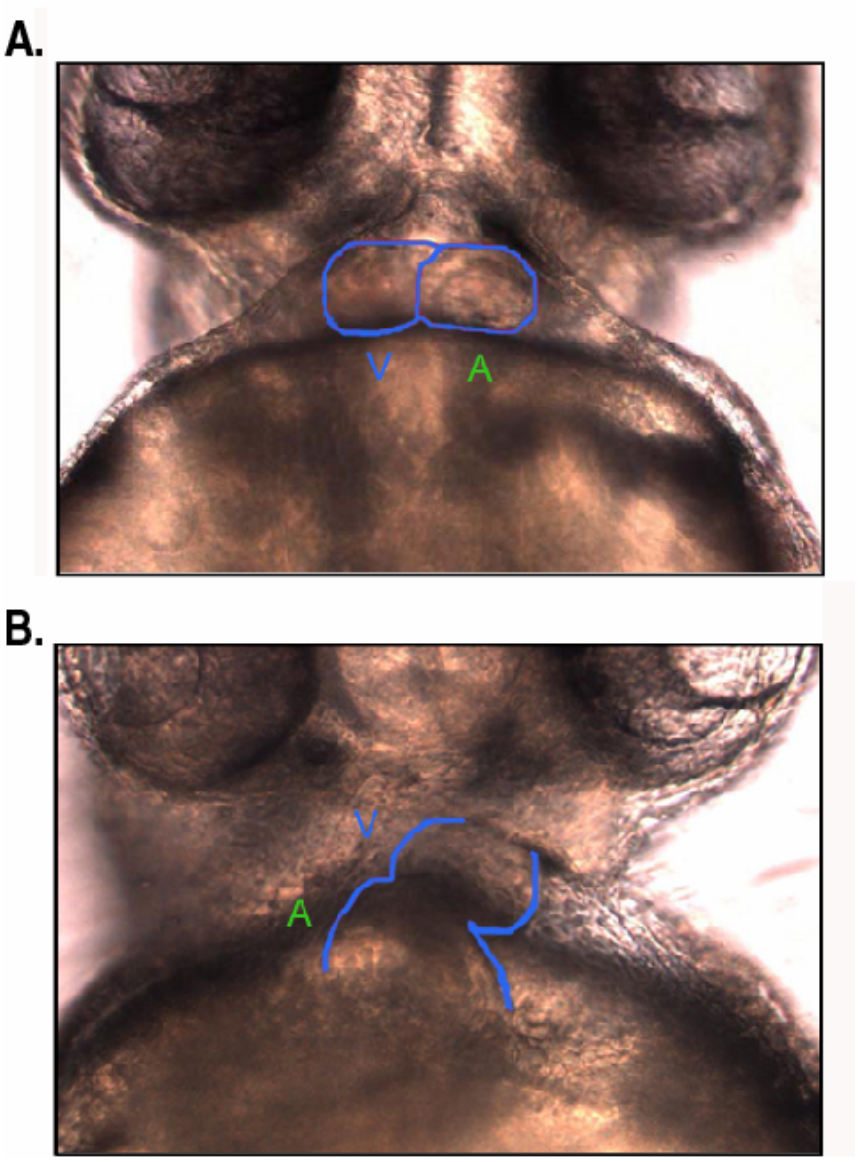


Figure 12. 50 µM Id1-MO injection results in abnormal heart looping. Embryos were manually dechorionated and photographed in 3% methylcellulose. Representative ventral photographs are shown of 48 hpf embryos injected with either **A.** 50 µM control-MO or **B.** 50 µM Id1-MO. Atrium (A) and ventricle (V) are outlined in blue to highlight differences in looping.

notochord sheath, which provides its rigidity (Fig. 11E). At 150 μM, embryos cease to develop and 61% are dead by 24hpf (Fig 11F). A second morpholino to Id1, which differs in sequence, (data not shown) was also used to verify binding specificity. Injections of this morpholino resulted in phenotypes similar to the original Id1-MO. For further experiments, 50 μM Id1-MO was used for injection, as this concentration showed a distinct phenotype without considerable mortality at 24 hpf. These data demonstrate that expression of Id1 is essential for early zebrafish embryo development, and suggest a role for Id1 in cardiovascular and central nervous system development.

Loss of Id1 in early embryogenesis effects larvae development.

To further characterize the effects of Id1 knockdown, Id1 morpholino injected embryos were allowed to develop for one week post fertilization. Although morpholinos are not susceptible to enzymatic degradation, they lose effectiveness through dilution and normal turnover and are most potent in the first few days of development (reviewed in (Heasman, 2002; Sumanas and Larson, 2002). As a result, effects seen at the larval stage of development are most likely a result of early loss of Id1. By day 3, zebrafish have completed embryogenesis and exist as free-swimming larvae. At one week, larvae swim actively and are capable of escape responses and prey capture (Kimmel et al., 1995). For our experiments, larvae were transferred to bowls and fed paramecium twice daily in order to observe feeding behaviors. Interestingly, we observed that Id1 morphants fail to exhibit normal prey capture activity upon feeding and decreased swimming activity. Those larvae that do swim, exhibit erratic spiral swimming patterns, unlike control larvae that show normal swimming behaviors. In addition, compared to control morpholino injected larvae, Id1 morphants have a 20% smaller overall body size (Fig. 13A).

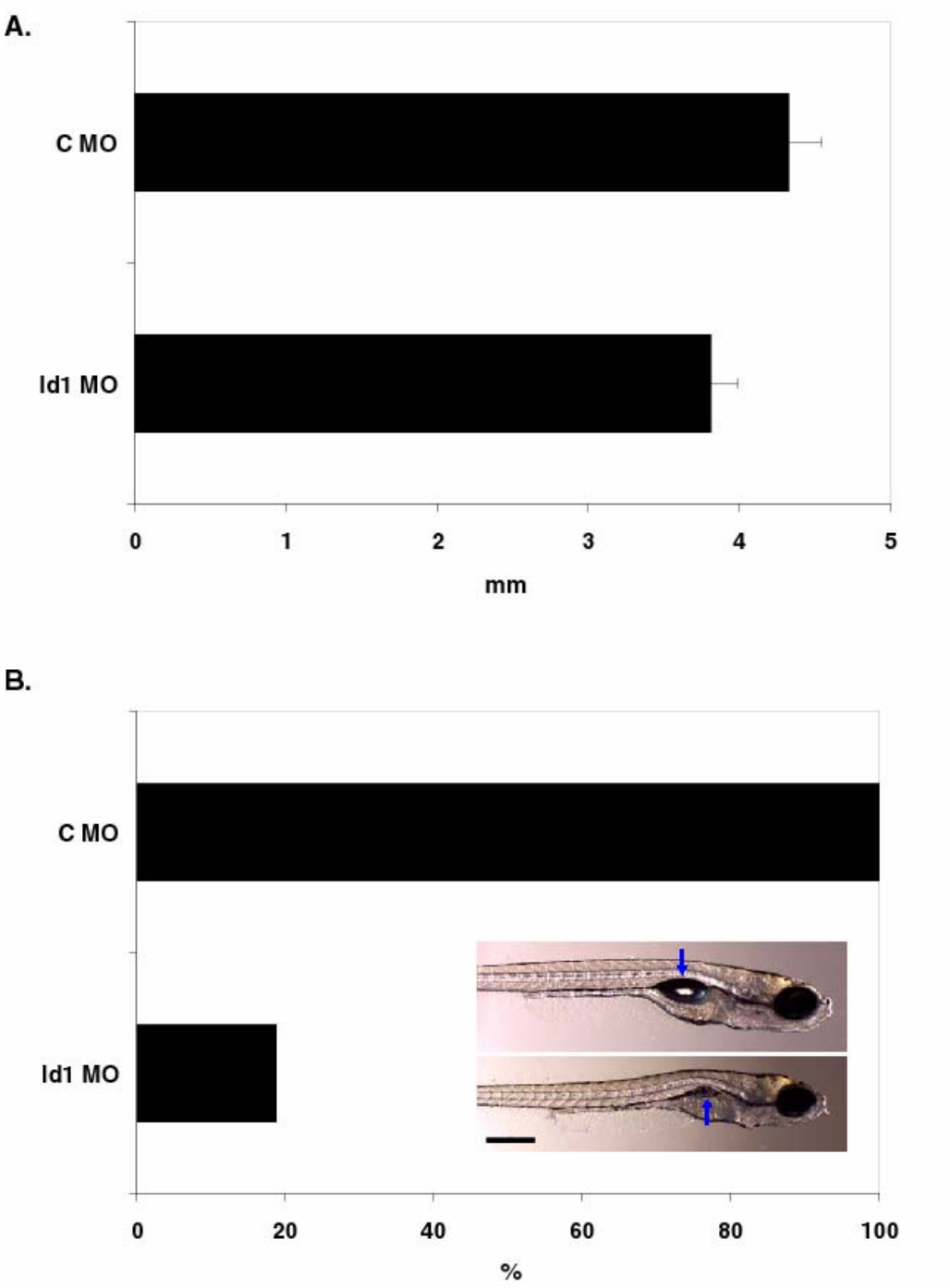


Figure 13. Effect of Id1-MO on larval zebrafish development. Morpholino injected embryos were allowed to develop for one week post fertilization. Control (50 μM) and Id1-MO (50 μM) injected larvae were anesthetized and photographed. Body length (A) was measured using Adobe Photoshop and each larva was scored for swim bladder inflation (B). Scale bar = 500 μm.

Additionally, all of the control-injected embryos had normally inflated swim bladders as compared to only 18% for Id1 MO injected (Fig. 13B).

We also observed differences in the formation of the mouth and jaw in the Id1 morphants. To further examine the differences observed in jaw structure, larvae were collected and fixed at 7 dpf and stained with Alcian blue to visualize cartilage development. Ventral photographs were taken of both control and Id1-MO injected larvae and three measurements were recorded, lower jaw length, ceratohyal angle, and interocular distance. A line was drawn between the posterior edges of the hyosymplectic cartilage and the distance from that line to the Meckel's cartilage was measured as lower jaw length (see Fig. 14A). Additionally, the angle formed by the two sides of the ceratohyal cartilage was measured to quantify changes in migration (see Fig. 14C). Our data show that there was a significant decrease in lower jaw length and an increase in ceratohyal cartilage angle in the Id1 injected larvae as compared to control (Fig. 14B, D). This suggests that the presence of Id1 in early embryogenesis is important for proper growth of the cartilage necessary for normal jaw development.

Interocular distance was measured to determine if Id1 morpholino knockdown resulted in changes in zebrafish neural plate development (see Fig. 14E). The zebrafish eyes are derived from a single field of cells in the anterior neural plate (Varga et al., 1999). Therefore, changes in interocular distance may represent changes in neural plate growth. Id1 expression can be detected in the ectoderm of the neural plate border in the chick (Kee and Bronner-Fraser, 2001) as well as the embryonic ectoderm during murine gastrulation (Jen et al., 1997). In zebrafish injected with 50 μM Id1-MO, no significant difference was observed compared to control embryos (Fig. 14F), suggesting that either

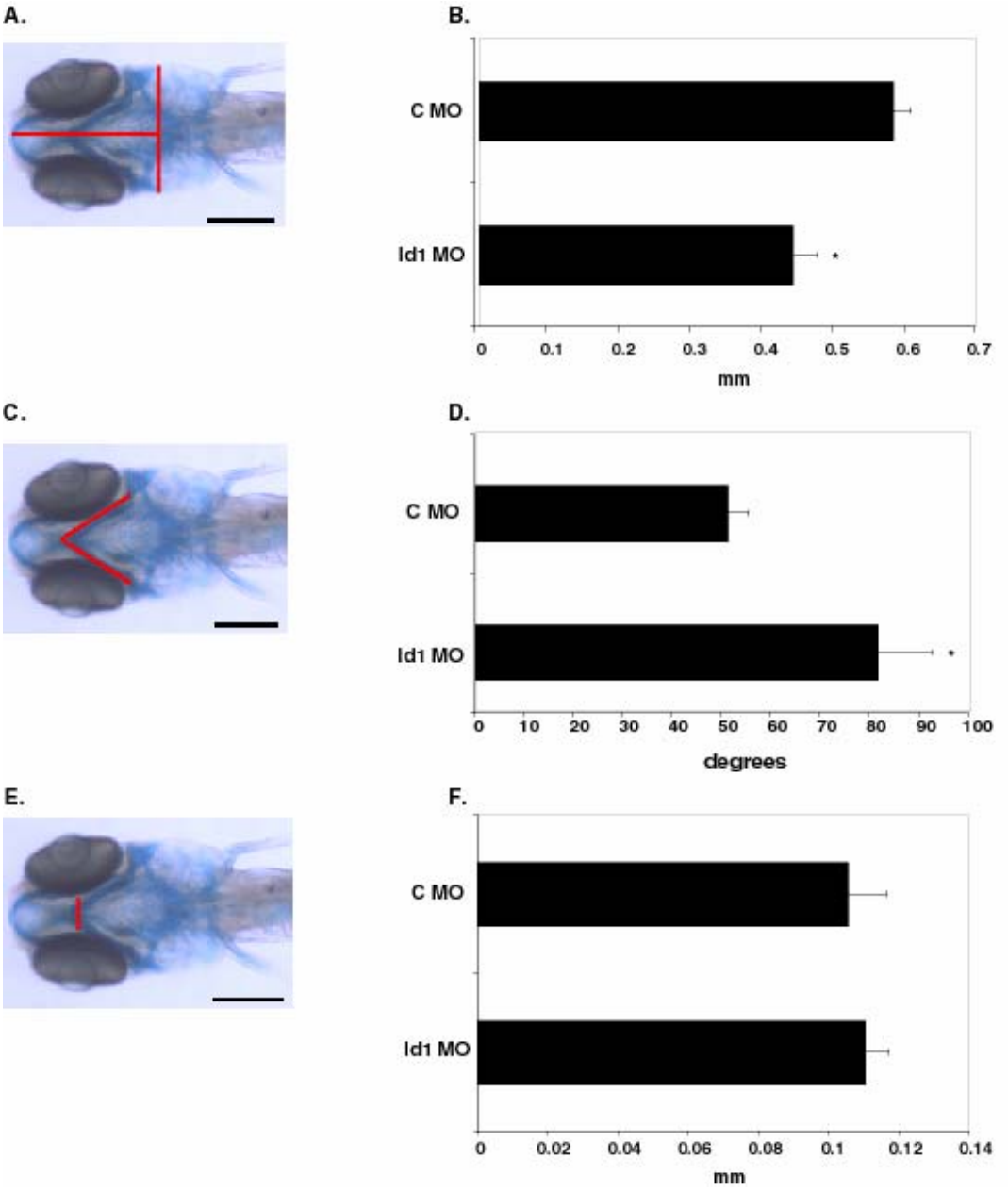


Figure 14. Early loss of Id1 leads to changes in craniofacial development. Larvae were collected and fixed at 7 dpf and stained with Alcian blue to visualize cartilage development. Ventral photographs were taken of both control and Id1-MO injected larvae in 100% glycerol. To measure lower jaw length (A,B), a line was drawn between the bottom edges of the hyosymplectic cartilage and the distance from that line to the Meckel's cartilage was measured. The angle formed by the two sides of the ceratohyal cartilage was measured to quantify changes in ceratohyal growth (C,D). Interocular distance was measured from the inner edges of the eyes (E,F). All measurements were completed in Adobe Photoshop. Scale bar = 500 μm.

Id1 alone may not be required for neural plate development or that higher concentrations of morpholino are required.

DISCUSSION

The data presented in this paper demonstrate that Id1 is expressed throughout zebrafish embryogenesis and that Id1 protein expression is critical for normal embryogenesis. Id1 knockdown results in pericardial and yolk sac edema and defects in heart looping, demonstrating that Id1 is critical for proper heart and vascular development. At higher concentrations of morpholino, embryos also show cranial edema and undulating notochords, implying that Id1 is involved in development of the brain and brain vasculature, as well as the developing nervous system. Furthermore, the loss of Id1 in early embryogenesis results in defects in the later larval stage, including craniofacial abnormalities, lack of swim bladder inflation, and decreased body length. Finally, Id1 MO is able to effectively knockdown Id1 protein, as confirmed by *in vitro* transcription/translation and a second morpholino of different sequence, demonstrating that the observed effects are due to loss of Id1.

Id1 knockout mice show no developmental abnormalities, while Id1/Id3 double knockout mice are nonviable and have collapsed brain vasculature and cranial hemorrhaging, as well as cardiac defects such at outflow tract atresia (Lyden et al., 1999; Fraidenraich et al., 2004). Our current observations are consistent with phenotypes seen in the Id double knockout mouse model, although data presented here suggest that knockdown of Id1 alone is sufficient for the disruption of embryogenesis. This implies a lack of redundancy in the zebrafish Id proteins, which is seen in the mouse and in human cell culture (Pan et al., 1999; Yokota et al., 1999; Sakurai et al., 2004). This difference

between the mouse and zebrafish knockdowns may be a result of the expression pattern differences of the Ids in these two species. In the mouse embryo, Id1, 2, and 3 have overlapping expression patterns in multiple organ tissues including the heart, kidney, pancreas, stomach, and tooth, while Id4 is mainly expressed in neuronal tissue (Jen et al., 1996). Id1 and Id3 are expressed in the endocardial cushion mesenchyme, epicardium, and endocardium, but not in the myocardium (Jen et al., 1996; Fraidenraich et al., 2004). In zebrafish, the expression patterns of the Ids are more distinct (Sawai and Campos-Ortega, 1997; Dickmeis et al., 2002; Chong et al., 2005). Id2 is involved more in later embryo development whereas Id1 is highly expressed in early stages of development. This suggests a requirement for Id1 in early differentiation processes and lineage commitment, which is supported by our data. Although Id3 is also detected in early embryonic stages, its expression is more restricted with prominent expression seen in the eye and brain. To date, no zebrafish Id4 has been identified.

Loss of Id1 in early zebrafish embryogenesis results in defects in later larval development, long after the effectiveness of the morpholino has diminished. This suggests that the effects observed in the larvae are likely a result of the loss of Id1 function during earlier embryonic stages, which may result in abnormal cellular differentiation or decreased proliferation, causing later structural defects. We show that early knockdown of Id1 inhibits craniofacial development, results in a decrease in overall body length, and considerably reduces swim bladder inflation. Our observed 20% decrease in overall body length is consistent with Id1/Id3 mouse knockout data that showed a 30% reduction in embryo size at E11.5 and 12.5 (Lyden et al., 1999). Since there is no noticeable difference in body length post-hatching, the observed reduction in

size may be attributed to the failure of the swim bladders to inflate. The zebrafish swim bladder is a two chambered organ, with the posterior chamber connected to the esophagus via a pneumatic duct (reviewed in (Finney et al., 2006). The swim bladder functions to allow the fish to maintain neutral buoyancy in the water column as well as to sense vibration (Kent, 1969b). Without proper swim bladder inflation, the larval zebrafish may not be able to adequately track prey, resulting in decreased feeding and reduced growth.

In the developing jaw, loss of Id1 reduces lower jaw length and growth of the ceratohyal cartilage. Craniofacial cartilage in the zebrafish arises from the pharyngeal arches (Yelick and Schilling, 2002). Cartilage development occurs in two stages, beginning with early rapid cartilage morphogenesis followed by an extended period of growth governed by regulated cell division (Kimmel et al., 1998). Growth of the hyosymplectic and ceratohyal cartilage occurs isometrically, beginning around 3 dpf. A variety of mutations have been identified in zebrafish that affect either the layout of the pharyngeal skeleton, cartilage differentiation and morphogenesis, or the spatial arrangement of the cartilage (Neuhauss et al., 1996). The phenotypes observed in the Id1 morphants are most similar to those mutations that affect layout of the pharyngeal skeleton. Interestingly, all of these mutants do not inflate their swim bladder and are unable to feed, similar to our observations in Id1-MO injected larvae. It has been observed that cartilage growth is also dependent on how well the larvae are feeding (Kimmel et al., 1998). Since we have observed a significant difference in feeding behaviors in the Id1 morphants, it is possible that decreased jaw length and growth of the ceratohyal cartilage are a result of decreased feeding ability.

In zebrafish and related teleosts, the neural plate develops into a neural tube, the anterior portion becoming the brain and the remainder forming the spinal cord (reviewed in (Strahle and Blader, 1994); (Kent, 1969a). *In situ* analysis has previously shown that zebrafish Id1 is strongly expressed in the three longitudinal stripes of the developing neural plate (Sawai and Campos-Ortega, 1997). Although zebrafish Id2 has also been shown to be expressed during neurogenesis, its expression is weak during early embryogenesis, with increased expression seen by 48 hpf at the lateral edge of the neural plate (Chong et al., 2005). Therefore, we measured interocular distance to determine if Id1 knockdown resulted in decreased neural plate development. Although we did not see any significant changes in interocular distance with 50 μM Id1-MO injection, it is possible that higher concentrations may have shown a significant difference. At 100 μM, MO-injected embryos show incidences of cranial edema, undulating notochord, and decreased head size suggesting that Id1 is involved in central nervous system development. However, embryos injected at this concentration do not survive out to 7 dpf, so a more in depth observation of later effects cannot be determined.

Data presented in this paper indicate that the function of Id1 is necessary for a variety of pathways in zebrafish development. In the hearts from Id1/Id3 double knockout mice, loss of Id is associated with increased expression of Stra13, skeletal myosin alkali light chain (skMLC), and cardiac α-myosin heavy chain (α-MHC), possibly resulting from the activation of other bHLH proteins which regulate them (Fraidenraich et al., 2004). Additionally, decreased expression of αvβ3-integrin and MMP2 was observed in $Id1^{+/-}Id3^{-/-}$ mice suggesting that Ids may be involved in the expression or recruitment of these genes (Lyden et al., 1999). The cardiac transcription factors gata4

and nkx2.5 have also been shown to interact with Id proteins in P19 cells, inhibiting transactivation of the expression of genes such as atrial natriuretic factor (ANF) and p204 (Ding et al., 2006). Therefore, the observed developmental defects seen here may be a result of the role Id1 plays in the regulation of genes involved in proliferation and differentiation.

Loss of Id1 during zebrafish development may result in alterations in growth factor signaling in a cell type-specific manner, ultimately leading to the developmental defects observed. Ids are downstream targets of the transforming growth factor β (TGF-β) and fibroblast growth factor (FGF) families, which include bone morphogenic protein (BMP), activin, TGFβ, and FGF. For example, during murine palate morphogenesis, Id1 is upregulated by BMP4 and expressed in overlapping patterns with the FGF-regulated transcription factors Twist and Snail (Rice et al., 2005). Ids are also required for BMP-induced bone formation in mice (Maeda et al., 2004). Interestingly, activin and TGF-β1 downregulate Id1 in keratinocytes while upregulating Id1 in fibroblasts (Rotzer et al., 2006), demonstrating that the regulation of Id proteins is also cell type specific. Bone morphogenetic protein signaling is required for migration, condensation, proliferation and differentiation during craniofacial development in vertebrates (reviewed in (Nie et al., 2006). In the zebrafish, BMP and FGF have been shown to be expressed in the early pharyngeal arches (Yelick and Schilling, 2002). Members of the TGF-β family have also been implicated in heart development (reviewed in (Armstrong and Bischoff, 2004). Specifically, BMP4 has been shown to regulate cardiac looping in zebrafish (Chen et al., 1997; Schilling et al., 1999). Taken together, this data suggests that developmental

defects observed in Id1 morphants may be a result of changes in the TGF-β or FGF signaling pathways.

In conclusion, the data presented in this paper demonstrate that Id1 is critical for zebrafish embryogenesis, and that the developmental defects observed with knockdown of Id1 are similar to those seen in murine knockouts. However, in the mouse, the function of the Ids appears to be overlapping, requiring the loss of at least two Id proteins to produce the developmental defects. Therefore, the zebrafish will be a useful model to dissect the distinct roles of the different Id proteins in development. Furthermore, the defects observed following Id1 knockdown are similar to those observed following exposure to teratogenic compounds, such as retinoic acid and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Stainier and Fishman, 1992; Prasch et al., 2003; Antkiewicz et al., 2005), and data from human cell culture indicates that the Id proteins are targets of both of these compounds (Zhang and Rosdahl, 2005; Villano and White, 2006; Akintobi et al., 2007). This suggests that some of the effects of these compounds may be mediated through changes in Id expression.

CHAPTER 5:

INHIBITOR OF DNA BINDING-1 IS A TARGET FOR RETINOIC ACID SIGNALING IN THE DEVELOPING ZEBRAFISH EMBRYO

INTRODUCTION

The inhibitor of DNA binding proteins are important regulators of organ development in the embryo, functioning as dominant negative inhibitors of the basic helix-loop-helix (bHLH) transcription factors, which regulate the transcription of cell cycle genes (Massari and Murre, 2000). Id proteins have also been shown to regulate cell cycle progression through interactions with the ETS family of transcription factors, Rb/p16^{INK4A} tumor suppressor proteins and the centrisome (Hasskarl et al., 2004; Norton, 2000; Zebedee and Hara, 2001). The importance of the Id proteins in development is supported by the findings that Id1/Id3 knockout mice are nonviable (Fraidenraich et al., 2004) and that overexpression of Id1 during mouse development inhibits neuronal development (Yokota, 2001). The Id family of transcriptional regulators is involved in embryogenesis, reproduction, proliferation and differentiation. Double and triple Id knockouts show severe cardiac defects leading to embryonic lethality at E13.5 (Fraidenraich et al., 2004). Our previous data (see Chapter 4) show that knockdown of Id1 in the developing zebrafish results in pericardial and yolk sac edema and defects in heart looping, suggesting that Id1 is critical for proper heart and vascular development. These observations are consistent with phenotypes seen in the Id double knockout mouse model, although our data suggest that knockdown of Id1 alone is sufficient for the disruption of embryogenesis.

Data from our laboratory, as well as others, indicate that Id expression is altered by retinoic acid (Villano and White, 2006; Zhang and Rosdahl, 2005; Nigten et al., 2005). Retinoic acid is a lipid soluble hormone derived from the metabolism of vitamin A, an essential nutrient obtained from food either as preformed vitamin A from animal products (eggs, liver, milk) or as pro-vitamin A (carotenoids) from fruits and vegetables (Sporn et al., 1994; Fisher and Voorhees, 1996). Dietary derived all-trans RA (atRA) is the main signaling retinoid in the body and is vital for biological functions such as embryogenesis, growth and differentiation, as well as vision and reproduction (Dragnev et al., 2000). In the developing embryo, the biosynthesis of atRA occurs as a stepwise oxidation from maternally derived retinol. The oxidation of retinal to atRA is catalyzed by the enzyme retinal dehydrogenase (raldh) and is thought to be the rate-limiting step in RA biosynthesis (reviewed in (Napoli, 1997)). The majority of RA's biological activity is mediated through regulation of gene expression, either directly through response elements or indirectly through other mechanisms such as mRNA stability (reviewed in (Balmer and Blomhoff, 2002)). RA signals through two families of nuclear receptors, the retinoic acid receptors (RARα, β, and γ), activated by both all-trans (atRA) and 9-cis forms of RA, and retinoid X receptors (RXRα, β, and γ), which bind 9-cis RA exclusively (reviewed in (Mangelsdorf et al., 1995; Chambon, 1996)). In the developing zebrafish embryo, there are four RARs and 6 RXRs identified in the zebrafish, which exhibit both overlapping and distinct expression patterns during development (Jones et al., 1995; Hale et al., 2006; Tallafuss et al., 2006; Waxman and Yelon, 2007). Six different retinoids can be detected in the zebrafish by HPLC including 4-oxo-RA, didehydroretinol, all-trans retinol and retinal, and atRA, which can be detected in all stages of development

(Costaridis et al., 1996). Interestingly, from the 8-cell stage through to 48 hpf, there is no significant change in atRA levels. Also, 9-cis RA is not detected and 13-cis RA is only detected occasionally in samples after 10 days post fertilization (Costaridis et al., 1996).

Because retinoic acid is a potent teratogen and that Id1 is necessary for normal zebrafish embryogenesis, we hypothesized that retinoic acid would also regulate Id expression in the developing embryo. We have chosen the zebrafish for our model because of its genetic and physiological similarity to mammals and because mechanisms of early embryogenesis are conserved between this model and mammalian systems (reviewed in (Grunwald and Eisen, 2002)). The utility of the zebrafish is enhanced by the complete sequencing of the zebrafish genome, and the availability of full-length cDNAs and DNA microarrays for expression analysis. Homologs to the mammalian Ids have been identified in the zebrafish, including homologs to human Id1 and human Id3 (zfId1 and zfId3) (Sawai and Campos-Ortega, 1997; Dickmeis et al., 2002). Therefore, we chose to use the developing zebrafish to continue our examination of atRA regulation of Id expression. The data presented in this chapter demonstrate that Id1 expression is altered by retinoic acid signaling in the developing zebrafish. Further, atRA-alterations of Id1 expression are observed in the developing heart, which has been shown to be a target for atRA-teratogenesis.

RESULTS

Id1 regulates normal expression levels of gata5 and nkx2.5.

Previously, we observed that Id1-MO injected embryos exhibit defects in heart development, mainly failure to loop and form distinct chambers (Fig. 12). To assess the role that Id1 plays in the developing cardiac tissue, the expression of specific markers of cardiac development was investigated. Zebrafish gata5 and nkx2.5 are essential for proper heart development (Harvey, 1996; Reiter et al., 1999). Gata5, a member of the GATA family of transcription factors, is required for myocardial differentiation in zebrafish and decreased gata5 expression alters the expression of nkx2.5, an Nk homeobox gene (Reiter, et al., 1999). Nkx2.5 is expressed in cardiac precursors and is one of the earliest genes involved in cardiac specification (Chen and Fishman, 1996). Knockout mouse data shows that nkx2.5 is necessary for normal heart morphogenesis, myogenesis, and function (Lyons et al., 1995).

Our data show that gata5 and nkx2.5 are expressed in low levels at 10 hpf, just prior to cardiac fusion, and are significantly increase by 24 hpf, after elongation of the cardiac cone into a heart tube occurs and contractions and circulation begin (Fig. 15). Gata5 expression levels are reduced by 48 and 72 hpf; however, nkx2.5 levels remain elevated at 48 and 72 hpf. At this time, the heart is completely formed, with the ventricle positioned to the right of the atrium and endocardial cushions present. Knockdown of Id1 by injection of the Id1-MO results in an increase in mRNA expression of gata5 and nkx2.5 at both 24 and 48 hpf (Fig. 16 A and B). It should be noted that injection of Id1-MO results in increased expression of gata5 and nkx2.5 from 24 to 48 hpf. This discrepancy may be a result of injection alone having an effect on expression levels. In

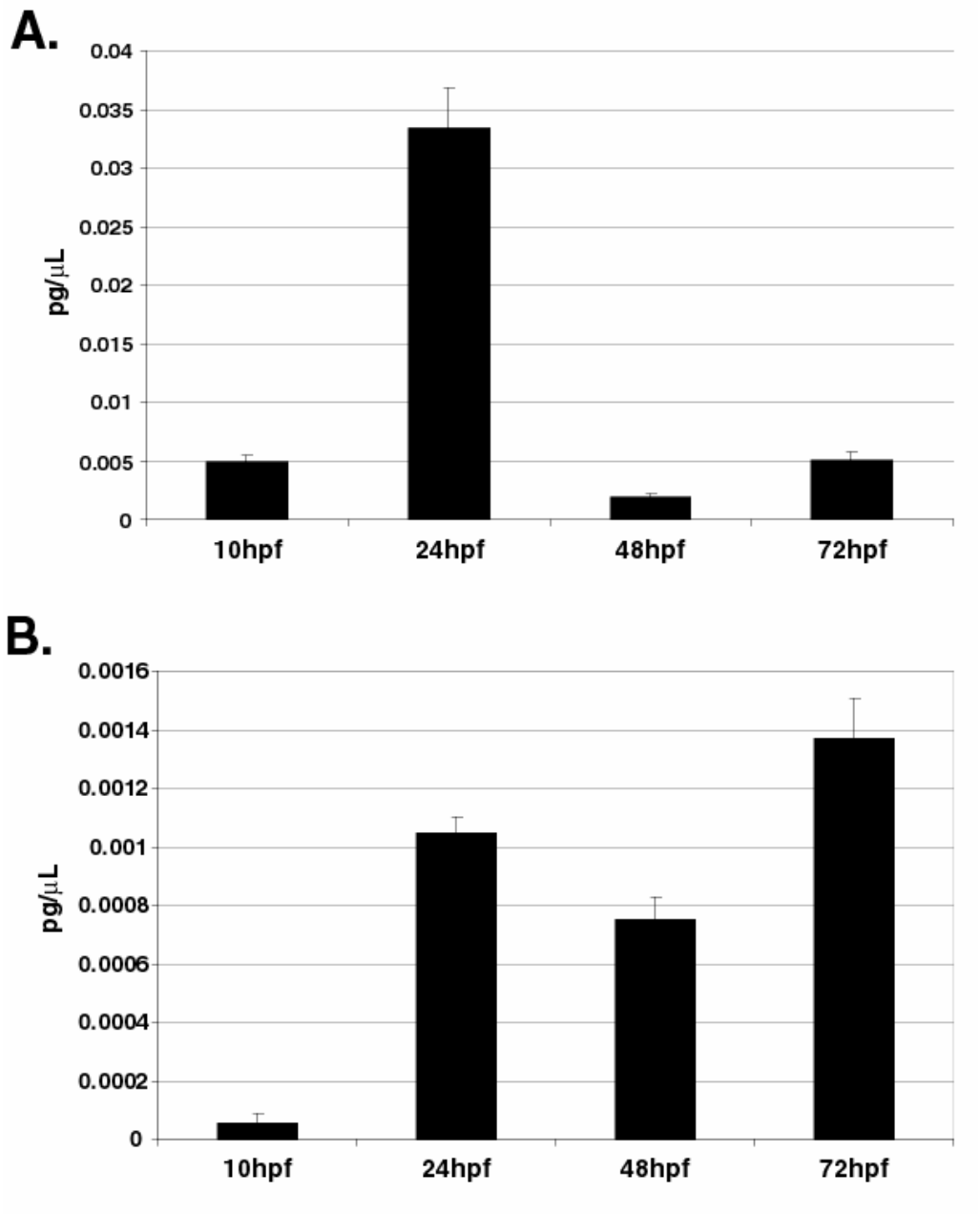


Figure 15. Gata5 and nkx2.5 timecourse of expression.
Total RNA was isolated from zebrafish at 10, 24, 48, and 72 hpf and used as template for qRT-PCR using primers specific for zebrafish gata5 (A), nkx2.5 (B) and 28S rRNA. Data were quantified by utilizing a standard curve generated using specific PCR products at concentrations ranging from 100pg to 0.1pg. Data were normalized to 28S rRNA. PCR reactions were performed in triplicate.

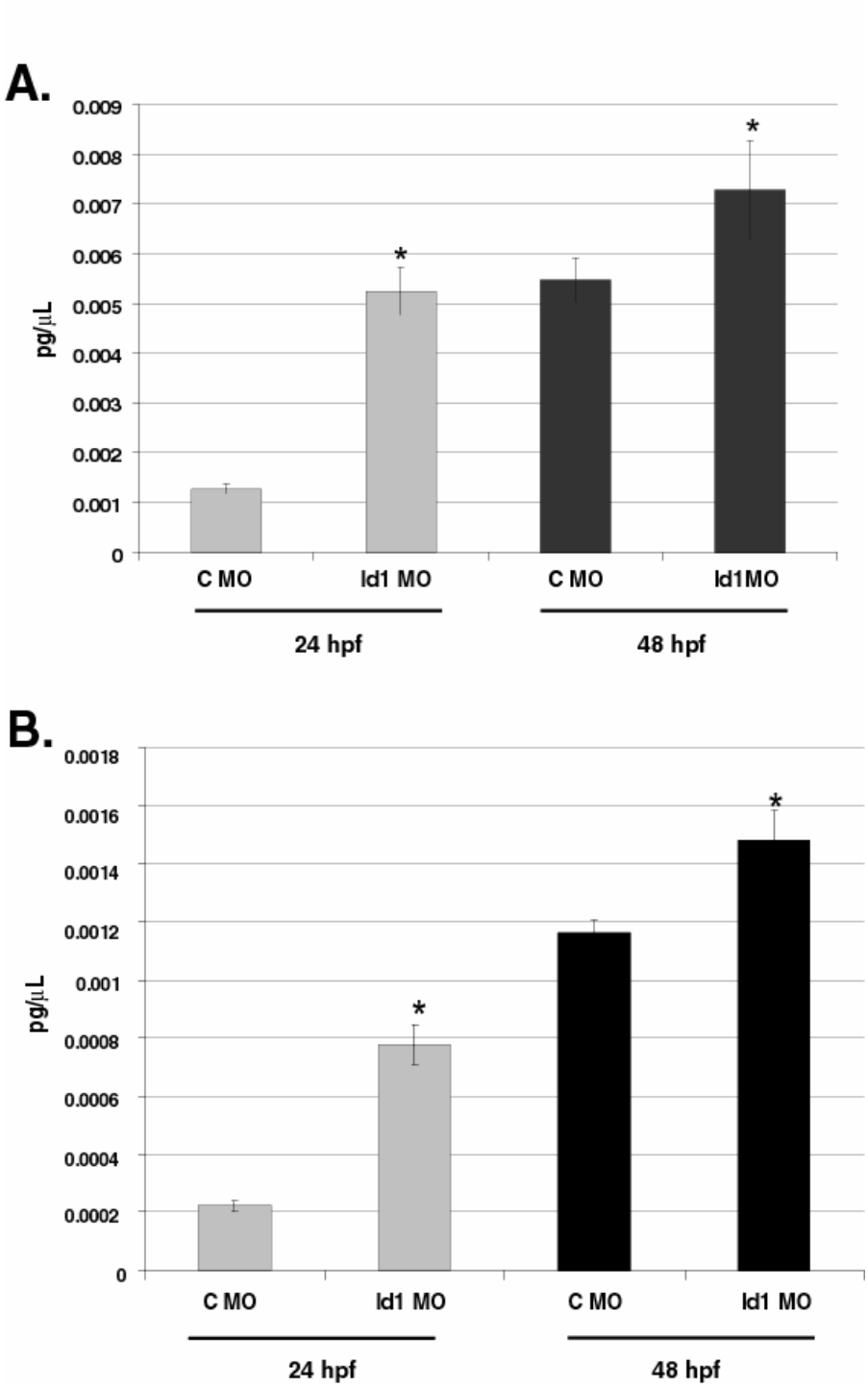


Figure 16. Id1 regulates expression of gata5 and nkx2.5.
Total RNA was isolated from Id1-MO injected zebrafish at 24 and 48 hpf and used as template for qRT-PCR using primers specific for zebrafish gata5 (A), nkx2.5 (B) and 28S rRNA. Data were quantified by utilizing a standard curve generated using specific PCR products at concentrations ranging from 100pg to 0.1pg. Data were normalized to 28S rRNA. PCR reactions were performed in triplicate. *$p<0.05$

order to determine whether or not this is the case, it will be necessary to compare sham injected control embryos (i.e. injection of 1X Danieau solution alone) or uninjected embryos to control MO injected embryos. These data indicate that Id1 regulates normal expression levels of these cardiac-specific transcription factors.

Exposure to all-trans retinoic acid causes heart malformations in the developing zebrafish.

Data from other laboratories demonstrate that exposure of developing zebrafish embryos to a low dose of retinoic acid for one hour results in decreased heart size or loss of heart chambers as well as decreased blood circulation (Stanier and Fishman, 1992). These defects are similar to what we have observed in Id1 morphants (Fig. 11). Different strains of zebrafish demonstrate varying sensitivities to toxicologic and pharmacologic compounds (Dlugos and Rabin, 2003; Loucks and Carvan III, 2004). Therefore, to rule out strain differences and ensure that our treatment regimen repeats this previous data, we subjected zebrafish embryos to atRA exposure from 3 hpf through 72 hpf and scored the developing embryos for percent mortality and heart defects (data not shown). Concentrations of atRA ranged from 1, 5, 10, and 100 nM atRA. By 24 hpf, embryos exposed to 100nM atRA were severely malformed and lacked differentiated anterior and posterior structures (Fig. 17A). These embryos never developed contractile cardiac tissue and were nonviable. At 10 nM atRA, embryos had severe pericardial and yolk sac edema and blood pooling (Fig. 17D). Their hearts fail to loop properly and retain early heart tube morphology. In addition, these embryos have a curved body axis, decreased head size and often a truncated tail. Embryos exposed to 5nM atRA have similar phenotypes as 10nM, although lesions were less severe (Fig. 17C). At 1nM atRA,

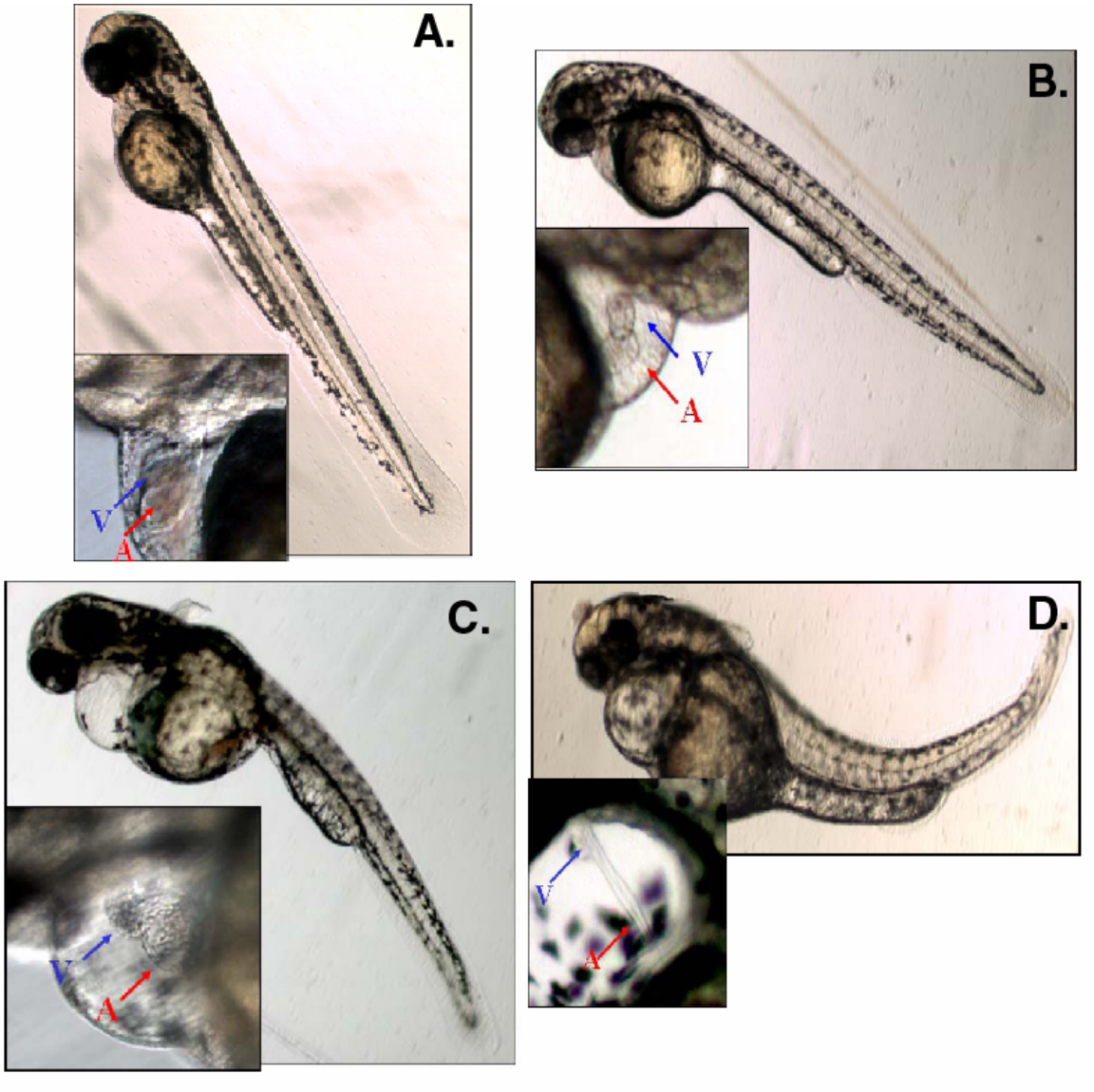


Figure 17. Excess All-trans retinoic acid causes dose dependent developmental defects. Representative photographs of embryos at 72hpf treated with varying concentrations of atRA. **A.** Control embryos were maintained in 0.01% EtOH. Close up of the heart depicts the normal side by side orientations of the atrium (A) and ventricle (V). **B.** 1nM atRA treatment. **C.** 5nM atRA treatment. **D.** 10nM atRA treatment.

embryos appear normal with a few occurrences of slight pericardial edema (Fig. 17B). These data demonstrate that lower doses of atRA can be used to induce heart malformations by constant exposure of embryos beginning at an early stage (~3 hpf). For the remainder of the experiments, we chose to use our lowest effective dose, which was 5 nM atRA.

Retinoic acid alters Id1 expression.

To determine if atRA was able to alter the expression of Id1 during development, zebrafish embryos were exposed to 5nM atRA at approximately 3 hpf. At 24 hpf no change in Id1 expression levels are observed, however, by 48 hpf a decrease in Id1expression is observed (Fig. 18A). To determine if the atRA-mediated decrease in Id1 in the whole embryo was observable in the cardiac tissue, we harvested heart tissue from 48 hpf embryos exposed to vehicle (EtOH) or 5nM atRA, and isolated total RNA for use in quantitative RT-PCR. Embryos exposed to 5nM atRA show a significant decrease in cardiac Id1 mRNA expression (Fig. 18B). This is confirmed by *in situ* hybridization with an Id1 specific anti-sense probe (Fig. 19A,B). In control embryos, Id1 can be detected in the heart whereas embryos exposed to 5nM atRA show no detectable Id1 signal in the heart. These data demonstrate that atRA-exposure results in a decrease in Id1 mRNA expression in the heart, and suggests that Id1 may play a role in retinoic acid-induced heart malformations.

Gata5 and nkx2.5 expression is altered by atRA.

Our data show Id1 is involved in the normal regulation of both gata5 and nkx2.5 (Fig 16). Retinoic acid signaling can inhibit differentiation in the developing heart and has been shown to affect the expression levels of Nk and Gata transcription factors in *Xenopus*

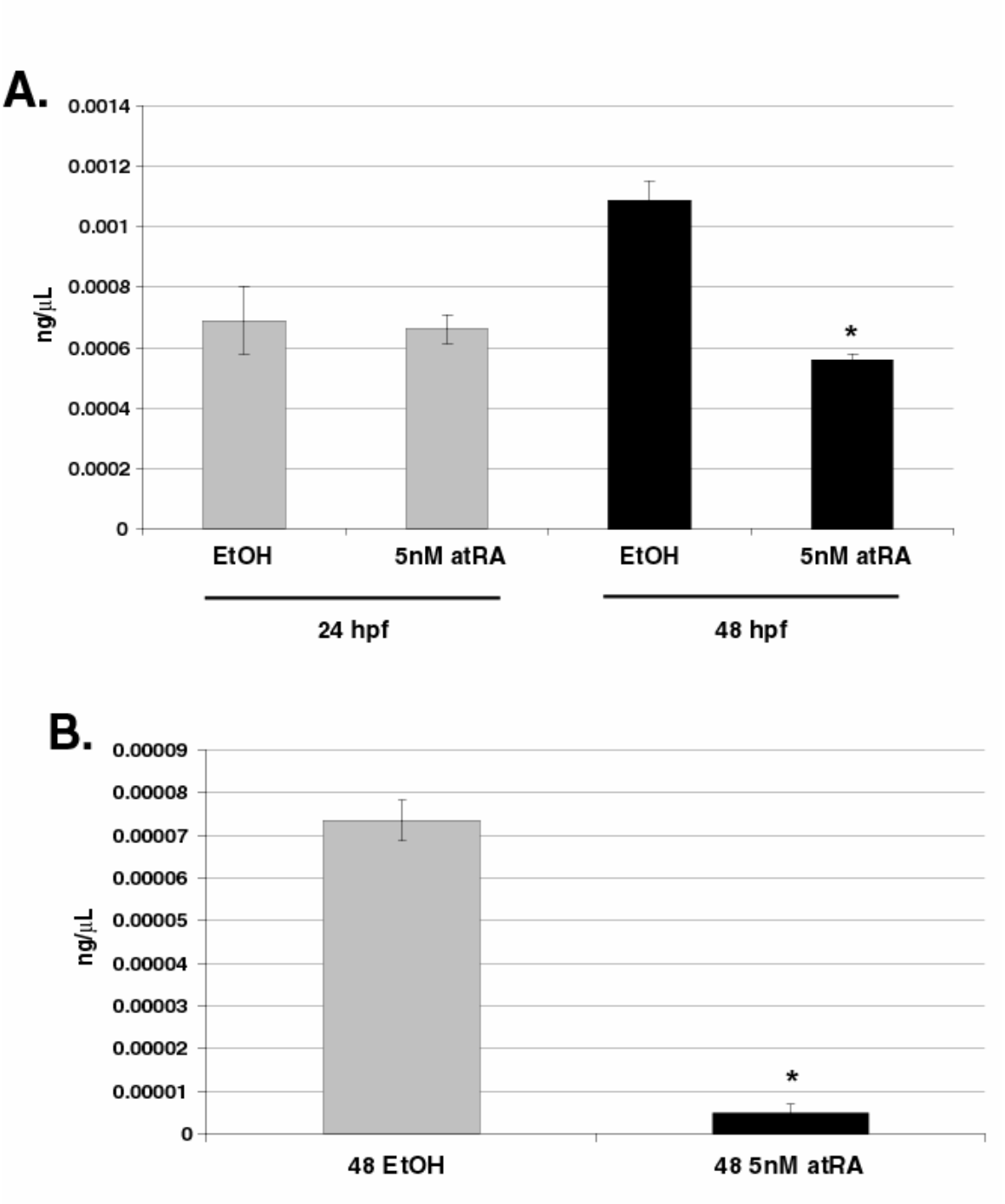


Figure 18. AtRA decreases zebrafish Id1 expression. Total RNA was isolated from zebrafish exposed to 5nM atRA at 24 and 48 hpf (**A)** or from hearts isolated at 48hpf from zebrafish exposed to 5nM atRA (**B.**) and used as template for qRT-PCR using primers specific for zebrafish Id1 and 28S rRNA. Data were quantified using a standard curve generated wtih specific PCR products ranging from 0.1ng to 1pg. Data were normalized to 28S rRNA. PCR reactions were performed in triplicate. * $p < 0.05$.

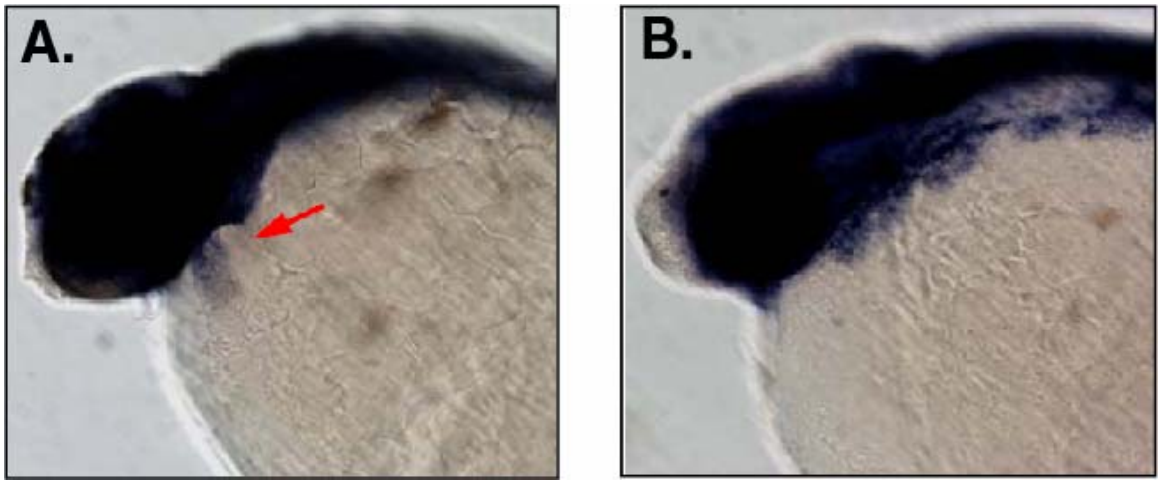


Figure 19. AtRA decreases zebrafish Id1 expression in the heart. *In situ* hybridization of control (**A**.) and 5nM atRA exposed (**B.**) embryos at 48hpf. Expression can be detected in the heart of control (arrow), but not in atRA-exposed embryos.

embryos (Jiang and Evans, 1996). Therefore, to determine the effect of atRA on gata5 and nkx2.5 in the developing zebrafish embryo, we examined the expression of these genes following exposure to atRA. At 24hpf, 5nM atRA results in an increase in gata5 and nkx2.5 mRNA (Fig. 20A, C). However, at 48hpf, a significant decrease in expression of these markers was observed. Additionally, atRA exposure results in decreased expression of gata5 and nkx2.5 at 48 hpf specifically in isolated cardiac tissue (Fig. 20B,D).

Retinoic acid deficiency increases the expression of Id1, gata5, and nkx2.5.

We have determined that excess atRA decreases Id1 expression, suggesting that Id1 is a downstream target of the RA signaling pathway (Fig. 18). To further examine the effect of RA signaling on Id1 expression, we investigated the effect of RA deficiency by the use of a retinaldehyde dehydrogenase 2 morpholino (raldh2-MO). The raldh2-MO has been previously used to produce RA depleted zebrafish embryos (Dobbs-McAuliffe et al., 2004; Stafford et al., 2006). Loss of this enzyme inhibits the transformation of retinal to retinoic acid. We observed significant pericardial and yolk sac edema, decreased heartbeat and abnormal heart looping following injection of 100 μM raldh2-MO, as well as incidences of tail edema (Fig. 21). At 24 hpf, qRT-PCR data shows that raldh2-MO injected embryos have more than a 2-fold increase in Id1 expression compared to control MO (Fig. 22). At 48 hpf, raldh2 morphants showed about a 9 fold increase in Id1 expression. Additionally, expression levels of both gata5 and nkx2.5 increase in raldh2-MO injected embryos. Taken together, these data provide further evidence that the retinoic acid signaling pathway regulates Id1 expression in the

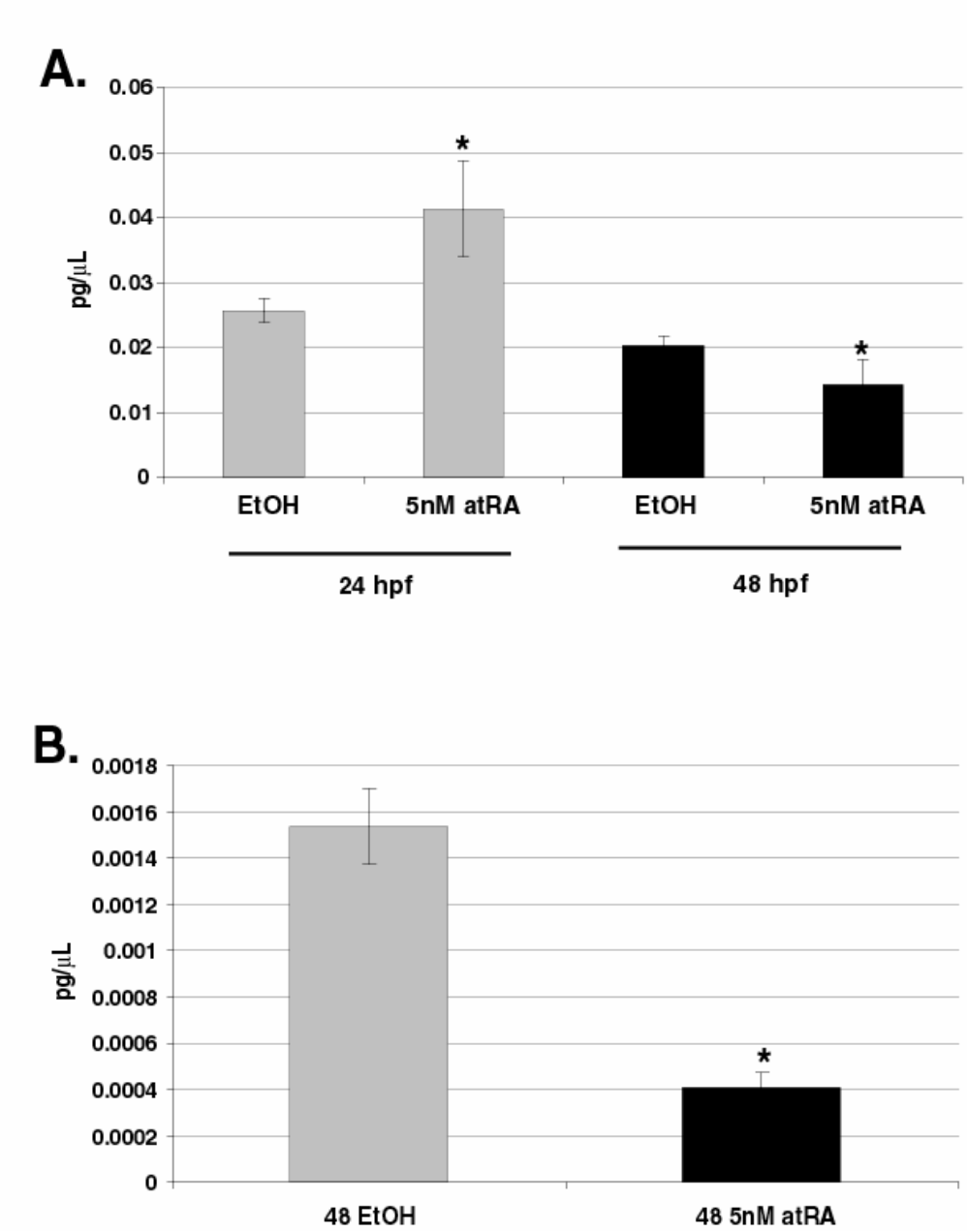


Figure 20. Excess atRA decreases zebrafish gata5 and nkx2.5 expression at 48hpf. Total RNA was isolated from zebrafish exposed to 5nM atRA at 24 and 48 hpf (**A,C)** or from hearts isolated at 48hpf from zebrafish exposed to 5nM atRA (**B,D.**) and used as template for quantitative RT-PCR using primers specific for zebrafish gata5, nkx2.5 and 28S rRNA. Data were quantified by utilizing a standard curve generated using specific PCR products at concentrations ranging from 100pg to 0.1pg. Data were normalized to 28S rRNA. PCR reactions were performed in triplicate. * $p<0.05$

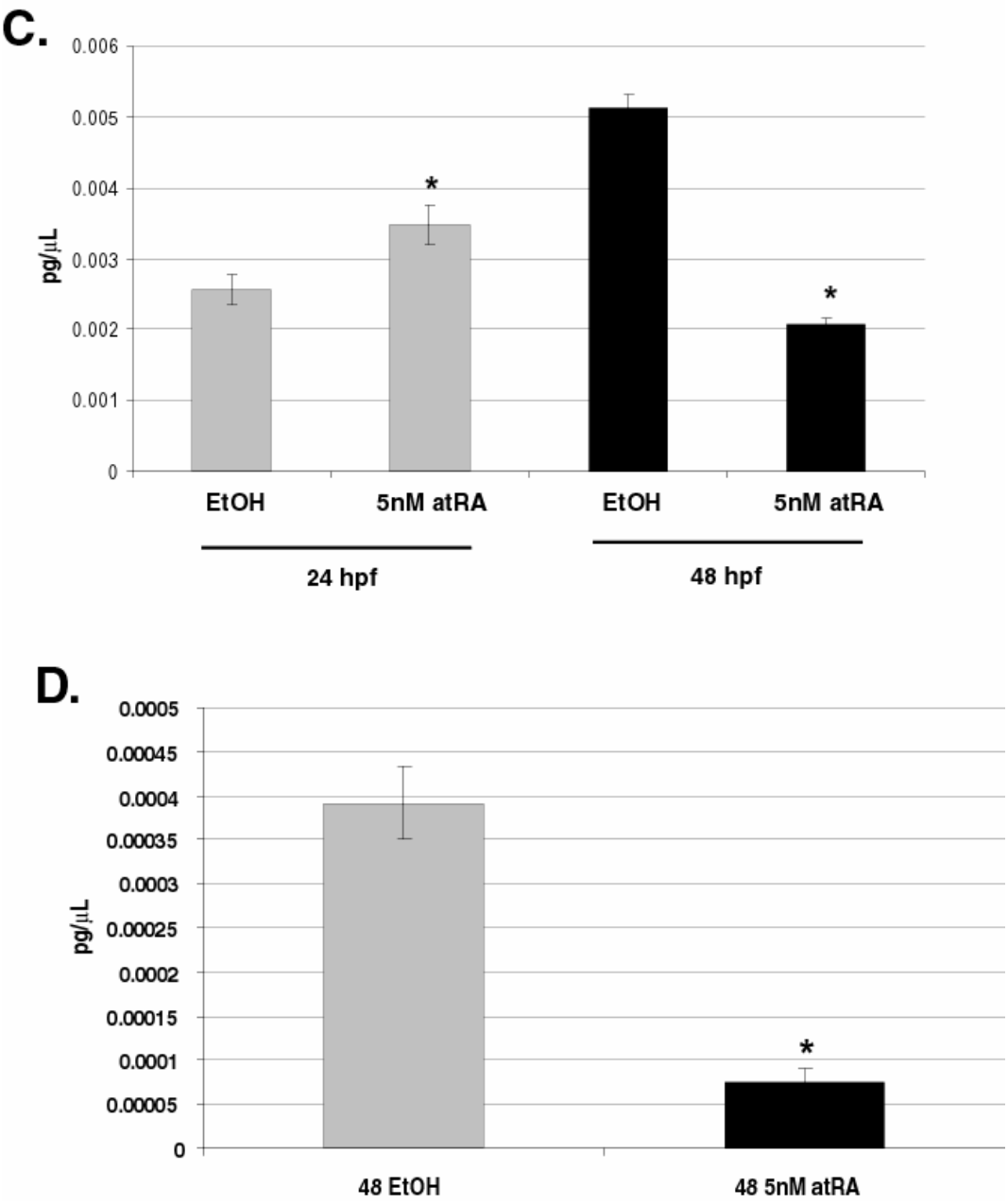


Figure 20. Excess atRA decreases zebrafish gata5 and nkx2.5 expression at 48hpf, cont'd.

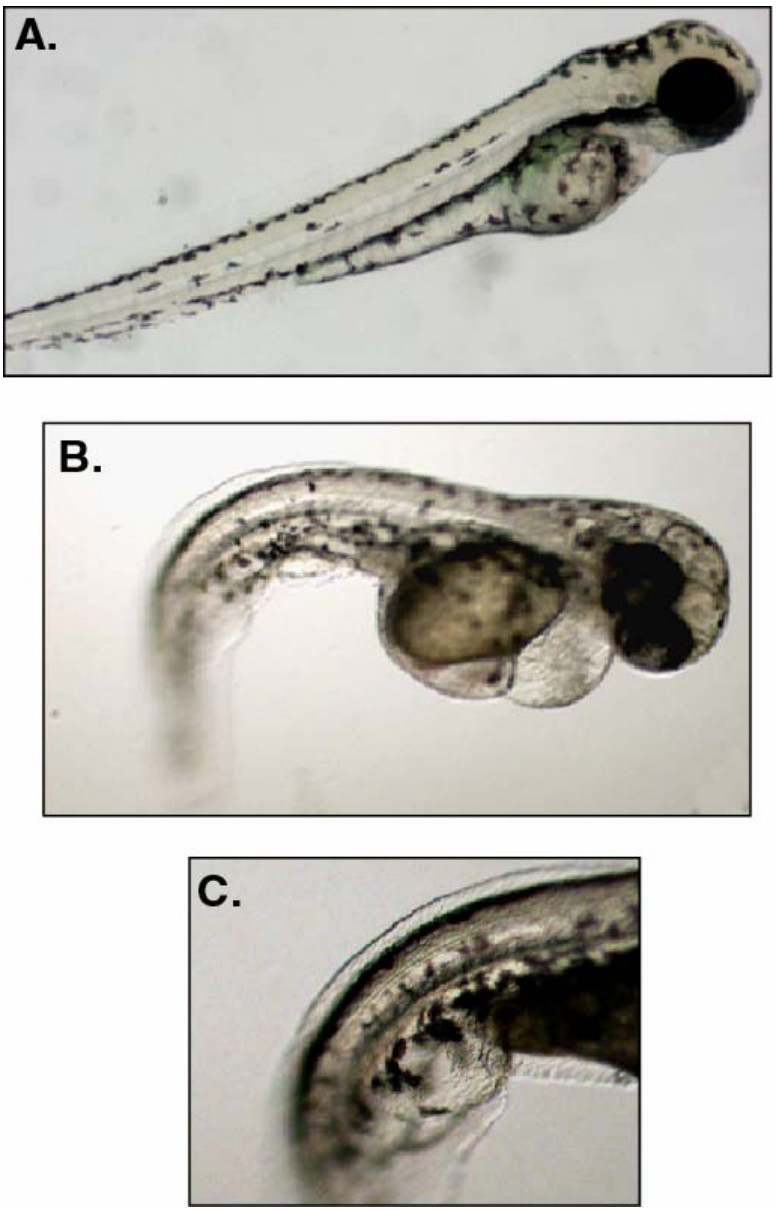


Figure 21. AtRA deficiency causes developmental defects in the zebrafish embryo. Representative photographs of raldh2-MO injected embryos at 72hpf **A.** Control MO injected embryos were **B.** 100 μM raldh2-MO injected embryo. **C.** Close up of tail edema observed in 100 μM raldh2-MO injected embryos.

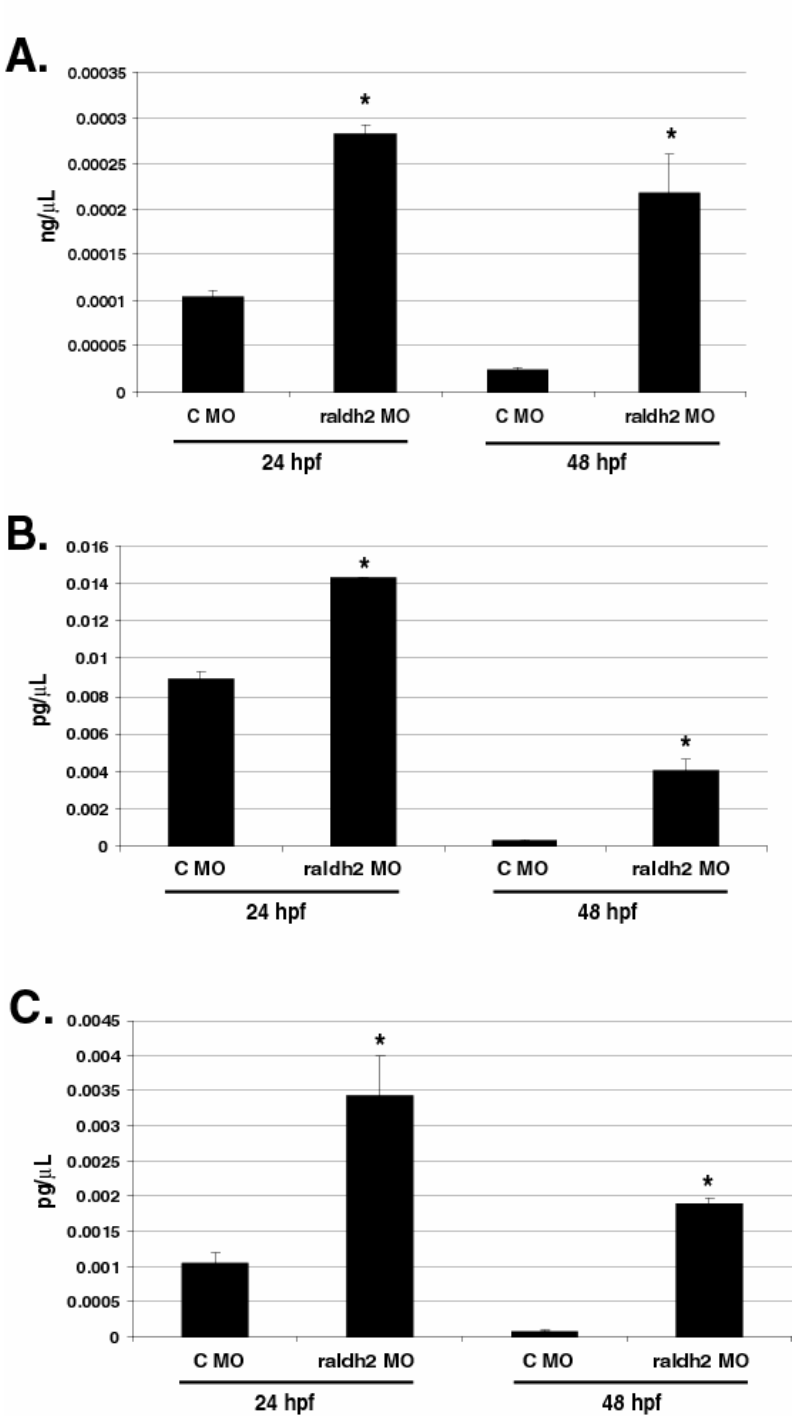


Figure 22. AtRA deficiency increases zebrafish Id1, gata5 and nkx2.5 expression. Total RNA was isolated from raldh2-MO injected zebrafish at 24 and 48 hpf and used as template for qRT-PCR using primers specific for zebrafish Id1 (A) gata5 (B), nkx2.5 (C) and 28S rRNA. Data were quantified by utilizing a standard curve generated using specific PCR products at concentrations ranging from 0.1ng to 1pg for Id1 and 100pg to 0.1pg for gata5 and nkx2.5. Data were normalized to 28S rRNA. PCR reactions were performed in triplicate. *$p<0.05$

developing zebrafish. Additionally, these data confirm that retinoic acid is required for normal expression levels of cardiac specific transcription factors gata5 and nkx2.5.

DISCUSSION

The data presented here demonstrate that exposure to atRA, a potent teratogen, decreases expression of Id1 in the developing zebrafish and specifically in the heart. In addition, RA deficiency results in increased Id1 expression. Together, these data indicate that Id1 is a target of the RA signaling pathway in the zebrafish heart. Our previous findings show that Id1 is critical for normal cardiac development in zebrafish embryos. Data here show that Id1 morpholino injection results in increased expression of the cardiac-specific transcription factors gata5 and nkx2.5. Morever, changes in RA levels alter the expression of gata5 and nkx2.5. We suggest that a regulatory negative feed-back loop exists between Id1, gata5 and nkx2.5 and atRA-induced heart malformations may result from disruption of this regulatory mechanism.

Findings here demonstrate that low doses of atRA can be used to induce heart malformations by constant exposure of embryos beginning at an early stage. Exposure to 5nM atRA causes severe pericardial and yolk sac edema, blood pooling, and a lack of proper heart looping. Similar defects were observed in RA deficient zebrafish embryos. Vitamin A deficiency in developing quail results in an enlarged heart that has failed to loop, and exhibits no distinct chambers (reviewed in Zile, 2004). Retinoic acid is also involved in the transition of cells to a complete heart tube in *Xenopus* and capable of regulating the expression of both nkx2.5 and gata4 (Collop et al., 2006). In the zebrafish, retinoic acid signaling is necessary for myocardial differentiation as well as acquisition of

heart tube polarity (Stainier and Fishman, 1992; Drysdale et al., 1997). Human embryogenesis is similarly affected following accidental exposure to isotretinoin, or 13-cis retinoic acid, which results in a wide range of malformations including conotruncal heart defects such as truncus arteriosis ((Lammer et al., 1985).Supporting our previous findings that Id1 expression is important for early embryogenesis is our data demonstrating that the potent teratogen atRA alters Id1 expression in the developing zebrafish. Proper spatial and temporal regulation of retinoid levels is critical for normal embryonic development and it is believed that atRA effects on development are mediated primarily through changes in gene expression. For example, many of the teratogenic effects of retinoids are mediated through changes in homeobox-containing (Hox) genes (reviewed in (Marshall et al., 1996)). Data here demonstrate that in the developing zebrafish, retinoic acid down-regulates Id1 expression not only in the whole embryo, but also in isolated cardiac tissue, suggesting that changes in Id1 contribute to atRA-induced heart malformation. Previous data from cell culture also indicate that the Ids are targets of the retinoic acid signaling pathway, although whether Id expression is upregulated or inhibited appears to be dependent on cell type (Villano and White, 2006; Nigten et al., 2005; Zhang and Rosdahl, 2005).

To examine the effect of Ids on heart development, we focused on two transcription factors that are involved in cardiac lineage commitment, gata5 and nkx2.5 (Harvey, 1996; Mably et al., 2003; Peterkin et al., 2005). These genes are representative of two families of homeodomain proteins that are important in cardiac lineage commitment. Nkx2.5 knockout mice exhibit abnormal heart morphogenesis, including disrupted heart looping and endocardial cushion formation (Lyons et al., 1995).

Although mouse gata5 knockouts show no cardiac defects, gata5 is required for differentiation of cell lines derived from cardiac mesoderm (reviewed in Peterkin et al., 2005). Our data show that knockdown of Id1 results in increased expression of both nkx2.5 and gata5 at 24 and 48 hpf, suggesting that Id1 functions to inhibit expression of these genes. The expression patterns of nkx2.5 in zebrafish correlate with the presence of cardiac precursor cells (Chen and Fishman, 1996) and nkx2.5 is essential for proper heart morphogenesis in the mammalian heart (Harvey, 1996). Gata5 is involved in growth and morphogenesis of the heart and endoderm in zebrafish and regulates the expression of nkx2.5 expression (Reiter et al., 1999). The increase in nkx2.5 and gata5 resulting from loss of Id1 most likely contributes to the abnormal heart morphology we have seen in Id1 morphants. A similar role for Id proteins has been shown in murine cardiac myocytes, where the Id protein binds to gata4 and nkx2.5 and inhibits their ability to transactivate the expression of cardiac genes such as p204 and atrial natriuretic factor (ANF) (Ding et al., 2006).

To further prove that retinoic acid signaling regulates Id1 expression in the zebrafish embryo, a morpholino designed to block the production of retinal dehydrogenase 2 was used to mimic a RA-deficient state (Stafford et al., 2006). Loss of raldh2 activity results in decreased synthesis of RA. We have shown here that morpholino knockdown of raldh2 results in an increase in Id1 expression. Also, in our raldh2-MO injected embryos, we observed pericardial, yolk sac, and tail edema along with a defect in heart looping. Data from other laboratories has shown that loss of raldh2 in the zebrafish mutants *neckess* and *no-fin* results in a truncation of the anteroposterior axis and defects in the mesodermal tissue that results in defects in the branchial arches

and a lack of pectoral fins (Grandel et al., 2002; Begemann et al., 2004). The loss of RA signaling in these mutants is also associated with heart defects such as pericardial and heart edema and weak heartbeat, confirming our data that raldh2 is needed for proper heart development. Further, raldh knockout mice properly develop a heart tube that fails to undergo rightward looping (Niederreither et al., 2001). These data demonstrate that Id1 is a downstream target of the retinoic acid signaling pathway and that changes in Id expression may play a role in heart defects resulting from a RA deficiency.

Our findings demonstrate that the retinoic acid signaling pathway regulates Id1 expression patterns in the developing zebrafish, and that these changes are associated with changes in gata5 and nkx2.5, transcription factors involved in regulation of heart morphogenesis. At 48 hpf, a significant decrease is observed in Id1 expression in the developing heart following atRA exposure, as demonstrated by expression analysis of RNA isolated from cardiac tissue, as well as *in situ* hybridization. Although RA slightly increased expression of gata5 and nkx2.5 at 24 hpf, a more substantial decrease can be seen at 48 hpf in whole embryo as well as in cardiac tissue isolates. The initial increase in expression can be accounted for by a possible increase in expression in tissues other than the heart. In the developing *Xenopus*, exogenous atRA can lead to ectopic expression of gata5 and gata6 (Jiang et al., 1999). *In situ* hybridization to localize gata5 and nkx2.5 transcript may help determine if the increased expression is due to ectopic expression outside the cardiac field. Our data at 48 hpf correlates with the effects of RA in other model systems. For example, excess RA in the developing *Xenopus* causes a reduction in the heart-forming region expressing nkx2.5 (Jiang et al., 1999).

Our data also show that RA deficiency results in a increase in Id1 expression, confirming that Id1 expression is regulated by the RA signaling pathway in the developing zebrafish. Raldh2-MO injected embryos show increased expression of Id1 at 24 and 48 hpf. Further, loss of RA signaling also resulted in increased expression of the cardiac specific markers gata5 and nkx2.5. In zebrafish, the use of a pan retinoic acid receptor agonist, BMS189453, resulted in expansion of nkx2.5 expression (Keegan et al., 2005). This is also consistent with data from *xenopus*, which shows that loss of RA signaling prior to gastrulation results in expanded nkx2.5 expression domain (Collop et al., 2005).

Although it is clear that atRA alters expression of Id1 the exact mechanism is yet unclear. One possibility is that gata5 and nkx2.5 may function, in part, to regulate Id1 expression levels. Data from other laboratories has shown that gata4 and nkx2.5 regulate Id expression levels in mouse P19CL6 cells, which differentiate into beating cardiomyocytes (Lim et al., 2007). Also, the mouse Id2 promoter was found to contain two nkx2.5 binding sites, and nkx2.5 along with tbx5 cooperatively activates Id2 transcription in the developing mouse myocardium (Moskowitz et al., 2007). Id1-MO data however, suggests that Id1 may also function as a negative regulator of gata5 and nkx2.5. From this work, we suggest the possibility of a regulatory negative feedback loop between Id1 and these two transcription factors, which functions to tightly regulate expression patterns during heart development in the zebrafish embryo (Fig. 28). Additionally, the effects of excess RA or loss of RA signaling on cardiac morphology may be a result of disruption of this regulatory mechanism. This information may

provides a new target gene for retinoic acid signaling during normal heart development and may aid in understanding the complex signaling pathways involved in heart defects.

CHAPTER: 6

INHIBITOR OF DNA BINDING-1 IS REQUIRED FOR NORMAL ZEBRAFISH CAUDAL FIN REGENERATION

INTRODUCTION

Wound healing and regeneration are complex processes involving cellular differentiation and migration, cell-cell interactions, and coordinated matrix remodeling. Epimorphic regeneration, the process that results in the functional reconstruction of the lost appendage, requires not only the regulation of cell proliferation and migration, but also cell differentiation leading to recapitulation of the limb pattern. In teleost fish, epimorphic regeneration begins with the formation of a blastema, a mass of heterogeneous mesenchymal-like cells located between the appendage stump and the wound epidermis (reviewed in (Akimenko et al., 2003)). Formation of the blastema is necessary for the regenerative process; however, the molecular mechanisms mediating blastema generation are still unclear. Recent data indicate that fibroblast growth factor (FGF) signaling is necessary for blastema formation (Poss et al., 2000; Lee et al., 2005), and that members of the wnt signaling family are also involved (Poss et al., 2000). Growth and elongation of the blastema occurs in an organized manner with the cells proximal to the blastema proliferating at a higher rate than those epithelial cells found distal (Santamaria and Becerra, 1991). Several gene families are implicated in cell cycle regulation in the regenerating tissue, including msxb, FGF, and the homeodomain proteins hoxd11 and hoxd12 (Akimenko et al., 2003). Finally, in order for the appendage regrowth to result in a functional duplicate of the lost limb, the pattern of the limb must be maintained, which is regulated by the expression of a variety of proteins including the

transcription factor evx1 (Borday et al., 2001), as well as the coordinated action of sonic hedgehog (shh), bone morphogenic protein 2b (bmp2b) and patch (ptc) (Borday et al., 2001).

In zebrafish fin regeneration, retinoic acid has been shown to be both teratogenic and morphogenic, causing either narrowing of the fins and bony ray fusion or increased number of ray segments (Geraudie et al., 1994; White et al., 1994). Dietary derived all-trans retinoic acid (atRA) is the main signaling retinoid in the body and is vital for biological functions such as embryogenesis, growth and differentiation, as well as vision and reproduction (Dragnev et al., 2000). The effects of retinoic acid during regeneration have been well studied in a variety of models including chick, frog, and zebrafish (reviewed in Lee et al., 2004). These effects are dependent on the concentration, time, and duration of atRA exposure. In zebrafish, exposure to atRA directly following amputation results in increased apoptosis in the wound epidermis and inhibition of blastema formation (Ferretti and Geraudie, 1995). Zebrafish RAR-γ is highly expressed in the blastemae associated with each individual ray of the regenerating tail fin (White et al., 1994). However, exposure to atRA results in decreased RAR-γ expression, which is not associated with a specific ray.

Previous findings from our lab show that retinoic acid signaling regulates the expression of inhibitor of DNA binding (Id) proteins, negative regulators of the basic helix–loop–helix (bHLH) family of transcription factors. The bHLH proteins are important transcriptional regulators of a variety of developmental processes such as lineage commitment, cell differentiation, and sex determination (Massari and Murre, 2000). The HLH region mediates homo and heterodimerization that is required for DNA

binding and conventional HLH transcription factors have a highly basic region immediately adjacent to this region that mediates binding to E-box sequences. The Id class of bHLH proteins is distinct from the other classes in that these proteins lack the basic DNA binding domain and form inactive heterodimers with other HLH proteins (Norton, 2000). To date, very little data exists describing the role of Ids during the regeneration process. Changes in Id1 expression have been observed in the regenerating rat liver (Le Jossic et al., 1994), muscle (Zador et al, 2002) and neurons (Kabos et al., 2002). Expression of Id2 and Id3 has been shown to be upregulated in the blastemae of the adult newt and *Xenopus* regenerating limb (Shimizu-Nishikawa et al., 1999). Homologs to the mammalian Ids have been identified in the zebrafish, including homologs to human Id-1 and human Id-3 (zfId1 and zfId3) (Sawai and Campos-Ortega, 1997; Dickmeis et al., 2002). We chose to use the adult zebrafish to examine the role of Id1 during regeneration and the effect of atRA on Id expression, specifically focusing on its role in proliferation during the process of regenerative outgrowth.

RESULTS

Id1 expression in the regenerating caudal fin.

In order to determine if Id1 expression was increased during regeneration, quantitative RT-PCR (qRT-PCR) was performed at 2 and 4 days post amputation (dpa). At 2 dpa at 33°C, the blastema has formed and outgrowth occurs for 1-2 weeks. The distal portion of the blastema is involved in regenerative outgrowth through cellular proliferation, while the proximal portion participates in differentiation required for formation of new structures (Johnson and Weston, 1995). In uncut caudal fins, Id1 is

expressed at low levels (Fig. 23). However, at 2 dpa Id1 expression is increased by 10 fold and this expression remains elevated at 4 dpa (Fig. 23). This suggests a role for Id1 in caudal fin regeneration, particularly during the regenerative outgrowth period.

Whole mount *in situ* hybridization was used to localize Id1 expression in the regenerating caudal fin. No Id1 was detectable in uncut fins (Fig. 24A); however, at 2 dpa Id1 expression was localized to the blastemal region of the regenerate located at the end of each amputated fin ray (Fig. 24C). By 4 dpa, Id1 expression was found at the distal tip of the regenerate in the region, which corresponds to the distal blastema (Fig. 24D). Taken together, these data indicate that increased Id1 expression during the regenerative process is associated with distal regions of the blastema, indicating that Id1 may play a role in regulation of proliferation. After determining that Id1 was associated with the blastema, experiments were carried out to determine if expression could be detected at 1 dpa, when the blastema is first being formed. Low levels of Id1 expression could be detected one day after amputation, suggesting that Id1 may also be involved in the initial formation of the blastema (Fig. 24B).

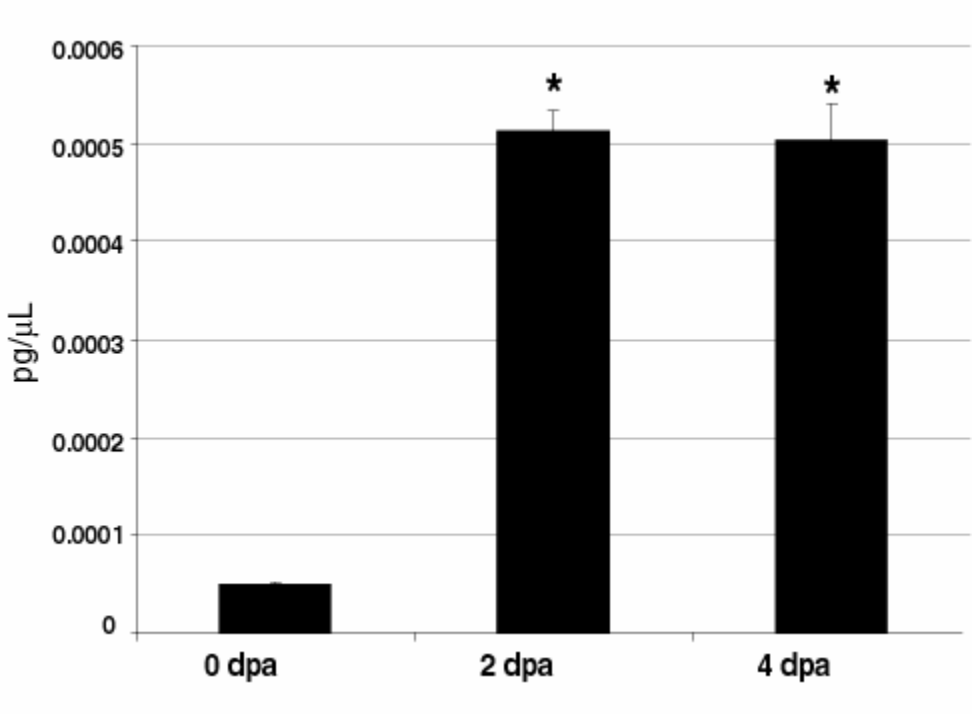


Figure 23. Expression of Id1 is increased during caudal fin regeneration. Total RNA was isolated from zebrafish at 0, 2 and 4 dpa and used as template for qRT-PCR using primers specific for zebrafish Id1 and 28S rRNA. Data were quantified by utilizing a standard curve generated using specific PCR products at concentrations ranging from 100pg to 0.1pg. Data were normalized to 28S rRNA. PCR reactions were performed in triplicate. For each timepoint, ten fins were used. *$p<0.05$

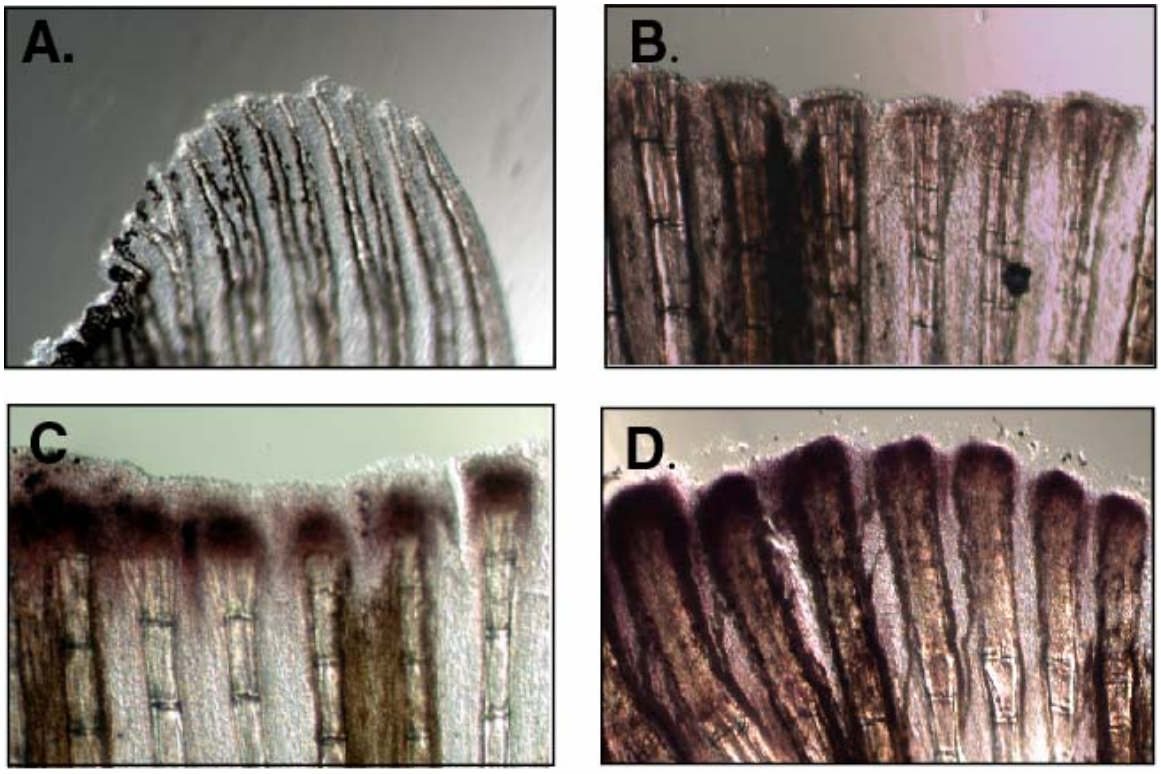


Figure 24. Expression of Id1 is associated with the blastema. Whole mount *in situ* hybridization with an Id1 probes was performed on regenerating fins at 0 dpa (A), 1 dpa (B) 2 dpa (C), and 4 dpa (D). For each timepoint, ten fins were used.

Exposure to atRA affects Id1 expression during regeneration.

Data from our laboratory demonstrate that atRA increases Id1 expression in normal human keratinocytes ((Villano and White, 2006); Fig. 1). To examine the effect of exogenous atRA on Id1 expression during zebrafish caudal fin regeneration, fins were amputated and 24 hours later 10^{-6} M atRA was added. This 24 hour recovery period is necessary because exposure to atRA immediately following amputation prevents regeneration throughout the entire experiment (White et al., 1994) As seen in previous studies, fish exposed to atRA show decreased regeneration and the regenerate tissue appears amorphous (White and Brinckerhoff, 1995). qRT-PCR data show that atRA significantly reduces expression of Id1 in the regenerated fin by approximately 3 fold at 2 dpa and 1.5 fold at 4 dpa (Fig. 25). Whole mount *in situ* hybridization data shows that at 2 dpa, Id1 expression is diffused throughout the regenerate tissue in contrast to control regenerates which show distinct blastemal expression (Fig. 26A-D). Similar results are observed at 4 dpa, however, at the outermost fin rays of the regenerates, expression appears to become restricted to the blastemal tissue (Fig. 26E-H).

Exposure to atRA alters blastemal proliferation.

The Id proteins play a significant role in regulation of cell cycle progression (Yokota and Mori, 2002). During regeneration, the blastema is a highly proliferative area, responsible for supplying cells necessary for replacing lost tissue. A proliferative gradient exists in the blastema of the regenerating caudal fin, with the more proximal cells proliferating at a high rate and the most distal cells not proliferating at all

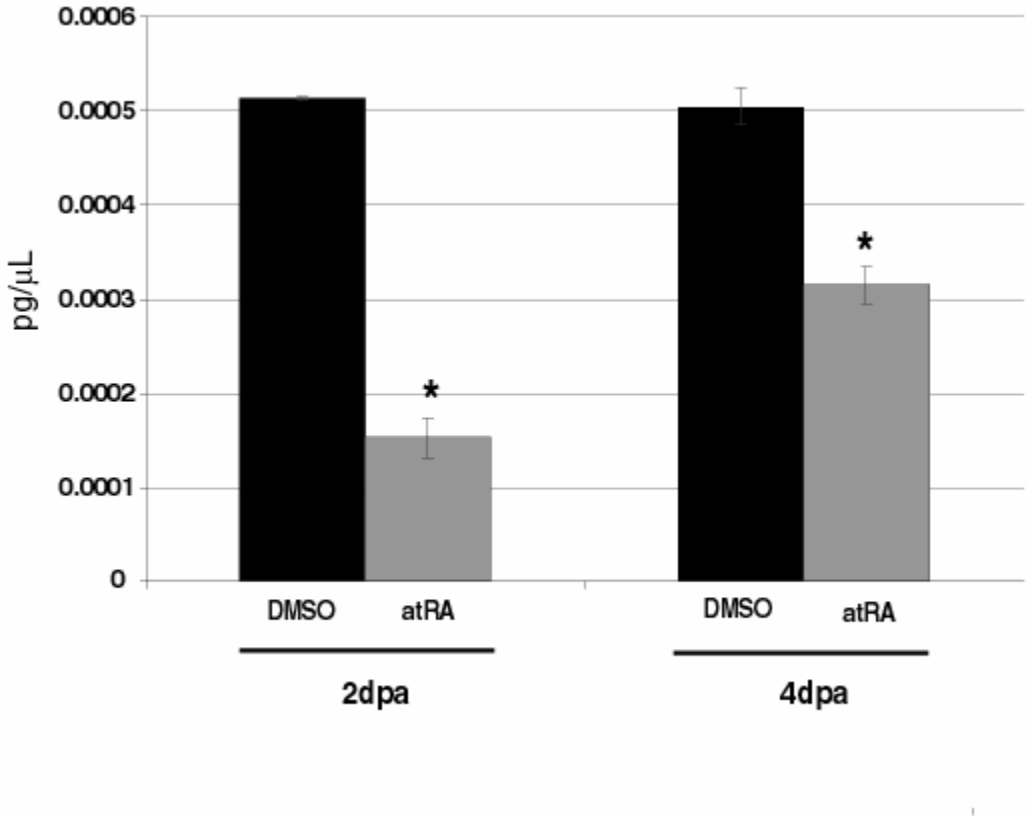


Figure 25. atRA downregulates expression of Id1 in the regenerating caudal fin. At 1 dpa, fish were exposure to either DMSO or 10^{-6} M atRA. Total RNA was isolated from zebrafish at 2 and 4 dpa and used as template for qRT-PCR using primers specific for zebrafish Id1 and 28S rRNA. Data were quantified by utilizing a standard curve generated using specific PCR products at concentrations ranging from 100pg to 0.1pg. Data were normalized to 28S rRNA. PCR reactions were performed in triplicate. Ten fins were used per treatment for each timepoint. *$p<0.05$

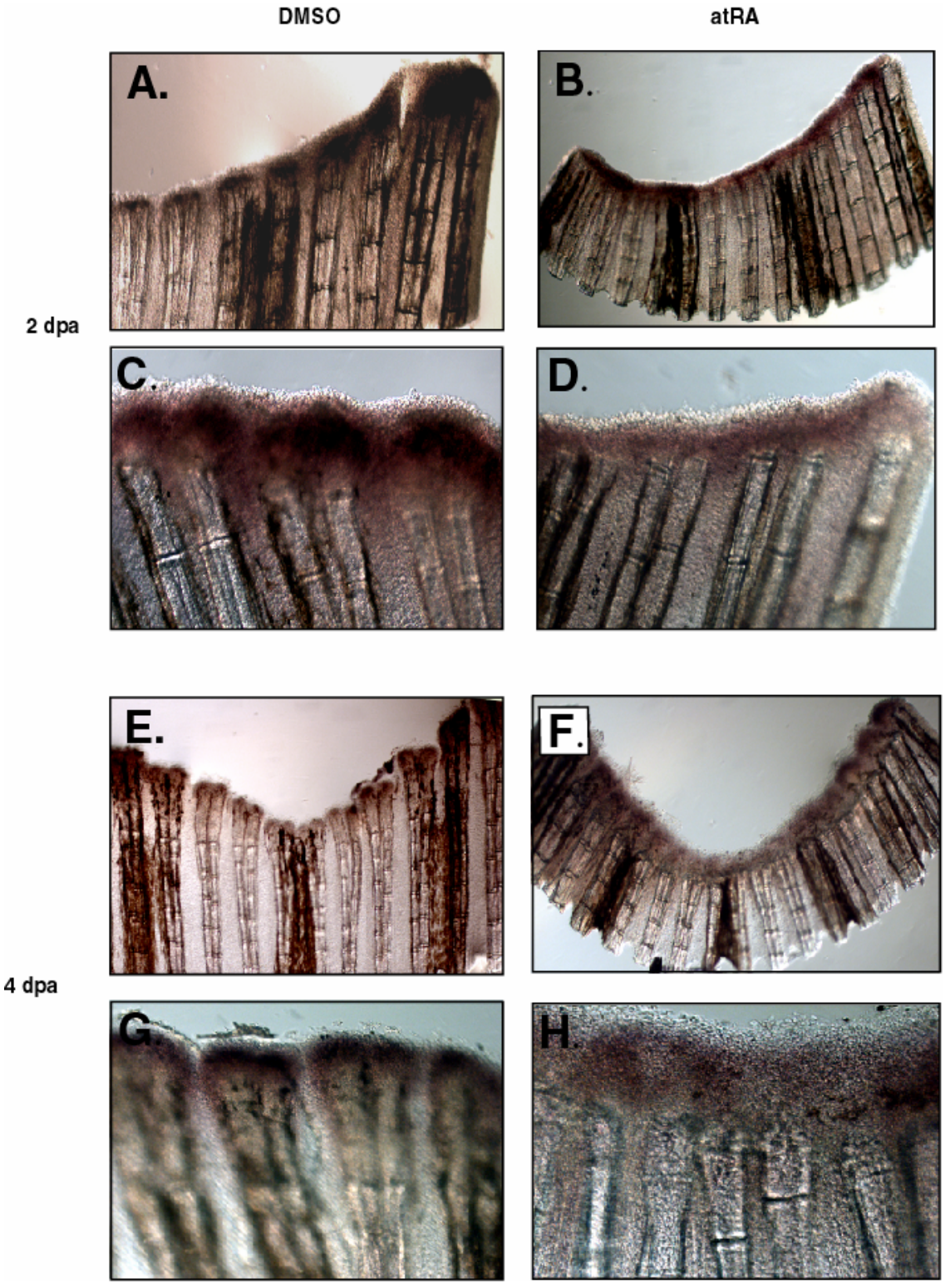


Figure 26. Expression patterns of Id1 in the regenerating fin are altered with atRA exposure. Whole mount *in situ* hybridization with an Id1 probes was performed on regenerating fins exposed to either DMSO at 2 dpa (A,C) and 4 dpa (E, G) or 10-6M atRA at 2 dpa (B,E) and 4 dpa (F,H). For each timepoint, ten fins were used.

(Nechiporuk and Keating, 2002). Previous data from the axolotl limb shows that retinoic acid inhibits blastemal cell proliferation (Maden, 1983; Pietsch, 1987). We hypothesize that Id1 expression patterns correlate with areas of increased proliferation and that the RA-induced decrease in Id1 levels are associated with a decrease in proliferation. At 3 dpa, control and atRA-exposed regenerating zebrafish were given intraperitoneal bromodeoxyuridine (5-bromo-2-deoxyuridine; BrdU) injections and allowed to recover for six hours. At this time, regenerating fins were amputated and fixed for immunohistochemistry. BrdU, a thymidine analogue, becomes incorporated in place of thymidine in actively replicating DNA, thus can be used for detecting actively proliferating cells. Control fins at 3 dpa show intense BrdU staining in the blastemal cells at the tip of each regenerating fin ray (Fig. 27A). Additionally, proliferating cells are seen in the intra-ray mesenchyme near the plane of amputation. In atRA exposed fins, there is a noticeable decrease in proliferation (Fig. 27B), confirming that the effect of RA on blastema proliferation in the regenerating zebrafish caudal fin is similar to that previously observed in the axolotl limb. In addition, the location of the proliferating cells in the regenerate are not distinctly associated with a particular fin ray and seem to be distributed in a manner similar to the expression pattern of Id1 in response to atRA. This suggests that atRA-induced changes in proliferation may be directly associated with altered Id1 expression in regenerating fin tissue.

DISCUSSION

Data presented here show that Id1 expression is induced during regeneration in the zebrafish caudal fin and that this increased expression is associated with the blastemal tissue. These data suggest a role for Id1 in the regenerative outgrowth phase of

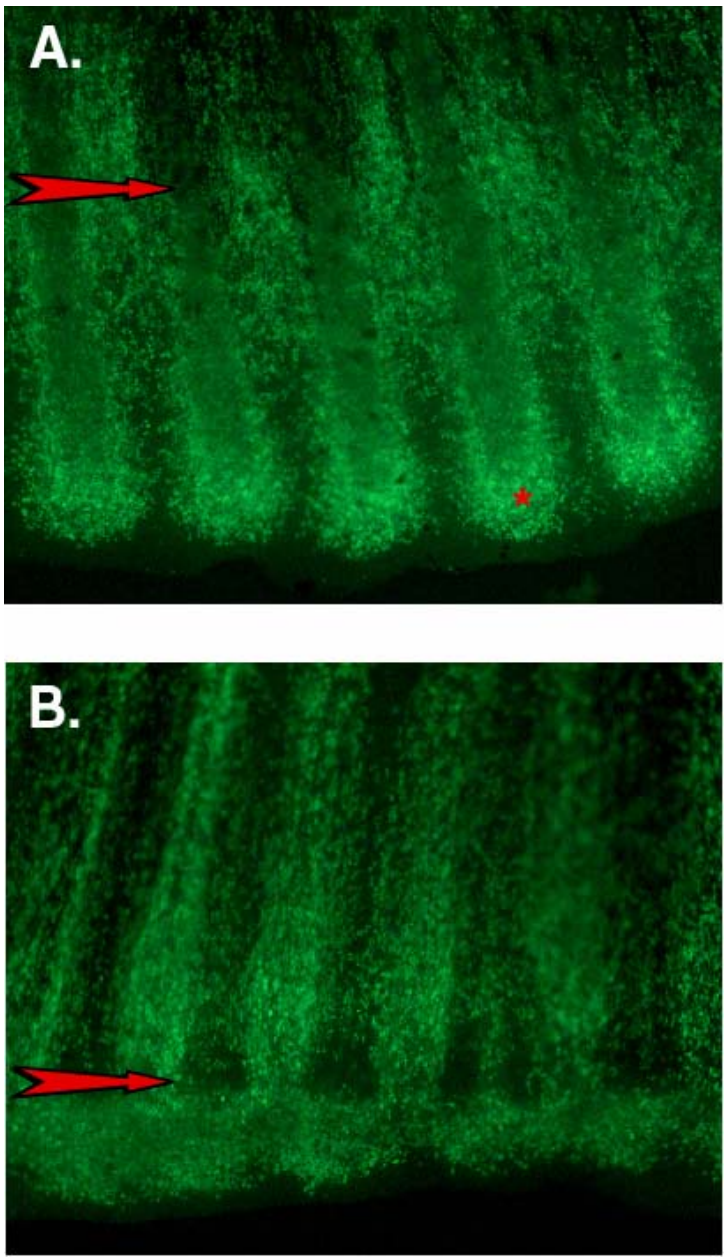


Figure 27. atRA decreases proliferation in the regenerating caudal fin. Adult fish were anesthetized and injected with BrdU. Six hours after injection, fins were reamputated, fix and used in whole-mount immunohistochemistry with an anti-BrdU primary antibody and a fluorescent secondary antibody. Representative images show caudal fins at 3 dpa in fish exposed to (A) DMSO or (B) 10-6 M atRA starting at 1 dpa. Arrows indicate the plane of amputation. Asterisk designates an early of highly proliferating blastemal tissue. For each treatment, ten fins were used.

regeneration, which involves increased cellular proliferation. Moreover, low-level expression of Id1 starting one day after amputation suggests that Id1 may also be involved in the initial formation of the blastema. Id1 has been shown to regulate proliferation and differentiation states of various epithelial cell types including those found in the epidermis, lung, liver, kidney, and mammary gland (reviewed in (Coppe et al., 2003)). In general, Id1 is upregulated during proliferation and downregulated during differentiation or in differentiated cells. In three-dimensional culture, Id1-overexpressing HaCat cells form a hyperthickened and disorganized epithelium, which was characterized by increased proliferation and apoptosis rate and abnormal differentiation (Rotzer et al., 2006). The Id proteins have been previously shown to be involved in the regeneration of various tissue types. A rat model of liver regeneration shows strong Id1 activation in the actively proliferating liver cells (Le Jossic et al., 1994). In the regenerating rat kidney, Id1 protein was detected in the regenerating tubule cells and subsequently decreased in more highly differentiated tubule cells (Matejka et al., 1998). Additionally, in excisional mouse wounds, Id1 expression was induced early after wounding as well as later in the hyperproliferative epithelium (Rotzer et al., 2006). Interestingly, Id1 was regulated differently than Id2 and Id3. Both Id2 and Id3 were detected in the regenerating limbs of the newt and frog (Shimizu-Nishikawa et al., 1999). However, upregulation of Id2 was observed mostly in the blastemal epidermis while Id3 was found in the blastemal epidermis and the mesenchyme of these regenerating limbs.

We have demonstrated that exposure to atRA during regeneration results in decreased Id1 expression at 2 and 4 dpa. At 4 dpa, Id1 expression in the outermost fin rays of the regenerates appears to become restricted to the blastemal tissue again. This

may be a result of decreased concentration of atRA by 4 dpa, since the treatment is not renewed daily. In addition to decreased expression, the expression pattern of Id1 is diffused across the poorly organized regenerated tissue and highlights the lack of distinct blastemae normally associated with individual fin rays. One explanation for this observation may be that retinoic acid treatment causes an increase in collagen fibrils, resulting in expansion of the extracellular space between blastemal cells (Ferretti and Geraudie, 1995). The expression patterns of Id1 in both normal and atRA-exposed regenerating tissue also seem to correlate with the expression patterns of RARγ (White et al., 1994). Moreover, receptor agonist studies have shown that teratogenic effects of RA in the developing limb are mediated preferentially by RARs (Kochhar et al., 1996). Taken together, this suggests that Id1 may be an important intermediate in retinoic acid signaling and play an essential role in the effect of atRA on caudal fin regeneration.

Extensive data exists demonstrating that both excessive and deficient retinoic acid results in a wide range of limb malformations (reviewed in Lee et al., 2004). In the regenerating axolotl limb, retinoic acid causes proximodistal pattern duplication and at high doses inhibits cell division in the blastemal cells until treatment is removed (Maden, 1983). Additionally, increased retinoic acid exposure time permanently inhibited regeneration due to decreased DNA synthesis and mitosis. When retinoic acid is locally applied to the chick wing bud, additional digits develop in the wings (Tickle et al., 1982). These data were among the first to recognize that retinoic acid could mimic the effects observed in grafts of the zone of polarizing activity (ZPA), a region of mesenchyme at the posterior distal margin of the limb bud that control anteroposterior axis patterning. In the zebrafish caudal fin, retinoic acid is not capable of inducing extra-long fins, but

results in fusion of fin rays and is dependent on concentration, time of exposure and duration (Geraudie et al., 1995).

In zebrafish caudal fins, the blastema forms from dedifferentiated cells that migrate locally and from more than two segments away from the site of amputation (Poleo et al., 2001). Proliferation assays using BrdU show that at 3 dpa, the blastema is significantly labeled, indicating a high rate of proliferation. This area seems to coincide with previously observed Id1 expression patterns. Further, exposure to atRA results in decreased BrdU staining. More specifically, the pattern of BrdU staining appears to be dispersed across the poorly established regenerated tissue. Again, this pattern resembles the altered expression pattern of Id1 in response to atRA exposure. We propose that expression of Id1 in the blastema may function to keep cells in an undifferentiated state while increasing the rate of proliferation and that the atRA-induced decrease in proliferation is directly related to a decrease in Id1 expression.

Although retinoic acid signaling is known to be critical for regeneration, little is known about the genes involved. Our data provides specific evidence that Id1 is a target of the retinoic acid signaling pathway in zebrafish caudal fin regeneration. In mammals, Id proteins regulate cell cycle progression primarily through binding to members of the E protein and forming inactive heterodimers (Massari and Murre, 2000). Id proteins have also been shown to dimerize with non-bHLH proteins including the retinoblastoma tumor suppressor protein (pRb), ETS transcription factors and paired-domain homeobox transcription factors (pax) (Norton, 2000; Yates et al., 1999). Id1 regulates cellular senescence in embryonic mouse fibroblasts and human keratinocytes through repression of the tumor suppressor protein $p16^{ink4a}$ (Alani et al., 2001; Nickoloff et al., 2000; You et

al., 2000). Decreased expression of Id1 during zebrafish caudal fin regeneration may result in aberrant binding of transcription factors that affect the expression of genes involved in proliferation, ultimately leading to the negative effects observed.

Ids are downstream targets of the transforming growth factor β (TGFβ) and fibroblast growth factor (FGF) families, which include bone morphogenic protein (BMP), activin, TGFβ, and FGF. FGF signaling has been shown to regulate blastemal proliferation during fin regeneration and loss of FGF signaling inhibits regenerative growth (Poss et al., 2000; Lee et al., 2005). Similarly, inhibition of BMP signaling inhibits regenerative outgrowth due to decreased blastemal cell proliferation (Smith et al., 2006). We have demonstrated here that Id1 is a target of retinoic acid signaling in the regenerating tail fin. Further, Id1 may also be a target of other growth factor signaling pathways involved in the regenerative process. Interestingly, TCDD also inhibits caudal fin regeneration, which is associated with decreased proliferation (Zodrow and Tanguay, 2003). Data from our laboratory has shown that TCDD downregulates Id1 expression in human dermal fibroblasts (Akintobi et al., 2007). It is possible that the ability of TCDD to inhibit proliferation in the blastema is in part due to changes in Id1 expression.

GENERAL DISCUSSION

Retinoic Acid regulation of Id1 in normal human keratinocytes

Findings from this study show that atRA induces the expression of Id1 and Id3 mRNA and protein in normal human keratinocytes (NHK). These results suggest that some of the effects of retinoic acid on cell proliferation are mediated in part by increased Id1 expression. This is supported by the fact that both atRA treatment and Id1 overexpression result in increased proliferation and delayed senescence, accompanied by repression of the cell cycle control gene $p16^{ink4a}$ in various cell types (Alani et al., 2001; Alisi et al., 2003). Our data confirm that atRA treatment not only increases Id1 expression, but also results in a significant down regulation of $p16^{ink4a}$ in NHKs.

We have determined that atRA-induced Id1 mRNA is a result of increased transcription. Results from cotreatment with actinomycin D, a transcriptional inhibitor, show that atRA induction of Id1 mRNA does not involve an increase in Id1 mRNA stability. Changes in heteronuclear RNA (hnRNA), prespliced nascent RNA, are indicative of transcriptional regulation. A significant increase in Id1 hnRNA was observed following atRA exposure of NHKs, providing additional evidence that atRA-induced Id1 expression is via transcription. Further, data using the protein synthesis inhibitor cycloheximide show that the transcriptional regulation of Id1 does not require *de novo* synthesis of a protein intermediate.

To investigate the regulatory regions specific for atRA-induced increase in Id1 transcription, we constructed a deletional series of the Id1 promoter. We determined that sequences in the distal region of the promoter are necessary for atRA-induced Id1 expression. This area contains many known sites, including several CAGA boxes

(SMAD binding sites), a binding site for CREB, and a cluster containing a YY1 site, a SP-1 element and a Egr1 site. Further, mobility shift analysis using oligonucleotides spanning this region demonstrates that atRA exposure induces protein binding to the Egr1/SP-1/YY1 region and to the CREB binding site. Incubation of extracts with a CREB-1 specific antibody resulted in a distinct supershifted complex, indicating that CREB proteins bind to this site. Interestingly, this region of the promoter was also indicated in serum and TGF-β responsiveness in epithelial, muscle, and metastatic breast cancer cells (Tournay and Benezra, 1996; Singh et al., 2002; Kang et al., 2003). The activating transcripton factor/cAMP response element binding protein (ATF/CREB) family of proteins represents a group of basic-region leucine zipper transcription factors that are involved in regulating cell growth (Hai and Hartman, 2001). Retinoic acid has been shown to activate CREB in normal bronchial epithelial cells through phosphorylation (Aggarwal et al., 2006). It is possible that in NHKs, atRA-mediated CREB phosphorylation increases binding to the Id1 promoter, thus inducing Id1 expression. Increased phosphorylation would be consistent with our data demonstrating that atRA upregulates Id1 expression without the requirement of a newly synthesized protein intermediate.

Our findings support several avenues of future research. First, our studies identify potential sites on the Id1 promoter that may be critical for RA's regulation of Id1 expression. Supershift assays suggest the CREB site as the most probable site required for Id1 activation by atRA. Additional transcriptional studies using a promoter construct containing a mutation in the CREB site would determine if this site is an essential cis-acting element for atRA-induced Id1 expression. Next, to further determine if Id1 is

necessary for the effect of atRA on proliferation, Id1 siRNA can be used to knockdown expression of Id1. Cell cycle analysis following atRA treatment of Id1 siRNA transfected NHKs will show if atRA is still able to increase the proliferative capacity of these cells. Lastly, the studies described here were carried out in a monolayer cell culture system. In intact skin, dermal fibroblasts secrete cytokines and growth factors that are essential for regulating keratinocytes proliferation and differentiation (Smola et al., 1993). The ability to grow skin equivalents through the use of 3D organotypic cultures is unique in that regulatory mechanisms of growth and differentiation of keratinocytes can be investigated under conditions mimicking those *in vivo*. Therefore, the use of organotypic cultures may provide a better model for understanding of the effects that RA-induced Id1 expression has in the skin model.

Retinoids are used in dermatology as therapeutics for a skin pathologies including acne vulgaris and keratinizing dermatoses such as psoriasis, to enhance wound healing, and as chemotherapies. The data on the use of retinoids for wound healing is controversial. While the use of retinoids preoperatively has been shown to enhance wound healing, the results of retinoid use during would healing are mixed (reviewed in (Abdelmalek and Spencer, 2006)). Clinical studies of retinoid chemotherapies have shown reduced tumor progression in a variety of non-melanoma skin cancers including malignancies in patients with xeroderma pigmentosa (Kraemer et al., 1988), oral leukoplakia (Hong et al., 1986), and squamous cell carcinoma (Moon et al., 1997). Animal studies of synthetic retiniods demonstrate possible chemopreventative activity in breast, bladder, lung, ovarian, and prostate cancers (reviewed in (Niles, 2000)). The frequent use of systemic retinoids, however, affects several organ systems including

skeletal, hepatic, and cardiovascular (Vahlquist, 1992; Desai et al., 2007). Therefore, understanding the downstream targets of retinoid signaling may provide new targets for therapeutic use in place of prolonged retinoid therapy. The role that Ids play in the regulation of cellular proliferation and new data demonstrating the role of Id1 in retinoic acid signaling suggests that Ids may be a novel target for cancer therapy as well as treatment of various skin pathologies and promotion of wound healing.

Retinoic Acid effects on Id1 in the developing zebrafish

The next goal of this thesis was to determine the affect of atRA on Id1 in the developing zebrafish embryo. First, we wanted to determine if Id1 alone played a critical role in zebrafish development. In the mouse model, loss of Id1 alone was not sufficient to cause abnormal phenotypes in the embryo (Fraidenraich et al., 2004). However, we show that morpholino knockdown of Id1 resulted in pericardial and yolksac edema, decreased blood flow, and improperly looped hearts. Cranial edema and undulating notochords were observed following injection of higher concentrations of morpholino. Additionally, early loss of Id1 affected zebrafish development well into the larval stage, after the concentration of the morpholino diminished. Larval Id1 morphants exhibited a variety of craniofacial malformations, decreased body size, and lack of swim bladder inflation. These results demonstrate that Id1 is essential for normal embryonic development and that loss of Id1 function during earlier embryonic stages may result in abnormal cellular differentiation or decreased proliferation causing structural defects in larval fish.

Exposure to excess atRA results in a phenotype similar to loss of Id1, including pericardial edema and an improperly looped heart. RA deficiency also resulted in cardiac

defects similar to atRA exposed embryos. Cardiovascular defects are also observed in vitamin A deficient mice as well as mice exposed to excess RA (reviewed in Pan and Baker, 2007). We show that atRA decreases Id1 expression in the zebrafish embryo and specifically in the heart tissue. Additionally, induced RA deficiency, by knockdown of raldh2, resulted in increased Id1 expression. In the zebrafish, retinoic acid signaling is necessary for patterning in the central nervous system and fin bud induction (Grandel et al., 2002; Maves and Kimmel, 2005; Gibert et al., 2006), myocardial differentiation (Drysdale et al., 1997), as well as acquisition of heart tube polarity (Stanier and Fishman, 1992). Moreover, Id combination knockout mice show severe cardiac defects leading to embryonic lethality at E13.5 (Fraidenraich et al., 2004). We proposed that the effects of retinoic acid on heart development may partially be a result of decreased Id1 expression.

Morpholino knockdown of Id1 resulted in increased expression of gata5 and nkx2.5, two cardiac-specific transcription factors. Our findings further demonstrate that RA mediated changes in Id1 expression are also associated with changes in gata5 and nkx2.5. More specifically, excess atRA decreased gata5 and nkx2.5 expression, while RA deficiency increased expression. This at first seemed to contradict our Id1 morpholino data. However, data from other laboratories demonstrate that mouse gata4 and nkx2.5 can regulate Id expression and that the mouse Id2 promoter contains nkx2.5 binding sites (Lim et al., 2007; Moskowitz et al., 2007). In addition, in mice Ids bind to gata4 and nkx2.5 and inhibit their ability to transactivate the expression of cardiac genes (Ding et al., 2006). These data, along with our findings, suggest a regulatory negative feedback loop between Id1, gata5 and nkx2.5, which functions to tightly regulate expression patterns during heart development in the zebrafish embryo (Fig. 28).

Additionally, we hypothesize that the effects of excess RA or RA deficiency on cardiac morphology may be a result of dysregulation in this feedback mechanism.

Further investigation is required to better understand the complex regulatory pathways that our findings suggest. Data regarding the interactions of atRA, gata5, nkx2.5 and Id1 represents what is observed in expression levels of the whole embryo. More information may be gained by localizing the expression of gata5 and nkx2.5 using *in situ* hybridization. This will clarify whether expression is simply increasing or decreasing or if there are changes in the tissue location and/or cell types where these transcription factors are being expressed. Further, the use of a gata5 and/or nkx2.5 morpholino may provide a more complete understanding of the ability of these two transcription factors to regulate Id1 levels. These experiments will test our hypothesis that a complex feedback loop exists between Id1, gata5, and nkx2.5.

Congenital heart defects are the clinical manifestation of anomalies in embryonic heart development. According to the American Heart Association, 9 occurrences of congenital heart defects per 1000 live births are expected each year in the United States. Additionally, more children die each year from congenital heart defects than are diagnosed with childhood cancer (Srivastava, 1999). However, the genetic mechanisms causing congenital heart defects are still unclear. A significant amount of research has been focused on the role of retinoids during embryonic development, especially development of the heart (for a review see (Pan and Baker, 2007)). RA signaling is required for regulating cardiomyocyte proliferation and differentiation during normal

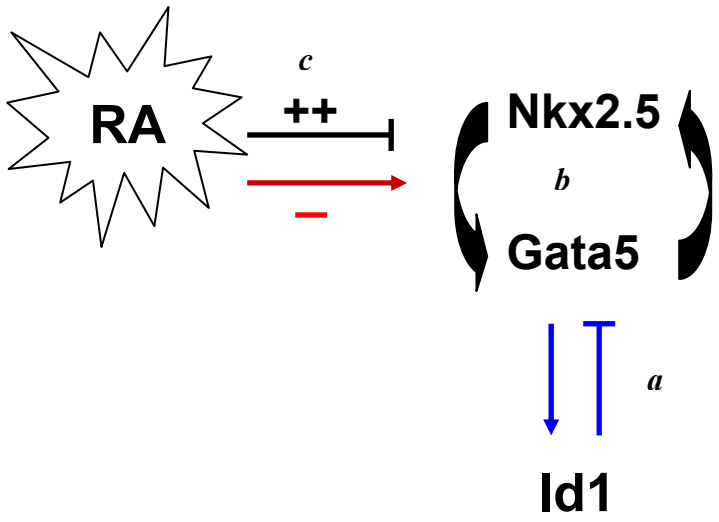


Figure 28. Proposed regulatory feedback loop involving Id1, gata5, and nkx2.5. Id1 inhibits the expression of gata5 and nkx2.5, while in turn, gata5 and nkx2.5 can activate the expression of Id1 (***a***) Blue arrows represent events which occur in normal development. Gata5 and nkx2.5 can also regulate the expression of one another (***b***). In addition, excess RA (black ++) results in decreased expression of gata5, and nkx2.5 and RA deficiency (red -) increases expression (***c***). RA-induced changes in gata5 and nkx2.5 expression may subsequently alter Id1 expression. Excess RA inhibits the expression of gata5 and nkx2.5, which in turn may decrease Id1 expression. Further, RA deficiency upregulates gata5 and nkx2.5, which may result in increased Id1 expression.

heart development. However, excess embryonic exposure to retinoic acid has teratogenic effects on heart development. For example, inadvertent retinoid exposure in human fetuses, in the form of prescribed isotretinoin (Accutane®), results in embryopathy characterized by craniofacial malformations, congenital heart defects, and central nervous system defects (Lammer et al., 1985). In laboratory mice, low doses of atRA is equally or more teratogenic than isotretinoin (Sulik et al., 1995; Campbell et al., 2004). A more complete understanding of the genetic mechanisms involved in congenital heart defects is necessary for the development of new therapeutic and preventative measures. The work presented here may add to the understanding of signaling events that occur during cardiac morphogenesis and help define the Ids as a family of genes required for normal heart development.

Our findings may also relate to the congenital heart defects that are a common among infants with fetal alcohol syndrome (FAS) (Chaudhuri, 2000). The exact mechanisms by which ethanol induces FAS are unknown. Interestingly, excessive alcohol intake is associated with decreased hepatic vitamin A levels (Wang, 2005). Alcohol can alter RA levels by competing for enzymes required for oxidation to RA, inducing P450s that catabolize RA, and mobilizing vitamin A stores in the liver. Given the ability of RA signaling to regulate Id expression, we hypothesize that ethanol exposure may result in changes in Id1 expression levels via altered RA levels.

An additional role for Id proteins may be in regenerating heart tissue, such as that seen after myocardial infarction (MI). Overexpression of Ids induces the expression and secretion of matrix metalloproteinases such as MMP2 and MMP7 (Desprez et al., 1998; Coppe et al., 2004; Ouyang et al., 2001; Lyden et al., 1999). Also, knockdown of Id1 in

the developing zebrafish results in increased MMP2 and MMP9 expression (Villano and White, unpublished data). Matrix metalloproteinases (MMPs) play a major role in extracellular matrix remodeling during the wound repair process that takes place post-MI (Fedak et al., 2005; Vanhoutte et al., 2006). MMP activity is tightly regulated by proteins the tissue inhibitor of MMPs (TIMPs). However, the mechanisms involved in the spatial and temporal regulation of MMP expression are not well understood. Given the role that Ids have been shown to play in heart development, it may be possible that Ids are involved in post-MI tissue remodeling, possibly through their interactions with MMPs. Further, the potential role of RA signaling and its effects on regenerating heart tissue can be investigated with Ids and MMPs as specific targets. Retinoic acid has been shown to attenuate cardiac remodeling after MI in rats, decreasing collagen accumulation which is associated with myocardial dysfuntion and poor prognosis (Padhi et al., 2004). The zebrafish is a valuable model for this type of study since they are able to fully regenerate their hearts in two months after a 20% ventricular resection (Poss et al., 2002).

Retinoic Acid effects on Id1 during zebrafish caudal fin regeneration

Our findings from the zebrafish regenerating caudal fin model show that Id1 is essential for normal regeneration and a target of atRA signaling. Id1 expression is induced during regeneration in the zebrafish caudal fin and this increased expression is associated with the blastemal tissue, suggesting a role for Id1 in the regenerative outgrowth phase, which involves increased cellular proliferation. Moreover, low-level expression of Id1 one day post amputation suggests that Id1 may also be involved in the initial formation of the blastema. Exposure to atRA during regeneration results in decreased Id1 expression at 2 and 4 dpa. The expression pattern of Id1 becomes diffused

across the poorly organized regenerated tissue and highlights the lack of distinct blastemae normally associated with individual fin rays. Investigation into changes in proliferation revealed that atRA not only decreases proliferation but the location of the proliferating cells in the regenerate is not restricted to distinct blastemae and seems to be distributed in a manner similar to the expression pattern of Id1 in response to atRA. We have proposed that expression of Id1 in the blastema may function to keep cells in an undifferentiated state while increasing the rate of proliferation and that the atRA-induced decrease in proliferation is directly related to a decrease in Id1 expression.

Our studies have demonstrated a role for Id1 in epimorphic zebrafish regeneration. However, future experiments will help determine the role that Ids play in RA-induced abnormalities in the regenerating caudal fin. First, to better understand the effects of atRA on cell cycle during regeneration flow cytometry can be performed on cells isolated from the regenerating caudal fin tissue to look for changes with atRA exposure. Although atRA is known to decrease proliferation, it is unknown whether the cells are in cell cycle arrest or undergoing apoptosis. If decreased Id1 expression is mediating the effects of atRA, we hypothesize that more cells will be seen in G1 arrest. This data will help determine the fate of cells in response to atRA during regeneration.

Next, the effects of Id1 expression in the presence of decreased RA signaling can be examined to support the findings in our regeneration experiments. Diethyl aminobenzaldehyde (DEAB), a raldh inhibitor can be used to block the synthesis of RA, similar to the use of the raldh2-MO in embryos. If Id1 is being regulated by atRA, we hypothesize that loss of RA signaling will result in an increase in Id1 expression. Since previous results show that exposure to atRA and injection of the raldh2-MO result in

similar defects during embryogenesis, we also expect to see a similar effect on caudal fin regeneration with DEAB as we do with atRA exposure.

Retinoids affect the wound healing process by influencing the production of cytokines and growth factors, regulating keratinocyte migration, as well as promoting the production of extracellular matrix components (reviewed in (Fu et al., 2007)). These effects can enhance wound healing in the inflammatory, proliferative, and remodeling phase. At present, there is very little data examining the role of Id during regeneration. We have provided a new functional role for Ids as well as a better understanding of RA signaling events during regeneration. Broadening our knowledge base in regards to the signaling pathways and their targets in the regenerative process will not only aid in our basic understanding, but provide new possibilities for therapies that can enhance regeneration and wound healing in humans.

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Curriculum Vitae

Caren M. Villano

EDUCATION

2003 - 2007 Rutgers University New Brunswick, NJ

Ph.D. /Joint Graduate Program in Toxicology

- Thesis: The effects of all-trans retinoic acid on the inhibitor of DNA binding (Id) family of proteins in cell cycle and development.

1999 - 2001 New York University New York, NY

M.S./Biology

- Thesis: A phylogenetic study of the order *Malpighiales* using phytochrome C sequencing.

1995 - 1999 Worcester Polytechnic Institute Worcester, MA

B.S. with Distinction/Biotechnology

- Thesis: TGF-beta signaling in A549 lung carcinoma cells.

PROFESSIONAL EXPERIENCE

2003-2006 Rutgers University New Brunswick, NJ

Departmental teaching assistant

- Helped set up faculty lectures and taught multiple sections of Introduction to Biochemistry Laboratory.

2004 Rutgers University New Brunswick, NJ

Part-time Lecturer

- Molecular Toxicology Laboratory

2004-present Rutgers University New Brunswick, NJ

Assistant Instructor

- Aid in teaching a five-day hands-on laboratory course in protein purification using green-fluorescent protein (GFP) run by the Center for Research and Education in Bioluminescence and Biotechnology (CREBB).

2001 - 2003 Rutgers University New Brunswick, NJ

Senior laboratory technician

- Helped set up a laboratory for a new faculty member in the Department of Biochemistry and Microbiology. Duties included conducting research, training undergraduate students, and ordering.

PROFESSIONAL PUBLICATIONS

Hillegass, J., Villano, C. M., Cooper, K., White, L. A. Matrix metalloproteinase-13 (MMP-13) is required for zebrafish development and is a target for glucocorticoids. Tox. Sci. (*Accepted).*

Elo, B., Govorko, D., Villano, C. M., White, L. A. Larval zebrafish as a model for glucose metabolism: expression of phophoenolpyruvate carboxykinase (PEPck) as a marker for exposure to anti-diabetic compounds. J. Mol. Endocrinology (*Accepted).*

Akintobi, A., Villano, C. M., White, L. A. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exposure of normal human dermal fibroblasts results in AhR-dependent and independent changes in gene expression. Tox. Applied Pharm. (*Accepted*).

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Murphy, K. A., Villano, C. M., Dorn, R., and White, L. A. 2004. Interaction between the aryl hydrocarbon receptor and retinoic acid pathways increases matrix metalloproteinase-1 expression in keratinocytes. J. Biol. Chem. **279**: 25284-25293.

PROFESSIONAL PRESENTATIONS

Villano, C. M., Hillegass, J. M., and White, L.A. Expression of the helix-loop-helix inhibitor of DNA binding 6 (Id6) is induced by all-trans retinoic acid in zebrafish embryos. 45th Annual Meeting Society of Toxicology, San Diego, March 5-9, 2006.

Akintobi, A., Villano, C. M., White, L. A. 2,3,7,8 -tetrachlorodibenzo-p-dioxin inhibits expression of the inhibitor of DNA binding (Id) -1 and -3 in normal human fibroblasts. 45th Annual Meeting Society of Toxicology, San Diego, March 5-9, 2006.

Hillegass, J. M., Villano, C. M., White, L.A., and Cooper, K. Matrix metalloproteinase expression and function during zebrafish embryogenesis: analysis of MMP-2, -9, and -13 following exposure to dexamethasone or hydrocortisone. 45th Annual Meeting Society of Toxicology, San Diego, March 5-9, 2006.

Villano, C. M., and White, L.A. Expression of the helix-loop-helix inhibitor of DNA binding-1 (Id-1) gene is regulated by retinoic acid in normal human keratinocytes. 44th Annual Meeting Society of Toxicology, New Orleans, March 6-10, 2005.

Murphy, K. M., Villano, C. M., and White, L.A. 2,3,7,8 -tetrachlorodibenzo-p-dioxin alters expression of retinoic acid receptors in normal human keratinocytes. 44th Annual Meeting Society of Toxicology, New Orleans, March 6-10, 2005.

Prince, V. L., LaPrete, V., Villano, C. M., White, L. A. Matrix metalloproteinases as biomarkers for dioxin exposure in developing Japanese medaka (*Oryzias latipes*). 44th Annual Meeting Society of Toxicology, New Orleans, March 6-10, 2005.

Hillegass, J. M., Villano, C. M., White, L.A., and Cooper, K. Matrix metalloproteinase inhibition during zebrafish embryogenesis following exposure to

dexamethasone or hydrocortisone. 44th Annual Meeting Society of Toxicology, New Orleans, March 6-10, 2005.

Villano, C. M. and White, L. A. Retinoic acid induces expression of the helix-loop-helix inhibitory protein Id-1 in normal human keratinocytes. 43rd Annual Meeting Society of Toxicology, Baltimore, March 21-25, 2004.

White, L. A., Murphy, K. A., Akintobi, and Villano, C. M. 2,3,7,8 - tetrachlorodibenzo-p-dioxin induces MMP expression and invasion in A2058 melanoma cells. 43rd Annual Meeting Society of Toxicology, Baltimore, March 21-25, 2004