Desease models in vertebrates : from hypoxia to cancer

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CHAPTER 5

SUMMARY AND DISCUSSION

Animal models have long been used in scientific research, some longer than others. Although in human disease studies, such as cancer, most research is usually done using mouse models, in the last decade a new vertebrate model has emerged. The zebrafish has proved to be very valuable in developmental studies, where its use has been mostly applied. Having a genome 98% similar to humans this small fish from the Indian rivers is nowadays a very common tool to study vertebrate development. In more recent years, the zebrafish started to be used as an animal model to help us better understand several human diseases, from hypoxia to cancer studies, or even immunological studies. In all these studies embryos and larvae, as well as the adult animal, have been used. The advantages of using such a model in human disease studies is becoming more clear as the number of research articles referring to this model increases: the large amount of eggs obtained from a single cross allows for larger sample to be used; the fast development from egg to adulthood (about three months) overcomes the long waiting periods to obtain results that other animal models, such as the mouse, have; the transparency of its embryos and larvae has proven very useful to follow the appearance and development of the disease in real time; finally, the absence of an adaptive immune system, in the early stages of development, circumvents the need to use immunosuppressor drugs, allowing the xenotransplantation of foreign bodies without the risk of rejection.

Along this thesis we demonstrated the value of the zebrafish model in the study of human diseases. We started on **chapter 2** by analyzing what changes occur in the zebrafish heart under chronic constant hypoxia (CCH). It has long been demonstrated that tissue hypoxia may be the result of insufficient blood supply and chronic ischemia. Understanding how teleost fish can withstand chronic

constant hypoxia may prove very valuable to prevent any permanent damage that such condition may cause. In our research we have demonstrated that although zebrafish can survive under CCH, its heart will have morphological and genetic alterations. Such modification may explain how fish adapt to low oxygen levels and how these changes can be used to improve survival and decrease long term effects of hypoxia in mammals. We showed that CCH of zebrafish caused smaller ventricular outflow tract, reduced lacunae and increased cardiac myocyte densities in the heart. Comparatively, in mammals the same type of exposure to hypoxia results in chronic heart failure and loss of cardiac myocytes. If the zebrafish heart responds to hypoxia in a similar manner as to mechanical dissection, this may demonstrate to be very useful in preventing apoptosis of cardiac myocytes, which will help prevent the development of anoxic cores in the cardiac myocytes. But besides the morphological changes that occurs in the heart of the zebrafish under chronic constant hypoxia, we were also interested in the underlying gene expression changes of these adaptations. Through a transcriptome analysis we found several genes were up- or down-regulated under CCH. We found gene regulations in a transcriptional network of the serum response element that were opposed to the ones described in mammals. For example, while c-fos was down-regulated in the zebrafish heart, in mammal studies it has been demonstrated to be up-regulated by hypoxia. We also uncovered novel gene expression changes induced by CCH, such has two notch receptors, whose expression was induced by CCH. Several genes linked to human heart pathology were also found to be up-regulated in this study. An example is the complement component C9 and haptoglobin, two markers for myocardial infarction. We also observed up-regulation of fetuin-α. Low levels of this gene

have been associated with heart failure in mice. Its higher expression levels in the zebrafish heart may help to better tolerate CCH. The changes identified in this study may add to the ability of teleosts to adapt to severe hypoxia. The better we understand how certain animals tolerate hypoxia, the better we can prevent its long term effects, or even use it to cure or control diseases linked to it. With this first study we demonstrated the versatility and resistance of the zebrafish model to withstand extreme conditions.

From the hypoxia study, we then demonstrated, on **chapter 3,** that the zebrafish is a useful *in vivo* animal model for rapid analysis of invasion and metastatic behavior of primary human tumor specimen. We used small explants from gastrointestinal human tumors, which were fluorescently labeled, and investigated their metastatic behavior after transplantation into zebrafish embryos. Using this model, we successfully followed the process of invasion, migration and micro-metastasis formation in real time. We started by implanting two sister human pancreatic cancer cell lines, one with invasive characteristics (PaTu8988t) and the other with a non-invasive behavior (PaTu8988s) into the yolk of the two day old zebrafish. We then followed these cell lines and observed that the cell line with invasive properties did, in fact, dislocate from the site of implantation, intravasated into the blood flow and latter extravasated and invaded distant tissues. These initial experiments validated the worth of our model to study the metastatic properties of human cancer cell lines. We then proceeded to use our model to test whether human tumors transplanted into the zebrafish also displayed metastatic behavior. We were able to establish that the early embryos and larvae used did not reject the primary tumor xenografts, and that cells would come loose from the implanted xenografts, enter the fish

circulation and, within 24h, would appear in distant tissues and organs, forming some micrometastasis, which were observed in histological sections. Furthermore, we investigated the effects of protease inhibitors on the invasiveness of implanted tumor cells and tumor tissue fragments and verified that two different protease inhibitors were able to inhibit invasiveness of the implanted cells and tissues. The panel of experiments performed along this work provided us with valid information for the future development of a screening methodology of drugs to prevent invasion and metastasis formation of human tumors.

We demonstrated here the usefulness of the zebrafish embryos as an *in vivo* model for the analysis of metastatic behavior of human tumor cells. We further established a novel assay in which resected human tumor tissue was successfully implanted into the zebrafish embryo, preserving its intrinsic metastatic ability. We followed *in vivo* and in real time the formation of micrometastasis, without the need to sacrifice the test animal, as it occurs with other mammalian models. And, above all, all of our experimental results could be obtained in a short period of time, avoiding the time consuming experiments that other animal models require. In conclusion, the zebrafish provides us with an advantageous short term model for cancer studies that could complement well established longer term tumor models, such as mouse models, which might be valuable and efficient tools to evaluate novel therapeutic strategies to fight cancer.

After validating the usefulness of the zebrafish model in cancer research, we then applied our model to two real case studies.

On **chapter 4** we used the zebrafish model to demonstrate the effect of retinoic acid receptor antagonists in pancreatic cancer. We identified microsRNA-10a as

an important mediator of metastasis formation in pancreatic tumor cells and proposed a model for its regulatory mechanisms. In a Northern blot analysis we verified that miR-10a expression is increased in pancreatic tumor cells lines and in chronic pancreatitis and pancreatic tumor tissue. Afterwards we implanted our samples of human pancreatic cell lines and human pancreatic tissue (tumor tissue, chronic pancreatitis and normal pancreatic tissue) into the zebrafish embryo to study the invasiveness and metastatic behavior of our samples. After assessing the cancerous properties of our material we investigated what role miR-10a played in the metastatic behavior of our cell lines and tissues. We verified that overexpression of miR-10a siRNA was sufficient for noninvasive cell lines to acquire invasiveness and metastatic behavior. On the other hand, blocking of miR-10a with morpholinos was enough to prevent the metastatic behavior characteristic of some of our cell lines and primary human tumors. Once we proved that miR-10a did indeed play a role in invasion and metastasis in pancreatic cancer, we proceeded to identify its regulators. Upstream, we discovered that miR-10a is regulated by RA signaling in pancreatic cancer cells. It had already been demonstrated that retinoids could be used as anticancer agents due to their pleiotropic regulatory function in cell differentiation, growth, proliferation and apoptosis. In our research we show that RARα antagonists have anti-metastatic properties and that it down-regulates miR-10a, demonstrating its potential as anti-metastatic drugs, at least in the case of pancreatic cancer.

We also found that miR-10a expression was up-regulated in conditions of chronic pancreatitis. It is however well known that chronic conditions of pancreatitis are a risk factor for the development of pancreatic cancer, which could possibly explain the higher expression of miR-10a in these cases. Although, at this time

we could not precise the role of miR-10a in the progression from chronic pancreatitis to pancreatic cancer, we theorized that miR-10a expression could be involved in more complex regulation mechanisms that control or counterparts the malignant transformation of a cell. To better understand the specific role of miR-10a in the formation of pancreatic cancer from chronic pancreatitis and the identification of miR-10a targets in chronic pancreatitis and pancreatic carcinoma, future experiments are required.

Downstream, we found that HOXB1 and HOXB3, as well as the cadherin/catenin complex proteins E-cadherin, α-catenin and β-catenin are targets for miR-10a. Using siRNAs to specifically knockdown HOXB1 and HOXB3, we found that suppression of these genes turned our non-metastatic cell line, Patu8988s, into an invasive and metastatic cell line, demonstrating that these genes function as metastasis suppressor genes. Similar results were previously observed, showing inhibition of metastatic behavior by miR-10b (154).

In view of our results we proposed a model in which suppression of HOXB1 and HOXB3 confers invasiveness and metastatic behavior to pancreatic cancer cell lines: on the one hand, RA stimulates the dimerization of RAR/RXR retinoid receptors, which bind to RAREs, triggering the increased miR-10a expression. This, in turn, suppresses HOXB1 and HOXB3, promoting invasion and metastatic behavior. On the other hand, transcriptional repression of miR-10a by a selective RARα inhibitor, releases the suppression of HOXB1 and HOX3, preventing invasiveness and metastatic behavior. Considering our findings, both RAR antagonists, as well as miR-10a inhibitors could be used as promising anti-metastatic compounds, as part of anti-metastatic therapy.

In conclusion, animals have long been used as models in scientific research, either as test subjects for new therapies against human diseases, or as models to help us better understand disease formation and progression. Vertebrates are the preferred animal model for research associated with human diseases due to their evolutionary proximity to us. The most commonly used animal model is probably the mouse, but in recent years a new model, the zebrafish, started to be used and is becoming more widely accepted as a research model in human disease studies. Through this thesis we demonstrated the usefulness of the zebrafish as a disease model in vertebrates. From its capacity to withstand and survive under hypoxia, which can help us to find newer therapies to revert this condition in humans and, as such, avert its harmful consequences. To the usefulness of the zebrafish in cancer studies, as a time saving model to complement well established models, such as the mouse, which can be rather valuable in evaluating novel therapies to fight tumor formation, progression and metastasis formation. And finally we used our vertebrate model in a case study, where we analyzed the role played by miR-10a in pancreatic cancer.