General and Comparative Endocrinology 277 (2019) 112–121

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Effects of sex hormones on liver tumor progression and regression in *Myc/xmrk* double oncogene transgenic zebrafish

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ARTICLE INFO

Keywords:
Zebrafish
Liver cancer
Hepatocellular carcinoma
Sex hormones
17β-Estradiol (E2)
11-Ketotestosterone (KT11)

ABSTRACT

Hepatocellular carcinoma (HCC) shows clear sex disparity with men being more prone to developing HCC and having higher mortality than women. Previous studies have indicated that sex hormones play important roles in HCC initiation and development, but the effects of sex hormones on HCC in clinical trials remain inconsistent. Using zebrafish liver tumor model co-induced by oncogenes *Myc* and *xmrk*, we observed similar sex disparity between male and female zebrafish in liver tumor progression and regression; i.e. male *Myc/xmrk* transgenic zebrafish developed HCC significantly faster and regressed HCC significantly slower than female *Myc/xmrk* transgenic zebrtafish. To investigate the effects of sex hormones on liver tumor progression and regression, *Myc/xmrk* fish were treated with either androgen or estrogen, we observed that androgen promoted HCC progression and retarded HCC regression in females, while estrogen attenuated HCC progression and accelerated HCC regression in males. Furthermore, androgen promoted cell proliferation while estrogen inhibited it. Overall, the present study suggested that sex hormones affected liver tumor progression and regression in the *Myc/xmrk* transgenic zebrafish.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most severe cancer types worldwide, ranking the fifth highest occurrence of cancer and being the second most common cause of death from cancer (Augustine and Fong, 2014). HCC shows apparent sex disparity in both incidence and mortality and is 2–3 times higher in men than in women (Ladenheim et al., 2016). This sex disparity of HCC may be associated with several factors such as alcohol abuse, virus infection and hepatic toxicants (El-Serag and Rudolph, 2007). Previous studies also suggest that sex hormones may contribute to the sex disparity of HCC.

It has been reported that androgens are positively related to the increased incidence of liver neoplasm. Male rodents have spontaneous occurrence of hepatic tumors and have increased incidence of liver tumor after exposure to androgens (Agnew and Gardner, 1952). Moreover, castration or anti-androgen treatment protects male rodents from liver tumor development (Toh, 1981; Vesselinovitch et al., 1980; Vesselinovitch and Mihailovich, 1967). In contrast, castrated female rodents receiving testosterone have an increased susceptibility to liver tumorigenesis; this susceptibility is easily reverted by the use of anti-androgenic drugs (Matsuura et al., 1994). Subsequent studies have revealed that male rodents are more susceptible to hepatocarcinogenesis not only by chemical induction, but also by chronic viral infection (Firminger and Reuber, 1961; Kemp et al., 1989); e.g., male transgenic mice expressing HBx or HCV core protein have HCC more frequently than female transgenic mice (Kim et al., 1991; Moriya et al., 1998). Results from *in vitro* studies are consistent with these findings: the growth and proliferation of normal or hepatic tumor cell lines are enhanced by testosterone and dihydrotestosterone (Ma et al., 2014). Moreover, clinical practice also suggests that the application of androgens is associated with an increased risk of developing liver neoplasm including HCC (Gupta et al., 2016; Sinclair et al., 2015). For example, in human patients with Fanconi's anemia and aplastic anemia, the substitute androgen intakes increased the risk of developing liver neoplasm such as adenoma and HCC (Velazquez and Alter, 2004; Westaby et al., 1983).

Apart from androgens, estrogens also play a role in hepatocarcinogenesis. Estrogens are associated with the regulation of hepatocyte proliferation. After partial hepatectomy in rats and humans, estrogen levels are increased and the testosterone level reduced, leading to a feminization of the hepatic environment (Mao et al., 2013). In early studies, estrogens have been shown to induce hepatocarcinogenesis in hamster and mice (Yager and Yager, 1980). In human, the chronic use of estrogens is associated with increased risk of developing liver neoplasms such as benign nodular hyperplasia and hepatic adenoma (Baum et al., 1973), suggesting that estrogens function as a tumor promoter in

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https://doi.org/10.1016/j.ygcen.2019.03.018
Received 27 November 2018; Received in revised form 21 March 2019; Accepted 24 March 2019
Available online 26 March 2019

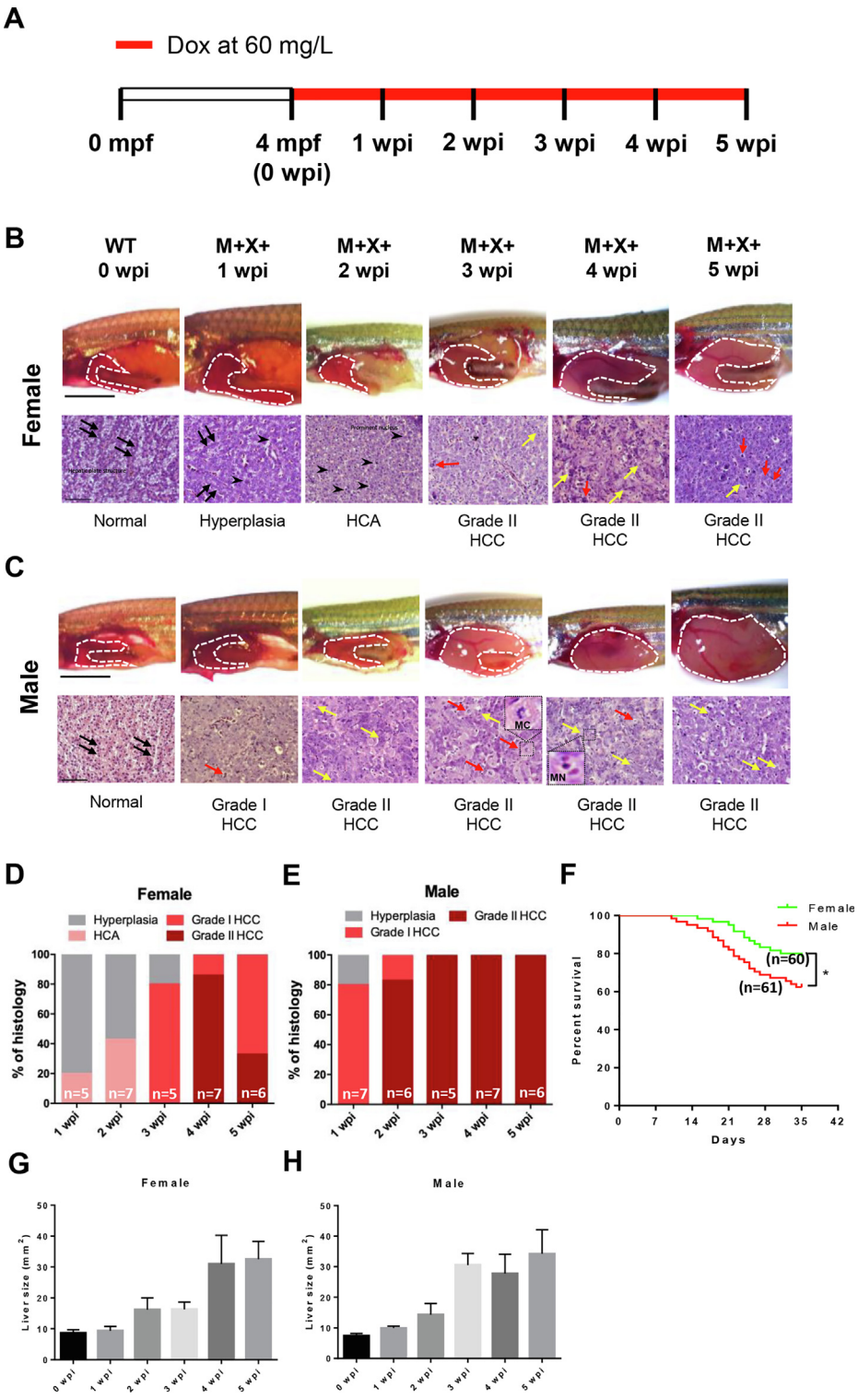

Fig. 1. HCC progression in female and male *Myc/xmrk* zebrafish during 5-week induction. (A) Diagram of experimental design and schedules of sample collection for the long-term tumor induction. (B and C) Gross observations (top) and liver histology (bottom) of female (B) and male (C) *Myc/xmrk* and wildtype siblings at different time points of Dox treatment. Livers in the gross observation pictures are outlined and the diagnosis of liver phenotype is described in the text. Some of the histological features used for classification of liver conditions are marked: double black arrows, haptic plate; arrowheads, prominent nuclei; red arrows, mitotic cells (MT); yellow arrows, multiple nucleoli (MN). Examples of MT and MN are shown in two insets respectively. Scale bars, 5 mm in the upper row and 50 μm in the lower row. (D and E) Quantitative summary of liver histology of female (D) and male (E) *Myc/xmrk* zebrafish at different time points of Dox treatment. Sample sizes (n) are indicated. (F) Kaplan-Meier survival curve of female and male *Myc/xmrk* zebrafish. (G and H) Quantification of 2-D liver size of female (G) and male (H) *Myc/xmrk* zebrafish during tumor progression. WT, wildtype; X + M +, *Myc/xmrk*; wpi, week post induction. *$p < 0.05$.

HCC. However, estrogen has also been found to suppress HCC though the anti-inflammation effect of estrogen (Shi et al., 2014). Since hepatoma has been considered as an inflammation-related cancer caused by chronic hepatitis (Lu et al., 2015), the role of estrogen in HCC changed from a risk factor to a suppressor of HCC. It has been shown that 17β-estradiol (E2) administration significantly diminished the inflammation and injury associated with a chemical carcinogen, diethylnitrosamine (Naugler et al., 2007). Among chronic inflammation-induced HCC, interleukin-6 (IL-6) is a key component (Johnson et al., 2012). It has been reported that IL-6 expression is associated with poor prognosis in HCC, and estrogen is shown to affect the IL-6 production significantly (He et al., 2013). Production of IL-6 also contributes to the gender disparity of HCC because IL-6 ablation protects male mice from HCC and estrogen inhibits IL-6 production (Lee et al., 2016). These studies all indicate that estrogen has an inhibitory effect on HCC.

Although sex hormones have been shown to be related to HCC, clinical trials of hormone replacement treatment on HCC patients received controversial results. An anti-estrogen drug tamoxifen was indicated as a possible therapeutic approach with a significant effect in HCC patients (Simonetti et al., 1997), but two major randomised phase

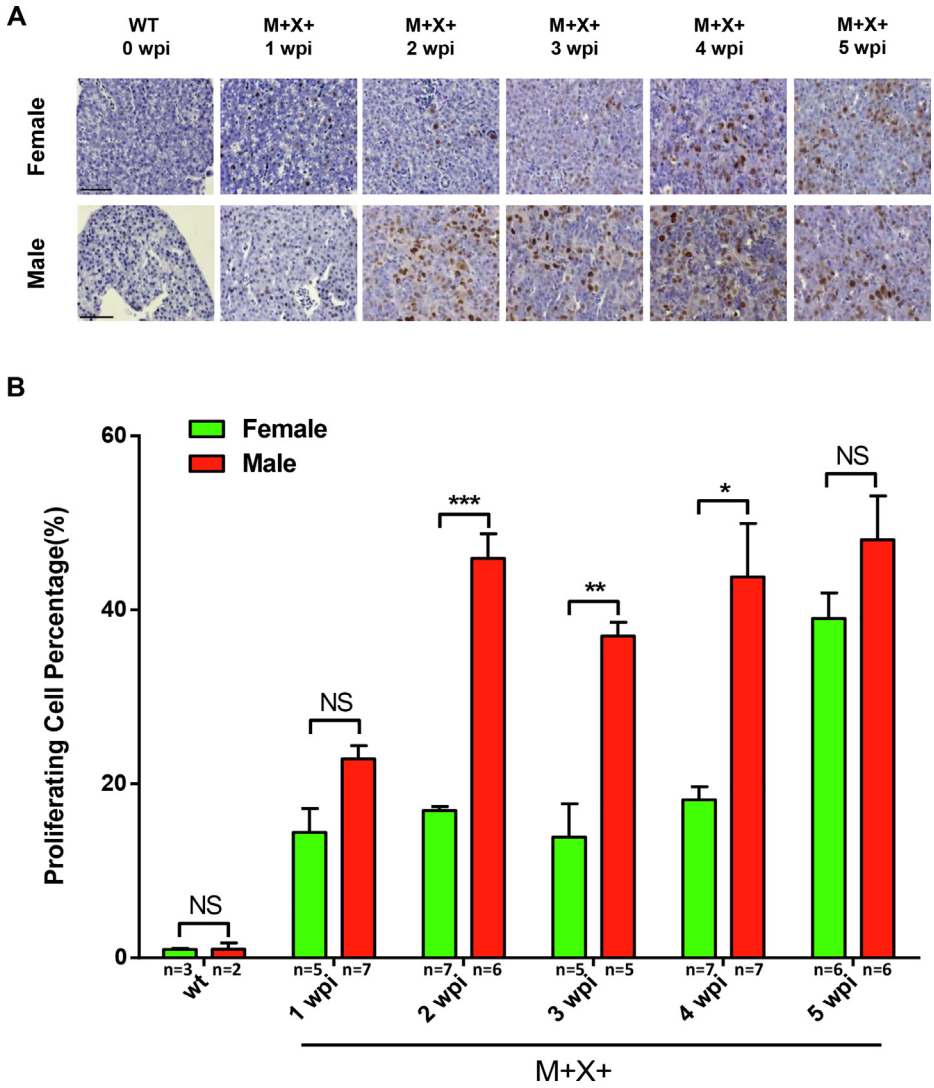

Fig. 2. Cell proliferation of *Myc/xmrk* zebrafish during 5 weeks of HCC progression. (A) PCNA staining in female and male *Myc/xmrk* zebrafish and wildtype siblings at different time points. Scale bars, 50 μm. (B) Quantitative summary of proliferating cell percentages in liver areas in female and male wildtype and *Myc/xmrk* zebrafish at different time points. Sample sizes (n) are indicated. Wt, wildtype; X+M+, *Myc/xmrk*; wpi, week post induction. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, NS: not significant.

III clinical trials of tamoxifen showed no evidence of a survival benefit in HCC patients (Barbare et al., 2005; Perrone et al., 2002). Cyproterone acetate, an anti-androgen, was also found to suppress HCC growth in male nude mice (Nagasue et al., 1996), but a single phase II trial of cyproterone acetate in patients with unresectable HCC showed no significant effect on survival (Kanda and Yokosuka, 2015). One explanation of these controversial results may be that in the randomised trials of sex hormones, patients were not separated in genders, as suggested by a previous study that tamoxifen may have strong effects on female, but not male, HCC patients (Ma et al., 2016).

In our laboratory, we are interested in using zebrafish to model human HCC and have previously demonstrated that HCC developed in the zebrafish highly resembles human HCC both histologically and transcriptomically (Lam et al., 2006; Zheng et al., 2014). In recent years, we have established several inducible HCC models using zebrafish by transgenic expression of selected oncogenes, including oncogenic *kras* (Chew et al., 2014; Nguyen et al., 2012), *xmrk* (Li et al., 2012), *Myc* (Li et al., 2013; Sun et al., 2015) and *tgf1b* (Yan et al., 2017b). HCC can be induced rapidly with essentially 100% penetrance by activation of an oncogene through a chemical inducer in these transgenic zebrafish; after removal of the chemical inducer, well developed HCC could regress rapidly due to suppression of the oncogene expression (Li et al., 2017; Li et al., 2012; Li et al., 2014; Nguyen et al., 2012; Sun et al., 2015). With these features, these zebrafish HCC models provide an excellent tool to investigate HCC progression and regression by manipulating the chemical inducer.

Recently, we have also observed an obvious sex disparity of HCC in *kras* transgenic zebrafish models, i.e. with the same duration of chemical induction, male transgenic zebrafish developed HCC significantly faster and more severely than females (Li et al., 2017; Yan et al., 2017a; Yang et al., 2017a; Yang et al., 2017b). We also found that sex hormones may contribute to the sex disparity of *kras*-induced HCC in transgenic zebrafish (Li et al., 2017), but the detailed effects of sex hormones during the course of HCC progression remain unelucidated. In particular, the effects of sex hormones on HCC regression upon suppression of transgenic oncogene expression are unknown. In the present study, we generated *Myc/xmrk* double oncogene transgenic zebrafish, which display more severe HCC phenotype than either single transgenic zebrafish (Li et al., 2015). We then investigated the effects of sex hormones on both HCC progression and regression. We found that male *Myc/xmrk* fish develop HCC much faster than female *Myc/xmrk* fish, but both sexes developed to similar high grade HCC by five weeks

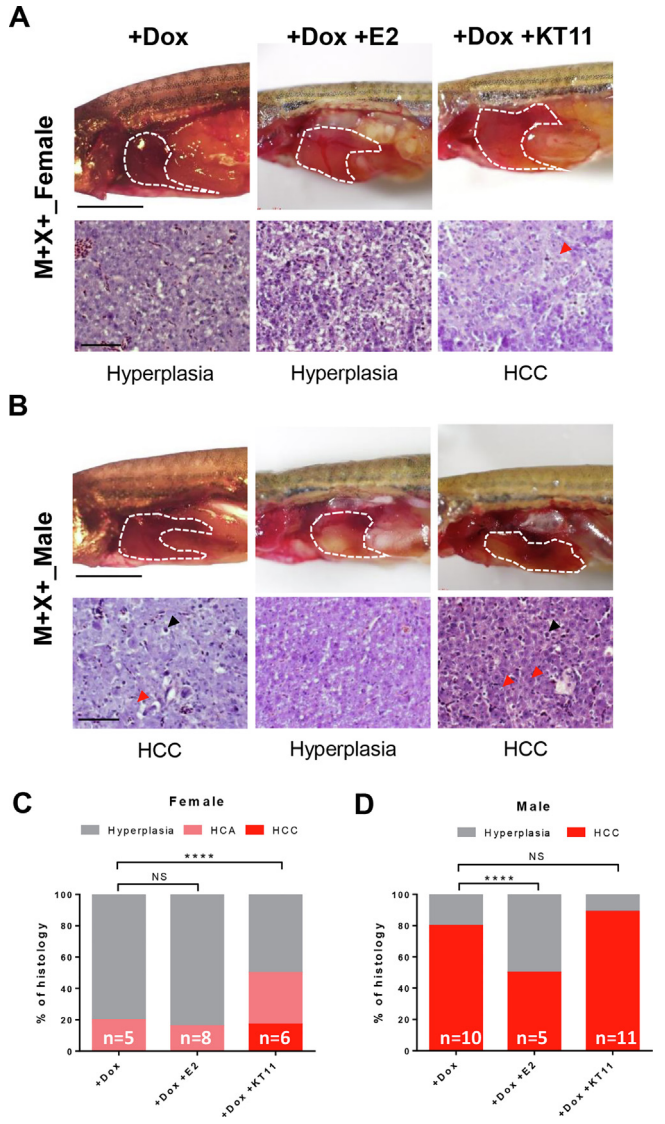

Fig. 3. Sex hormone treatments on female and male *Myc/xmrk* zebrafish during HCC progression. Dox and sex hormones were introduced simultaneously for one week. (A and B) Gross observations (top) and liver histology (bottom) of female (A) and male (B) *Myc/xmrk* and wildtype siblings after sex hormone treatment during HCC progression. Representative gross observations and histology pictures of female (A) and male (B) *Myc/xmrk* fish after treatment with Dox, Dox and E2, and Dox and KT11 are shown. Livers in the gross observation pictures are outlined. Scale bars, 5 mm in the upper row and 50 μm in the lower row. Red arrowheads point representative mitotic cells and black arrowheads point apoptotic cells. (C and D) Quantitative summary of histology in the female (C) and male (D) *Myc/xmrk* zebrafish in different treatment groups. Sample sizes (n) are indicated. X + M +, *Myc/xmrk* zebrafish. ****$p < 0.0001$; NS: not significant.

of oncogene induction. Interestingly, HCC induced by the double oncogenes could also be regressed after suppression of the expression of these oncogenes. Male hormone KT11 (11-ketotestosterone) had a general stimulating effect on HCC progress and delayed HCC regression in female *Myc/xmrk* fish while female hormone E2 could retard HCC progression and accelerate HCC regression in male *Myc/xmrk* fish, suggesting that sex hormones play important roles in HCC progression and regression, contributing toward the sex disparity.

2. Materials and methods

2.1. Zebrafish maintenance and chemical treatments

All experiments on zebrafish were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the National University of Singapore (Protocol Number: 096/12). *Myc/xmrk* transgenic zebrafish were generated by crossing *Myc* transgenic zebrafish *Tg(fabp10:TA; TRE:Myc; krt4:GFP)* (Li et al., 2013) with *xmrk* transgenic zebrafish *Tg(fabp10:TA; TRE:xmrk; krt4:GFP)* (Li et al., 2012). For induction of transgenic oncogene expression, chemical treatments of adult fish (4 months postfertilization, mpf) were conducted in 6-liter tanks and water was changed every other day from 4 mpf for 5 weeks. For sex hormone treatments, 5 μg/ml 17β-estradiol (E2) (Sigma) or 11-ketotestosterone (KT11) (Steraloids) was used for 4 mpf adult zebrafish. During Dox induction, E2 or KT11 was used to treat male and female zebrafish for 1 week. After removal of Dox, E2 was used to treat male zebrafish and KT11 was used to treat female zebrafish for 1 week.

2.2. Paraffin sectioning and histological analysis

Fish abdominal regions were dissected and fixed in formalin solution (Sigma-Aldrich), dehydrated and embedded into paraffin. Sections at 5-μm thickness were processed using a microtome. These sections were deparaffinised, rehydrated and stained with Mayer's hematoxylin (Vector Laboratories) and eosin (Sigma-Aldrich). The stained slides were mounted in Micromount (Leica) and imaged using a light microscope (Zeiss, Axiovert 200 M). Hepatocellular neoplasms grown in zebrafish show similar histology to those of humans. Therefore we used the same criteria for classifying rodent hepatocellular neoplasms to classify liver neoplasms developed in zebrafish. Classification of tumor types were based on established criteria as previously reported (Ishak et al., 2001; Schlageter et al., 2014; Spitsbergen et al., 2000a, b). Normal livers in zebrafish showed typical 2-cell hepatic plate structure, uniformed cell shape and size, and distinct cell boundary. Hyperplasia maintains hepatic plate arrangement but shows increased prominent nuclei. Hepatocellular adenoma (HCA) shows unclear hepatic plates but still have clear cell boundary and relatively uniformed cell shape. Grade I HCC phenotype, characterized by the loss of cell boundaries and hepatic plate structure, and increase of mitotic cells and appearance of multiple nucleoli. Grade II HCC has similar features as Grade I HCC but with more mitotic cells and multiple nucleoli.

2.3. Immunohistochemistry staining

Paraffin sections were deparaffinised and rehydrated. Antigen retrieval was performed using sodium citrate buffer by heating in a boiling water bath for 20 min. Sections were then treated with 3% H_2O_2 for 10 min to inactivate the endogenous peroxidase and blocked with 5% BSA (Sigma) in 1 × PBS (vivantis) for 2 h. After blocking, primary antibody (rabbit anti-PCNA, Santa Cruz) were diluted at 1:500 and incubated at 4 °C overnight. Sections were incubated with HRP (horseradish peroxidase)-conjugated secondary antibodies at room temperature for one hour, followed by color development using the DAKO Real Envision Detection System (Agilent, Singapore).

2.4. Statistical analyses

Statistical significance between two groups was evaluated by two-tailed unpaired Student *t* test (GraphPad). Statistical data were presented as mean value ± standard error of mean (SEM). $P < 0.05$ was chosen to be statistically significant.

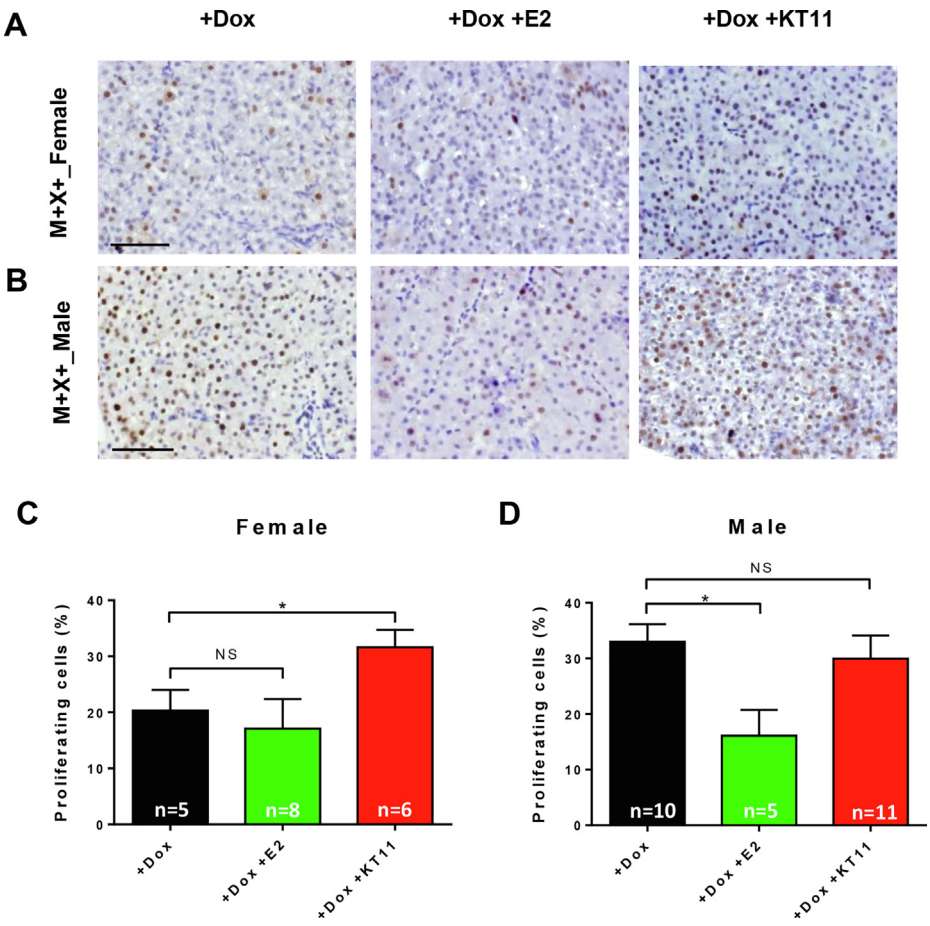

Fig. 4. Cell proliferation altered by sex hormone treatments during HCC progression. (A and B) PCNA staining of liver sections of female (A) and male (B) *Myc/xmrk* zebrafish in different treatment groups. Scale bars, 50 μm. (C and D) Quantification of cell proliferation in female (C) and male (D) *Myc/xmrk* zebrafish in different treatment groups. Sample sizes (n) are indicated. X+M+_Female, female *Myc/xmrk* zebrafish; X+M+_Male, male *Myc/xmrk* zebrafish; +Dox, treated with Dox only; +Dox+E2, treated with Dox and E2; +Dox +KT11, treated with Dox and KT11. *$p < 0.05$; NS: not significant.

3. Results

3.1. Sex disparity in HCC progression in Myc/xmrk transgenic zebrafish

To compare HCC development in the two sexes, male and female *Myc/xmrk* zebrafish (4 mpf) were exposed to Dox for five weeks, and samples were collected weekly for histological examination (Fig. 1A). Normal livers in zebrafish showed typical 2-cell hepatic plate structure, uniformed cell shape and size, and distinct cell boundary. After 1-week of Dox induction, the majority of the female *Myc/xmrk* zebrafish showed hyperplasia in liver (Fig. 1B), with normal cell plate arrangement but increased cell numbers. In comparison, majority of the male *Myc/xmrk* zebrafish already showed Grade I HCC phenotype, characterized by the loss of cell boundaries and hepatic plate structure, and a few abnormal mitotic cells (Fig. 1C). After 2-week induction, about half of the female *Myc/xmrk* fish remained liver hyperplasia and the other half showed unclear hepatic plates but still uniformed cell shapes, indicating HCA (Fig. 1A). At the same treatment stage, male *Myc/xmrk* fish showed mostly Grade II HCC, which was featured with a large population of abnormal mitotic cells and multiple nucleoli (Fig. 1B and C). From 3-week to 5-week induction, the severity of HCC was increased in both sexes. By the end of 4-week induction, in both female and male *Myc/xmrk* fish, the majority of them had Grade II HCC with unclear cell boundaries and prominent nucleoli (Fig. 1B-E). Thus, HCC developed significantly faster in male than female *Myc/xmrk* zebrafish (Fig. 1D and E). It appears that there was a drop of Grade II HCC in female *Myc/xmrk* fish at 5 wpi in this experiment, but the difference between 4 wpi and 5 wpi was not statistically significant ($p = 0.444$ by two-tailed unpaired Student *t* test). Kaplan-Meier survival curve of female and male *Myc/xmrk* zebrafish also showed that during the 5-week induction, the male *Myc/xmrk* zebrafish had significantly higher mortality than female *Myc/xmrk* zebrafish (Fig. 1F). In both female and male *Myc/xmrk* zebrafish, the liver size was apparently increased during the 5 weeks of tumor progression (Fig. 1G and H).

To further compare the severity of HCC between female and male *Myc/xmrk* fish, cell proliferation in the livers was examined by PCNA staining. As shown in Fig. 2, after induction of transgenic oncogene expression, liver cell proliferation was dramatically increased in both female and male *Myc/xmrk* zebrafish (Fig. 2). Consistent with the histological observations, the numbers of proliferating cells were increased more rapidly in male *Myc/xmrk* fish than the female *Myc/xmrk* fish from 1-week to 4-week induction (Fig. 2); however, after 5-week induction, the numbers of liver proliferating cells were similar between female and male *Myc/xmrk* zebrafish (Fig. 2). These observations suggested that cell proliferation was also a basis for the sex disparity during HCC progression in *Myc/xmrk* zebrafish.

3.2. Effects of sex hormone on HCC progression in Myc/xmrk transgenic zebrafish

To investigate the effects of sex hormones on adult *Myc/xmrk* zebrafish during HCC progression, the female and male *Myc/xmrk* zebrafish were treated with KT11 or E2 during HCC progression. After one week treatment with KT11 in female *Myc/xmrk* zebrafish, the histology of liver showed increased HCA and HCC compared with the control female *Myc/xmrk* zebrafish treated with only Dox (Fig. 3A and C), but E2 treatment in female *Myc/xmrk* zebrafish showed no significant alternation on liver histology (Fig. 3A and C). In male *Myc/xmrk* zebrafish, one week of E2 treatment decreased the incidence of HCC while KT11 treatment in male *Myc/xmrk* zebrafish had slight or no significant deterioration of the liver histology (Fig. 3B and D). For female and male wildtype siblings, neither Dox treatment nor Dox and sex hormone

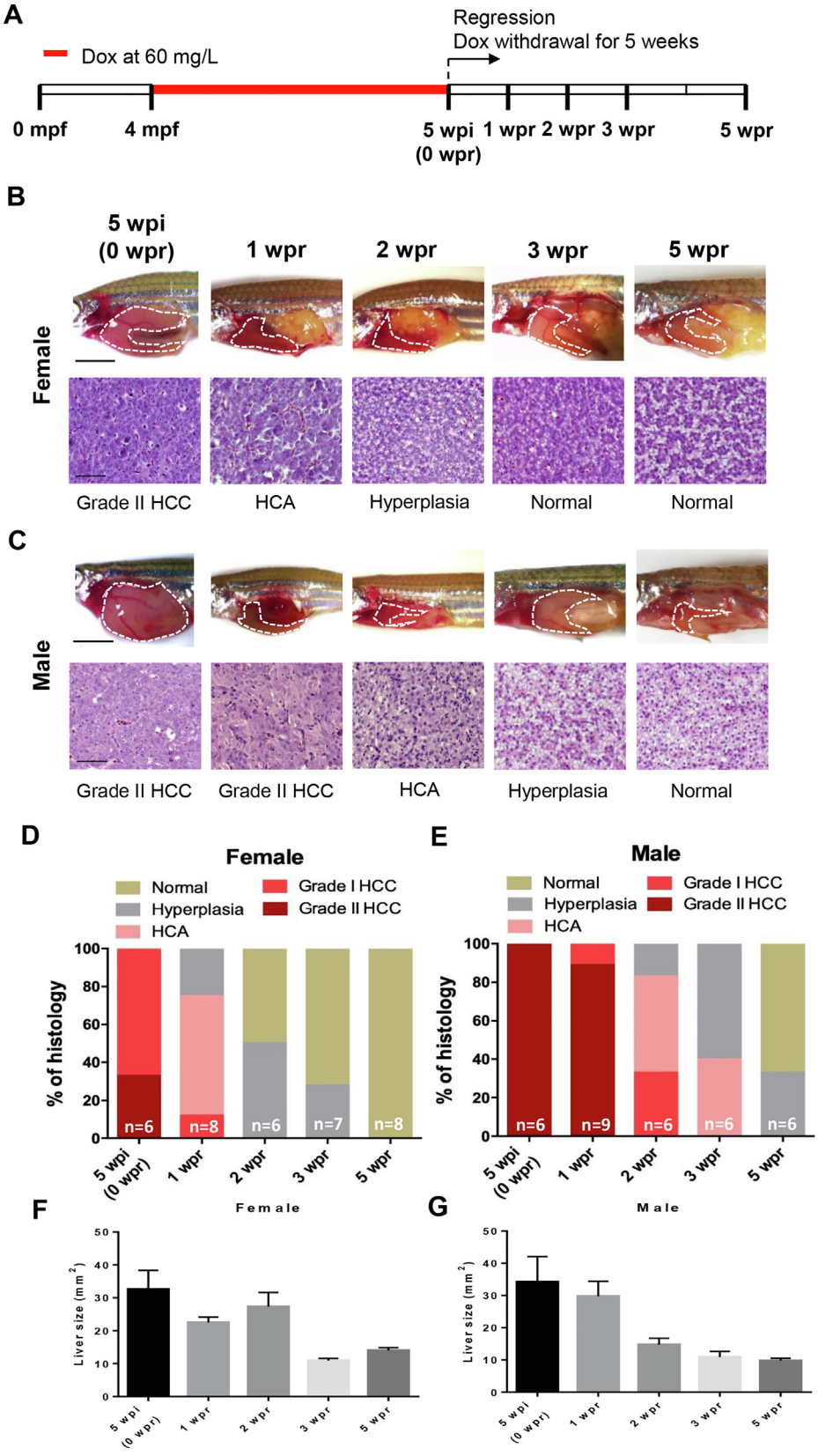

Fig. 5. Sex disparity of the regression of HCC in *Myc/xmrk* zebrafish. (A) Diagram of experimental design and schedules of sample collection for tumor regression. (B and C) gross observation (top) and liver histology (bottom) of female (B) and male (C) *Myc/xmrk* zebrafish during liver tumor progression. Livers in the gross observation pictures are outlined. Scale bars, 5 mm in the upper row and 50 μm in the lower row. (D and E) Quantification of the histological phenotypes of livers of female (D) and male (E) *Myc/xmrk* zebrafish at different time points during HCC regression. Sample sizes (n) are indicated. (F and G) Quantification of 2-D liver size of female (F) and male (G) *Myc/xmrk* zebrafish during liver tumor regression. Wpi, week post induction; wpr, week post regression.

treatments had oncogenic effects based on gross observations and histology (Fig. S1), indicating that sex hormone treatments for one week did not alter the normal liver histology. Thus, it appears that E2 could attenuate HCC progression in male *Myc/xmrk* zebrafish while KT11 could accelerate this process in female *Myc/xmrk* zebrafish.

To further study the effects of sex hormones on HCC progression, cell proliferation was examined via PCNA staining. KT11 treatment in female *Myc/xmrk* zebrafish significantly increased the cell proliferation rate in the liver (Fig. 4A and C), but in male *Myc/xmrk* zebrafish, KT11 treatment showed no significant effect on cell proliferation (Fig. 4B and

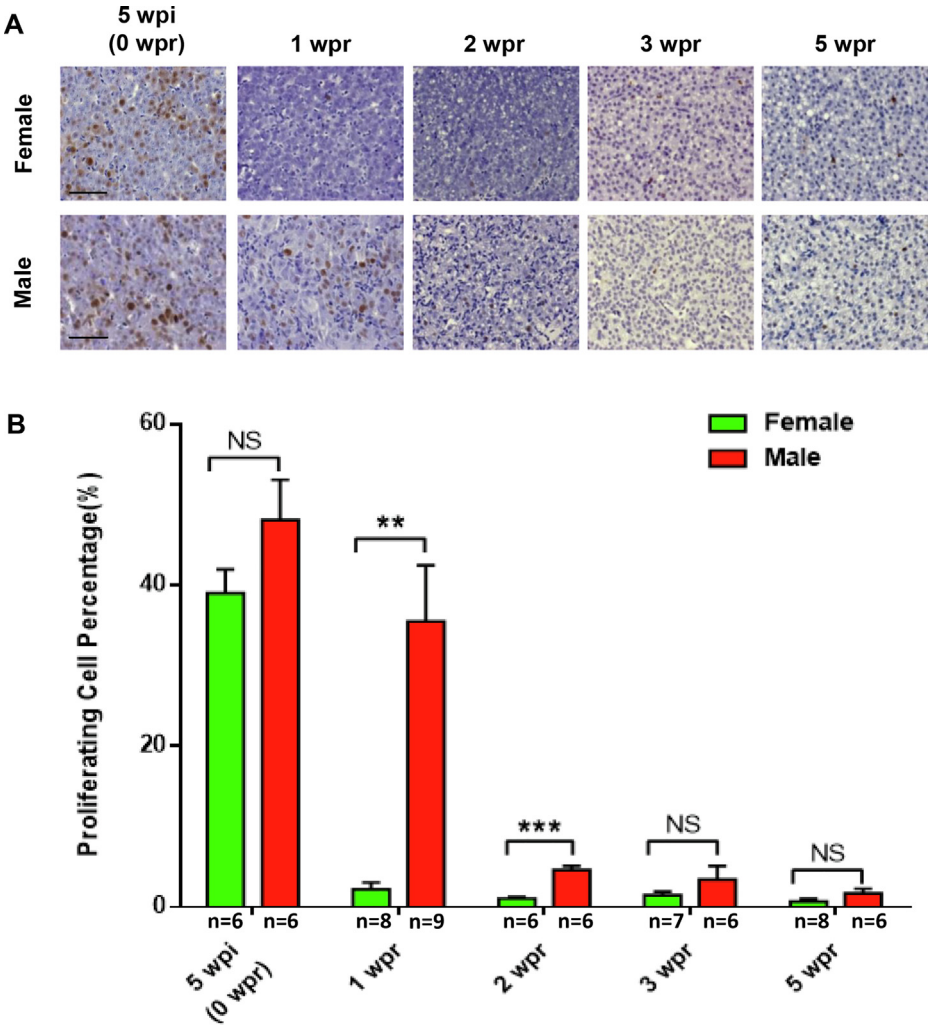

Fig. 6. Cell proliferations in female and male *Myc/xmrk* zebrafish during 5-week tumor regression. (A) PCNA staining of liver sections of female (top) and male (bottom) *Myc/xmrk* zebrafish during 5 weeks of HCC regression. Scale bars, 50 μm. (B) Quantification of cell proliferation in female and male *Myc/xmrk* zebrafish during 5 weeks of HCC regression. Sample sizes (n) are indicated. Wpi, week post induction; wpr, week post regression. $^{**}p < 0.01$, $^{***}p < 0.001$, NS: not significant.

D). E2 treatment in male *Myc/xmrk* zebrafish decreased liver cell proliferation significantly (Fig. 4B and D); however, E2 treatment had no effect on liver cell proliferation in female *Myc/xmrk* zebrafish (Fig. 4A and C). In comparison, neither KT11 nor E2 had significant effect on liver cell proliferation in both male and female wild type zebrafish (data not shown). These observations showed that sex hormones significantly affected cell proliferation during HCC progression in *Myc/xmrk* zebrafish with E2 inhibiting liver cell proliferation in males and KT11 promoting liver cell proliferation in females. These trends are consistent with the histological examination of sex hormone treated *Myc/xmrk* zebrafish. The effects of sex hormones on cell proliferation are also consistent with previous studies on cell lines and other liver tumor models (Chang-Lee et al., 2017; Li et al., 2017).

3.3. HCC regression in Myc/xmrk HCC transgenic zebrafish

In our inducible transgenic expression models, liver tumors induced from *Myc* and *xmrk* single transgenic zebrafish can be regressed upon removal of the chemical inducer (Li et al., 2012; Sun et al., 2015). To examine whether liver tumors from *Myc/xmrk* double transgenic zebrafish could be similarly regressed, Dox was removed after 5-week induction when both male and female *Myc/xmrk* fish developed advanced HCC (Fig. 5A). The liver samples were collected at 1-week post regression (wpr), 2 wpr, 3 wpr and 5 wpr (Fig. 5A). After removal of Dox, the gross observations of the male and female *Myc/xmrk* zebrafish showed a rapid shrinking of the abdomen sizes (Fig. 5B and C). Later, pathological examination confirmed the reduction of the liver size in both female and male *Myc/xmrk* zebrafish (Fig. 5B and C) and their histology was reverted to the normal liver phenotype by 5 wpr. However, the speed of HCC regression showed significant differences between female and male *Myc/xmrk* zebrafish. Before tumor regression, both male and female *Myc/xmrk* zebrafish showed overwhelmingly Grade II HCC (Fig. 5B-E). After 1 week of tumor regression, male *Myc/xmrk* fish remained mostly Grade II HCC features while in female *Myc/xmrk* zebrafish the livers were mainly HCA (Fig. 5B-E). At 2 wpr, the male *Myc/xmrk* fish were reverted to Grade I HCC and HCA while the female *Myc/xmrk* fish showed hyperplasia and normal livers (Fig. 5B-E). At 3 wpr, most of male *Myc/xmrk* zebrafish turned into hyperplasia while most female *Myc/xmrk* fish showed normal liver histology. By 5 wpr, all female and most male *Myc/xmrk* fish completely recovered (Fig. 5C and E). In both female and male *Myc/xmrk* zebrafish, the liver size was apparently decreased during the 5 weeks of tumor regression (Fig. 5F and G). Thus, double *Myc/xmrk* oncogene-induced HCC can also be regressed upon suppression of oncogene expression and the regression of liver cancer in female *Myc/xmrk* zebrafish is faster than in male *Myc/xmrk* zebrafish.

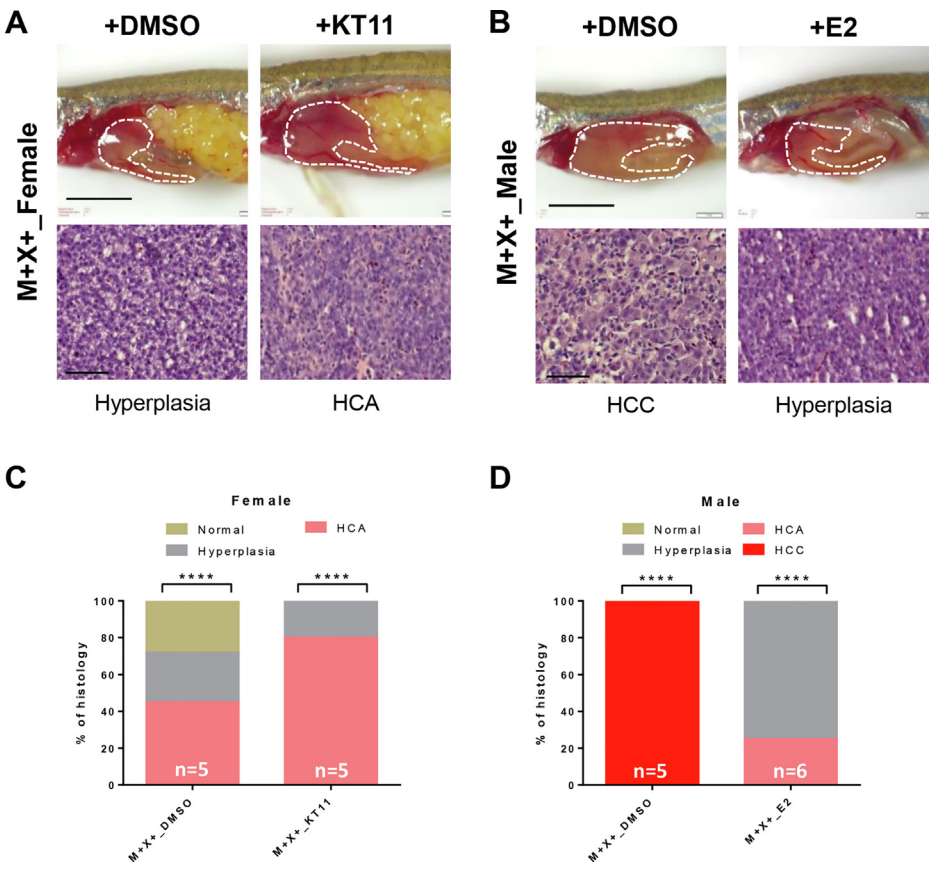

Fig. 7. Sex hormone treatments on female and male *Myc/xmrk* zebrafish during HCC regression. Sex hormones were introduced for one week following Dox withdrawal after five weeks of liver tumor induction as indicated in Fig. 5A. (A and B) Gross observation (top) and liver histology (bottom) of female (A) and male (B) *Myc/xmrk* zebrafish after sex hormone treatment during HCC regression. Livers in the gross observation pictures are outlined. Scale bars, 5 mm for the upper row and 50 μm for the lower row. (C and D) Quantitation of histological features of female (C) and male (D) *Myc/xmrk* zebrafish in different treatment groups. Sample sizes (n) are indicated. +DMSO, control *Myc/xmrk* zebrafish with DMSO vehicle; +KT11, *Myc/xmrk* zebrafish with KT11 treatment; +E2, *Myc/xmrk* zebrafish with E2 treatment. ****$p < 0.0001$.

To further characterize HCC regression in female and male *Myc/xmrk* zebrafish, cell proliferation was examined at different time points. During HCC regression, the proliferating cell number decreased in both female and male *Myc/xmrk* zebrafish liver (Fig. 6A). At 1 wpr, the number of proliferating cells in the livers in male *Myc/xmrk* fish was significantly higher than that in female *Myc/xmrk* fish. At 2 wpr, cell proliferation in male *Myc/xmrk* zebrafish was still higher than that in female *Myc/xmrk* fish, but in the following two time points, 3 and 5 wpr, proliferation of liver cells was low and there was no significant difference between male and female *Myc/xmrk* fish (Fig. 6B). Thus, during HCC regression, male *Myc/xmrk* zebrafish had a significantly higher cell proliferation level than female *Myc/xmrk* zebrafish in the first two weeks of HCC regression.

3.4. Effects of sex hormones on HCC regression in Myc/xmrk transgenic zebrafish

To investigate the effects of sex hormones on regression of *Myc/xmrk*-driven HCC, sex hormone treatments were performed during regression of the *Myc/xmrk*-driven HCC. During tumor regression, the male *Myc/xmrk* zebrafish were exposed to E2 while the female *Myc/xmrk* fish were exposed to KT11 for one week. E2 treatment in female *Myc/xmrk* zebrafish and KT11 treatment in male *Myc/xmrk* zebrafish were not performed during HCC regression because these treatments showed no significant effect on HCC progression in female and male *Myc/xmrk* zebrafish.

In female *Myc/xmrk* zebrafish, the histology of liver showed increased HCA and decreased normal liver histology in the KT11 treatment group compared with the DMSO vehicle control group after one week of tumor regression (Fig. 7A and C). In male *Myc/xmrk* zebrafish, at the end of one week tumor regression, the fish with E2 treatment showed faster recovery with no HCC histology and low ratio of HCA histology, while the histology in the fish in DMSO treatment group still showed Grade II HCC (Fig. 7B and D). Meanwhile, in wildtype siblings of *Myc/xmrk* zebrafish, either vehicle (DMSO) treatment or sex hormone treatment did not affect normal liver histology (Fig. S2). These observations suggested that sex hormones affected HCC regression in both female and male *Myc/xmrk* zebrafish. E2 helped HCC regression while KT11 retarded this process.

To further investigate the effects of the sex hormone treatments on this process, cell proliferation assay was performed for different groups. After E2 treatment, cell proliferation of the livers in male *Myc/xmrk* fish decreased greatly compared with the livers of male *Myc/xmrk* fish without E2 treatment (Fig. 8B and D), while KT11 treatment in female *Myc/xmrk* fish showed the opposite effect, i.e. a higher proliferation rate in the KT11 treatment group than that in the control group (Fig. 8A and C). These results indicated that the cell proliferation could be altered by sex hormone treatments during HCC regression, which is consistent with the histological examinations. Both histological and cell proliferation data suggested the sex hormones might affect the sex disparity of HCC regression through regulation of cell proliferation.

4. Discussion

In this study, we confirmed the sex diversity of HCC developed from a double oncogene transgenic zebrafish model by co-inducing *Myc* and *xmrk* oncogenes in the liver. *MYC* is one of the best known oncogenes involving in almost all types of human cancers (Kuzyk and Mai, 2014) while *xmrk* is a fish oncogene isolated from *Xiphophorus* (platyfish and swordtails) and it codes for a hyperactive form of EGFR (Gomez et al., 2001), another well characterized oncoprotein in human (Arteaga, 2001). Another important finding of this study is the demonstration of the feasibility of regression of HCC induced by two collaborating oncogenes, suggesting a strong and robust effect of oncogene addition for HCC despite a synergistic effect of the two oncogenes, *Myc* and *xmrk*, to cause a more severe HCC phenotype than a single oncogene alone (Li

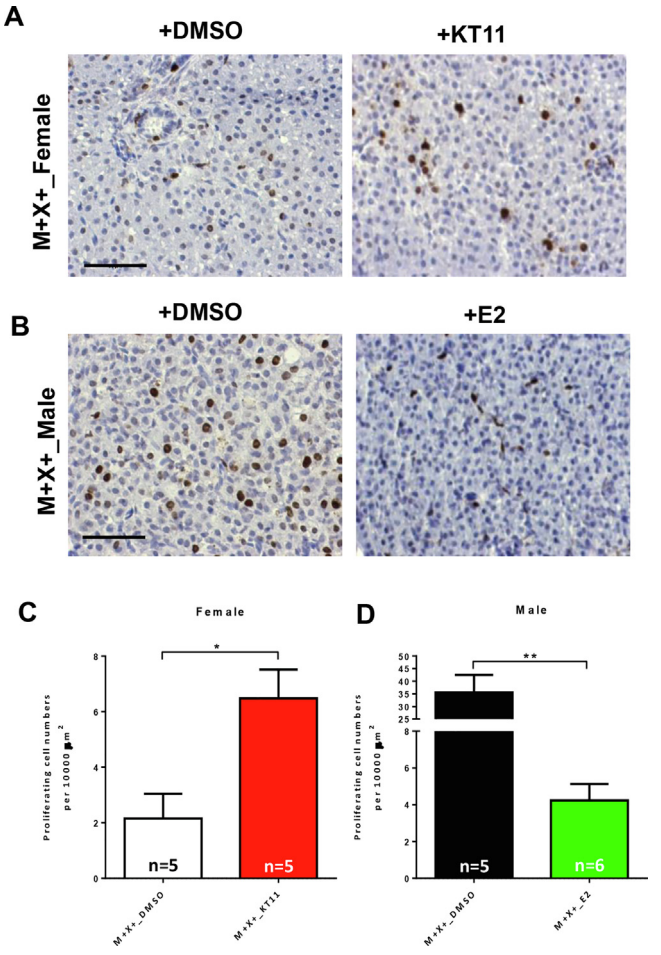

Fig. 8. Cell proliferation altered by sex hormone treatments during HCC regression. The cell proliferation in *Myc/xmrk* zebrafish with vehicle (DMSO) and sex hormone treatment during HCC regression. (A) PCNA staining of liver sections of female *Myc/xmrk* zebrafish after vehicle (DMSO) and KT11 treatment during HCC regression. (B) PCNA staining of liver sections of male *Myc/xmrk* zebrafish after vehicle (DMSO) and E2 treatment during HCC regression. Scale bars, 50 μm. (C) Quantification of cell proliferation in female *Myc/xmrk* zebrafish after KT11 treatment during HCC regression. (D) Quantification of cell proliferation in male *Myc/xmrk* zebrafish after E2 treatment during HCC regression. Sample sizes (n) are indicated. +DMSO, *Myc/xmrk* zebrafish with DMSO treatment; +KT11, *Myc/xmrk* zebrafish with KT11 treatment; +E2: *Myc/xmrk* zebrafish with E2 treatment. *$p < 0.05$, **$p < 0.01$.

et al., 2015).

In the present study, we also demonstrated that sex hormones play significant roles in both HCC progression and regression. Specifically, KT11 could stimulate HCC progression particularly in female *Myc/xmrk* transgenic zebrafish while E2 could retard HCC progression particularly in male *Myc/xmrk* transgenic zebrafish. During HCC regression, KT11 could delay the process in female *Myc/xmrk* transgenic fish while E2 could accelerate the process in male *Myc/xmrk* transgenic fish. Thus, sex hormones apparently contribute to the sex disparity of liver tumors in zebrafish.

In all liver tumor progression and regression experiments, we also examined cell proliferation and found that the numbers of proliferating cells are generally correspondent to the severity and sex disparity of liver tumors. Interestingly, cell proliferation in female *Myc/xmrk* fish was lower than that of male counterpart, explaining the more rapid progression of liver tumors in males (Fig. 2B). By the end of 5 weeks of oncogene induction, the proliferation rates between the two sexes were indistinguishable, corresponding similar severity of liver tumors developed in the two sex at this stage; thus, cell proliferation provides an important basis in liver tumor progression. Similarly, during liver tumor regression, decrease of cell proliferation in male *Myc/xmrk* fish was slower than female counterparts. For example, in female *Myc/xmrk* zebrafish, cell proliferation decreased to a low basal level only within one week of regression; however, in male *Myc/xmrk* zebrafish, cell proliferation was as high as that before tumor regression (5-week tumor induction) and the drop of cell proliferation to the basal level only by the end of the second week of tumor regression (Fig. 6B).

Moreover, the differential effects of the two sex hormones are also consistent with levels of cell proliferation in the liver. In general, E2 decreases the number of proliferating cells while KT11 increases the number of proliferating cells, consistent with their inhibitory and promoting effects in liver tumor progression. Our observations are consistent with previous *in vitro* studies on HCC cell lines in which cell proliferation could be promoted by androgen and inhibited by estrogen (Ma et al., 2008; Xu et al., 2012). However, the hormonal effects appear to be also sex dependent, i.e. E2 usually has stronger effects in males while KT11 had more obvious effects in females. This phenomenon might be partially explained by a previous transcriptomic study as that E2 treatment changed transcriptome of adult male zebrafish liver more compared with adult female zebrafish liver; however, in adult female zebrafish liver, transcriptome showed resistant to either KT11 or E2 treatment (Zheng et al., 2013).

Sex hormones have long been implicated in the sex disparity in human HCC and it has been suggested that androgen has a stimulatory role while estrogen has a protective role in HCC (Yeh and Chen, 2010). Consistent with this, indeed there are correlations of high levels of androgen and low level of estrogen in HCC patients, which are also consistent with many animal experimental data (Kalra et al., 2008; Yeh and Chen, 2010). Our current data from the zebrafish study are consistent with these observations. Several sex hormone based clinical trials (e.g. by using the estrogen receptor modulator tamoxifen, synthetic progestin [megestrol] and androgen antagonist flutamide) have been conducted for HCC patients in the past two decades (Chow et al., 2011; Chow et al., 2002; Yeh et al., 2013), but the results are generally disappointing because of subtle and no benefit from these randomized controlled trials. This fact points potentially additional factors involved in the sex disparity of human HCC. Indeed, we recently also found at least two non-sex hormones, cortisol, a glucocorticoid hormone, and serotonin, a neurotransmitter, also play important roles in the sex disparity of HCC in the zebrafish model (Yan et al., 2017a; Yang et al., 2017b). The relevance of these non-sex hormone factors have also been validated from human liver disease samples (Yan et al., 2017a; Yang et al., 2017b). Thus, the zebrafish model is emerging as a powerful animal model to help understanding molecular mechanisms of HCC and diseases in general in human.

Acknowledgements

This work was supported by grants from Ministry of Education of Singapore (R154000A23112).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.03.018.

References

Agnew, L.R.C., Gardner, W.U., 1952. The incidence of spontaneous hepatomas in C3H, C3H (Low Milk Factor), and CBA mice and the effect of estrogen and androgen on the occurrence of these tumors in C3H mice. Cancer Res. 12, 757–761.
Arteaga, C.L., 2001. The epidermal growth factor receptor: from mutant oncogene in nonhuman cancers to therapeutic target in human neoplasia. J. Clin. Oncol. 19, 32S–40S.
Augustine, M.M., Fong, Y., 2014. Epidemiology and risk factors of biliary tract and primary liver tumors. Surg. Oncol. Clin. N. Am. 23, 171–188.

Barbare, J.C., Bouche, O., Bonnetain, F., Raoul, J.L., Rougier, P., Abergel, A., Boige, V., Denis, B., Blanchi, A., Pariente, A., et al., 2005. Randomized controlled trial of tamoxifen in advanced hepatocellular carcinoma. J. Clin. Oncol. 23, 4338–4346.
Baum, J.K., Bookstein, J.J., Holtz, F., Klein, E.W., 1973. Possible association between benign hepatomas and oral contraceptives. Lancet (London, England) 2, 926–929.
Chang-Lee, S.N., Hsu, H.-H., Shibu, M.A., Ho, T.-J., Tsai, C.-H., Chen, M.-C., Tu, C.-C., Viswanadha, V.P., Kuo, W.-W., Huang, C.-Y., 2017. E2/ERβ inhibits PPARα to regulate cell-proliferation and enhance apoptosis in Hep3B-hepatocellular carcinoma. Pathol. Oncol. Res. 23, 477–485.
Chew, T.W., Liu, X.J., Liu, L., Spitsbergen, J.M., Gong, Z., Low, B.C., 2014. Crosstalk of Ras and Rho: activation of RhoA abates Kras-induced liver tumorigenesis in transgenic zebrafish models. Oncogene 33, 2717–2727.
Chow, P.K., Machin, D., Chen, Y., Zhang, X., Win, K.M., Hoang, H.H., Nguyen, B.D., Jin, M.Y., Lobo, R., Findlay, M., et al., 2011. Randomised double-blind trial of megestrol acetate vs placebo in treatment-naive advanced hepatocellular carcinoma. Br. J. Cancer 105, 945–952.
Chow, P.K., Tai, B.C., Tan, C.K., Machin, D., Win, K.M., Johnson, P.J., Soo, K.C., Asian-Pacific Hepatocellular Carcinoma Trials, G., 2002. High-dose tamoxifen in the treatment of inoperable hepatocellular carcinoma: A multicenter randomized controlled trial. Hepatology 36, 1221–1226.
El-Serag, H.B., Rudolph, K.L., 2007. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology 132, 2557–2576.
Firminger, H.I., Reuber, M.D., 1961. Influence of adrenocortical, androgenic, and anabolic hormones on the development of carcinoma and cirrhosis of the liver in A x C rats fed N-2-fluorenyldicetamide. J. Natl Cancer Inst. 27, 559–595.
Gomez, A., Wellbrock, C., Gutbrod, H., Dimitrijevic, N., Schartl, M., 2001. Ligand-independent dimerization and activation of the oncogenic Xmrk receptor by two mutations in the extracellular domain. J Biol Chem 276, 3333–3340.
Gupta, S., Naini, B.V., Munoz, R., Graham, R.P., Kipp, B.R., Torbenson, M.S., Mounajjed, T., 2016. Hepatocellular neoplasms arising in association with androgen use. Am. J. Surg. Pathol. 40, 454–461.
He, G., Dhar, D., Nakagawa, H., Font-Burgada, J., Ogata, H., Jiang, Y., Shalapour, S., Seki, E., Yost, Shawn, E., Jepsen, K., et al., 2013. Identification of liver cancer progenitors whose malignant progression depends on autocrine IL-6 signaling. Cell 155, 384–396.
Ishak, K.G., Goodman, Z.D., Stocker, J.T., 2001. Tumors of the Liver and Intrahepatic Bile Ducts (Amer Registry of Pathology).
Johnson, C., Han, Y., Hughart, N., McCarra, J., Alpini, G., Meng, F., 2012. Interleukin-6 and its receptor, key players in hepatobiliary inflammation and cancer. Transl. Gastrointestinal Cancer 1, 58–70.
Kalra, M., Mayes, J., Assefa, S., Kaul, A.K., Kaul, R., 2008. Role of sex steroid receptors in pathobiology of hepatocellular carcinoma. World J. Gastroenterol. 14, 5945–5961.
Kanda, T., Yokosuka, O., 2015. The androgen receptor as an emerging target in hepatocellular carcinoma. J. Hepatocellular Carcinoma 2, 91–99.
Kemp, C.J., Leary, C.N., Drinkwater, N.R., 1989. Promotion of murine hepatocarcinogenesis by testosterone is androgen receptor-dependent but not cell autonomous. PNAS 86, 7505–7509.
Kim, C.-M., Koike, K., Saito, I., Miyamura, T., Jay, G., 1991. HBx gene of hepatitis B virus induces liver cancer in transgenic mice. Nature 351, 317–320.
Kuzyk, A., Mai, S., 2014. c-MYC-induced genomic instability. Cold Spring Harbor Perspect. Med. 4, a014373.
Ladenheim, M.R., Kim, N.G., Nguyen, P., Le, A., Stefanick, M.L., Garcia, G., Nguyen, M.H., 2016. Sex differences in disease presentation, treatment and clinical outcomes of patients with hepatocellular carcinoma: a single-centre cohort study. BMJ Open Gastroenterol. 3, e000107.
Lam, S.H., Wu, Y.L., Vega, V.B., Miller, L.D., Spitsbergen, J., Tong, Y., Zhan, H., Govindarajan, K.R., Lee, S., Mathavan, S., et al., 2006. Conservation of gene expression signatures between zebrafish and human liver tumors and tumor progression. Nat. Biotechnol. 24, 73–75.
Lee, S., Lee, M., Kim, J.B., Jo, A., Cho, E.J., Yu, S.J., Lee, J.-H., Yoon, J.-H., Kim, Y.J., 2016. 17β-estradiol exerts anticancer effects in anoikis-resistant hepatocellular carcinoma cell lines by targeting IL-6/STAT3 signaling. Biochem. Biophys. Res. Commun. 473, 1247–1254.
Li, Y., Li, H., Spitsbergen, J.M., Gong, Z., 2017. Males develop faster and more severe hepatocellular carcinoma than females in kras(V12) transgenic zebrafish. Sci. Rep. 7, 41280.
Li, Z., Huang, X., Zhan, H., Zeng, Z., Li, C., Spitsbergen, J.M., Meierjohann, S., Schartl, M., Gong, Z., 2012. Inducible and repressable oncogene-addicted hepatocellular carcinoma in Tet-on xmrk transgenic zebrafish. J. Hepatol. 56, 419–425.
Li, Z., Luo, H., Li, C., Huo, X., Yan, C., Huang, X., Al-Haddawi, M., Mathavan, S., Gong, Z., 2014. Transcriptomic analysis of a transgenic zebrafish hepatocellular carcinoma model reveals a prominent role of immune responses in tumour progression and regression. Int. J. Cancer 135, 1564–1573.
Li, Z., Zheng, W., Li, H., Li, C., Gong, Z., 2015. Synergistic induction of potential warburg effect in zebrafish hepatocellular carcinoma by co-transgenic expression of Myc and xmrk oncogenes. PLoS One 10, e0132319.
Li, Z., Zheng, W., Wang, Z., Zeng, Z., Zhan, H., Li, C., Zhou, L., Yan, C., Spitsbergen, J.M., Gong, Z., 2013. A transgenic zebrafish liver tumor model with inducible Myc expression reveals conserved Myc signatures with mammalian liver tumors. Dis Model Mech. 6, 414–423.
Lu, X., Ma, P., Shi, Y., Yao, M., Hou, L., Zhang, P., Jiang, L., 2015. NF-κB increased expression of 17β-hydroxysteroid dehydrogenase 4 promotes HepG2 proliferation via inactivating estradiol. Mol. Cell. Endocrinol. 401, 1–11.
Ma, J., Malladi, S., Beck, A.H., 2016. Systematic analysis of sex-linked molecular alterations and therapies in cancer. Sci. Rep. 6, 19119.
Ma, W.-L., Lai, H.-C., Yeh, S., Cai, X., Chang, C., 2014. Androgen receptor roles in hepatocellular carcinoma, fatty liver, cirrhosis and hepatitis. Endocr. Relat. Cancer 21, R165–R182.
Ma, W.L., Hsu, C.L., Wu, M.H., Wu, C.T., Wu, C.C., Lai, J.J., Jou, Y.S., Chen, C.W., Yeh, S., Chang, C., 2008. Androgen receptor is a new potential therapeutic target for the treatment of hepatocellular carcinoma. Gastroenterology 135, 947–955 955 e941-945.
Mao, W.L., Shi, X.P., Lou, Y.F., Ye, B., Lu, Y.Q., 2013. The association between circulating oestradiol levels and severity of liver disease in males with hepatitis B virus infection. Liver Int. 33, 1211–1217.
Matsuura, B., Taniguchi, Y., Ohta, Y., 1994. Effect of antiandrogen treatment on chemical hepatocarcinogenesis in rats. J. Hepatol. 21, 187–193.
Moriya, K., Fujie, H., Shintani, Y., Yotsuyanagi, H., Tsutsumi, T., Ishibashi, K., Matsuura, Y., Kimura, S., Miyamura, T., Koike, K., 1998. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat. Med. 4, 1065–1067.
Nagasue, N., Yu, L., Yamaguchi, M., Kohno, H., Tachibana, M., Kubota, H., 1996. Inhibition of growth and induction of TGF-β1 in human hepatocellular carcinoma with androgen receptor by cyproterone acetate in male nude mice. J. Hepatol. 25, 554–562.
Naugler, W.E., Sakurai, T., Kim, S., Maeda, S., Kim, K., Elsharkawy, A.M., Karin, M., 2007. Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. Science 317, 121–124.
Nguyen, A.T., Emelyanov, A., Koh, C.H.V., Spitsbergen, J.M., Parinov, S., Gong, Z., 2012. An inducible kras(V12) transgenic zebrafish model for liver tumorigenesis and chemical drug screening. Dis. Models Mech. 5, 63–72.
Perrone, F., Gallo, C., Daniele, B., Gaeta, G., Izzo, F., Capuano, G., Adinolfi, L., Mazzanti, R., Farinati, F., Elba, S., 2002. Tamoxifen in the treatment of hepatocellular carcinoma: 5-year results of the CLIP-1 multicentre randomised controlled trial. Curr. Pharm. Des. 8, 1013–1019.
Schlageter, M., Terracciano, L.M., D'Angelo, S., Sorrentino, P., 2014. Histopathology of hepatocellular carcinoma. World J. Gastroenterol. 20, 15955–15964.
Shi, L., Feng, Y., Lin, H., Ma, R., Cai, X., 2014. Role of estrogen in hepatocellular carcinoma: is inflammation the key? J. Transl. Med. 12, 93.
Simonetti, R.G., Liberati, A., Angiolini, C., Pagliaro, L., 1997. Treatment of hepatocellular carcinoma: a systematic review of randomized controlled trials. Ann. Oncol. 8, 117–136.
Sinclair, M., Grossmann, M., Gow, P.J., Angus, P.W., 2015. Testosterone in men with advanced liver disease: abnormalities and implications. J. Gastroenterol. Hepatol. 30, 244–251.
Spitsbergen, J.M., Tsai, H.W., Reddy, A., Miller, T., Arbogast, D., Hendricks, J.D., Bailey, G.S., 2000a. Neoplasia in zebrafish (Danio rerio) treated with 7,12-dimethylbenz[a] anthracene by two exposure routes at different developmental stages. Toxicol. Pathol. 28, 705–715.
Spitsbergen, J.M., Tsai, H.W., Reddy, A., Miller, T., Arbogast, D., Hendricks, J.D., Bailey, G.S., 2000b. Neoplasia in zebrafish (Danio rerio) treated with N-methyl-N'-nitro-N-nitrosoguanidine by three exposure routes at different developmental stages. Toxicol. Pathol. 28, 716–725.
Sun, L., Nguyen, A.T., Spitsbergen, J.M., Gong, Z., 2015. Myc-induced liver tumors in transgenic zebrafish can regress in tp53 null mutation. PLoS One 10, e0117249.
Toh, Y.C., 1981. Effect of neonatal castration on liver tumor induction by N-2-fluorenylacetamide in suckling BALB/c mice. Carcinogenesis 2, 1219–1221.
Velazquez, I., Alter, B.P., 2004. Androgens and liver tumors: Fanconi's anemia and non-Fanconi's conditions. Am. J. Hematol. 77, 257–267.
Vesselinovitch, S.D., Itze, L., Mihailovich, N., Rao, K.V., 1980. Modifying role of partial hepatectomy and gonadectomy in ethylnitrosourea-induced hepatocarcinogenesis. Cancer Res. 40, 1538–1542.
Vesselinovitch, S.D., Mihailovich, N., 1967. The effect of gonadectomy on the development of hepatomas induced by urethan. Cancer Res. 27, 1788–1791.
Westaby, D., Portmann, B., Williams, R., 1983. Androgen related primary hepatic tumors in non-Fanconi patients. Cancer 51, 1947–1952.
Xu, H., Wei, Y., Zhang, Y., Xu, Y., Li, F., Liu, J., Zhang, W., Han, X., Tan, R., Shen, P., 2012. Oestrogen attenuates tumour progression in hepatocellular carcinoma. J. Pathol. 228, 216–229.
Yager Jr., J.D., Yager, R., 1980. Oral contraceptive steroids as promoters of hepatocarcinogenesis in female Sprague-Dawley rats. Cancer Res. 40, 3680–3685.
Yan, C., Yang, Q., Gong, Z., 2017a. Tumor-associated neutrophils and macrophages promote gender disparity in hepatocellular carcinoma in zebrafish. Cancer Res. 77, 1395–1407.
Yan, C., Yang, Q., Shen, H.M., Spitsbergen, J.M., Gong, Z., 2017b. Chronically high level of tgfb1a induction causes both hepatocellular carcinoma and cholangiocarcinoma via a dominant Erk pathway in zebrafish. Oncotarget 8, 77096–77109.
Yang, Q., Yan, C., Gong, Z., 2017a. Activation of liver stromal cells is associated with male-biased liver tumor initiation in xmrk and Myc transgenic zebrafish. Sci. Rep. 7, 10315.
Yang, Q., Yan, C., Yin, C., Gong, Z., 2017b. Serotonin activated hepatic stellate cells contribute to sex disparity in hepatocellular carcinoma. Cell Mol. Gastroenterol. Hepatol. 3, 484–499.
Yeh, S.H., Chen, P.J., 2010. Gender disparity of hepatocellular carcinoma: the roles of sex hormones. Oncology 78 (Suppl. 1), 172–179.
Yeh, Y.T., Chang, C.W., Wei, R.J., Wang, S.N., 2013. Progesterone and related compounds in hepatocellular carcinoma: basic and clinical aspects. Biomed. Res. Int. 2013, 290575.
Zheng, W., Li, Z., Nguyen, A.T., Li, C., Emelyanov, A., Gong, Z., 2014. Xmrk, Kras and Myc transgenic zebrafish liver cancer models share molecular signatures with subsets of human hepatocellular carcinoma. PLoS One 9, e91179.
Zheng, W., Xu, H., Lam, S.H., Luo, H., Karuturi, R.K.M., Gong, Z., 2013. Transcriptomic analyses of sexual dimorphism of the zebrafish liver and the effect of sex hormones. PLoS One 8, e53562.