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Estrogen accelerates heart regeneration by promoting inflammatory responses in zebrafish

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Abstract

Sexual differences are frequently observed in the onset and prognosis of human cardiovascular diseases, but the underlying mechanisms are not clearly known. Here, we report that zebrafish heart regeneration is faster in females, can be accelerated by estrogen and suppressed by estrogen-antagonist tamoxifen. Transcriptomic analyses suggested heart injuries triggered more pronounced immune and inflammatory responses, previously shown to enhance heart regeneration, in females, leading to increased STAT1 expression and leukocyte filtration. These responses could be enhanced by estrogen treatment in males. We also showed that injuries to the heart, but not other tissues, increased plasma estrogen level and expression of estrogen receptors, especially *esr2a*, in zebrafish hearts. Although this effect was stronger in female fish, the resulting endocrine disruption was sufficient to induce the expression of female-specific protein vitellogenin in male zebrafish. Altogether, this study reveals that heart regeneration is modulated by an estrogen-inducible inflammatory response to heart injury, which in turn stimulates estrogen signalling predominately in females. These findings elucidate a previously unknown layer of control in zebrafish heart regeneration and provides a new model system for the study of sexual differences in human cardiac repair.

Key words

sexually dimorphic, heart regeneration, estrogen, inflammation, endocrine disruption, estrogen receptor, feminisation

Introduction

Cardiovascular diseases (CVD) are the primary cause of death worldwide: causing the death of 17.9 million people in 2015, more than 30% of the global mortality (Roth et al., 2017). Gender differences have been reported on risk factors, clinical manifestation and recovery of CVD (reviewed by Maas and Appelman, 2010; Ostadal and Ostadal 2014; EUGenMed et al., 2015). In various mammalian models of cardiac defects, females consistently demonstrate a lower mortality, less severe disease phenotypes and better functional recovery than the male counterparts (Du 2004, Czubryt et al., 2006; Du et al., 2006). An understanding of the molecular mechanism underlying these gender differences will provide an important gateway towards better CVD prevention and treatment. While the protective effects of estrogen on the cardiovascular system have been widely reported, most of these studies focused on how estrogen ameliorates CVD risk factors, by the maintenance of vasodilation and in the prevention of atherosclerosis (Pare et al., 2002; Mendelsohn 2002; Babiker et al., 2002; Fliegner et al., 2010). But estrogen may play more direct roles in cardiac development, functions and pathology, as estrogen can protect cardiomyocytes from apoptosis caused by ischaemia–reperfusion in vitro (Liu et al., 2011). Gene expression profiles of mammalian cardiomyocytes (CM) are sexually dimorphic (Isensee et al, 2008, Witt et al, 2008, Tsuji et al, 2017), and estrogen receptor expression is deregulated in some cardiomyopathies (Mahmoodzadeh et al., 2006). Despite the apparent involvement of estrogen in CVD, clinical translation of these findings has been lagging, partly due to inconclusive results from large-scale studies on the benefits of post-menopausal hormone replacement treatment on CVD prevention (Low et al., 2002). This is attributed, at least in part, to the lack of a mechanistic understanding of the roles of estrogen in cardiomyocyte biology, which prevents optimal study design and subject selection in clinical studies (Whayne & Mukherjee, 2015).

While adult human cardiomyocytes are virtually unable to re-enter the cell cycle, many vertebrates, including zebrafish, newts and axolotls, demonstrate a remarkable ability to regenerate their hearts

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(reviewed by Garcia-Gonzalez and Morrison 2014; Vivien et al., 2016). Among these organisms, zebrafish has emerged as one of the most well-established model organisms for the study of heart regeneration. Even after the resection of up to 20% of its ventricle, a zebrafish can completely regenerate its heart within 2 months with no scarring (Poss et al., 2002). Apart from physical amputation, cryoinjury (Chablais et al., 2011; González-Rosa et al., 2011) and genetic ablation (Wang et al., 2011) of cardiomyocytes can also trigger proliferation, usually measured by the expression of proliferating cell nuclear antigen (PCNA) or phosphor-histone H3. Heart regeneration is not localized to the proximity of the wound, but involves the precise coordination of cardiomyocytes proliferation, migration (Itou et al., 2012) and extracellular matrix remodelling (Wang et al., 2013). Fate mapping experiments have established that the proliferating cardiomyocytes originated from mature cardiomyocytes (Jopling et al., 2010; Senyo et al., 2013; Zhang et al., 2013). The rapid disappearance of the sarcomeric structures and re-expression of embryonic genes, such as embryonic cardiac myosin heavy chain gene (embCMHC), suggest that regeneration largely involves the expansion of dedifferentiated cardiomyocytes (Jopling et al., 2010; Sallin et al., 2015). Biochemical and gene expression analyses have identified the involvement of growth factors such as *tgfb1, pdgf, shh* and *igf2b*, and signalling pathways such as Notch, Jak1/Stat3 and NFkB in zebrafish heart regeneration (reviewed by Kikuchi et al., 2015; González-Rosa et al., 2018). A recent study has attributed the remarkable regenerative capacity of zebrafish heart to this organism's enhanced inflammatory and immune responses, in comparison with other fish species (Lai et al., 2017). Hypoxia, as a result of cardiac damage, also plays a positive role in cardiomyocyte proliferation (Jopling et al., 2012), whereas hyperoxia and miR-133 inhibit regeneration (Yin et al., 2012).

While the zebrafish heart is different from the human counterpart on cellular and anatomical levels (Hu et al., 2000; Jensen et al., 2013), the conservation of the genetic circuitry between zebrafish and mammals (Howe et al., 2013), and the technical and genetic resources offered by the zebrafish research community, make this organism an ideal model for the study of the gender bias associated with human CVD. Some observations have suggested a role of sex hormones in zebrafish cardiac development and functions. For instance, inhibitors of estrogen synthesis induced phenotypes similar to congestive heart failure and tamponade in zebrafish embryos (Allgood et al., 2013). These conditions could be reversed by estradiol (E2) treatment, which has also been shown to affect heart rates during zebrafish embryonic development (Romano et al., 2017). Despite these findings, the entire literature on zebrafish heart regeneration is based on studies on a single sex (usually male) and sexual differences in regeneration have never been investigated. In this study, we examine, for the first time, the sexual dimorphism of zebrafish in heart regeneration and in response to factors that influence the rate of cardiac regeneration.

Results

Zebrafish heart regeneration is sexually dimorphic

Female and male zebrafish, matched for age and weight, were subjected to cardiac damage by cryoinjury. On day 7 post cryoinjury (7 dpc), female hearts contained a significantly higher number of PCNA-positive cells (Fig 1A) and less vimentin immunoreactivity (Fig 1C) than in the male counterpart (Fig 1B and 1D), indicating more cell proliferation and fewer scar-forming fibroblasts in the regenerating female heart. After one month of regeneration, female hearts contained significantly smaller scar tissues compared to males (Fig 1E, F). These observations prompted us to investigate the effect of estrogen on zebrafish heart regeneration. Male zebrafish exposed to E2 after cryoinjury displayed a ~5-fold increase in cardiomyocyte proliferation in the vicinity of the injured area (Fig 1G, H). Male cardiomyocytes were highly sensitive to E2: the number of PCNA-positive cells in the wound increased by ~4-folds when exposed to E2 at a concentration as low as 0.01nM (Fig S1A, B). E2 treatment of male fish also increased cardiomyocyte dedifferentiation, as judged by the expression of embCMHC (Fig 1I, J, Fig S1E), and scar reduction (Fig 1K, L). Although E2 treatment could also accelerate heart regeneration in females (Fig 1K, L), the effect was less pronounced (~2-fold reduction of scar size) than in males (~3-fold reduction of scar size, Fig 1L). Conversely, treatment of female fish with tamoxifen, an antagonist of estrogen receptors (Jordan, 2006; Xia et al., 2016), resulted in a ~4-fold decrease in cardiomyocyte proliferation (Fig 1G, H), ~2-fold decrease in embCMHC expression (Fig 1I, J, Fig S1E), and ~2-fold increase in scar size (Fig 1K, L). Moreover, echocardiographic measurements, that allowed the non-invasive measurement of cardiac performance during regeneration (Wang et al., 2017), indicated that E2 treatment of male fish after cryoinjury accelerated the restoration of Fractional Shortening (FS) time to the pre-injury level (Fig S1F). FS is a parameter that indicates the cardiac contractile force and has previously been shown to correlate with zebrafish cardiac recovery during regeneration (Hein et al., 2015). The increase in FS recovery by E2 treatment confirms the

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promoting effect of this hormone on the recovery of physiological functions after cardiac damage in male zebrafish.

To determine whether female and male zebrafish responded differently to environmental conditions that alter heart regeneration rates, heart-injured fish were exposed to hyperoxia, a condition known to suppress the induction of HIF1a after cardiac injury and decelerate regeneration (Jopling et al., 2012). As expected, hyperoxia reduced the occurrence of PCNA-positive cells in the regenerating hearts of both sexes (Fig S2). However, the effect of hyperoxia on the frequency of PCNA-positive cells was stronger in females (~40% in females, less than 30% in males). As a result, the sexual difference in cardiomyocyte proliferation was significantly reduced in hyperoxic condition. As hyperoxia also eliminated the effect of E2 on cardiomyocyte proliferation in male zebrafish (Fig S2), estrogen is likely to regulate mechanisms that are upstream of HIF1a in the heart regeneration regime.

Enhanced immune and inflammatory responses to heart injury in female zebrafish

To elucidate the mechanism underlying the sexual difference in zebrafish heart regeneration, we performed comparative transcriptomic analyses on RNA extracted from female and male hearts at 7 dpc. A total of 1050 genes were found to be differentially expressed (by more than two-fold) between two sexes with statistical significance across 3 biological replicates (Table S1). Gene ontology (GO) analyses revealed that a majority of biological processes enriched for the female-biased genes are related to immunological processes, such as immune response, inflammatory response and chemotaxis for immune cells. On the other hand, male-biased genes are more diverse in function, ranging from protein homeostasis, stress response to muscle contraction (Fig 2A). Gene set enrichment analysis (GSEA, Subramanian et al., 2005) confirmed that immune-related pathways are among the most sexually dimorphic in post-injured zebrafish hearts (Fig S3). As female heart regeneration is faster, the over-representation of immune-related genes in heart-injured females might be a mere reflection of the fact that at 7 dpc the female hearts were at more advanced stage of regeneration than the males. A comparison of the published transcriptomic profiles of zebrafish hearts collected at 2 and 5 dpc (Lai et al., 2017), however, shows that immune-related genes were not among the most differentially expressed genes between these two days (Fig S4), indicating that the observed female-biased expression of immune-related genes after heart injury was far more pronounced than can be explained by the difference in heart regeneration rate between sexes.

To test how much the observed female-specific gene expression pattern in heart regeneration was shaped by estrogen, the respective effects of E2 treatment of male fish, and tamoxifen treatment of female fish, on the transcriptome of injured hearts were investigated. Fig 2B shows that tamoxifen treatment reversed almost all female-specific gene expression signatures in the injured heart, whereas genes involved in immune response and neutrophil chemotaxis were upregulated in estrogen-treated males. Among the genes whose expression level in injured hearts are sexually dimorphic, and/or reciprocally regulated by E2 and tamoxifen, are interferon-gamma (IFNg), interferon regulatory factor 1b (IRF1b) and various isoforms of CXCL11 (Fig 2C).). In mammals, both IRF1 and CXCL11 are IFNg-inducible (Yang et al., 2007; Flodstro□m & Eizirik, 1997), suggesting the interferon-gamma pathway is instrumental to the sexual dimorphism in heart regeneration. In consistent with the transcriptomic data, Q-PCR analysis showed that the expression level of IFNg in the female heart increased by ~20-folds rapidly (1 dpc) after injury, while remained unchanged in males (Fig 2D). IFNg expression after heart injury was significantly stimulated by E2 treatment of male fish and suppressed by tamoxifen treatment of females (Fig 2E, F), in consistent with the role of estrogen in IFNg expression in other tissues (Fox et al., 1991; Hao et al., 2013). There was a significant increase of STAT1, a target of IFNG (Horvath, 2004), at 7 dpc in female hearts but not in males (Fig 2G), consistent with the female-specific induction of IFNg induction (Fig 2D). The upregulation of STAT1 in female hearts was reversed by tamoxifen. In males, E2 treatment resulted in a small but significant increase in STAT1 (Fig 2G, H). STAT3 was also induced in female hearts after heart injury, but this induction was not tamoxifen-sensitive (Fig 2G), suggesting that, although STAT3 is essential to injury-induced cardiomyocyte proliferation in general (Fang et al., 2013), STAT1 may be more important for orchestrating sexual difference in regeneration rates. Interestingly, STAT1 has recently been identified as an estrogen-responsive gene in mammals (Young et al., 2017). As IFNg and STAT1 are important for the activation (Ohmori & Hamilton, 2001) and retention (Hu et al., 2008) of macrophages and neutrophils in the inflamed area, we asked how the sexually dimorphic activation of STAT1 affected the occurrence of leukocytes in cardiac wound. Figure 2I shows that, at 1 dpc, the number of L-Plastin positive leukocytes (Shinomiya, 2012) detected in the injured area was significantly higher in females than in males (Fig 2I, J).

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Estrogen receptor esr2a is induced by heart injury

In our transcriptomic analyses, we noticed that the expression level of an estrogen receptor (ER) was significantly upregulated in male hearts at 7 dpc (data not shown). To investigate this phenomenon in more detail, the expression of all three nuclear ERs (Lu et al., 2017) was examined in zebrafish hearts after cryoinjury or sham operation, as well as in the heart of fish that had not undergone any surgery (uninjured). Sham operation was performed by the identical procedures that preceded cryoinjury, including the opening of chest skin and tissue to expose the heart. Fig 3A shows that at 7 days post-surgery, the expression of *esr1* and *esr2a* was induced (~3-fold compared to uninjured) in male hearts. The increase in expression of these two ERs in female hearts was even more pronounced (up to 4-folds for *esr1* and 9-folds for *esr2a*, Fig 3B). Sham operation also induced in expression of *esr2a* (in female) and *esr1* (in both sexes), but to a lesser extent compared to the effect of cryoinjury. *Esr2b* was suppressed by both cryoinjury and sham operation in either sex. Fig 3C shows that E2 treatment led to a dramatic increase in *esr2a* expression in injured male hearts (~6-folds higher than injured males without E2 treatment), whereas the effect on *esr1* and *esr2b* was comparatively minor. The expression of *esr2a*, but not *esr1* and *esr2b*, in injured female hearts was suppressed by tamoxifen (Fig 3B). Cryoinjury appeared to sensitise *esr2a* towards E2, as the induction of *esr2a* expression by E2 treatment was much less pronounced in uninjured male hearts (~2-folds) than at 7 dpc (~6-folds) (Fig 3E).

Cardiac injury leads to endocrine disruption in zebrafish

As ERs are estrogen inducible genes, the upregulation of *esr2a* in heart-injured fish prompted us to investigate whether the level of E2 in those fish have also changed. Fig 3F shows that the plasma E2 level in male cryoinjured and sham-operated fish was 20% and 10% higher than that of the control respectively. Both cryoinjury and sham operation led to an even larger increase in E2 level in females (~5-fold) (Fig 3G). The induction of ER expression did not generally occur in all types of injured tissue, as the plasma E2 levels in male and female fish on day 7 after caudal fin amputation were virtually unchanged as compared to untreated controls (Fig 3H), and the expression of *esr1* and *esr2a* in the fin was not significantly altered after fin amputation (Fig 3I, J). Hence, heart injury and sham operation increased estrogen level and ER expression in zebrafish hearts, especially in females, and this endocrine disruption appeared to be specifically linked to injuries of the heart and its proximity, but not other tissue regeneration/repair systems. We hypothesised that hypoxia, due to heart injury and/or blood loss, may be the stimulus of estrogen induction. However, hyperoxia did not significantly affect the increase of E2 after heart damage in either sex (Fig 3K).

To examine the biochemical consequence of this hormonal change, we utilized the sexual dimorphism in zebrafish plasma proteins (Babaei et al., 2013; Li et al., 2016). Quantitative proteomic analysis was used to study the relative abundances of 18 known sexually dimorphic plasma proteins in the plasma collected from untreated, sham-operated and ventricular amputated male zebrafish (Fig S5 and Table S2). Most female-biased plasma proteins increased after cardiac damage, as compared to sham operation, except Nucleoside Diphosphate Kinase (NDPK), a protein known to be marginally female-biased (Li et al., 2016). Conversely, all but two male-biased plasma proteins were downregulated after ventricular amputation. As the two outliers, Myoglobin and Phosphoglycerate Kinase 1, are expressed predominately in muscle, the increase of these proteins in plasma might be a result of the release of CM cellular content into circulation. These data indicate that although the impact of heart injury on estrogen level in males was relatively minor, it was sufficient to shift the gender characteristic of the male zebrafish plasma towards a more feminised composition.

Vitellogenin accumulate in the regenerating heart of adult male zebrafish

Our plasma proteomic analysis indicates the presence of vitellogenin isoforms in the plasma of male zebrafish after heart injury (Fig S5). Western blotting detected vitellogenin in the plasma of male zebrafish as early as day 1 after both cryoinjury (Fig 4A) and heart amputation (data not shown), as compared to sham or untreated fish. Vitellogenin is specifically expressed in females (reviewed by Hara et al., 2016) and are routinely used as biomarkers for endocrine disruption in males (García-Reyero et al., 2004; Scott and Robinson, 2008). As vitellogenin has been reported as an acute phrase response protein (Tong et al., 2010; Zhang et al., 2011), it is possible that vitellogenin was induced in cardiac-damaged males as a result of infection. However, the abundance of vitellogenin in the plasma

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was much higher in heart-damaged than in sham-operated fish, even though the latter also suffered from extensive tissue damages. Moreover, plasma proteomics showed that most of the known acute phase response proteins (Roy et al., 2017), such as the complement and coagulating factors, were down-regulated in the heart regenerating fish as compared to the sham (Fig S6 and Table S3). This suggests that the presence of vitellogenin in male plasma after heart injury is not a consequence of injury-related infection or inflammation; but is specifically associated with heart regeneration.

We examined the tissue distribution of vitellogenin in male zebrafish during heart regeneration. On day 5 after cardiac injury, vitellogenin was detected in the heart, but not in the gill, kidney and liver (Fig 4B). Vitellogenin was observed in the entire regenerating heart, not restricted to the wound. Whole mount immunohistochemistry confirmed that vitellogenin accumulated in the injured heart of male fish (Fig 4C) and was not observed in the proximity of the chest wound of the sham-operated fish. This indicates that the vitellogenin accumulation in male fish is specifically associated with cardiac damages and is not related to general wound healing. Moreover, vitellogenin was not undetectable on the male caudal fin on day 7 after amputation (Fig 4D), confirming that vitellogenin accumulation is not a general consequence of tissue regeneration or repair. Furthermore, E2 treatment of male fish increased the level of vitellogenin in the regenerating hearts, while tamoxifen treatment of female fish reduced this accumulation (Fig 5).

Discussion

In this study, we revealed the roles of estrogen in several aspects of zebrafish heart regeneration. First, we demonstrated by cellular (Fig 1, 2), anatomical (Fig 1, 4), physiological (Fig 3) and biochemical (Fig 3) parameters that zebrafish heart regeneration is sexually dimorphic. As far as we know, this is the first evidence of sexual dimorphism in heart regeneration. Sexual differences in the regeneration of other tissues has been observed in mammals but has not been documented for the heart (Harada et al., 2003; Blankenhorn et al., 2003; Deasy et al., 2007). In zebrafish, the pectoral fin is the only tissue that has been shown to demonstrate a sexually dimorphic regenerative capacity (Nachtrab et al., 2011). Like their hearts, males regenerate the pectoral fin more slowly and often incompletely. However, this phenomenon was not observed in other fins (Nachtrab et al., 2011), suggesting that this sexual dimorphism is related to the unique role of pectoral fins in reproduction (Kang et al., 2013; McMillan et al., 2013).

The observed female superiority in heart regeneration is likely due to the effect of estrogen: E2 accelerated heart regeneration in males (Fig 1), and tamoxifen retarded it in females (Fig 1). Our gene expression analyses suggested that female hearts demonstrated a stronger immune and inflammatory responses to cryoinjury. In particular, the expression of IFNg, as well as IFNg-inducible factors, in regenerating hearts was highly sexually dimorphic and E2 sensitive (Fig 2). As inflammation is essential for tissue regeneration (Eming et al., 2017), including cardiac repair (Frangogiannis, 2015), our observation is consistent with the higher heart regeneration rate in female zebrafish. Importantly, a comparison between fish species with different heart regeneration rates attributes the high heart regeneration capacity of zebrafish to this organism's enhanced inflammatory response to heart injury (Lai et al., 2017). These authors also discovered that treatment with poly I:C, a viral mimic known to stimulate IFNg-responsive genes (Farina, et al., 2010), can enable heart regeneration in medaka, a species that is normally unable to repair heart injuries. The immune-promoting effects of estrogen have been well documented in fish and mammals (Burgos-Aceves et al., 2016 Taneja, 2018). This study reveals, for the first time, that the sexual differences in immune functions can modulate tissue regeneration.

In this study, we have also discovered a previously unknown aspect of tissue regeneration in zebrafish: the spontaneous endocrine disruption after cardiac damage towards feminisation. Our data reveal that cardiac damage triggers the secretion of E2 (Fig 3F, G) and expression of estrogen receptors, notably *esr2a* (Fig 3A, B), in both sexes. Interestingly, a genetic variant of *esr2* has been identified as a risk factor of myocardial infraction (Domingues-Montanari et al., 2008). In addition, ERb, a mammalian homologue of *esr2a*, has recently been found to locate in mitochondria and play bioenergetic roles (Liao et al., 2015). We observed a ~9-fold increase in *esr2a* expression in female hearts at 7 dpc. Such dramatic increase in ER expression, compounded by the ~5-fold increase of plasma E2 level, will likely sensitise estrogen-responsive genes and amplify the estrogen-dependent inflammatory response of the female heart towards injury. We postulate that this can further enhance the female prominence in heart regeneration, and firmly establish the sexual dimorphism of this process (see Fig 6 for a model that summarises the data presented in this study).

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The stimulation of estrogen level and receptor by heart injury also operates in male fish, hitherto to a lesser extent. Interestingly, the level of serum estrogen among acute myocardial infarction and unstable angina patients were found significantly higher than in the normal group and intensive care patients (Aksut et al., 1986), suggesting that cardiac lesions, but not other traumas, can lead to an increase in estrogen level in humans. The clinical significance of this observation is undermined by the lack of understanding of the underlying mechanism. We now show that this phenomenon is evolutionarily conserved.

Sham operation can precondition zebrafish for heart regeneration (de Preux Charles et al., 2016a, b). Lai et al (2017) postulated that the preconditioning was mediated by the upregulation of immune-related genes in the heart of sham-operated zebrafish, but could not identify the cause of this change in gene expression. Here, we discovered that sham operation could induce plasma E2 level and the expression of ER in zebrafish hearts, suggesting that an estrogen-dependent inflammatory mechanism might be the cause of preconditioning. It is not known how cardiac injury and sham operation induces E2 level in zebrafish. It is not caused by hypoxia, a physiological consequence of heart damage, as hyperoxic treatment did not prevent E2 increase (Fig 3K). Moreover, environmental hypoxia is known to decrease, rather than increase, estrogen levels in zebrafish (Shang et al., 2006). Zebrafish offers an excellent animal model for delineating the molecular mechanism of this process, as biochemical pathways for sex hormone synthesis have been studied in detail in this organism (Segner, 2009).

It is interesting to examine how the systemic increase of estrogen as a result of heart injury may impact the functions of other organs during heart regeneration. One of the consequences of the endocrine disruption is the detection of vitellogenin in males, an occurrence generally regarded as the hallmark of endocrine disruption by environmental agents (García-Reyero et al., 2004; Scott & Robinson, 2008). The accumulation of vitellogenin in regenerating hearts, but not in tissues (Fig 4), suggests a functional role in cardiac regeneration, rather than as a collateral consequence of estrogen secretion. The canonical function of vitellogenin is to transport lipids, calcium and phosphate to the developing oocytes (Arukwe & Goksøyr, 2003, Hara et al., 2016). We hypothesise that vitellogenin might play similar roles in the regenerating heart. Lipids are instrumental to cardiac remodelling in reptiles (Riquelme et al., 2011), and our previous micro-CT imaging of cardiac-damaged male zebrafish has shown the accumulation of lipids around the regenerating heart (Babaei et al., 2016). Future investigations will involve the characterisation of the vitellogenin cargoes isolated from the plasma and heart of fish after cardiac damage.

Acknowledgements

SHC and YWL conceived and coordinated the study; SSX, SF and FB designed, performed and analyzed the experiments; FJX, KFW, and LS analyzed the echocardiographic data; TML and RR conducted the proteomic analyses; LNZ and XW do GSEA analysis; SSX and YWL wrote the paper; All authors reviewed the results and approved the final version of the manuscript. This work was supported by a general research fund grant [Project No. CityU 160213] from the Research Grants Council (RGC) of the Hong Kong Special Administrative Region, China, to SHC, and a Strategic Research Grant [Project No. CityU 7004661] from City University of Hong Kong to YWL. We thank the University Research Facility in Life Sciences of Hong Kong Polytechnic University for providing Vevo 2100 for this research. We thank all members of the SHC and YWL labs for fruitful discussions.

Competing Interests

The authors declare that they have no conflicts of interest with the contents of this article.

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Figures

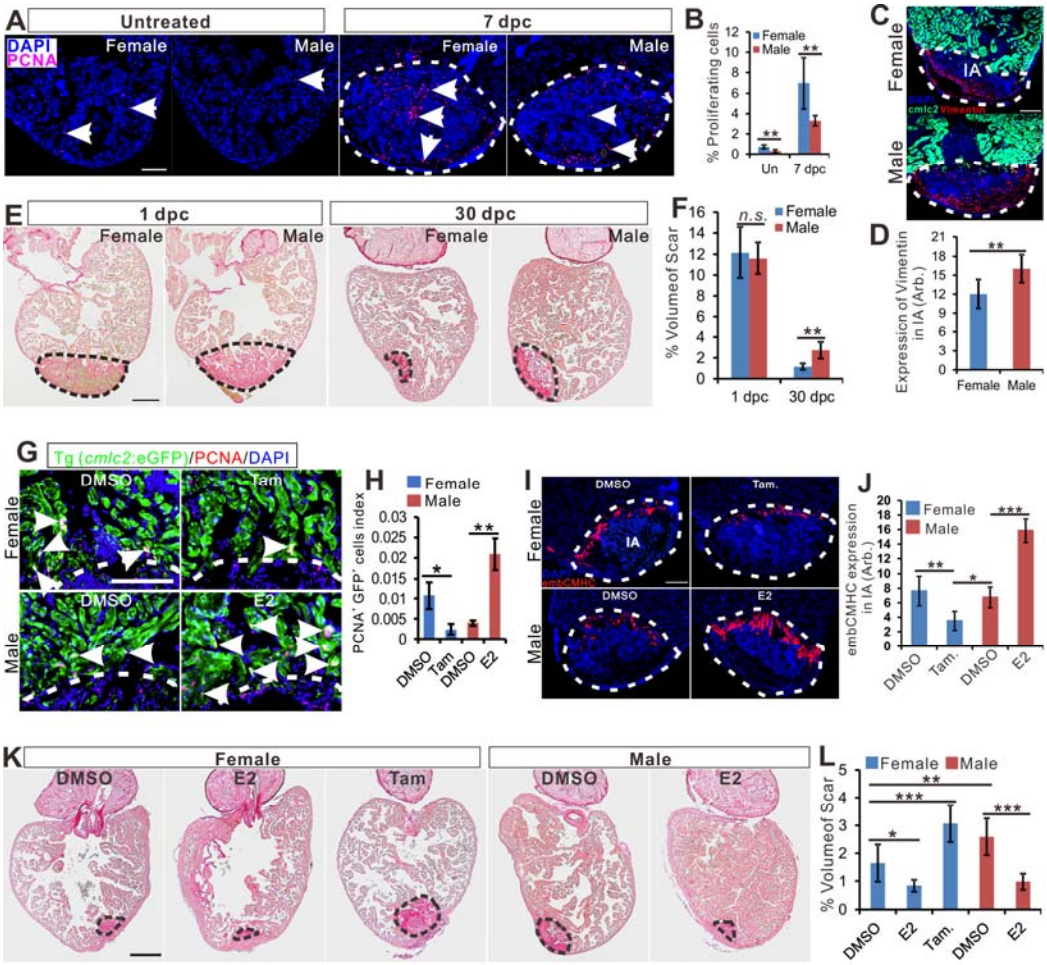

Figure 1. Zebrafish heart regeneration is sexually dimorphic. A. PCNA immunofluorescence (red) in the heart of female and male zebrafish, uninjured (Un) and at 7 dpc. **B**. Quantification of percentage of PCNA positive cells (mean±SD, n=7) in panel A. **C**. Vimentin immunofluorescence (red) in female and male Tg (cmlc2: eGFP, green) zebrafish hearts at 7 dpc. **D**. Quantitation of vimentin staining in the injured area of female and male fish (marked by dashed lines, mean±SD, n=8). **E**. Picrosirus red staining of female and male hearts at 1 dpc and 30 dpc. **F**. Quantitation of scar volume (marked by dashed lines, mean±SD, n=9~12) between females and male fish. Scale bars: 200μm. Two-tail t-test, **p<0.01. Un: untreated, Dpc: days post-cryoinjury, CI: Cryoinjury. **G**. PCNA immunofluorescence (red) in the heart of female and male Tg (cmlc2: eGFP) zebrafish exposed to 1μM tamoxifen (Tam) and 1nM E2 respectively at 7 dpc. Arrows highlight the PCNA staining among differentiated cardiomyocytes. **H**. Quantification of PCNA-positive cells in panel A (mean±SD, n=5) in injured areas (marked by dash lines). **I**. embCMHC immunofluorescence (red) in the heart of untreated female, female treated with 1μM tamoxifen (Tam), untreated male, and male treated with 1nM E2 at 7 dpc. **J**. Quantification of embCMHC staining in panel C (mean±SD, n=4~5) in injured areas. **K**. Picrosirus red staining of the heart from female zebrafish treated with DMSO, E2 (1 nM) and Tamoxifen (1 μM); and male zebrafish treated with DMSO and E2 (1 nM) at 30 dpc. Scale bar: 200μm. **L**. Quantitation of scar volume of the samples in panel A (marked by dash lines, mean±SD, n=5~6). One-way ANOVA test, *p<0.05, **p<0.01, and ***p<0.001

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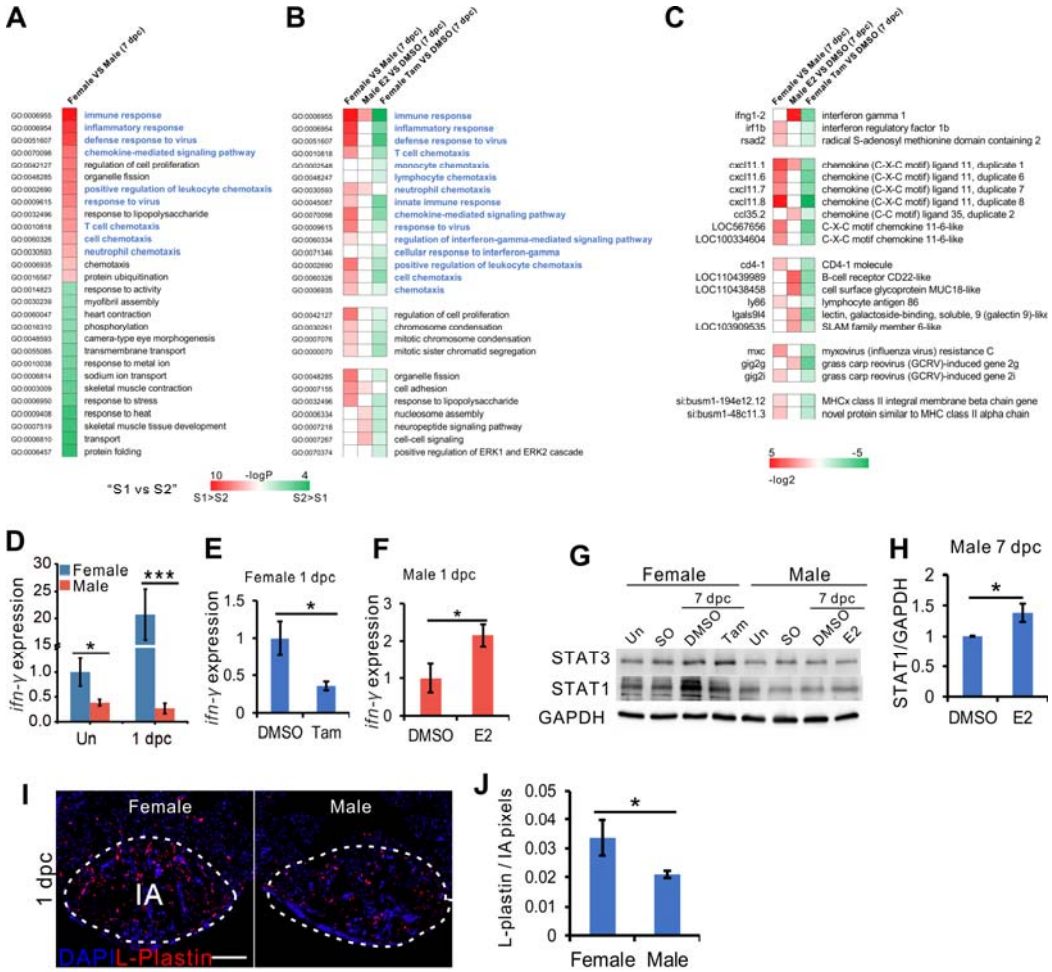

Figure 2. Estrogen induces inflammation in the injured zebrafish heart. **A**. Gene Ontogeny (GO) terms significantly enriched in genes differentially expressed in female vs male hearts at 7 dpc. **B**. Comparison of gene expression patterns in the hearts of female vs male, E2 (1 nM) -treated male vs untreated male, and Tamoxifen (1 μM) -treated female vs untreated female, all at 7 dpc. **C**. Expression of immune- and inflammation-related genes in these datasets. **D-F**, Expression of ifn-γ in female and male heart of zebrafish at uninjured fish and 1dpc (D), tamoxifen decreased (E) and E2 increased (F) the expression of ifn-γ in female and male at 1 dpc. N=3, two tail t test, *p<0.05, **p<0.01, ***p<0.001. **G-H**, Western blot of STAT1 and SATA3 in female and male heart at 7dpc after different treatment. H, bar chart to show the quantification of the expression of STAT1 in male fish in panel G, n=3, two tail t test, *p<0.05. I-J. L-plastin labelled leukocytes in the injured area at 1 dpc in female and male fish. N=5, two tail t test, *p<0.05.

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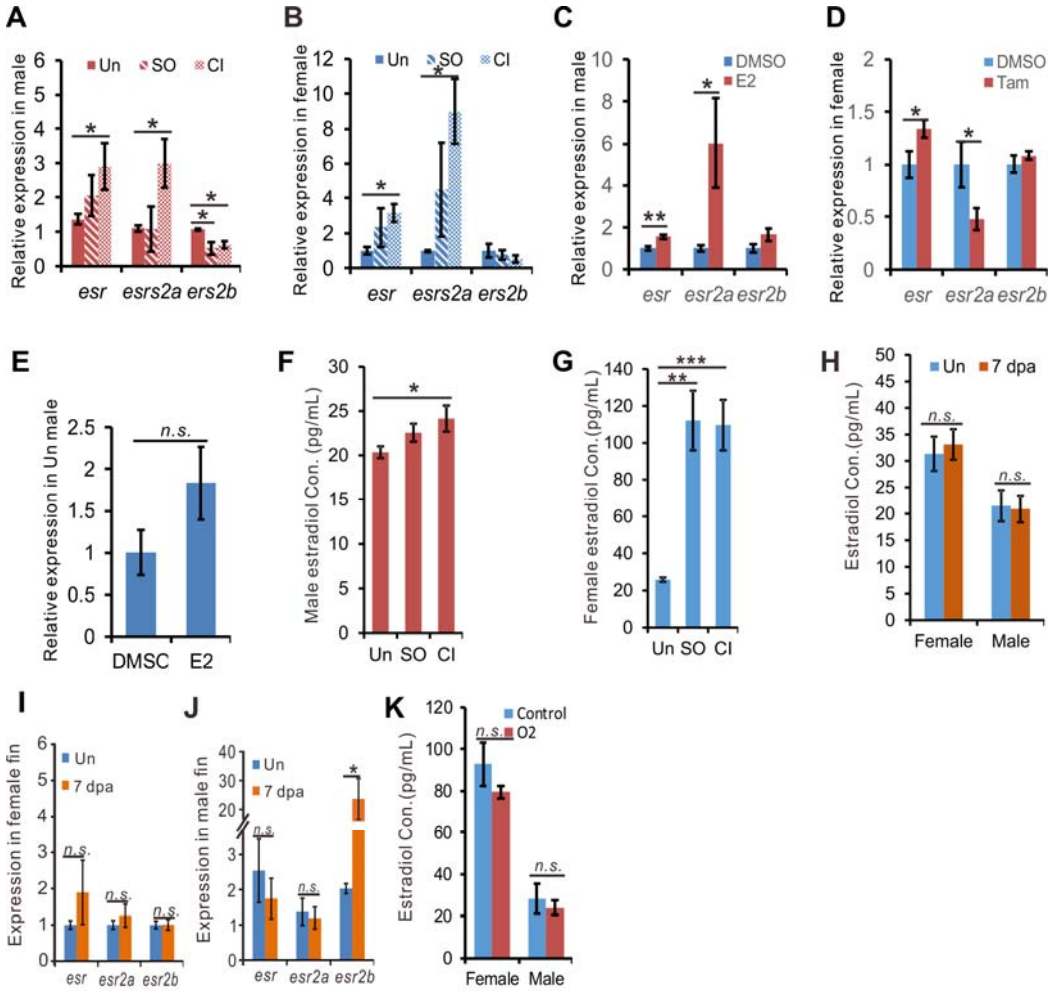

Figure 3. Cardiac damage induces feminisation of zebrafish. A-E, q-PCR shows the expression of estrogen receptor genes in zebrafish 7 days after surgery. N=3, two-tail t test, *p<0.05, **p<0.01. **F-H.** Plasma E2 concentration in zebrafish with heart injury (F, G) or fin amputation (H) 7 days after surgery. Un: uninjured control, SO: sham-operated, CI: cryoinjured. **I, J,** q-PCR shows the expression of estrogen receptor genes in zebrafish fin at 7 days after amputation. N=3, two-tail t test, *p<0.05. **K**. Plasma E2 concentration in normoxia zebrafish (control) of hyperoxia (O2) at 7 days after cryoinjury. N=3, two-tail t test, n.s. nonsignificant different.

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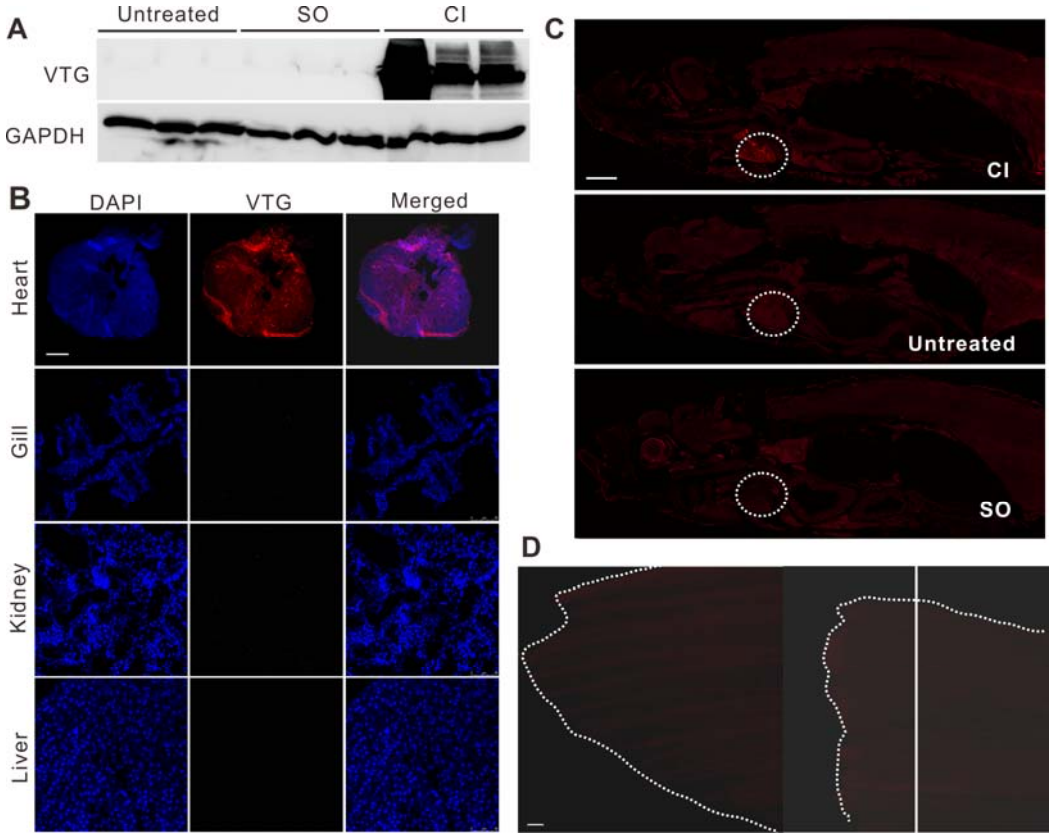

Figure 4. Vitellogenin accumulates in the male zebrafish heart after cardiac damage. A. Detection of vitellogenin by Western blotting in plasma collected from untreated male zebrafish and fish one day after sham-operation (SO) and cryoinjury (CI). **B**. Vitellogenin (VTG) immunofluorescence (green) in male zebrafish heart, gill, kidney and liver on day 5 after VA. Scale bars: 200um. **C**. Detection of VTG (red) in an untreated male zebrafish and in male zebrafish on day 7 after heart cryoinjury (CI) and sham operation (SO). Scale bars: 1mm. **D**. Detection of VTG in uninjured caudal fin and on 7 days after fin amputation. The dashed white line showed the shape of fin, and the white line showed the amputation site. Bars: 1mm.

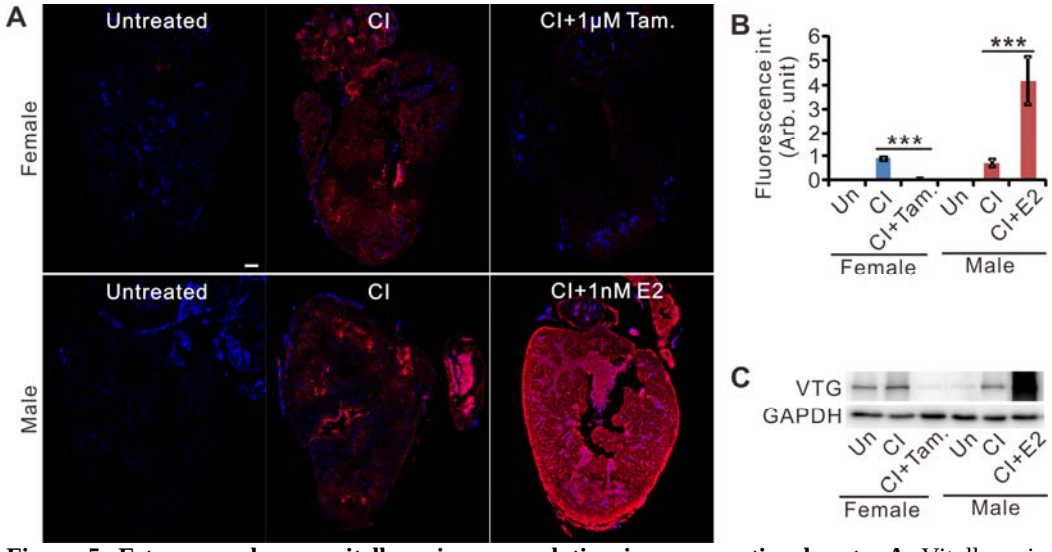

Figure 5. Estrogen enhances vitellogenin accumulation in regenerating hearts. A. Vitellogenin (VTG) immunofluorescence (red) in the heart of untreated female, 7 dpc female, 7 dpc female treated with 1μM Tamoxifen, untreated male, 7 dpc male, and 7 dpc male treated with 1nM E2. B.

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Quantification of VTG staining (mean±SD, n=4~5) in panel A. Scale bar: 200μm. One-way ANOVA test, ***p<0.001. **C**. Expression of VTG in the samples shown in panel A, as detected by Western blotting. Dpc: days post-cryoinjury.

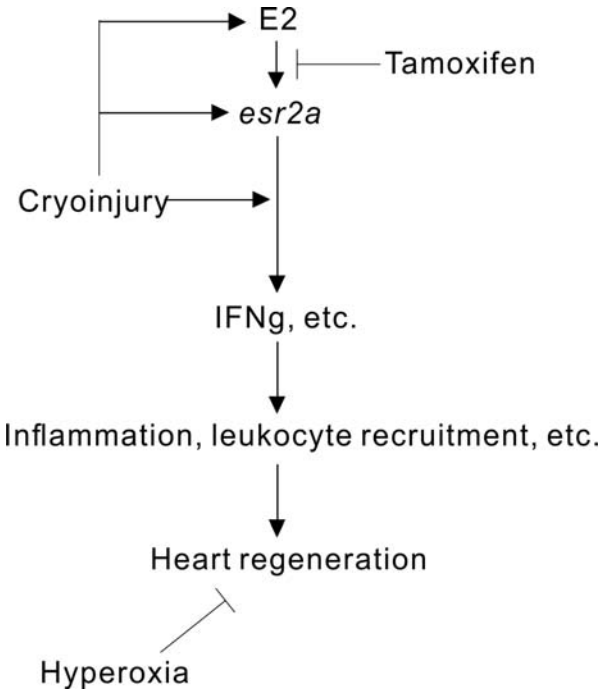

Figure 6. Proposed model on the roles of estrogen in zebrafish heart regeneration

Methods

Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
mouse anti-GAPDH (1:10000 for WB)	Proteintech Group, Inc.	60004-1
mouse anti-zebrafish vitellogenin, JE-2A6 (1:2000 for WB)	Bio-sense laboratories	V01408102-100
mouse anti-embCMHC (1:50 for IF, 1:500 for WB)	DSHB	N2.261
rabbit anti-SATA1(1:2000 for WB)	Huabio Inc.	R1408-2
rabbit anti-STAT3(1:2000 for WB)	Huabio Inc.	ET1607-38
HRP-conjugated rabbit anti-mouse IgG (1:5000 for WB)	Millipore	AP160P
HRP-conjugated goat anti-rabbit IgG (1:5000 for WB)	Millipore	AP307P
mouse anti-vimentin (1:200 for IF)	Abcam	ab8978
mouse anti-PCNA (1:200 for IF)	Santa Cruz	sc-56
rabbit polyclonal anti-GFP (1:200 for IF)	Abcam	ab13970
rabbit polyclonal anti-GFP (1:200 for IF)	Abcam	ab13970
mouse anti-L-plastin (1:100 for IF)	Santa Cruz	sc-133218
Cy3-conjugated goat anti-mouse (1:500 for IF)	Invitrogen	A10521

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Alexa Fluor 488-conjugated goat anti-rabbit (1:500 for IF)	Invitrogen	A11034
Chemicals and reagents		
MS-222 (ethyl-3-aminobenzoate methanesulfonate salt)	Sigma-Aldrich	E10521
17β-estradiol (E2)	Sigma-Aldrich	E1132
tamoxifen	Sigma-Aldrich	T9262
Dimethylsulphoxide (DMSO)	Sigma-Aldrich	D5879
RIPA Lysis and Extraction Buffer	Thermo scientific	89901
BCA Protein Assay kit	Thermo scientific	23225
EMD Luminata Western HRP chemiluminescence substrate	Millipore	WBLUF0500
dithiothreitol	Thermo Scientific	R0861
iodoacetamide	Thermo Scientific	90034
LysC	Roche	11058533103
Trypsin	Roche	06369880103
ammonium bicarbonate	Sigma-Aldrich	09830-500G
trifluoroacetic acid	Sigma-Aldrich	302031
ZipTip	Millipore	MA01821
acetonitrile	Sigma-Aldrich	34998
isopropanol	Sigma-Aldrich	650447
paraformaldehyde	Sigma-Aldrich	30525-89-4
picrosirus red	Abcam	ab150681
HistoChoice	Amresco Inc.	H120
Estradiol ELISA Kit	Cayman	582251
NucleoZOL reagent	MACHEREY-NAGEL GmbH & Co. KG	740404
RQ1 RNA-free DNase	Promega Co.	M6101
PrimeScript reverse transcription (RT) reagent kit	Takara Bio. Inc.	6210B
SYBR Premix Ex Taq	Takara Bio. Inc.	RR402A
Experimental Models: zebrafish		
Danio Renio (strain AB)	ZRIC	N/A
Tg(cmlc2: eGFP)	ZRIC	N/A
Oligonucleotides		
esr F: CAGGACCAGCCCGATTCC	Chandrasekar et al., 2010	N/A
esr R: TTAGGGTACATGGGTGAGAGTTTG	Chandrasekar et al., 2010	N/A
esr2a F: CTCACAGCACGGACCCTAAAC	Chandrasekar et al., 2010	N/A
esr2a R: GGTTGTCCATCCTCCCGAAAC	Chandrasekar et al., 2010	N/A

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esr2b F: CGCTCGGCATGGACAAC	Chandrasekar et al., 2010	N/A
esr2b R: CCCATGCGGTGGAGAGTAAT	Chandrasekar et al., 2010	N/A
ifn-γ F: CTATGGGCGATCAAGGAAAA	Hou et al., 2016	N/A
ifn-γ R: CTTTAGCCTGCCGTCTCTTG	Hou et al., 2016	N/A
β-actin-F: GCTGACAGGATGCAGAAGGA	Xu et al., 2018	N/A
β-actin-R: TAGAAGCATTTGCGGTGGAC	Xu et al., 2018	N/A
Software		
ImageJ	NIH	https://imagej.nih.gov/ij/download.html

Experimental Model

Zebrafish used for heart cryoinjury, ventricle amputation, fin amputation, immunofluorescence, western blot, echocardiography, Mass spectrometry, Quantitative PCR (q-PCR) was acquired from the Zebrafish International Resource Center (ZIRC; University of Oregon, Eugene, OR, USA). Fish were maintained in a recirculating system at 28°C with a photoperiod of 14 h of light/10 h of darkness, and fed with dry meal (TetraMin) three times a day, supplemented with live brine shrimp once a day (Westerfield, 2000). All the animal procedures used in this study were approved by the Department of Health, Hong Kong, SAR, China (refs (17-18) in DH/HA&P/8/2/5 Pt.1), and the experiments were conducted in accordance with the relevant guidelines and regulations in Hong Kong, SAR, China.

Method Details

Animal surgery

Adult zebrafish (12 to 18-month-old, the weight is 0.35-0.37g, with cardiac weight accounting for 0.5% of body weight) were anesthetised by immersion in system water containing 0.04% MS-222 (ethyl-3-aminobenzoate methanesulfonate salt; E10521; Sigma-Aldrich) for 3−5 min and then immobilized on a Petri dish. After the removal of the ventral scales by forceps, a small incision was made through the body wall and the pericardium by using forceps and micro-dissection scissors, tearing the tissue rather than making a clean cut in order to facilitate healing. Once the pericardial sac was opened, the heart ventricle was exposed by gently squeezing the abdomen. For sham operation, the heart was gently pushed back into the body without any further treatment. For ventricular amputation, a portion of the ventricle was excised by using surgical fine scissors (Poss et al., 2002). For cryoinjury, the ventricle was touched for 10-12 seconds by metal probe pre-chilled in liquid nitrogen (Chablais et al., 2011). For fin amputation, the caudal fin of zebrafish was cut using a blade after anesthetization (Nachtrab et al 2011). After the operation, fish were placed in a tank of fresh water, and reanimation was enhanced by pipetting water onto the gills for a couple of minutes. At time points indicated in the Results, the zebrafish were sacrificed by immersion in overdose concentration of MS-222. Hearts were dissected from the fish and their plasma were collected as previously described (Babaei et al., 2013).

Chemical exposure

Adult male and female zebrafish, untreated or after surgery, were incubated in water containing 17β-estradiol (E2; E1132, Sigma-Aldrich) at various concentrations (0, 0.01, 1 and 100 nM), tamoxifen (T9262, Sigma-Aldrich) at various concentrations (0 nM, 10 nM, 100 nM, 1000 nM), or DMSO (D5879, Sigma-Aldrich), which was used to prepare the E2 and tamoxifen, at matching concentrations. The water was changed every day, and the fish were continuously exposed to the vehicle or drugs for durations indicated in Fig 1-5.

Hyperoxic treatment

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Zebrafish were exposed to hyperoxia by using the method described by Jopling et al (2012). Briefly, zebrafish were placed in an autoclave bag (EURTUBO) with 2L fish water. 100% oxygen was used to fill the bag. The bag was tightly sealed with a rubber band and placed in an incubator at 28°C. The average O2 concentration in the water was 20 mg/L. The control zebrafish was maintained in the bag with normoxic condition, and left bag open. The average O2 concentration in the water was 6.5 mg/L.

Western blotting

Proteins from zebrafish hearts were extracted by using RIPA Lysis and Extraction Buffer (89901, Thermo scientific) according to manufacturer's instruction. BCA Protein Assay (23225; Thermo Scientific) was used to determine the total protein concentration of zebrafish plasma and heart protein extract, according to the manufacturer's instruction. 20μg of protein sample per lane was separated in a 10% SDS polyacrylamide gel, transferred to polyvinylidene difluoride (PVDF) membrane (10600023; GE Healthcare life science) by using Mini-Trans Blot Electrophoretic Transfer Cell system (1703930; Bio-Rad Laboratories) according to the manufacturer's instructions. The blot was blocked in 5% no fat milk in PBST (0.05% Tween 20 in 1X PBS) for 1 hour at room temperature, following by incubation in primary antibodies diluted in PBST overnight at 4□. The following primary antibodies were used: mouse anti-GAPDH (60004-1; Proteintech Group, Inc.) at 1:10000, mouse anti-zebrafish vitellogenin, JE-2A6 at 1:2000, mouse anti-embCMHC at 1:500, rabbit anti-SATA1(R1408-2, Huabio Inc.) and rabbit anti-STAT3(ET1607-38, Huabio Inc) at 1:2000. For secondary antibodies, HRP-conjugated rabbit anti-mouse IgG (AP160P; Millipore) at 1:5000, and HRP-conjugated goat anti-rabbit IgG (AP307P; Millipore) at 1:5000 were used. The proteins were detected with the EMD Millipore Luminata Western HRP chemiluminescence substrate (WBLUF0500; Millipore) and the signals were visualized with the Western blotting system (C600; Azure Biosystems, Inc.). The band densities were quantified using Image J.

Mass spectrometry

Proteins from plasma collected from individual fish (typically about 20ug) were precipitated in 1 ml of acetone for 60 min at -20°C and pelleted at 1000g for 10 min at 4°C. The pellet was air-dried (30 min) and then dissolved in 8M urea in 10mM Tris-HCl (pH 8.0). After clarification (10000g, 10 min, RT), the supernatant was reduced by 10mM dithiothreitol (R0861, Thermo Scientific) in 50mM ammonium bicarbonate (30 min, RT), and alkylated in 50mM iodoacetamide (90034, Thermo Scientific) in 50mM ammonium bicarbonate (20 min, RT). LysC (Roche) was then added to the protein mixtures and incubated for 120 min at RT. The samples were then diluted with ammonium bicarbonate (Sigma-Aldrich; 50 mM, pH 8.0) so that the final concentration of urea was 1 M, followed by the addition of 1 ug trypsin (Roche). The mixtures were incubated overnight at 37°C and dried in a vacuum centrifuge. Peptides were dissolved in a small amount of 0.1% trifluoroacetic acid (Sigma-Aldrich; TFA), followed by purification with the use of ZipTip (Millipore), following manufacturer's instruction.

Mass spectrometry was performed on the Thermo Scientific Q-Exactive Plus Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled to a Thermo Ultimate 3000 RSLCnano HPLC system. About the 0.5 ug of the peptide mixture was loaded onto a 250nl OPTI-PAK trap (Optimize Technologies, Oregon City, OR) custom packed with Michrom Magic C18 5um solid phase (Michrom Bioresources, Auburn, CA). Chromatography was performed a Thermo EasySpray 25cm × 75um C18 2um column, using 0.2 % formic acid in both Solution A (98%water/2% acetonitrile) and Solution B (80% acetonitrile/10% isopropanol/10% water), in a gradient from 2%B to 35%B over 140 min at a flowrate of 325 nl/min. The Q-Exactive Plus mass spectrometer was set up with a FT survey scan from 340-1500 m/z at resolution 70,000 (at 200m/z), followed by HCD MS/MS scans on the top 15 ions at resolution 17,500. The MS1 AGC target was set to 1e6 and the MS2 target was set to 2e5 with max ion inject times of 50ms and 75ms respectively. Dynamic exclusion placed selected ions on an exclusion list for 30 seconds. Charge exclusion was used to perform MS/MS only on +2, +3, and +4 ions.

MS/MS peak lists were exported as an .mgf file and proteins were identified via automated Mascot database searching (Matrix Science 2.2.04) of all tandem mass spectra against of the Danio rerio Protein Index protein sequence database that contained all zebrafish protein entries (143,725 sequences) from NCBI RefSeq (version 51). The instrument setting for the Mascot search was specified as "ESI-Trap." Parameters used for the database search were as follows: a maximum of two missed cleavages; carbamidomethylation of cysteine as a fixed modification and oxidation of methionine, acetylation of protein N-term and Gln→pyro-Glu conversion as variable modifications; trypsin as the enzyme; a

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peptide mass tolerance of 10 ppm; a fragment mass tolerance of 0.6 Da; and an ion score of 35 as the cut-off, using a significance threshold of $p < 0.05$. After peptide identification, any peptides that had conflicting assignments were resolved, either one of identical proteins or by assignment to proteins with the largest number of peptides already present (by following Occam's Razor principle).

The acquired MS data (in Thermo ".raw" format) from 3 biological replicates in the SO and Untreated groups (i.e., three separate plasma samples each collected from one individual fish) and 4 biological replicates in the VA group were quantitated by using Progenesis LC-MS (version 2.5, Nonlinear) as previously described (Babaei et al., 2013).

Histology

Organs dissected from zebrafish were fixed with 4% paraformaldehyde at 4°C overnight, dehydrated and embedded in paraffin as previously described (Chablais et al., 2011). To measure scar size, the entire heart was serially sectioned at the thickness of 5μm. The sections were deparaffined, rehydrated and stained with picrosirus red (ab150681, Abcam). On each section, the labeled area and the area of the whole ventricle were measured by using ImageJ (National Institutes of Health, Bethesda, MA, USA), and the percentage of the scar volume to the entire ventricle was calculated by the summation of the data from all the sections (Xu et al., 2018). For the whole mount paraffin sections, the fish were fixed with 2% PFA and 0.05% Glutaraldehyde in 80% HistoChoice (H120, Amresco Inc.) with 1% sucrose and 1% $CaCl_2$ at 4□ for 24h, and the paraffin sections were prepared according to Kong et al. (Kong et al., 2008).

For immunohistochemistry, antigen retrieval was performed on dewaxed sections in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) at 95°C for 15 min. Primary antibodies used were: mouse anti-vimentin (ab8978; Abcam) at 1:200; mouse anti-PCNA (sc-56; Santa Cruz) at 1:200; mouse anti-zebrafish vitellogenin, JE-2A6 (V01408102; Biosense) at 1:200, mouse anti-embCMHC (N2.261;Developmental Studies Hybridoma Bank) at 1:50, rabbit polyclonal anti-GFP (ab13970; Abcam) at 1:200, mouse anti-L-plastin (sc-133218; Santa Cruz Biotechnology Inc.) at a dilution of 1:100. The secondary antibodies used were: Cy3-conjugated goat anti-mouse (A10521; Invitrogen) or Alexa Fluor 488-conjugated goat anti-rabbit (A11034; Invitrogen) antibodies at 1:500. The sections were amounted with cover slide in 50% Glycerol in PBS and images were acquired using Olympus BX61 microscope.

Plasma E2 concentrations measurement

Plasma from three individual females or males were pooled, and three biological replicates were performed. The concentration of E2 and testosterone in plasma were measured using Estradiol ELISA Kit (582251, Cayman).

Quantitative PCR (q-PCR) and RNA sequencing

Total RNA was extracted from zebrafish heart, fin or liver using NucleoZOL reagent (740404; MACHEREY-NAGEL GmbH & Co. KG). Three individuals were pooled together as one biological replicate, and three biological replicates were performed. 1μg total RNA was decontaminated using RQ1 RNA-free DNase (M6101; Promega Co.) and then cDNA was synthesized using the PrimeScript reverse transcription (RT) reagent kit (6210B; Takara Bio. Inc.) according to the manufacturer's instructions. The expression of each gene was determined by q-PCR using the SYBR Premix Ex Taq (RR402A; Takara Bio. Inc.), and *β-actin* was used as the reference gene. q-PCR was performed in triplicate for each gene, and the results were analyzed using the $\Delta\Delta^{CT}$ method. All levels of expression of genes were normalized to *β-actin* and fold change was calculated by setting the normal female to 1.

For RNA sequencing, total RNA was extracted and decontaminated, the sequencing was performed using BGISEQ-500 platform, averagely generating about 21.83 M reads per sample. The reference genome can be accessed at: http://www.ncbi.nlm.nih.gov/genome/50?genome_assembly_id=210873. The average mapping ratio with reference genome is 90.81%, the average mapping ratio with gene is 71.22%; A total of 25,900 genes were detected. The sequencing and the primary analysis were performed by BGI (Shenzhen, China), three bio-replicates of each samples were performed. We did comparative GSEA (Gene set enrichment analysis) and drew the union enrichment maps using an R package, HTSanalyzeR2 (https://github.com/CityUHK-CompBio/HTSanalyzeR2). Here, we focus on

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Biological Process Gene Ontology. By permutating 1000 times, we get the statistical significance and choose adjusted P value < 0.05 as significant results.

Echocardiography

Zebrafish was anaesthetized with 0.02% MS-222 and fixed in a sponge upside-down. Echocardiographic studies were performed using Vevo LAZR Multi-modality Imaging Platform (FUJIFILM VisualSonics) under B-mode (50 MHz, 77 fps) and PW Doppler mode (40 MHz, 25 kHz PRF) at 20□ as previously described (González-Rosa et al., 2014; Hein et al., 2015; Wang et al., 2017). Ejection Fraction (EF) and Fractional Shortening (FS) were acquired using a plug-in of Vevo LAB (Vevo Strain) under B-mode images. The window was set to be 300 frames long, starting with diastalsis. PW Doppler images collected were analyzed using Vevo LAB (FUJIFILM VisualSonics) for E/A ratio calculation. 5 respective sets of E/A values were calculated for each sample.

Quantification and Statistical Analysis

Three different images were taken for each heart. The percentages of proliferating cells in ventricle or injured area were the ratio of PCNA positive cells/DAPI. For untreated fish, all nuclei in the whole ventricle were counted; for cryoinjured fish, only the nuclei in the injured area (marked by white dash lines) in Fig 1, Fig S1, and Fig S2 were counted. The area of vimentin, embCMHC, and L-plastin expressions were quantified in the injured area using Image J and the percentage of the expression with respect to the area of the injured area was calculated (de Preux Charles et al., 2016a). For quantification of proliferating cardiomyocyte, the $PNCA^{+}/GPF^{+}$/ double positive cardiomyocyte within 200 μm of the vicinity of the injured area were quantified, and normalized with injured area in Fig 1G, H. The data were expressed as the mean±S.D.(standard deviation). Statistical analysis was performed using Student's two-tailed *t*-test and one way-ANOVA.

Supplementary fiels

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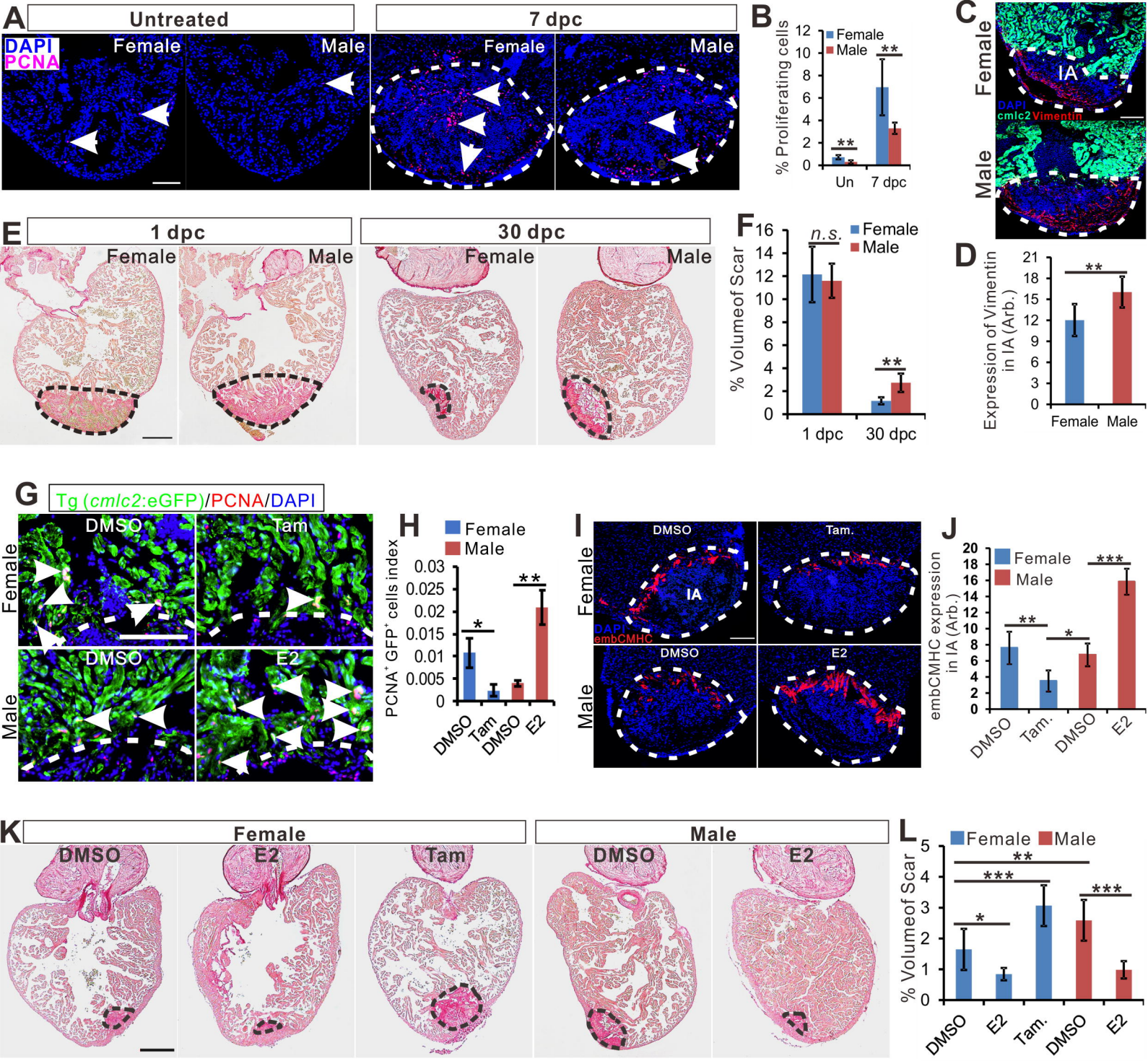
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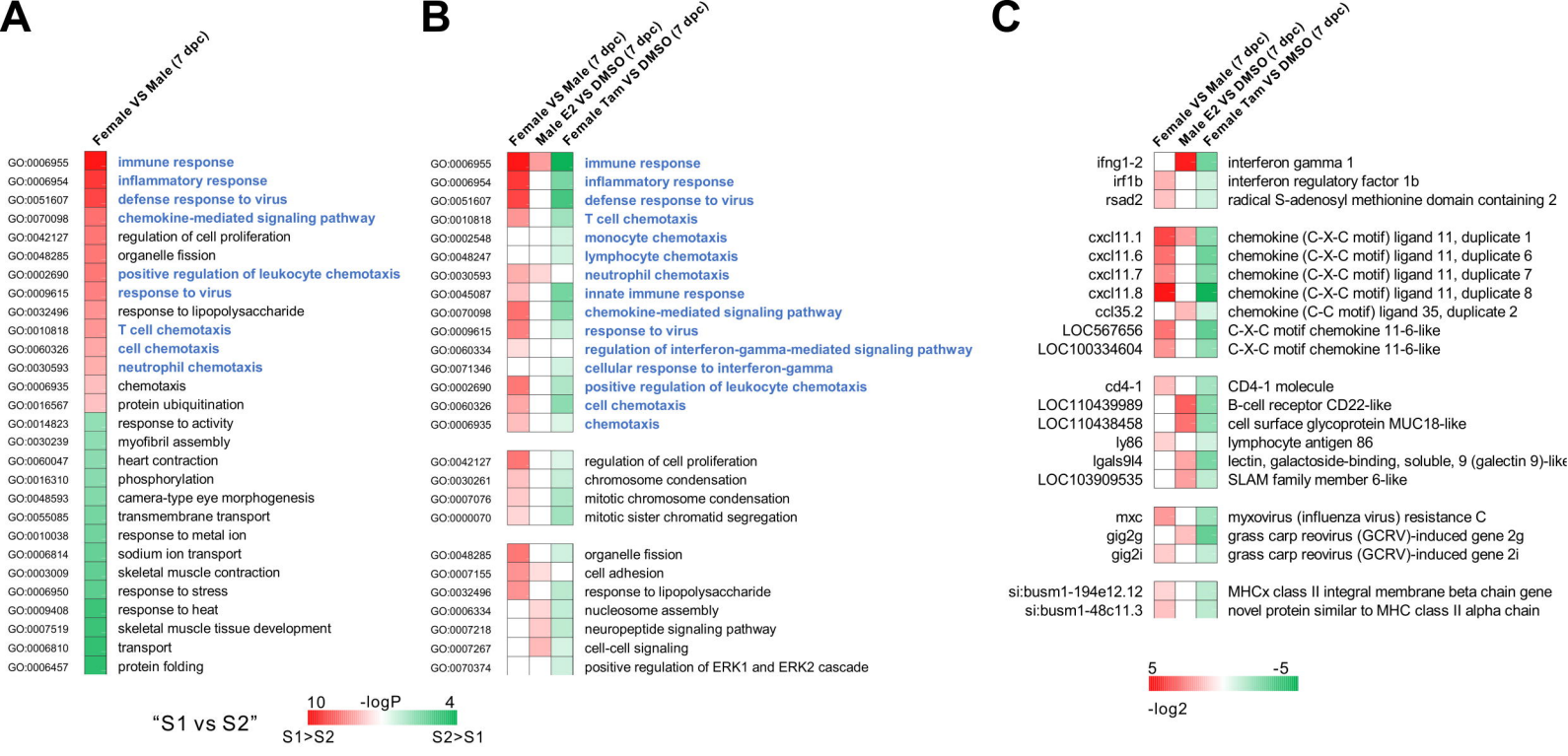
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GO:0006954 inflammatory response
GO:0051607 defense response to virus
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GO:0048285 organelle fission
GO:0002690 positive regulation of leukocyte chemotaxis
GO:0009615 response to virus
GO:0032496 response to lipopolysaccharide
GO:0010818 T cell chemotaxis
GO:0060326 cell chemotaxis
GO:0030593 neutrophil chemotaxis
GO:0006935 chemotaxis
GO:0016567 protein ubiquitination
GO:0014823 response to activity
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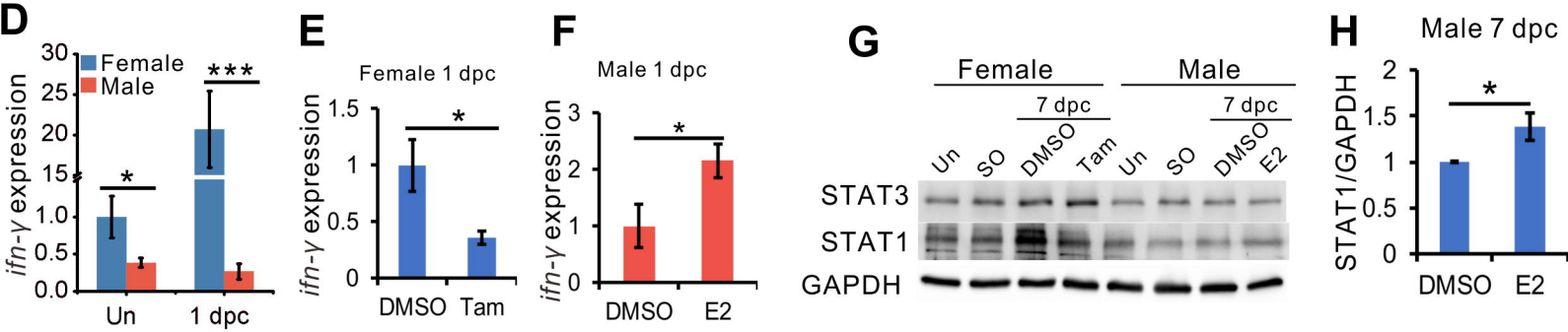
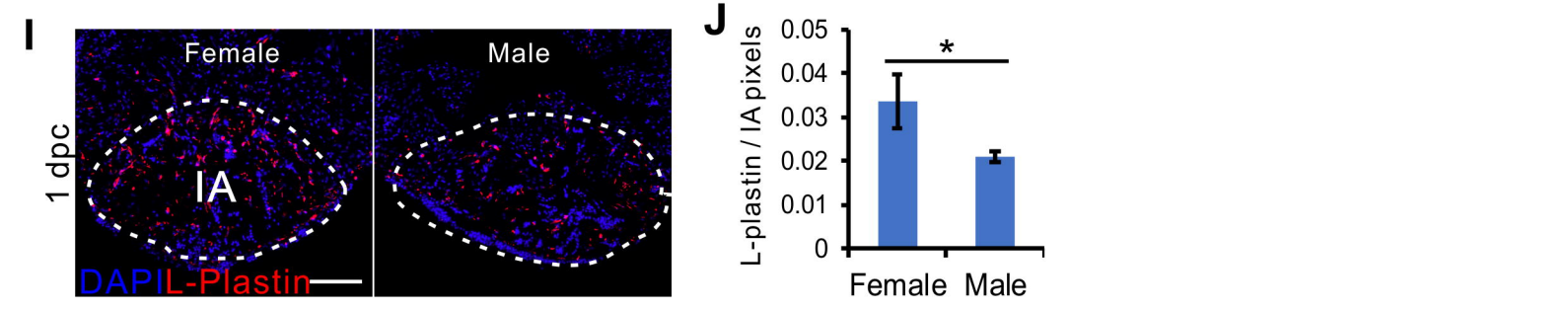
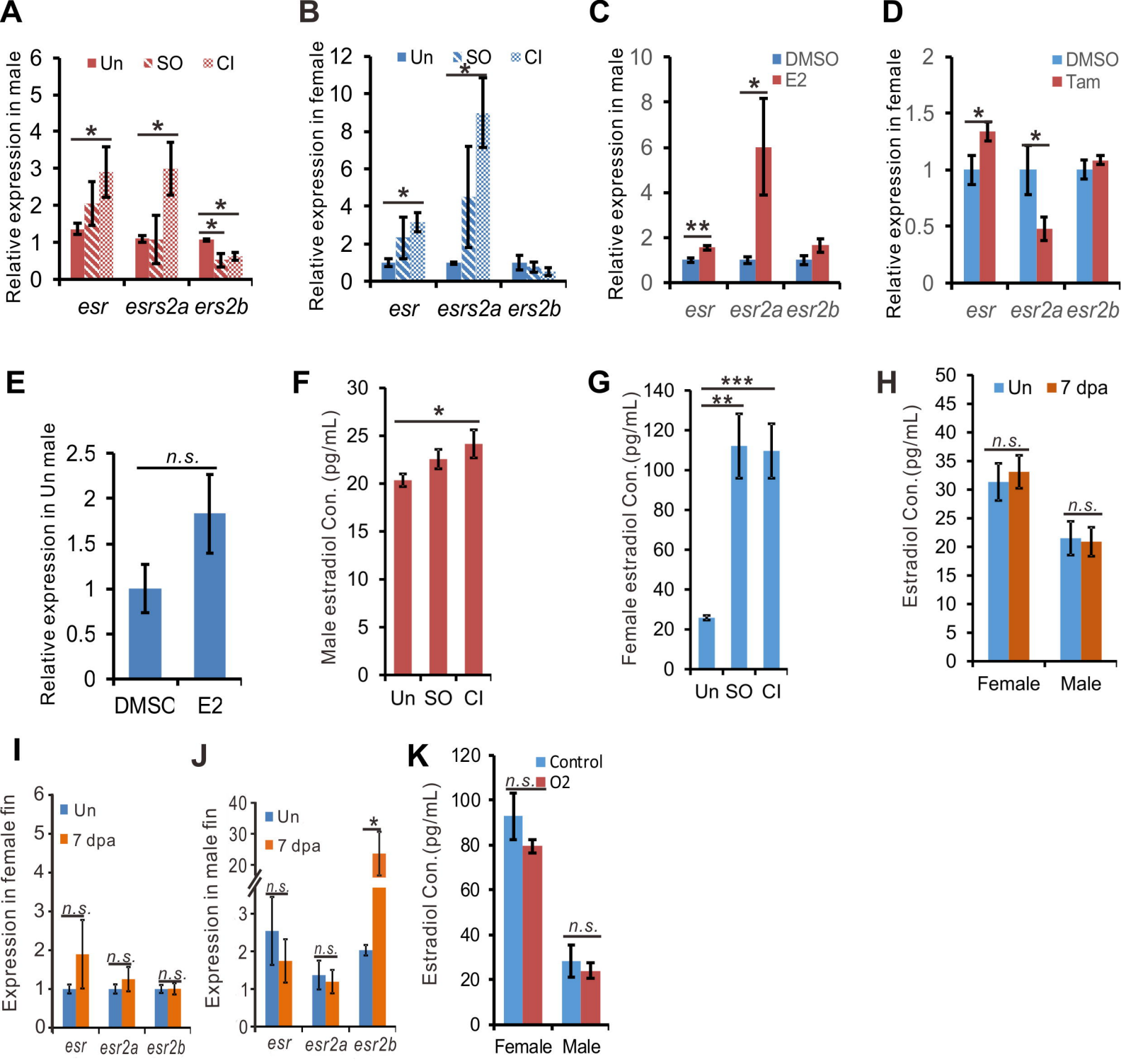
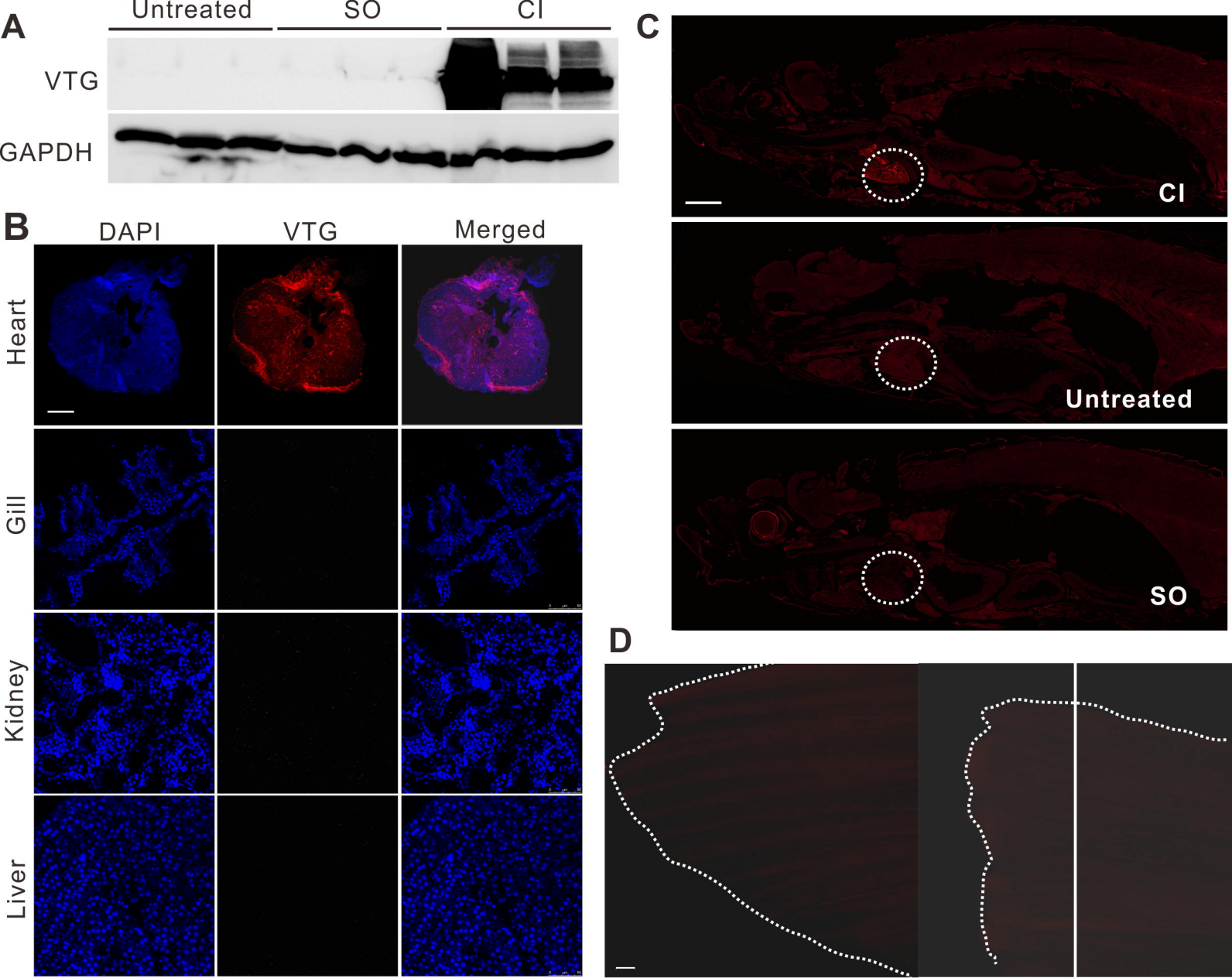
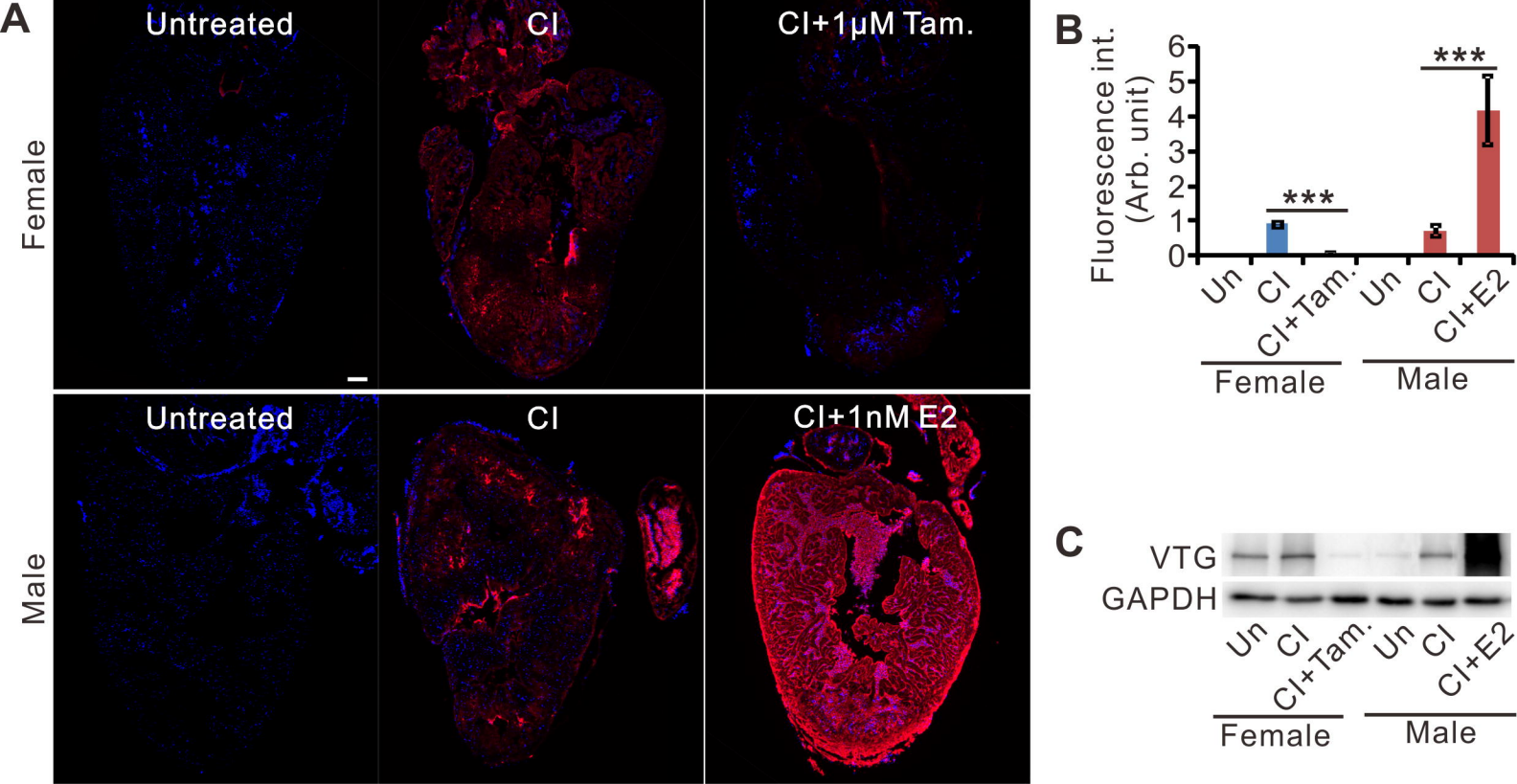
GO:0048593 camera-type eye morphogenesis
GO:0055085 transmembrane transport
GO:0010038 response to metal ion
GO:0006814 sodium ion transport
GO:0003009 skeletal muscle contraction
GO:0006950 response to stress
GO:0009408 response to heat
GO:0007519 skeletal muscle tissue development
GO:0006810 transport
GO:0006457 protein folding
"S1 vs S2"
10 -logP 4
S1>S2 S2>S1
B
Female VS Male (7 dpc)
Male E2 VS DMSO (7 dpc)
Female Tam VS DMSO (7 dpc)
GO:0006955 immune response
GO:0006954 inflammatory response
GO:0051607 defense response to virus
GO:0010818 T cell chemotaxis
GO:0002548 monocyte chemotaxis
GO:0048247 lymphocyte chemotaxis
GO:0030593 neutrophil chemotaxis
GO:0045087 innate immune response
GO:0070098 chemokine-mediated signaling pathway
GO:0009615 response to virus
GO:0060334 regulation of interferon-gamma-mediated signaling pathway
GO:0071346 cellular response to interferon-gamma
GO:0002690 positive regulation of leukocyte chemotaxis
GO:0060326 cell chemotaxis
GO:0006935 chemotaxis
GO:0042127 regulation of cell proliferation
GO:0030261 chromosome condensation
GO:0007076 mitotic chromosome condensation
GO:0000070 mitotic sister chromatid segregation
GO:0048285 organelle fission
GO:0007155 cell adhesion
GO:0032496 response to lipopolysaccharide
GO:0006334 nucleosome assembly
GO:0007218 neuropeptide signaling pathway
GO:0007267 cell-cell signaling
GO:0070374 positive regulation of ERK1 and ERK2 cascade
C
Female VS Male (7 dpc)
Male E2 VS DMSO (7 dpc)
Female Tam VS DMSO (7 dpc)
ifng1-2 interferon gamma 1
irf1b interferon regulatory factor 1b
rsad2 radical S-adenosyl methionine domain containing 2
cxcl11.1 chemokine (C-X-C motif) ligand 11, duplicate 1
cxcl11.6 chemokine (C-X-C motif) ligand 11, duplicate 6
cxcl11.7 chemokine (C-X-C motif) ligand 11, duplicate 7
cxcl11.8 chemokine (C-X-C motif) ligand 11, duplicate 8
ccl35.2 chemokine (C-C motif) ligand 35, duplicate 2
LOC567656 C-X-C motif chemokine 11-6-like
LOC100334604 C-X-C motif chemokine 11-6-like
cd4-1 CD4-1 molecule
LOC110439989 B-cell receptor CD22-like
LOC110438458 cell surface glycoprotein MUC18-like
ly86 lymphocyte antigen 86
lgals9l4 lectin, galactoside-binding, soluble, 9 (galectin 9)-like
LOC103909535 SLAM family member 6-like
mxc myxovirus (influenza virus) resistance C
gig2g grass carp reovirus (GCRV)-induced gene 2g
gig2i grass carp reovirus (GCRV)-induced gene 2i
si:busm1-194e12.12 MHCx class II integral membrane beta chain gene
si:busm1-48c11.3 novel protein similar to MHC class II alpha chain
5 -5
-log2

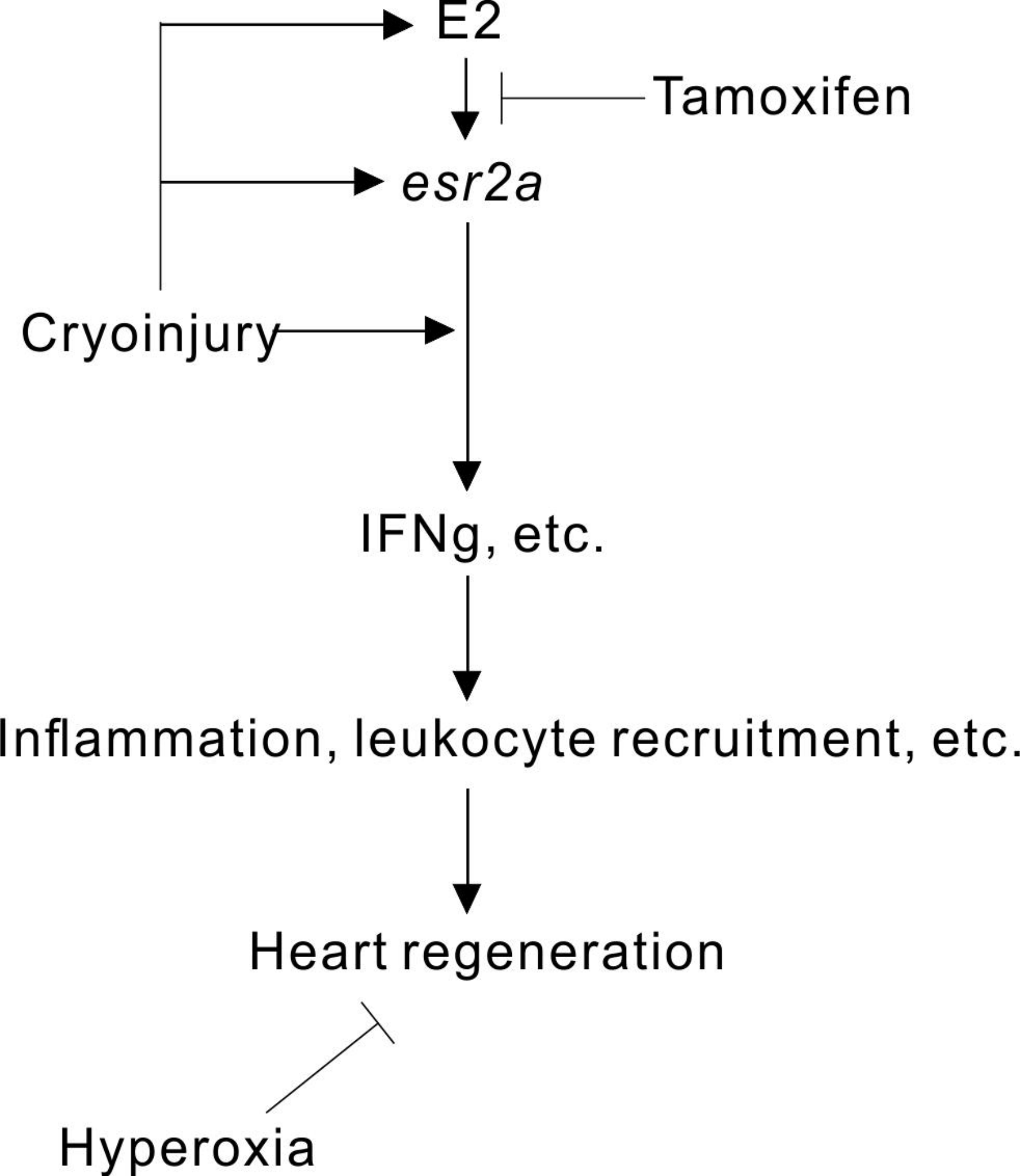

D
Female
Male
ifn-γ expression
30
25
20
15
1.0
0.5
0.0
*
***
Un
1 dpc
E
Female 1 dpc
ifn-γ expression
1.5
1
0.5
0
*
DMSO
Tam
F
Male 1 dpc
ifn-γ expression
3
2
1
0
*
DMSO
E2
G
Female
Male
7 dpc
7 dpc
Un
SO
DMSO
Tam
Un
SO
DMSO
E2
STAT3
STAT1
GAPDH
H
Male 7 dpc
STAT1/GAPDH
2
1.5
1
0.5
0
*
DMSO
E2

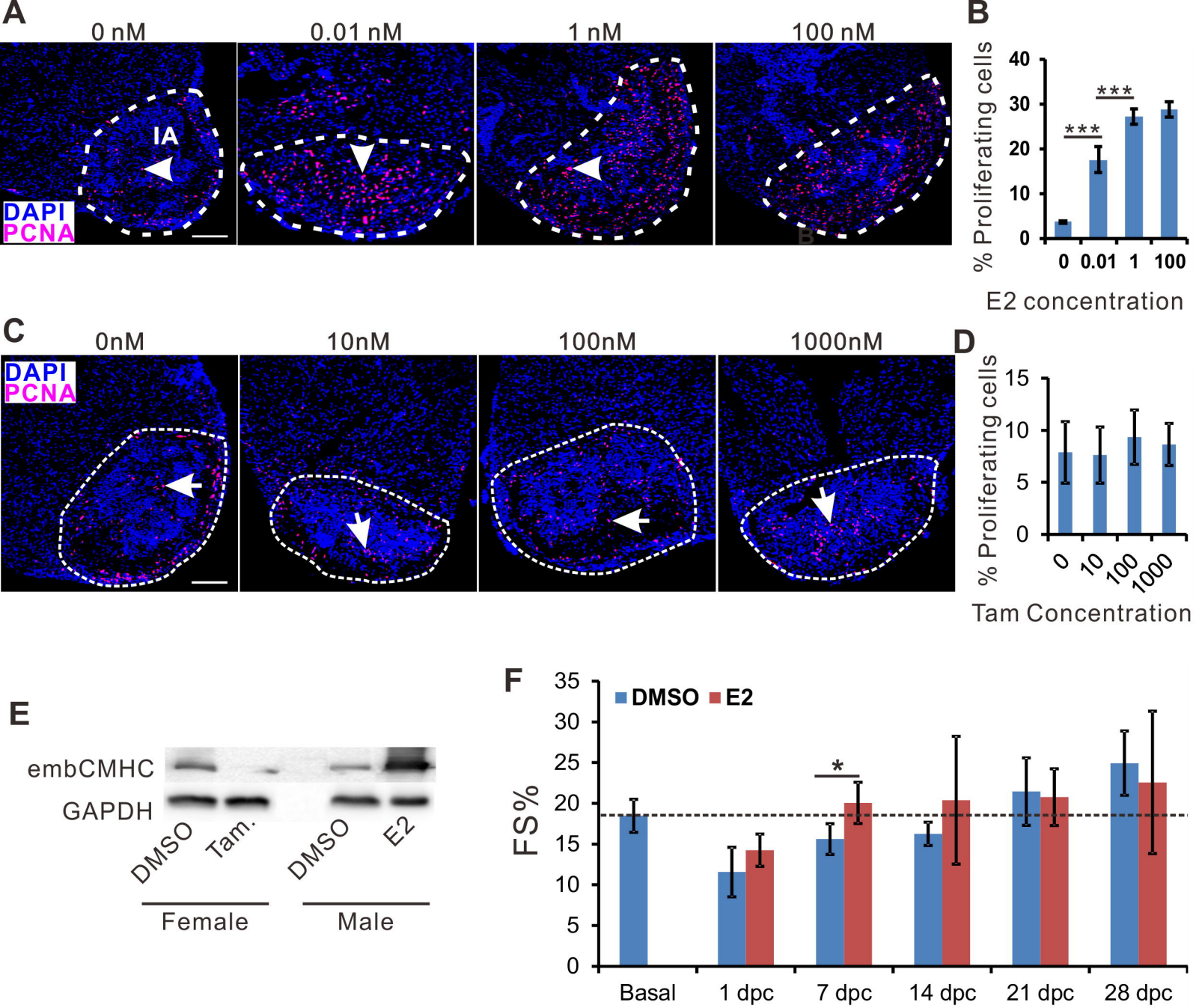

I
Female
Male
1 dpc
IA
DAPI L-Plastin
J
L-plastin / IA pixels
0.05
0.04
0.03
0.02
0.01
0
*
Female
Male

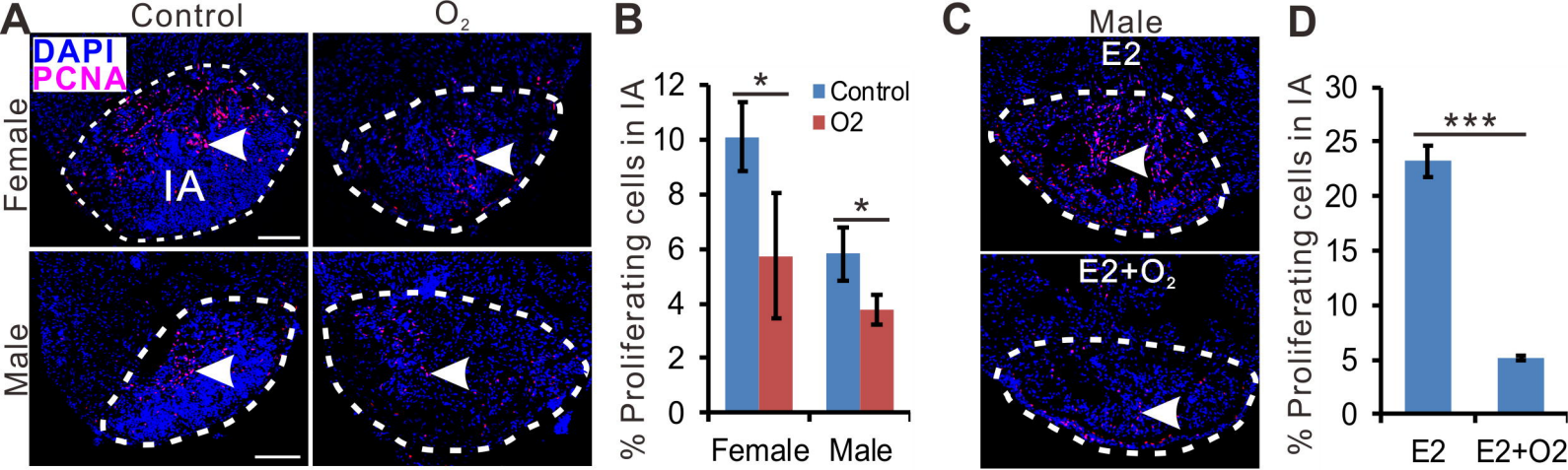

A
Un
SO
CI
Relative expression in male
esr
esrs2a
ers2b
B
Un
SO
CI
Relative expression in female
esr
esrs2a
ers2b
C
DMSO
E2
Relative expression in male
esr
esr2a
esr2b
D
DMSO
Tam
Relative expression in female
esr
esr2a
esr2b
E
Relative expression in Un male
n.s.
DMSC
E2
F
Male estradiol Con. (pg/mL)
Un
SO
CI
G
Female estradiol Con.(pg/mL)
Un
SO
CI
H
Un
7 dpa
Estradiol Con.(pg/mL)
n.s.
Female
Male
I
Un
7 dpa
Expression in female fin
n.s.
esr
esr2a
esr2b
J
Un
7 dpa
Expression in male fin
n.s.
esr
esr2a
esr2b
K
Control
O2
Estradiol Con.(pg/mL)
n.s.
Female
Male

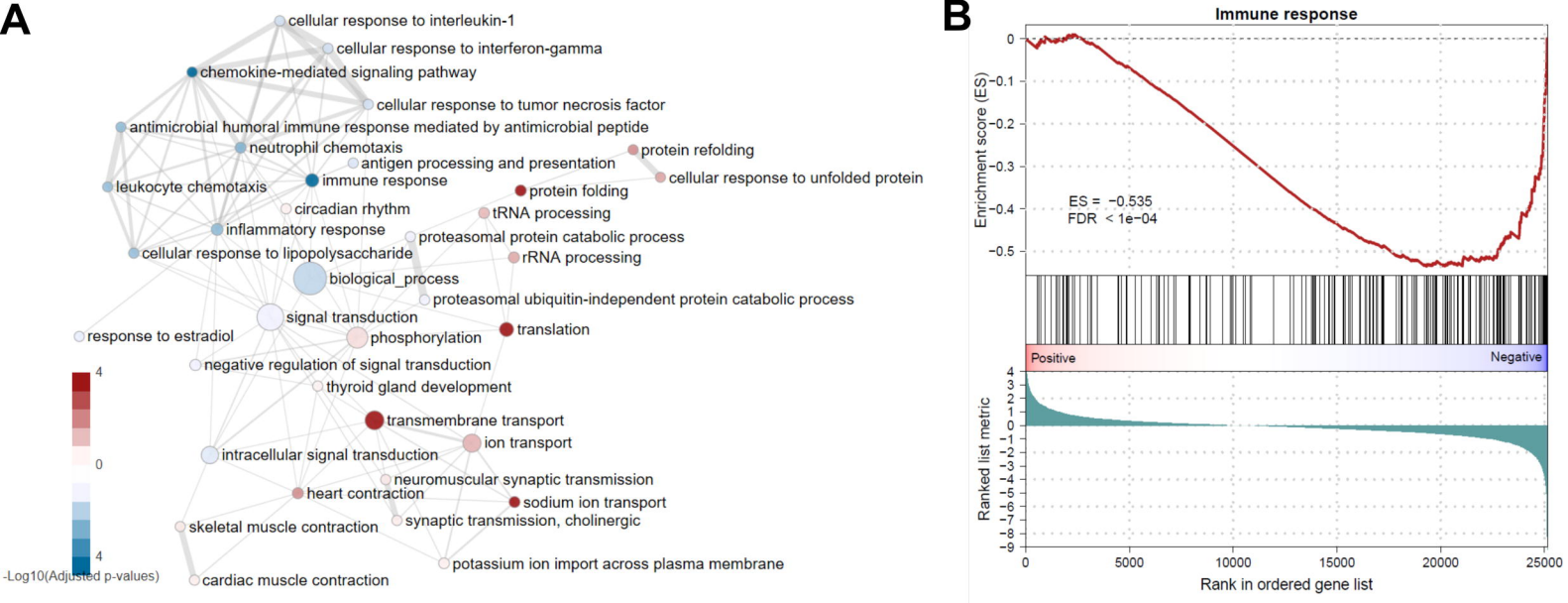

A
Untreated
SO
CI
VTG
GAPDH
B
DAPI
VTG
Merged
Heart
Gill
Kidney
Liver
C
CI
Untreated
SO
D

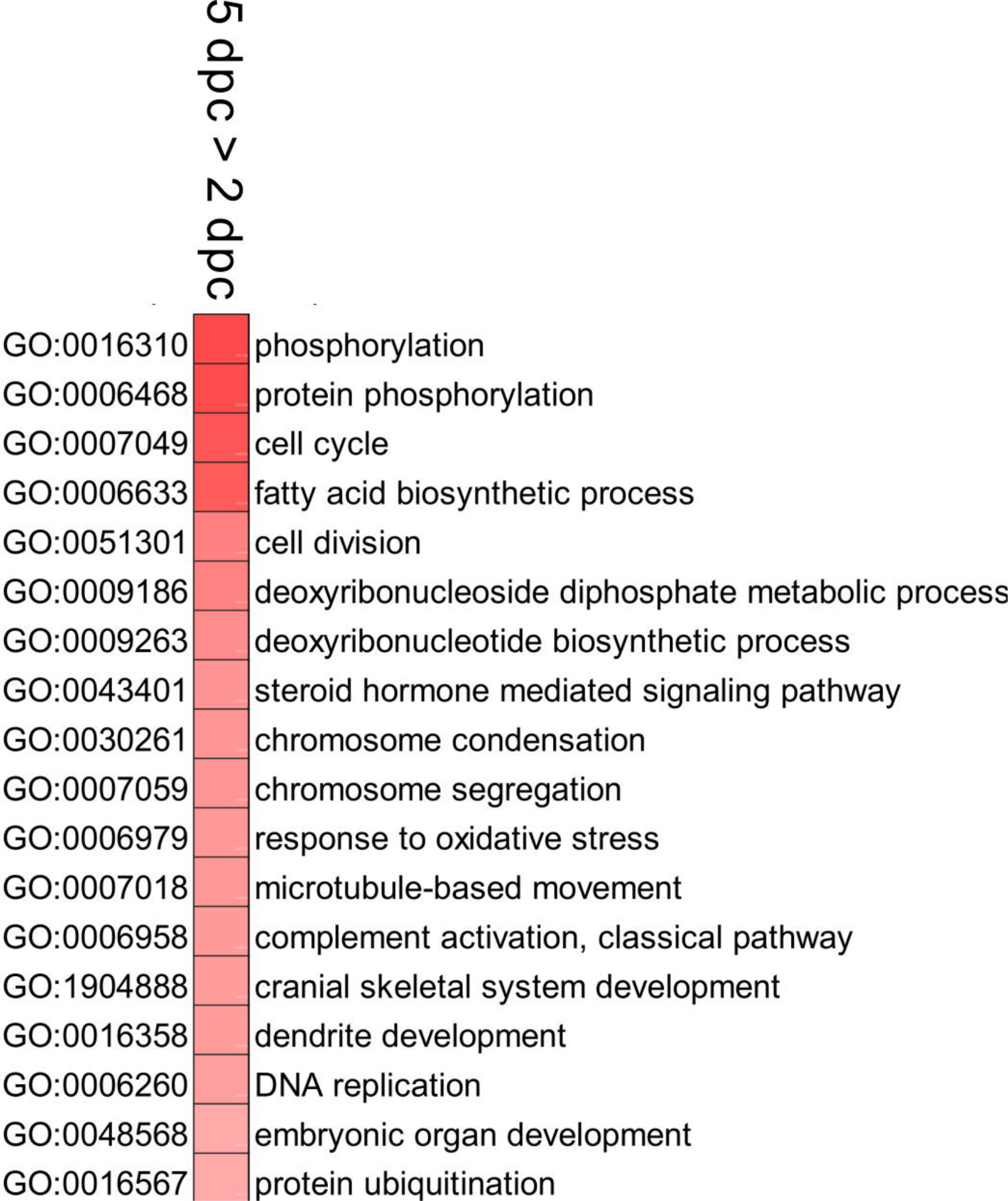

A
Untreated
CI
CI+1μM Tam.
Female
Untreated
CI
CI+1nM E2
Male
B
Fluorescence int.
(Arb. unit)
0
1
2
3
4
5
6
***
***
Un
CI
CI+Tam.
Un
CI
CI+E2
Female
Male
C
VTG
GAPDH
Un
CI
CI+Tam.
Un
CI
CI+E2
Female
Male

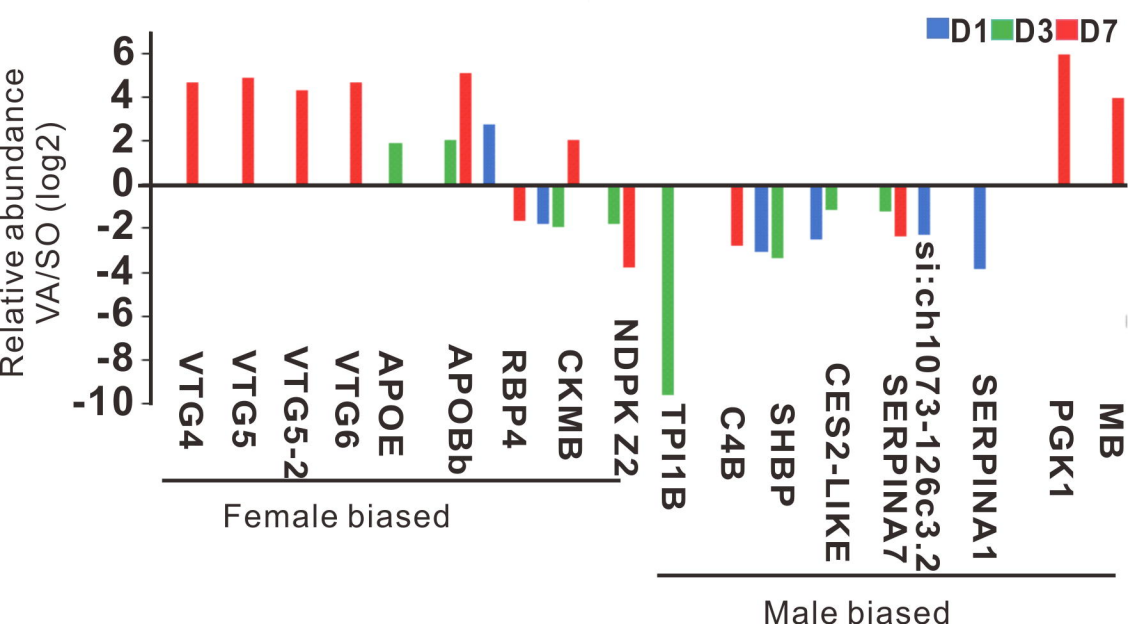

E2
Tamoxifen
esr2a
Cryoinjury
IFNg, etc.
Inflammation, leukocyte recruitment, etc.
Heart regeneration
Hyperoxia

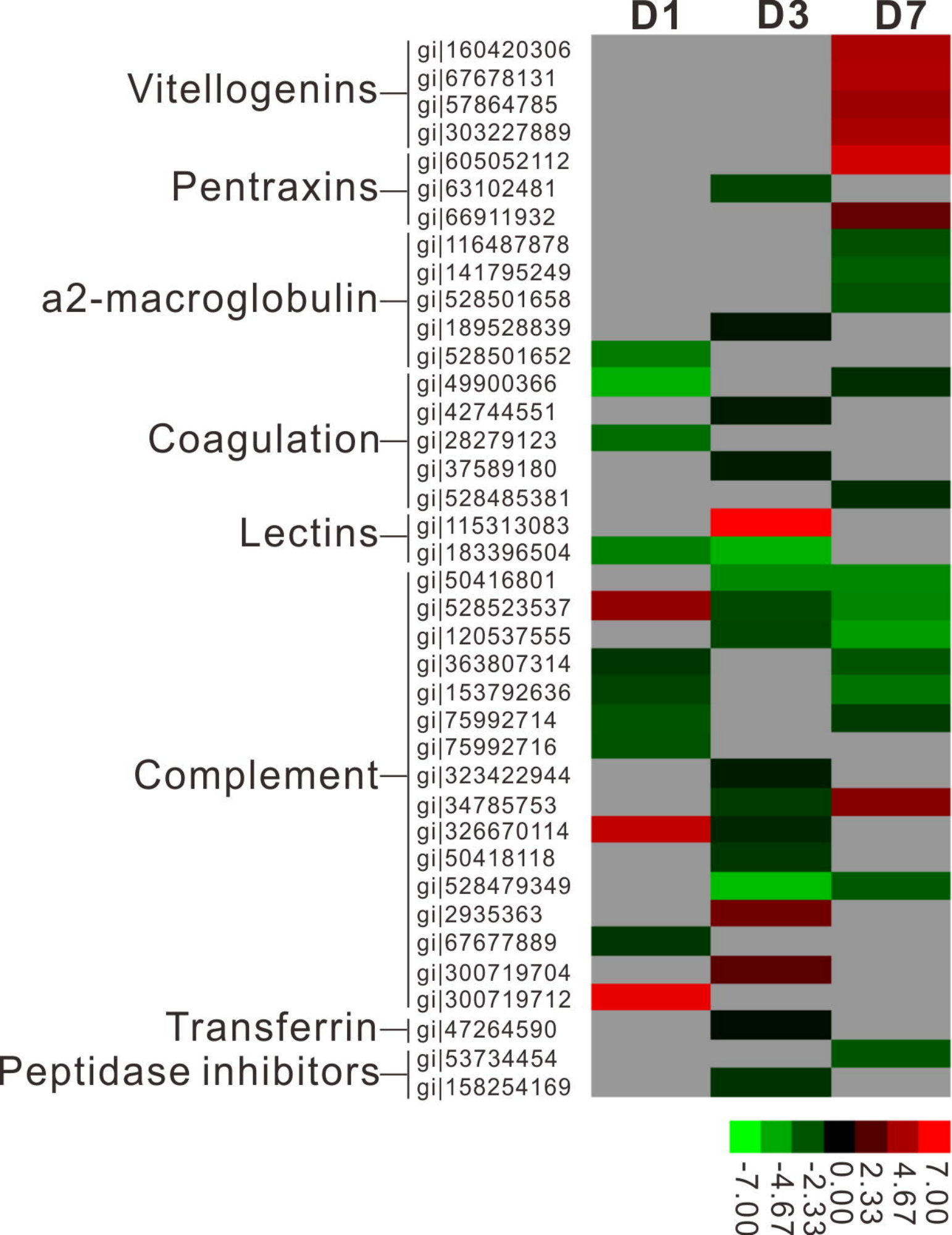

A
0 nM
0.01 nM
1 nM
100 nM
IA
DAPI
PCNA
B
% Proliferating cells
40
30
20
10
0
***
***
0
0.01
1
100
E2 concentration
C
0nM
10nM
100nM
1000nM
DAPI
PCNA
D
% Proliferating cells
15
10
5
0
0
10
100
1000
Tam Concentration
E
embCMHC
GAPDH
DMSO
Tam.
DMSO
E2
Female
Male
F
DMSO
E2
FS%
35
30
25
20
15
10
5
0
*
Basal
1 dpc
7 dpc
14 dpc
21 dpc
28 dpc


A
Control
O2
DAPI
PCNA
Female
Male
IA
B
% Proliferating cells in IA
12
10
8
6
4
2
0
*
*
Control
O2
Female
Male
C
Male
E2
E2+O2
D
% Proliferating cells in IA
30
25
20
15
10
5
0
***
E2
E2+O2


A
cellular response to interleukin-1
cellular response to interferon-gamma
chemokine-mediated signaling pathway
cellular response to tumor necrosis factor
antimicrobial humoral immune response mediated by antimicrobial peptide
neutrophil chemotaxis
protein refolding
antigen processing and presentation
leukocyte chemotaxis
immune response
cellular response to unfolded protein
protein folding
circadian rhythm
tRNA processing
inflammatory response
proteasomal protein catabolic process
cellular response to lipopolysaccharide
rRNA processing
biological_process
proteasomal ubiquitin-independent protein catabolic process
signal transduction
response to estradiol
phosphorylation
translation
negative regulation of signal transduction
thyroid gland development
transmembrane transport
ion transport
intracellular signal transduction
neuromuscular synaptic transmission
heart contraction
sodium ion transport
skeletal muscle contraction
synaptic transmission, cholinergic
potassium ion import across plasma membrane
cardiac muscle contraction
4
0
4
-Log10(Adjusted p-values)
B
Immune response
Enrichment score (ES)
ES = −0.535
FDR < 1e−04
Positive
Negative
Ranked list metric
Rank in ordered gene list


5 dpc < 2 dpc
GO:0016310 phosphorylation
GO:0006468 protein phosphorylation
GO:0007049 cell cycle
GO:0006633 fatty acid biosynthetic process
GO:0051301 cell division
GO:0009186 deoxyribonucleoside diphosphate metabolic process
GO:0009263 deoxyribonucleotide biosynthetic process
GO:0043401 steroid hormone mediated signaling pathway
GO:0030261 chromosome condensation
GO:0007059 chromosome segregation
GO:0006979 response to oxidative stress
GO:0007018 microtubule-based movement
GO:0006958 complement activation, classical pathway
GO:1904888 cranial skeletal system development
GO:0016358 dendrite development
GO:0006260 DNA replication
GO:0048568 embryonic organ development
GO:0016567 protein ubiquitination


D1
D3
D7
Relative abundance
VA/SO (log2)
6
4
2
0
-2
-4
-6
-8
-10
VTG4
VTG5
VTG5-2
VTG6
APOE
APOBb
RBP4
CKMB
NDPK Z2
TPI1B
C4B
SHBP
CES2-LIKE
SERPINA7
si:ch1073-126c3.2
SERPINA1
PGK1
MB
Female biased
Male biased


D1
D3
D7
Vitellogenins
gi|160420306
gi|67678131
gi|57864785
gi|303227889
Pentraxins
gi|605052112
gi|63102481
gi|66911932
a2-macroglobulin
gi|116487878
gi|141795249
gi|528501658
gi|189528839
gi|528501652
Coagulation
gi|49900366
gi|42744551
gi|28279123
gi|37589180
gi|528485381
Lectins
gi|115313083
gi|183396504
Complement
gi|50416801
gi|528523537
gi|120537555
gi|363807314
gi|153792636
gi|75992714
gi|75992716
gi|323422944
gi|34785753
gi|326670114
gi|50418118
gi|528479349
gi|2935363
gi|67677889
gi|300719704
gi|300719712
Transferrin
gi|47264590
Peptidase inhibitors
gi|53734454
gi|158254169
7.00
4.67
2.33
0.00
-2.33
-4.67
-7.00