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Chronic *Mycobacterium marinum* Infection Acts as a Tumor Promoter in Japanese Medaka (*Oryzias latipes*)

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Abstract

An accumulating body of research indicates there is an increased cancer risk associated with chronic infections. The genus *Mycobacterium* contains a number of species, including *M. tuberculosis*, which mount chronic infections and have been implicated in higher cancer risk. Several non-tuberculosis mycobacterial species, including *M. marinum*, are known to cause chronic infections in fish and like human tuberculosis, often go undetected. The elevated carcinogenic potential for fish colonies infected with *Mycobacterium* spp. could have far reaching implications because fish models are widely used to study human diseases. Japanese medaka (*Oryzias latipes*) is an established laboratory fish model for toxicology, mutagenesis, and carcinogenesis; and produces a chronic tuberculosis-like disease when infected by *M. marinum*. We examined the role that chronic mycobacterial infections play in cancer risk for medaka. Experimental *M. marinum* infections of medaka alone did not increase the mutational loads or proliferative lesion incidence in all tissues examined. However, we showed that chronic *M. marinum* infections increased hepatocellular proliferative lesions in fish also exposed to low doses of the mutagen benzo[a]pyrene. These results indicate that chronic mycobacterial infections of medaka are acting as tumor promoters and thereby suggest increased human risks for cancer promotion in human populations burdened with chronic tuberculosis infections.

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Keywords

benzo[a]pyrene; carcinogenesis; hepatic neoplasia; inflammation; *Oryzias latipes*; tuberculosis; non-tuberculosis mycobacteria

Introduction

Both epidemiological and experimental evidence have revealed increased cancer risks in individuals with chronic infectious diseases (reviewed in Ames et al., 1995; Lu et al., 2006). A number of studies suggest that this greater risk of tumor induction with chronic infections may result from one or more mechanisms (Ames et al., 1995; Friedberg et al., 2006; Lu et al., 2006). The observed differences likely reflect different characteristics of a given infectious agent, the host response to infection, and the pathology of infection (Ames et al., 1995). Much evidence has advanced the model that tumorigenesis occurs in stages: first, an initiation stage, usually involving DNA damage; second, a promotion stage, with proliferation of cells and the fixation of mutations from premutational lesions; and finally, tumor progression by expansion of mutant cells leading to the development of growing tumors (Bowden, 2004). Chronic infections that provoke inflammatory responses may have tumor initiation or promotion effects by attracting macrophages to sites of infections (Weitzman et al., 1985; Yamashina et al., 1986; Shacter et al., 1988; Christen et al., 1999; Lu et al., 2006). Activated macrophages and other leukocytes produce a number of toxic agents, such as reactive oxygen and reactive nitrogen intermediates (ROI and RNI), which may damage surrounding tissue (Zhuang et al., 2002; Kim & Wogan, 2006). Extensive damage may provoke cell death, while lesser damage may induce mutations in the surviving cells, initiating cells for tumor formation. The provocation of the inflammatory response and subsequent tissue damage may also increase cancer risk by acting as a tumor promoter. With heavy tissue damage, compensating cell proliferation or healing may follow, allowing for error-prone replication across premutational lesions and the clonal expansion of cells carrying fixed mutations (Rosin et al., 1994a, 1994b; Cohen, 1999; Ennis, 2001; Bowden, 2004; Friedberg et al., 2006). For some chronic infections, tissues may remain infected for decades, encountering multiple rounds of inflammation and necrosis/wounding followed by healing. Hence, the cancer risk would be amplified at each cycle (Ames et al., 1995; Mostafa et al., 1999; Friedberg et al., 2006; Lu et al., 2006; Izzotti et al., 2007).

The connection between chronic infection, resulting in persistent inflammation, and cancer has been clearly documented for a few infectious diseases. For example, *Helicobacter pylori* infection has been correlated with higher risk of gastric cancer, has been classified as a type I carcinogen since 1994, and may serve the role of both tumor initiator and promoter (Mostafa et al., 1999; Uemura et al., 2001; Kusters et al., 2006). Schistosomal infections have long been associated with higher rates of bladder and colon cancers. However, both epidemiology and experimental evidence indicate that these parasitic infections act mainly as tumor promoters requiring mutagen exposure to lead to tumor development (Hashem and Boutros, 1961; Domingo et al., 1967; Hicks et al., 1980; Rosin & Hofseth, 1999). Furthermore, other animal models of infection have documented that many chronic infections can similarly act as tumor promoters (Bailey, 1979; Thamavit et al., 1996; Morotona et al., 2001).

Epidemiological evidence has implicated chronic mycobacterial infections in increased cancer risk. For example, higher lung cancer rates were documented among people with pulmonary *Mycobacterium tuberculosis* infections (Alavanja et al., 1992; Brown and Almanoff, 1992; Wu et al., 1995; Brenner et al., 2001). In addition, other non-tuberculous mycobacterial (NTM) infections have also been implicated in increased cancer risk. For example, *M. ulcerans* has been correlated with scar carcinogenesis in skin and other organs (Evans et al., 1999; Cope et

al., 2004), *M. leperae* with scar carcinoma (Richardus et al., 1991; Adams & Krahenbuhl, 2003) and *M. paratuberculosis* with tumors secondary to granulomas of Crohn's disease (Hermon-Taylor et al., 1998).

Several environmental NTM cause chronic, tuberculosis-like infections in a variety of teleost fish (Aronson, 1926; Kaattari et al., 2006; Ranger et al., 2006) and mycobacteriosis is a prominent problem in aquaculture as well as wild fish populations (Heckert et al., 2001; Ucko et al., 2002). Furthermore, several outbreaks of mycobacteriosis have been documented in research facilities housing zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*), important laboratory models in toxicology and carcinogenesis (Teska et al., 1997; Astrofsky et al., 2000; Sanders & Swaim, 2001; Kent et al., 2004; Watral & Kent, 2007). This is a concern considering that mycobacteriosis in fish colonies is common and may go undetected, possibly compromising interpretations of research results, especially for genotoxicity and carcinogenesis studies (Abner et al., 1994; Kent et al., 2004). Because of the possible role of chronic inflammation in neoplasm formation and the increased use of small fish models to study human health, it is important to evaluate both the mutagenic and carcinogenic potential of chronic infections that plague fish models including medaka.

One particular species of *Mycobacterium*, *M. marinum*, poses a significant problem in both wild and domesticated fish (Aronson, 1926; Ucko, 2002; Swaim et al., 2006) and experimental *M. marinum* infections of medaka have recently been established as a model for tuberculosis (Broussard & Ennis, 2007). Medaka provide an excellent *in vivo* model for the study of *M. marinum* as a potential promoter of neoplasia for several reasons: (1) experimental induction of chronic infection with *M. marinum* is highly reproducible (Broussard & Ennis, 2007), (2) carcinogenesis in medaka is well characterized and they are highly responsive to tumor induction with various carcinogens, (3) spontaneous tumor frequencies in livers and other tissues are exceptionally low (Abner et al., 1994; Hawkins et al., 1995; Boorman et al., 1997; Hawkins et al., 2003), (4) the λ transgenic medaka line has been established to detect mutations in tissues, a proximal event in carcinogenesis (Winn et al., 2000; Winn & Norris, 2005a). The present study using the λ transgenic medaka line together with *M. marinum* explores the role of chronic infections in cancer risk. We provide evidence that chronic *M. marinum* infection acts as a tumor promoter in liver tissues of animals also exposed to low doses of the mutagen benzo[a]pyrene (BaP).

Materials and Methods

Bacterial strains and culture

M. marinum strain G13R (1218R/pG13), a strain derived from a fish outbreak isolate (ATCC 927), was provided by Lucia Barker (University of Minnesota Medical School, Duluth, MN) and was used in all experimental infections of fish (Barker et al., 1999). The pG13 plasmid carried by this strain of *M. marinum* allows for high constitutive expression of Green fluorescent protein (Gfp) and confers kanamycin resistance. Cultures of *M. marinum* were grown at 30 °C in Difco™ Middlebrook 7H9 (M7H9) broth, with the addition of ADS (BSA Fraction V 5%, Dextrose 2%, NaCl 0.81%), Tween 80 (0.2%), kanamycin (50 µg/mL), and cycloheximide (100 µg/mL). When *M. marinum* was grown on solid media, cultures were streaked or spread onto Middlebrook 7H10 (M7H10) plates supplemented with ADS, kanamycin (50 µg/mL), and cycloheximide (100 µg/mL) and incubated at 30 °C for 7–12 days prior to determination of colony forming units (cfu).

Preparation of infection doses

Prior to inoculation of fish, cultures of *M. marinum* were collected at an optical density at 600 nm (O.D.₆₀₀) of ~0.6, washed twice in phosphate buffered saline (PBS) (pH 7.4), and

concentrations were adjusted to the appropriate infection dose. Doses were confirmed by plating serial dilutions onto M7H10 agar plates, as described above, to determine viable counts.

Exposure of medaka to benzo[a]pyrene and *M. marinum*

A transgenic medaka (*Oryzias latipes*, Adrianichthyidae, Beloniformes; fishbase.org name: Japanese rice fish) line, carrying the λ LIZ bacteriophage construct (Winn et al., 2000), a shuttle vector for molecular detection of mutations, was used in this experiment. Fish were reared from eggs collected within a 5 day period. At 2 months of age, all fish were transferred to animal biosafety level 2 (ABL2) rated aquatics lab facilities and maintained in 40 L aquaria at a density of 28–30 fish per aquarium and held under conditions of 28°C, 16 h light/8 h dark throughout the experiment. The fish were initially split into four conditional groups: (1) 150 fish infected with *M. marinum*, (2) 150 fish exposed to BaP, (3) 170 fish infected with *M. marinum* and exposed to BaP, and (4) 130 untreated control fish that were not exposed to *M. marinum* or BaP.

At 3.5 months of age, the two groups exposed to *M. marinum* were intraperitoneally injected with 10^4 cfu *M. marinum* suspended in 20 μ L PBS, using a 26-gauge needle. Prior to injection, fish were anesthetized with tricaine methanesulfonate (MS-222) (0.02%). The infection was confirmed by dissecting two fish from each *M. marinum* infection groups at 4 weeks post-injection and viewing the viscera and peritoneal cavity by fluorescence microscopy to confirm Gfp expression. This was followed by homogenizing sampled kidneys and livers in PBS and plating onto M7H10 plates as described previously to determine viable counts of Gfp-expressing *M. marinum* in infected organs (Broussard & Ennis, 2007). After shipment of fish to Oregon State University (OSU), Corvallis, OR (USA) (see below) infection was further confirmed by culture on mycobacterial selective media.

At 4.5 months of age, both BaP exposed groups were placed at a density of 10 fish/L in separate 40 L aerated aquaria at 28°C. Fish were exposed to 300 mg/L BaP for 96 h, with complete water change and dose renewal every 24 h. At the end of the 96 h period, fish were returned to their original tanks. Two weeks post exposure to BaP, 100 fish from each of the four groups were transferred from the University of Louisiana (UL), Lafayette, LA (USA) to OSU for continued maintenance and histological evaluation. Here fish were also maintained in 3 tanks/exposure group (i.e. ~30 fish/tank). Fish were held in similar conditions as those at UL; 40 L aquaria at 28°C in an ABL2 approved laboratory. All procedures were conducted as prescribed by NIH BMBL guidelines (5th ed, 2007) and were approved by both the Institutional Biosafety Committee and by the Institution Animal Care and Use Committee.

Histology

At 31 weeks post-injection with *M. marinum* three separate samples were taken per group, each sample consisted of ~10 fish and were taken from different tanks to control for any tank effect. Fish were fixed in Dietrich's solution and processed for histology using standard techniques. Three step-wise sagittal sections (100 μ m apart) per animal were cut and stained with hematoxylin and eosin, a matching set of sections were stained with Ziehl-Neelson acid fast stain. In this way all fish were tested for proliferative lesions, histopathology matching mycobacteriosis and for acid fast bacilli. Sections were evaluated for the presence and types of proliferative lesions based on guidelines of Boorman et al. (1997). Furthermore, histological sections were scored for incidence and severity of mycobacteriosis: acid fast bacilli and granuloma formation. Incidence was scored for presence or absence of mycobacteriosis, while severity for each section was scored on a scale of 0 to 4 (0 = no mycobacteriosis, 4 = most severe cases. Scoring was done independently by JWF and MLK.

Mycobacterium species identification

Upon arrival of fish to OSU, intermittent mortalities were observed in all groups of fish. Because of this, histological evaluation was carried out on moribund fish. In order to investigate possible contamination of control fish with our *M. marinum* strain or possible wide spread infection with other bacterial pathogens, fish tissues from animals of our medaka colony, that were not shipped to OSU, along with those at OSU were homogenized and plated onto M7H10 and other mycobacterial enrichment media. Some unexposed animals were found to have tissues carrying bacteria which grew on these enrichment media and formed nonpigmented colonies with morphology matching mycobacteria, suggestive of mycobacterial infection. Species determination of isolates from unshipped fish was carried out by partial PCR amplification of the 16S *rRNA* gene followed by diagnostic restriction digestion as described previously (Talaat et al., 1997) indicating the presence of *M. chelonae*. The presence of *M. chelonae* was independently confirmed at OSU by partial sequencing (~550 bp) of the *hsp65* gene, which was found to be identical to *M. chelonae* isolated previously from zebrafish (GenBank accession no. [DQ866784](#)).

cII Mutagenesis Assay

Nine weeks after fish were exposed to BaP, 15 fish from each of the 4 study groups were transferred from UL to the University of Georgia, Athens, GA to assess mutant frequencies (MF) of the *cII* gene using the *cII* mutagenesis assay. Fish were dissected upon arrival and separate tissues were immediately placed in liquid nitrogen and stored at -80°C . The *cII* mutagenesis assay was conducted on liver and kidney tissues as previously described and MF is expressed as the number of mutants $\times 10^{-5}$ pfu (Winn et al., 2000).

Statistical Analysis

The mean MFs from each treatment were compared using the COCHARM program (Troy Johnson, Proctor and Gamble, Cincinnati, OH, USA), a modification of the generalized Cochran-Armitage test (Carr and Gorelick, 1995). A value of $p < 0.05$ was considered significant. Fisher's Exact Test was used to compare incidence of mycobacteriosis between two treatment groups, as recommended by Ramsey and Schafer (1997). Pearson chi-squared analysis was used to determine differences in mortality frequencies across the four treatment groups. T-test was used to determine differences in severity of mycobacteriosis between treatment groups. The odds of developing proliferative liver lesions in the fish exposed to BaP compared to the fish exposed to BaP and *M. marinum* was also calculated. All p -values are two-tailed.

Results

M. marinum does not increase the mutagenic load of chronically infected livers

After 13 weeks of chronic *M. marinum* infection of the λ transgenic line of medaka, we investigated whether infected tissues carried higher mutant frequencies (MF), thereby raising the cancer risk. We also sought to evaluate if mutation levels induced by a low dose benzo[a]pyrene (BaP) exposure could be augmented by chronic mycobacterial infection. We focused our mutagenesis measurements on the liver because *M. marinum* colonizes this organ (Broussard & Ennis, 2007) and it is the site that BaP is metabolized to its reactive form, thus suffering relatively high levels of BaP-induced mutagenesis (Shane et al., 2000; Helleberg et al., 2001; Winn et al., 2005b). We compared the MF of the *cII* transgene carried in liver DNA of *M. marinum*-infected fish to the unexposed study group. Interestingly, in the infected fish MF was low ($6.4 \pm 0.9 \times 10^{-5}$) and was not significantly different ($p = 0.68$) than the spontaneous MF values measured in the untreated controls ($6.2 \pm 1.1 \times 10^{-5}$) (Fig. 1). We next tested if BaP-induced MF could be augmented by chronic *M. marinum* infections. BaP-

exposure yielded a highly significant, eightfold increase in liver MF ($48.8 \pm 7.4 \times 10^{-5}$) compared to untreated controls ($p < 0.01$) (Fig. 1). Average MF ($53.3 \pm 8.8 \times 10^{-5}$) in animals exposed to both agents was 1.09-fold higher than the BaP-treated group (Fig. 1). However, this change was determined to be insignificant ($p = 0.16$). We also compared MF in kidney tissues from unexposed control fish and those infected with *M. marinum*, because this organ is known to carry high infectious loads. The MF in the infected fish ($3.0 \pm 1.4 \times 10^{-5}$, $n = 4$) was not significantly different ($p = 0.64$) than the spontaneous MF values measured in the untreated controls ($3.8 \pm 0.6 \times 10^{-5}$, $n = 7$). In summary, *M. marinum* infection did not significantly increase the mutagenic loads in either untreated or BaP-exposed liver tissues, or untreated kidney tissue.

Histological detection of proliferative liver lesions

Proliferative liver lesions were observed in medaka from both groups exposed to low BaP doses, these included foci of cellular alterations, hepatocellular adenomas, and carcinomas (Fig. 2). Lesions were not observed in the unexposed and *M. marinum* infected groups (Fig. 3). Out of 29 fish exposed to both BaP and *M. marinum*, one had a hepatocellular carcinoma, three had adenomas, and seven had altered foci. Out of the 28 fish exposed to BaP alone, none of the fish had hepatocellular carcinomas, two had adenomas, and one had altered foci.

At 31 weeks post-injection, the fraction of fish with liver lesions in the BaP exposed group was 3 of 28 (~11%) fish, while the fraction with lesions in the *M. marinum*-BaP exposed group was greater, 12 of 29 (~41%) fish (Fig. 3). We compared the odds of developing lesions in the fish exposed to BaP or BaP and *M. marinum*, finding 5.8 times greater chance of developing proliferative lesions in the fish exposed to both BaP and *M. marinum* compared to BaP alone ($p = 0.0016$, 95% confidence intervals for the odds ratio range from 1.4 to 24.0). Together, these results show that *M. marinum* infection alone did not increase the incidence of proliferative liver lesions, but increased the chances of developing such lesions with subsequent BaP exposure.

Disease and mortality associated with Mycobacterium infection

Because of the mortality following shipping and detection of *M. chelonae* in tissues of some fish (see Materials and Methods), all fish from each group were evaluated for mortality, as well as incidence and severity of mycobacteriosis. It was hoped that these data would allow the determination of any effect of the unexpected *M. chelonae* infection on liver lesion incidence or mutant frequency. Mortality was different among treatments ($\chi^2 = 12.57$, d.f. = 3, $p = 0.0057$) with the fish exposed to BaP (50 of 101) and BaP and *M. marinum* (49 of 100) being higher than the unexposed fish (30 of 100) and the *M. marinum* exposed fish (35 of 102). We found significantly higher incidence of mycobacteriosis in the BaP exposed group compared to the control (Fisher's, $p = 0.02$) (Fig. 4). However, mycobacteriosis incidence in the *M. marinum* and *M. marinum*-BaP exposed groups was not different (Fisher's, $p = 0.23$) (Fig. 4). The same trend was observed for the severity index; the *M. marinum* infected groups had higher numbers of granulomas and scored higher for severity of mycobacteriosis. Exposure to BaP did not have an effect on the severity of mycobacteriosis in the *M. marinum* infected groups (T-test, $p = 0.46$). However, in the non-*M. marinum* infected groups, severity of infection was higher in the BaP group compared to the unexposed control (T-test, $p = 0.02$). In considering the histopathology, fish intentionally exposed to *M. marinum* showed pathological changes matching previously reported *M. marinum* infection in medaka (Broussard and Ennis, 2007). Granulomatous lesions were detected within kidneys and livers (Fig. 5A & B) and contained acid fast bacilli (Fig. 5B). However, some fish from the two groups not injected with *M. marinum* also showed histological signs of mycobacteriosis and the presence of acid fast bacilli, presumed to be an indication of *M. chelonae* (Fig. 4 & 5C).

A clear correlation is observed between the incidence of mycobacteriosis and the severity of mycobacteriosis; the *M. marinum* infected groups scored higher for both (Fig. 4). However, no apparent patterns emerged between the proportion of mycobacteriosis incidence/severity in the four treatment groups (Fig. 4) and the occurrence of liver lesions (Fig. 3) or the mutant frequencies (Fig. 1). Furthermore, in evaluating the histological data, 3 of the 28 fish in the BaP exposed group developed liver lesions (Fig. 3), however only one of the three lesion-containing fish showed any indication of mycobacteriosis. Thus, there was not a one-to-one correlation between the incidence of *M. chelonae* infection and induction of liver lesions. These analyses indicate that the relevant variables leading to induction of proliferative liver lesions (Fig. 2 & 3) were exposures to both *M. marinum* and BaP.

Histological changes not attributed to BaP or mycobacterial exposures

Histological changes not attributed to BaP exposure included aggregates of spherical, intracellular protists consistent with rosette agent (*Sphaerothecum destruens*) which were observed in the liver of two fish collected from the *M. marinum*-BaP group (Fig. 6A). Two control fish exhibited testicular oocytes in which developing eggs were scattered throughout an enlarged testis (Fig. 6B). Spongiosis hepatitis occurred in both the BaP and unexposed groups, and a large spindle cell sarcoma occurred in a *M. marinum*-BaP fish at 22 weeks post-BaP-exposure (Fig. 6C & D). This sarcoma extended from the muscle through the skin at the dorsal fin.

Discussion

Chronic infections have been well-documented tumor initiators and tumor promoters in some diseases; however they do not always participate in both events. Besides the two best-studied models, *Helicobacter pylori* and schistosomal infections, additional evidence exists for the roles of other chronic infectious agents in increased cancer risk. Infections by the liver fluke, *Fasciola hepatica*, have not only been associated with cancer in rodents, but experimental infections in the Big Blue transgenic mouse have documented increased mutations in the *lacI* transgene of infected livers, supporting the idea that chronic inflammation may play the role of a tumor initiator (Morotona et al., 2001). This trend of association between chronic infection and cancer is also suspected in fish. A common intestinal nematode of cyprinid fishes, *Pseudocapillaria tomentosa*, is suspected to be a promoter of intestinal cancers in zebrafish that were previously exposed to a low initiating dose of 7,12-dimethylbenz[a]anthracene (DMBA) (Kent et al., 2002). Although epidemiological evidence points to a potential role of mycobacterial infection in the promotion of cancer, the actual stage of tumor formation and/or progression in which mycobacteriosis may be acting is unclear. Furthermore, the length of time in which an individual needs to carry a chronic mycobacterial infection is not known (Alavanja et al., 1992; Brown and Almanoff, 1992; Wu et al., 1995; Brenner et al., 2001, Gandhi et al., 2006). Epidemiological studies present little information on the time of inoculation and duration of infection. Other uncertainties include whether the apparent higher cancer incidence may result from general poor health of individuals or exposures to environmental pollutants. The confounding uncertainties presented by epidemiological studies and the lack of experimental evidence in this regard was a major motivation for the present study; an effort to address these fundamental health questions, in particular the potential connection between cancer and mycobacterial infections.

Because mutations are critical proximal events in the initiation of cancer, we investigated whether mutational loads were increased in chronically infected livers. Interestingly, no significant difference in liver MF was observed in *M. marinum*-infected animals compared to unexposed animals. Furthermore, there was no significant difference in MF of uninfected and infected kidney tissues, despite substantial inflammation. The lack of a MF-induction indicates

that the mycobacterial infection and its associated inflammation, was not mutagenic, at least during the time points used in this study. Likewise, the MF values for both the BaP-exposed group and the group exposed to both *M. marinum* and BaP were indistinguishable, indicating that *M. marinum* infection does not enhance BaP-induced mutagenesis. These results, suggest that mycobacterial infections do not increase cancer risk by a concomitant augmentation of mutational loads.

As shown in Fig. 3, proliferative liver lesions were observed in more than 40% of the animals that were experimentally exposed to *M. marinum* and low BaP levels; a much higher lesion incidence than animals exposed to either agent individually. The observed incidence of hepatic lesions in animals exposed to both agents gain greater significance considering that spontaneous levels in untreated medaka livers were previously reported to be 0.1–0.3% (Hawkins et al., 1995). Although the sample size of the unexposed animals (29 fish) was insufficient to detect lesions at this low frequency, we note that our results are consistent with these published values, because lesions were not observed in any of the animals studied (<3%). We also closely examined the other tissues for proliferative lesions, with a special focus on kidneys. Except for the one spindle cell sarcoma, in a BaP-*M. marinum* exposed fish, proliferative lesions were only observed in livers. The failure to observe an induction of lesions and increased MF in kidneys is of special interest, especially in experimentally infected animals, because medaka kidneys are known to be a target tissue for infection, carrying very high infectious loads of *M. marinum* and experiencing extensive inflammation (Fig. 5A) (Broussard & Ennis, 2007). We conclude that chronic *M. marinum* infections failed to enhance MF and did not result in detectable induction of neoplasms in infected tissues on its own.

In light of these data, it is unlikely that chronic *Mycobacterium* infections in fish lead to increased cancer incidence without the influence of secondary factors such as exposure to tumor initiators such as mutagens. Mutagenesis in BaP-exposed vertebrates first requires the metabolism by liver cytochrome P450s (e.g. Cyp1A) to highly reactive compounds like Benzopyrene diol Epoxide that produce DNA adducts. The nucleotide sequence for the medaka gene encoding cytochrome P450 Cyp1A has been determined (Kim et al., 2004) and is known to be inducible by BaP as well as other polycyclic aromatic hydrocarbons (PAHs) (Kashiwada et al., 2007). Hence, the highest levels of adduct formation and mutations are expected in livers, especially with low BaP exposures (Friedberg et al., 2006). Moreover, the lack of MF- or lesion-induction in kidneys despite higher infection levels, underscores the importance of the infections occurring in tissues where these DNA adducts are also generated. Based on both MF and histological data, we propose that chronic mycobacterial infections are acting as a tumor promoter of cells carrying BaP-induced DNA damage.

A requirement for exogenous DNA damage was surprising considering previous studies that suggested that the inflammatory response increases the effectiveness of PAHs, such as BaP, to form DNA adducts, not to mention DNA damage induced directly through the inflammatory response (Shacter et al., 1988; Borm et al., 1997; Zhuang et al., 2002; Kim & Wogan, 2006). Although this would suggest that inflammation alone or in the presence of BaP could lead to increased mutagenic lesions, we failed to observe such an effect. Interestingly, we note that the requirements for mutagenic initiation with infection may be consistent with some epidemiological evidence which demonstrates a significant relationship between tuberculosis and lung cancer, but only in people with long-term smoking histories (Alavanja et al., 1992). Cigarette smoke is known to carry multiple carcinogens, including BaP, each with the potential to form DNA adducts (Hecht, 2003). Although, it is tempting to link increased cancer risk with exposures to both cigarette smoke and tuberculosis, the epidemiological evidence in this regard is conflicting (Alavanja et al., 1992; Wu et al., 1995; Brenner et al., 2001).

It may be relevant that in separate experiments utilizing a medaka microarray (Zhenlin et al., 2007a, 2007b), in which we compared medaka mRNA expression levels in *M. marinum* infected medaka against uninfected controls, expression levels of the retinoblastoma gene (*RB*) were decreased by 3.7-fold and 2.0-fold in infected kidney and liver tissues, respectively, at 8 weeks post infection (data not shown). The retinoblastoma protein (pRB) is a well-studied tumor suppressor and inactivation of pRB can lead to many types of cancer. pRB is necessary for progression of the cell cycle from G1 into S phase and it is important for the elimination of abnormal cells through apoptosis. This tumor suppressor is highly conserved in medaka (Morgenbesser et al., 1994; Butler et al., 2007) and considering the role that pRB plays in a large number of cancers suggests one possible means in which *M. marinum* infection may promote tumor formation, possibly by down-regulating transcription of the *RB* gene.

As with schistosomal and *H. pylori* infections, it is likely that chronic infections can lead to carcinogenesis by several pathways mainly derived from the inflammatory response. Comparing the possible contributions of mycobacterial infection to tumorigenesis with the above mentioned infection models, it seems that *Mycobacterium* infection most closely resembles the results of schistosomiasis, in which chronic infections perform the role of a promoter (Hashem and Boutros, 1961; Domingo et al., 1967; Hicks et al., 1980). This is not unexpected considering both schistosomiasis and mycobacteriosis produce granulomatous lesions. In such inflammatory lesions, constant or relapsing states of inflammation with tissue damage and recovery are likely to occur. It is the following compensatory cell turnover, together with DNA damage, which is believed to provide an environment likely to promote increased mutagenesis, clonal expansion of mutant cells, and possibly induction of tumor progression (Ames et al., 1995; Bowden, 2004; Friedberg et al., 2006; Lu et al., 2006).

During the course of these experiments, it was determined that some of the fish in our colony carried a background *M. chelonae* infection. To gain some insights into what if any effect this background infection might have on the four study groups, these groups were scored for incidence and severity of mycobacteriosis (Fig. 4). Both of these measurements of infection were compared to both end points: MF induction and the promotion of neoplasms in each group. Unexpectedly, both the severity and incidence of background infection seemed to be enhanced in animals that were exposed to BaP compared to the control. This apparent increase in severity/incidence of mycobacteriosis with BaP exposure was not observed between the group intentionally infected with *M. marinum* only and the BaP-*M. marinum* exposed group. We have also concluded that since MF was only increased in the two study groups exposed to BaP, that exposures by either mycobacterial species did not increase mutational loads. A similar analysis was made for the occurrence of proliferative liver lesions and background infections. This second end-point (proliferative lesions) was only observed in the study groups exposed to BaP and lesion incidence was greater with exposure to both *M. marinum* and BaP than with BaP alone. Since some of the BaP-exposed animals carried *M. chelonae*, we were interested to learn if all three animals carrying hepatic neoplasms also had acid fast rods or histopathology matching mycobacteriosis. However, histological analyses revealed that two of the three animals with proliferative lesions were not burdened by mycobacterial infection. This lack of a one-to-one correlation between the occurrence of liver lesions in the BaP-exposed group and detection of mycobacteriosis would suggest that the presence of *M. chelonae per se* did not likely provoke these lesions. Instead we suspect that the few induced lesions were likely due to exposure to BaP, since previous studies have shown that this low dose induced a similar low incidence of hepatic neoplasms (Hawkins et al., 1998). Taken together, these data indicate that the increases in MF resulted from BaP and incidence of liver lesions are likely the products of the experimental exposures to BaP and *M. marinum*. Although the outcome of this particular experiment did not appear to be affected by the unintended mycobacterial infection in this particular cohort of fish; our experience underscores the importance of variation in

experimental conditions and the need to be able to identify infected fish and prevent or treat mycobacterial infections in test animals.

Other nonbacterial background infections were also observed in a few animals; two fish were infected with an intracellular protist similar to *Sphaerothecum destruens*, (Class Mesomycetozoea), which is a lethal parasite of salmonids (Arkush et al., 2003). A very similar parasite is apparently responsible for population declines in the *Leucaspis delineatus* (Cyprinidae) in Europe (Gozlan et al., 2005). Without nucleotide sequence data, it is not possible to determine if the observed intracellular protists represent one organism, or multiple morphologically indistinguishable species. Considering the potential lethality of this parasite, diagnosticians should be aware of this infection in laboratory-reared medaka. Spongiosis hepatitis has been observed in a number of fish and while some research attributes this to carcinogen exposure, it often occurs in medaka not exposed to carcinogens (Boorman et al., 1997). Two of the 120 fish examined by histology exhibited testicular oocytes. This is a relatively common finding in medaka. For example, Grim et al. (2007) found testicular oocyte tissues in control medaka from 14 of 41 studies. The basis for these unusual ovarian testicular structures remains unclear and may represent a developmental aberration rather than a response to mutagen exposures.

In conclusion, mycobacteriosis is one of the most common diseases in laboratory fishes (Abner et al., 1994; Kent et al., 2004; Watral and Kent, 2007) and it is typically an infection which goes undetected. The high occurrence of mycobacterial infections in aquatic species and the demonstrated effect of infections on increasing cancer risk, underscores the need for animal husbandry and surveillance measures to assure disease-free fish colonies and especially control of mycobacteriosis in these populations. Further experimental research will be required to investigate the apparent increased cancer risks uncovered here, in animals carrying chronic *M. marinum* infections. Additional studies will also be needed to better understand the mechanisms of why some infectious agents, like mycobacteria and schistosomes appear to act as tumor promoters, while agents like *H. pylori* appear to act as full carcinogens. The fact that medaka, like other teleost fish, carry homologues to many mammalian genes linked to DNA repair (e.g. AlkB, Apex1, Mgmt, Mre11a, Msh2, XPA), carcinogenesis (e.g. Brca1, CDKN2A/B, c-Ki-ras-1, p53, RAB1, RAB2, RB1, Rev1, Rev3, XPV), and immunity (e.g. CD4, CD8, CXCR4, IgM, IL-10, IL-12, INF- γ , MHC I, MHC II, Rag1, Rag2, TCR- α , TCR- β , TLR) will allow in depth studies using medaka to be extrapolated to mammalian systems (Van der Sar et al., 2004; Taniguchi et al., 2006; Butler et al., 2007; Kasahara et al., 2007; Ortutay et al., 2007; NBRP Medakafish Genome Project (<http://www.shigen.nig.ac.jp/medaka/genome/top.jsp>); NCBI (<http://www.ncbi.nlm.nih.gov>); unpublished data). Future results obtained from this highly tractable medaka-*M. marinum* model will likely gain insights into the current epidemiological evidence linking human tuberculosis and cancer.

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References

- Abner, SR.; Frazier, CL.; Scheibe, JS.; Krol, RA.; Overstreet, RM.; Walker, WW.; Hawkins, WE. Chronic inflammatory lesions in two small fish species, medaka (*Oryzias latipes*) and guppy (*Poecilia reticulata*), used in carcinogenesis bioassays. In: Stolen, JS.; Fletcher, TC., editors. *Modulators of Fish Immune Responses: Models for Environmental Toxicology, Biomarkers, and Immunostimulators*. SOS Publications; Fair Haven, NJ, USA: 1994. p. 219-233.
- Adams, LB.; Krahenbuhl, JL. Human leprosy. In: Boros, DL., editor. *Granulomatous Infections and Inflammations: Cellular and Molecular Mechanisms*. ASM Press; Washington, DC: 2003. p. 207-244.
- Alavanja MCR, Brownson RC, Boice JD, Hock E. Preexisting lung disease and lung cancer among nonsmoking women. *Am J Epidemiol* 1992;136:623–632. [PubMed: 1442729]
- Ames BN, Gold LS, Willett WC. The causes and prevention of cancer. *Proc Natl Acad Sci USA* 1995;92:5258–5265. [PubMed: 7777494]
- Arkush KD, Mendoza L, Adkinson MA, Hedrick RP. Observations on the life stages of *Sphaerothecum destruens* n. g., n sp., a mesomycetozoean fish pathogen formally referred to as the rosette agent. *J Eukaryot Microbiol* 2003;50:430–438. [PubMed: 14733435]
- Aronson JD. Spontaneous tuberculosis in saltwater fish. *J Infect Dis* 1926;39:315–320.
- Astrofsky KM, Schrenzel MD, Bullis RA, Smolowitz RM, Fox JG. Diagnosis and management of atypical *Mycobacterium* spp. infections in established laboratory zebrafish (*Brachydanio rerio*) facilities. *Comp Med* 2000;50:666–672. [PubMed: 11200575]
- Bailey WS. *Spirocerca*-associated esophageal sarcomas. *J Am Vet Med Ass* 1979;175:148–50. [PubMed: 500437]
- Barker LP, Porcella SF, Wyatt RG, Small PLC. The *Mycobacterium marinum* G13 promoter is a strong sigma 70-like promoter that is expressed in *Escherichia coli* and mycobacteria species. *FEMS Microbiol Lett* 1999;175:79–85. [PubMed: 10361711]
- Boorman GA, Botts S, Bunton TE, Fournie JW, Harshbarger JC, Hawkins WE, Hinton DE, Jokinen MP, Okinhiro MS, Wolfe MJ. Diagnostic criteria for degenerative, inflammatory, proliferative and neoplastic liver lesions in medaka (*Oryzias latipes*): consensus of a National Toxicology Program pathology working group. *Toxicol Pathol* 1997;25:202–210. [PubMed: 9125779]
- Borm PJA, Knaapen AM, Schins RPF, Godschalk RWL, Van Schooten FJ. Neutrophils amplify the formation of DNA adducts by benzo[a]pyrene in lung target cells. *Environ Health Perspect* 1997;105:1089–1093. [PubMed: 9400705]
- Bowden GT. Prevention of non-melanoma skin cancer by targeting ultraviolet-b-light signalling. *Nat Rev Cancer* 2004;4:23–35. [PubMed: 14681688]
- Brenner AV, Wang Z, Kleinerman RA, Wang L, Zhang S, Metayer C, Chen K, Lei S, Cui H, Lubin JH. Previous pulmonary diseases and risk of lung cancer in Gansu Province, China. *Int J Epidemiol* 2001;30:118–124. [PubMed: 11171871]
- Broussard GW, Ennis DG. *Mycobacterium marinum* produces long-term chronic infections in medaka: a new animal model for studying human tuberculosis. *Comp Biochem Physiol C* 2007;145:45–54.
- Brown ST, Almenoff PL. Pulmonary mycobacterial infections associated with neoplasia. *Semin Respir Infect* 1992;7:104–113. [PubMed: 1439320]
- Butler AP, Trono D, Coletta LD, Beard R, Fraijo R, Kazianis S, Nairn R. Regulation of CDKN2A/B and Retinoblasoma genes in *Xiphophorus* melanoma. *Comp Biochem Physiol C* 2007;145:145–155.
- Carr GJ, Gorelick NJ. Statistical design and analysis of mutation studies in transgenic mice. *Environ Mol Mutagen* 1995;25:246–255. [PubMed: 7737142]
- Christen, S.; Hagen, TM.; Shigenaga, MK.; Ames, BN. Chronic inflammation, mutation, and cancer. In: Parsonet, J., editor. *Microbes and malignancy: infection as a cause of human cancers*. Oxford Press; New York, NY: 1999. p. 313-345.
- Cohen, SM. Infection, cell proliferation, and malignancy. In: Parsonet, J., editor. *Microbes and malignancy: infection as a cause of human cancers*. Oxford Press; New York, NY: 1999. p. 313-345.
- Cope RB, Stang B, Valentine BA, Bermudez LE. Topical pre-exposure to cis-urocanic acid enhances *Mycobacterium ulcerans* infection in a Crl: IAF(HA)-hrBR hairless guinea pig model of Buruli ulcer disease. *Photodermatol Photoimmunol Photomed* 2004;20:14–20. [PubMed: 14738528]

- Domingo EO, Warren KS, Stenger RJ. Increased incidence of hepatoma in mice with chronic schistosomiasis mansoni treated with a carcinogen. *Am J Pathol* 1967;51:307–321. [PubMed: 4292194]
- Ennis, DG. Encyclopedia of Life Sciences. Vol. 559. Scientific American/John Wiley and Sons, Ltd; 2001. Mutagenesis; p. 1-8.
- Evans MR, Etuful SN, Amofah G, Adjei O, Lucas S, Wansbrough-Jones MH. Squamous cell carcinoma secondary to Buruli ulcer. *Trans R Soc Trop Med Hyg* 1999;93:63–64. [PubMed: 10492793]
- Friedberg, EC.; Walker, GC.; Siede, W. DNA Repair and Mutagenesis. ASM Press; Washington, D.C., USA: 2006.
- Gandhi NR, Moll A, Sturm AW, Pawinski R, Govender T, Lallo U, Zeller K, Andrews J, Friendland G. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. *Lancet* 2006;368:1575–1580. [PubMed: 17084757]
- Gozlan RE, St-Hilaire S, Feist SW, Martin P, Kent ML. Disease threat to European fish. *Nature* 2005;435:1046. [PubMed: 15973399]
- Grim KC, Wolfe M, Hawkins W, Johnson R, Wolf J. Intersex in Japanese medaka (*Oryzias latipes*) used as negative controls in toxicologic bioassays: A review of 54 cases from 41 studies. *Environ Toxicol Chem* 2007;26:636–1643.
- Hashem M, Boutros K. The influence of bilharzial infection on the carcinogenesis of the bladder. An experimental study. *J Egypt Med Assoc* 1961;44:598–606. [PubMed: 13905175]
- Hawkins, WE.; Walker, WW.; Overstreet, RM. Carcinogenicity tests using aquarium fish. In: Rand, GM., editor. *Fundamentals of Aquatic Toxicology*. Taylor and Francis; New York, NY: 1995. p. 225-263.
- Hawkins WE, Walker WW, Overstreet RM, Lytle TF, Lytle JS. Dose-related carcinogenic effects of water-borne benzo[a]pyrene on livers of two small fish species. *Ecotoxicol Environ Safety* 1998;16:219–231. [PubMed: 2852591]
- Hawkins WW, Walker VW, Fournie JW, Manning CS, Krol RM. Use of the Japanese medaka (*Oryzias latipes*) and guppy (*Poecilia reticulata*) in carcinogenesis testing under National Toxicology Program protocols. *Toxicol Pathol* 2003;31(suppl):88–91. [PubMed: 12597435]
- Hecht SS. Tobacco carcinogens, their biomarkers and tobacco-induced cancer. *Nat Rev Cancer* 2003;3:733–744. [PubMed: 14570033]
- Heckert RA, Elankumaran S, Milani A, Baya A. Detection of a new *Mycobacterium* species in wild striped bass in the Chesapeake Bay. *J Clin Microbiol* 2001;39:710 – 715. [PubMed: 11158132]
- Helleberg H, Xu H, Ehrengerg L, Hemminki K, Rannug U, Törnqvist M. Studies of dose distribution, premutagenic events and mutation frequencies for benzo[a]pyrene aiming at low dose cancer risk estimation. *Mutagenesis* 2001;16:333–337. [PubMed: 11420402]
- Hermon-Taylor J, Barnes N, Clark C, Finlayson C. *Mycobacterium paratuberculosis* cervical lymphadenitis, followed by terminal ileitis similar to Crohn's Disease. *Brit Med J* 1998;316:449–453. [PubMed: 9492675]
- Hicks RM, James C, Webbe G. Effect of Schistosomiasis haematobium and N-butyl-N-(4-hydroxybutyl)nitrosamine on the development of urothelial neoplasia in baboon. *Br J Cancer* 1980;42:730–755. [PubMed: 7459210]
- Izzotti A, De Flora S, Cartiglia C, Are BM, Longobardi M, Camoirano A, Mura I, Dore MP, Scanu AM, Rocca PC, Maida A, Pian A. Interplay between *Helicobacter pylori* and host gene polymorphisms in inducing oxidative DNA damage in the gastric mucosa. *Carcinogenesis* 2007;28:892–898. [PubMed: 17127715]
- Kaattari IM, Rhodes MW, Kaattari SL, Shotts EB. The evolving story of *Mycobacterium tuberculosis* clade members detected in fish. *J Fish Dis* 2006;29:509–520. [PubMed: 16948700]
- Kasahara M, Naruse K, Sasaki S, et al. The medaka draft genome and insights into vertebrate genome evolution. *Nature* 2007;447:714–719. [PubMed: 17554307]
- Kashiwada S, Goka K, Shiraishi H, Arizono K, Ozato K, Wakamatsu Y, Hinton DE. Age-dependent in situ hepatic and gill CYP1A activity in the see-through medaka (*Oryzias latipes*). *Comp Biochem Physiol C* 2007;145:96–102.
- Kent ML, Bishop-Stewart JK, Matthews JL, Spitsbergen JM. *Pseudocapillaria tomentosa*, a nematode pathogen of zebrafish (*Danio rerio*) kept in research colonies and associated neoplasms. *Comp Med* 2002;52:362–367.

- Kent ML, Matthews JL, Bishop-Stewart JK, Whipps CM, Watral V, Poort M, Bermudez L. Mycobacteriosis in zebrafish (*Danio rerio*) research facilities. *Comp Biochem Physiol C* 2004;138:383–390.
- Kim IC, Kim YJ, Yoon YD, Kawamura S, Lee YS, Lee JS. Cloning of cytochrome P450 1A (CYP1A) genes from the hermaphrodite fish *Rivulus marmoratus* and the Japanese medaka *Oryzias latipes*. *Mar Environ Res* 2004;58:125–129. [PubMed: 15178024]
- Kim MY, Wogan GN. Mutagenesis of the supF gene of pSP189 replicating in AD293 cells cocultivated with activated macrophages: roles of nitric oxide and reactive oxygen species. *Chem Res Toxicol* 2006;19:1483–1491. [PubMed: 17112236]
- Kusters JG, van Vliet AHM, Kuipers EJ. Pathogenesis of *Helicobacter pylori* infection. *Clin Microbiol Rev* 2006;19:449–490. [PubMed: 16847081]
- Lu H, Ouyang W, Huang C. Inflammation, a key event in cancer development. *Mol Cancer Res* 2006;4:221–233. [PubMed: 16603636]
- Morgenbesser SD, Williams BO, Jacks T, Depinho RA. p53-dependent apoptosis produced by Rb-deficiency in the developing mouse lens. *Nature* 1994;371:72–74. [PubMed: 8072529]
- Morotona OO, Martin H, Gentile GJ, Gentile JM. Enhanced liver cell mutations in trematode-infected Big Blue Mice. *Mutation Res* 2001;484:69–76. [PubMed: 11733073]
- Mostafa MH, Sheweita SA, O'Connor PJ. Relationship between schistosomiasis and bladder cancer. *Clin Microbiol Rev* 1999;12:97–111. [PubMed: 9880476]
- Ortutay C, Siemala M, Vihinen M. Molecular characterization of the immune system: emergence of proteins, processes, and domains. *Immunogenetics* 2007;59:333–348. [PubMed: 17294181]
- Ramsey, FL.; Schafer, DW. *The Statistical Sleuth*. Wadsworth Publishing Company; Balmont, CA: 1997. p. 742
- Ranger BS, Mahrous EA, Mosi L, Adusumilli S, Lee RE, Colorni A, Rhodes M, Small PLC. Globally distributed mycobacterial fish pathogens produce a novel plasmid-encoded toxic macrolide, mycolactone F. *Infect Immun* 2006;74:6037–6045. [PubMed: 16923788]
- Richardus JH, Smith TC. Squamous cell carcinoma in chronic ulcers in leprosy: a review of 38 consecutive cases. *Lepr Rev* 1991;62:381–388. [PubMed: 1784153]
- Rosin, M.; Hofseth, LJ. Schistosomiasis, bladder, and colon cancer. In: Parsonet, J., editor. *Microbes and malignancy: infection as a cause of human cancers*. Oxford Press; New York, NY: 1999. p. 313–345.
- Rosin MP, El Din Zaki SS, Ward AJ, Anwar WA. Involvement of inflammatory reactions and elevated cell proliferation in the development of bladder cancer in schistosomiasis patients. *Mutation Res* 1994a;305:283–292. [PubMed: 7510039]
- Rosin MP, Anwar WA, Ward AJ. Inflammation, chromosomal instability, and cancer: the schistosomiasis model. *Cancer Res (suppl)* 1994b;54:1929s–1933.
- Sanders GE, Swaim LW. Atypical piscine mycobacteriosis in Japanese medaka (*Oryzias latipes*). *Comp Med* 2001;51:171–175. [PubMed: 11922182]
- Shacter E, Beecham EJ, Covey JM, Kohn KW, Potter M. Activated neutrophils induce prolonged DNA damage in neighboring cells. *Carcinogenesis* 1988;9:2297–2304. [PubMed: 2847879]
- Shane BS, de Boer J, Watson DE, Haseman JK, Glickman BW, Tindall KR. LacI mutation spectra following benzo[a]pyrene treatment of Big Blue® mice. *Carcinogenesis* 2000;21:715–725. [PubMed: 10753208]
- Swaim LE, Connolly LE, Volkman HE, Humbert O, Born DE, Ramakrishnan L. *Mycobacterium marinum* infection of adult zebrafish causes caseating granulomatous tuberculosis and is moderated by adaptive immunity. *Infect Immun* 2006;74:6108–6117. [PubMed: 17057088]
- Talaat AM, Reimschuessel R, Trucksis M. Identification of mycobacteria infecting fish to the species level using polymerase chain reaction and restriction enzyme analysis. *Vet Microbiol* 1997;58:229–237. [PubMed: 9453133]
- Taniguchi Y, Takeda S, Furutani-Seiki M, Kamei Y, Todo T, Sasado T, Deguchi T, Kondoh H, Mudde J, Yamazoe M, Hidaka M, Mitani H, Toyoda A, Sakaki Y, Plasterk RHA, Cuppen E. Generation of medaka gene knockout models by target-selected mutagenesis. *Genome Biol* 2006;7:R116. [PubMed: 17156454]
- Teska JD, Twerdok LE, Beaman J, Curry M, Finch RA. Isolation of *Mycobacterium abscessus* from Japanese medaka. *J Aquatic Anim Health* 1997;9:234–238.

- Thamavit W, Tiwawech D, Moore MA, Ito N, Shirai T. Equivocal evidence of complete carcinogenicity after repeated infection of syrian hamsters with *Opisthorchis viverrini*. *Toxicol Pathol* 1996;24:493–497. [PubMed: 8864191]
- Ucko M, Colorni A, Kvitt H, Diamant A, Zlotkin A, Knibb WR. Strain variation in *Mycobacterium marinum* fish isolates. *Appl Envir Microbiol* 2002;68:5281–5287.
- Uemura N, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, Taniyama K, Sasaki N, Schlemper R. *Helicobacter pylori* infection and the development of gastric cancer. *N Engl J Med* 2001;345:784–789. [PubMed: 11556297]
- Van der Sar AM, Appelmelk BJ, Vandenbroucke-Grauls CMJE, Bitter W. A star with stripes: zebrafish as an infection model. *Trends Microbiol* 2004;12:451–457. [PubMed: 15381194]
- Watral V, Kent ML. Pathogenesis of *Mycobacterium* spp. in zebrafish (*Danio rerio*) from research facilities *Comp Biochem Physiol C* 2007;145:55–60.
- Weitzman SA, Weitberg AB, Clark EP, Stossel TP. Phagocytes as carcinogens: malignant transformation produced by human neutrophils. *Science* 1985;227:1231–1233. [PubMed: 3975611]
- Winn RN, Norris MB, Brayer KJ, Torres C, Muller SL. Detection of mutations in transgenic fish carrying a bacteriophage lambda cII transgene target. *P Natl Acad Sci USA* 2000;97:12655–12660.
- Winn, RN.; Norris, MB. Analysis of mutations in lambda transgenic medaka using the cII mutation assay. In: Ostrander, GK., editor. *Techniques in aquatic toxicology*. Vol. 2. CRC Press; New York, NY: 2005a. p. 705-734.
- Winn RN, Kling H, Norris MB. Antimutagenicity of green tea polyphenols in the liver of transgenic medaka. *Environ Mol Mutagen* 2005b;46:88–95. [PubMed: 15880736]
- Wu AH, Fontham ETH, Reynolds P, Greenberg RS, Buffler P, Liff J, Boyd P, Henderson BE, Correa P. Previous lung disease and risk of lung cancer among lifetime nonsmoking women in the United States. *Am J Epidemiol* 1995;141:1023–1032. [PubMed: 7771438]
- Yamashina K, Miller BE, Heppner GH. Macrophage-mediated induction of drug resistant variants in a mouse mammary tumor cell line. *Cancer Res* 1986;46:2396–2401. [PubMed: 3084067]
- Zhenlin J, Wells MC, Heater SJ, Walter RB. Multiple tissue gene expression analyses in Japanese medaka (*Oryzias latipes*) exposed to hypoxia. *Comp Biochem Physiol C* 2007a;145:134–144.
- Zhenlin J, Wells MC, Walter RB. DNA microarray technology in toxicogenomics of aquatic models: methods and applications. *Comp Biochem Physiol C* 2007b;145:5–14.
- Zhuang JC, Lin D, Lin C, Jethwaney D, Wogan GN. Genotoxicity associated with NO production in macrophages and co-cultured target cells. *Free Rad Biol Med* 2002;33:94–102. [PubMed: 12086687]

Fig. 1. *M. marinum* infection does not significantly increase the mutant frequency induced by BaP Mutant frequencies ($\pm$ standard error) in livers of medaka within 4 conditional groups: unexposed control, BaP exposed, *M. marinum* exposed, and *M. marinum*-BaP exposed. Mutant frequencies are expressed in number of mutants $\times 10^{-5}$ pfu (Winn et al., 2000). n = number of fish sampled in each group.

Fig. 2. Proliferative lesions in medaka liver tissues

H&E. A–C, fish exposed to both BaP and *M. marinum*; D is from a fish exposed to BaP only. (A) Adenoma (arrows) at edge of liver. M = macrophage aggregate with acid fast bacteria. Bar = 50 μm . (B) Large hepatocellular carcinoma. Neoplasm (arrows) has replaced most of normal parenchyma (seen at lower left). Note neoplastic hepatocytes forming columnar rosettes around blood vessels. Bar = 20 μm . (C) Small adenoma (arrows). Bar = 20 μm . (D) Large clear cell focus showing centrally located nuclei and clear cytoplasm due to accumulation of glycogen. Bar = 100 μm

Fig. 3. Incidence of proliferative liver lesions in medaka exposed to *M. marinum* and BaP
 The average proportion ($\pm$ standard error) of proliferative liver lesions as determined by histological evaluation of medaka within 4 conditional groups: unexposed control, BaP exposed, *M. marinum* exposed, and *M. marinum*-BaP exposed. n = number of fish sampled in each group.

Fig. 4. Incidence and severity of mycobacteriosis in medaka exposed to *M. marinum* and BaP
 The average proportion ($\pm$ standard error) of mycobacteriosis incidence (dark bars) and the severity index ($\pm$ standard error) of mycobacteriosis (light bars) as determined by histological evaluation of medaka within 4 conditional groups: unexposed control, BaP exposed, *M. marinum* exposed, and *M. marinum*-BaP exposed. The severity index is an average from individuals in each study group that was scored from 0 to 4 (0 = no observable mycobacteriosis, 4 = most severe mycobacteriosis histopathology). The number of fish sampled in each group are as follows: control = 29, BaP = 28, *M. marinum* = 30, *M. marinum* + BaP = 29.

Fig. 5. Histological sections of *Mycobacterium* infections in medaka

(A) *Mycobacterium marinum* infection of the kidney, with multiple, coalescing granulomas denoted by “G”. H&E. Bar = 100 μ m. (B) Pigmented macrophage aggregate in liver with acid fast bacilli (arrows). Ziehl-Neelsen Acid Fast. Bar = 10 μ m. (C) Pancreas of fish with *M. chelonae* infection showing numerous acid fast bacteria (arrows) in macrophages. Ziehl-Neelsen acid fast. Bar = 10 μ m.

Fig. 6. Histological sections from medaka showing incidental histological changes H&E. A & C–D, fish exposed to both BaP and *M. marinum*; B, unexposed control; (A) Protists resembling “rosette agent” (arrows) within macrophages in liver. Bar = 10 μ m. (B) Testis ova showing both perinuclear (small arrow) and vitellogenic (large arrow) oocytes. Bar = 100 μ m. (C & D) Spindle cell sarcoma. Bar = 100 μ m and 50 μ m.