Desease models in vertebrates : from hypoxia to cancer

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CHAPTER 1

GENERAL INTRODUCTION AND THESIS OUTLINE

Cancer is probably the biggest epidemic of the 21st century (Figure 1). Despite considerable resources spent on cancer research and the better and marked improvement of cancer therapies, cancer is still one of the leading causes of death worldwide. According to the World Health Organization (WHO), cancer-associated mortality is predicted to continue rising, with an estimated 12 million yearly deaths by 2030 (WHO, 2011). Lifestyle changes and early detection are still the best way to prevent and treat cancer.

In 2000, Hanahan and Weinberg foresaw that a more logical approach should be followed in cancer research, in order to better understand the complexities of the disease in terms of a small number of underlying principles. In their review article they summarized the six alterations that most, if not all, cancers must have undergone (Figure 2): the ability to control its own growth (self-sufficiency of growth signals); resistance to anti-growth signals; evasion of programed cell-death (no apoptosis); unlimited replication; the ability to form new blood vessels around it, (angiogenesis); the ability to invade other tissues and metastasize to different areas of the body (Hanahan, et al., 2000). Nonetheless, metastasis is a very complex process in which primary tumor cells must first invade the neighboring tissues; then intravasate into the circulation; translocate through the vasculature until they arrest in distant capillaries; extravasate into the perivasculature; and finally proliferate from micrometastasis into macroscopic secondary tumors (Fidler, 2003). And, in order for us to fight this deadliest aspect of cancer, we must understand and find a way to prevent each of the steps required for a cancer to metastasize.

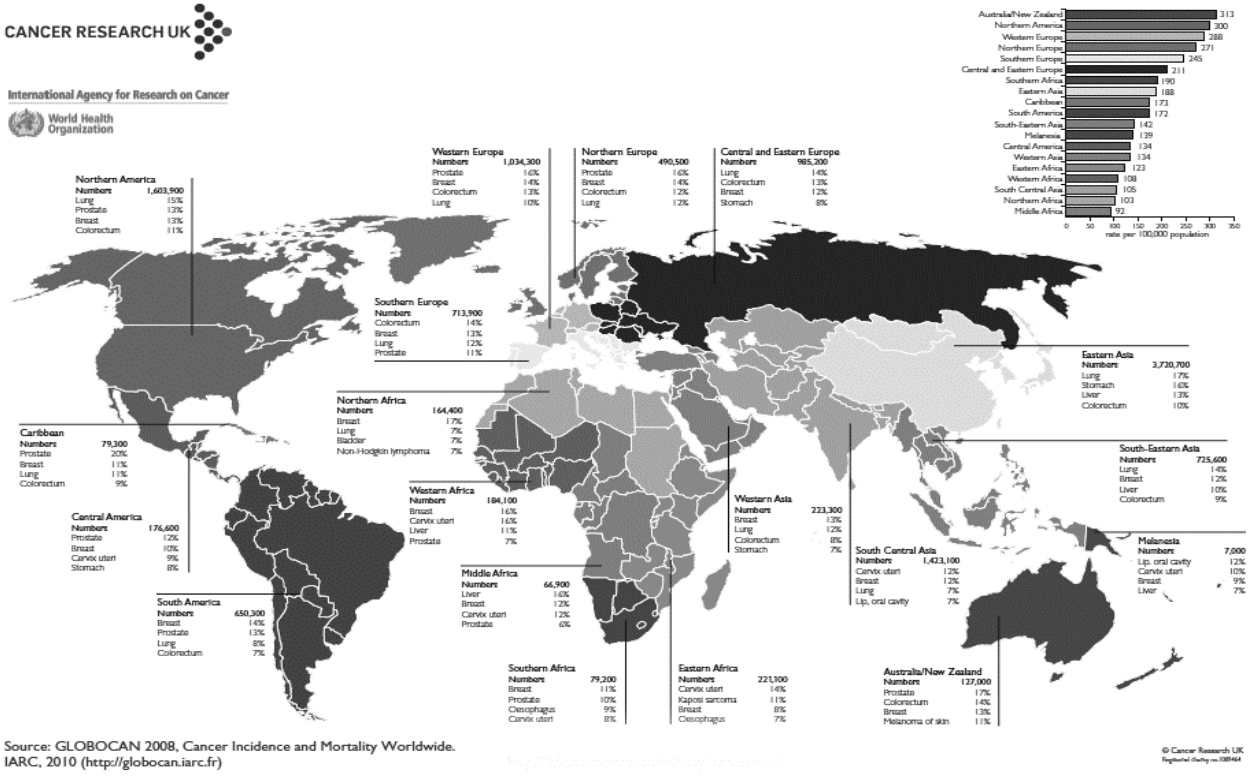

Figure 1 Cancer Incidence Worldwide: this map illustrates cancer incidence worldwide, standardized by age and the most commonly diagnosed cancers. (Source: GLOBOCAN 2008 (Cancer Research UK).)

Nowadays pancreatic cancer is considered one of the deadliest forms of cancer. Patients diagnosed with this type of cancer have usually a very poor prognosis, mainly due to the late detection of the tumor. This delay in diagnosis is largely attributable to the absence of symptoms and early stages of the disease can often be mistaken for chronic pancreatitis. Additionally, resistance to conventional treatments, like chemotherapy and radiation, also contributes to the high mortality rate. Survival after diagnosis is usually three to six months, and the 5-year survival is only 5% (WHO, 2011). The best way to increase early detection of pancreatic cancer is to find specific biomarkers for this type of cancer, which still do not exist. Novel biomarkers should be able to distinguish between chronic pancreatitis and pancreatic cancer. A review of literature reveals several other biomarkers that can be used to identify pancreatic cancer in early stages (Tanase, et al., 2010)

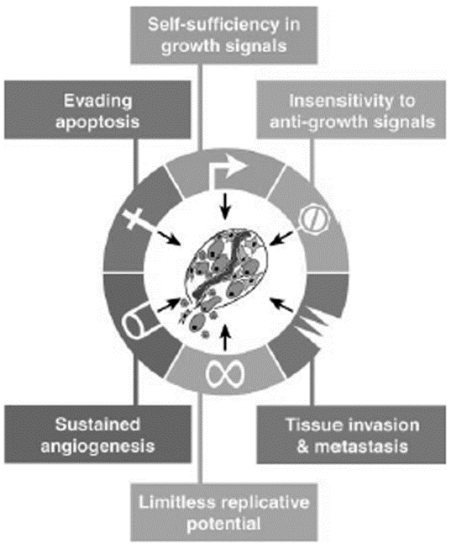

Figure 2 Summary of the mutations a normal cell must undergo in order to become a cancer cell (Hanahan, et al., 2000)

The Zebrafish as a vertebrate disease model

The use of zebrafish as a model system is first mentioned in the literature in 1958, (coincidentally this was in the context of cancer research). KK Hisaoka (1958) describes the effects of the carcinogen 2-acetylaminofluorene in the embryonic development of the zebrafish. Since then it has been mentioned in over thirteen thousand articles. Although evolution has placed some distance between humans and zebrafish, many signaling pathways have been conserved. These include pathways involved in development, proliferation, cell movements, apoptosis, etc. (Amatruda, et al., 2008), as well as, cell-cycle genes, tumor suppressor genes and oncogenes (Amatruda, et al., 2002). The low maintenance costs, fecundity, transparency of the embryos and fast development make the zebrafish a very versatile animal model that can be used in a broad array of research studies. Also, the genetic manipulation to create fish lines with specific genetic characteristics, such as the fli1::egfp line that develops GFP-labeled vasculature (Lawson, et al., 2002) or the *casper* line which is characterized by transparent adult fish (White, et al., 2008), makes it a very amenable model for developmental and cancer studies. Zebrafish are also very easy to use in forward genetic screening and reverse genetics techniques, such as morpholino injections (inhibitory of mRNA translation). Furthermore, they have a developed innate and adaptive immune system (the last one appearing only in later stages of development, around 4-6 weeks). The late development of adaptive immunity has allowed us to carry out successful xenotransplantation experiments of human pancreatic cancer cells and implantation of primary tumor tissue, in embryonic stages (Chapter 3 of this thesis).

The zebrafish was the first fish model to be used for carcinogenesis studies (Stanton, 1965). In his research Stanton describes how he chemically induced

hepatic degeneration and neoplasia in the zebrafish. In the last decade several advances were made in the cancer research field using this model. In 2000 Spitsbergen demonstrated that the zebrafish responds in a similar way to mammals to carcinogens, and develop tumors that are histopathologically similar to their human counterparts (Spitsbergen, et al., 2000a; Spitsbergen, et al., 2000b). Subsequently Lee *et al* (2005) and Topczewska *et al* (2006) showed that human tumor cells can be easily injected into zebrafish embryos and survive through to adulthood. Since then, several xenotransplantation studies of human and mouse tumors into zebrafish embryos and larvae have been published, proving that these cells were able to proliferate, invade and form tumor masses in zebrafish embryos (Lee, et al., 2006; Nicoli, et al., 2007; Stoletov, et al., 2007; Hendrix, et al., 2007). The use of zebrafish allows for a very simple monitoring of cancer formation and progression. They also allow fast genetic screening as well as rapid tests to evaluate new anti-cancer drugs and small molecule compounds that selectively target tumor tissue and cells (L.I., et al., 2005; den Hertog, 2005; Kari, et al., 2007; Lally, et al., 2007; Geiger, et al., 2008). The incorporation of such compounds into the zebrafish is done simply by adding them to the water where the embryos are swimming, so that they are absorbed by the organism through the skin or ingested.

In conclusion, the use of the zebrafish in cancer research is a major catch, presenting a number of advantages over the mouse model. For example, it avoids time-consuming transplantation studies, which require large numbers of cells, and which require the sacrifice of the animal; it also allows cancer formation and development to be followed *in vivo.* Finally, it provides a rapid screen for new anti-cancer therapies.

From hypoxia to cancer

Hypoxia, or a low level of oxygen, has been recognized as playing a key role in several cellular physiological processes, from cell proliferation, to cell survival, angiogenesis, metabolism and tumor progression and metastasis. Hypoxia can be divided into acute and chronic hypoxia. The acute variant is usually caused by a temporary disruption to the blood flow and does not last long. On the other hand chronic hypoxia is durable and can have lasting effects (Dewhirst, 1998).

Besides the epigenetic, genetic and somatic changes that occur in cancer, it is also important to understand the tumor microenvironment, since it may also play a significant role in tumor progression and metastasis and influence the response to conventional cancer treatments, like chemo- and radiotherapy. One of the micro environmental stresses that play a role in cancer is hypoxia. Hypoxic cells have been shown to be more resistant to radiotherapy and chemotherapy, and they usually originate more unstable and aggressive phenotypes (Rademakers, et al., 2008).

Adaptation of cancer cells to low oxygen levels has been shown to be dependent on the expression of HIF-1 (hypoxia inducible factor 1 (Semenza, et al., 1992)). HIFs are transcription factors that play a key role in the regulation of oxygen homeostasis and the response of cells to hypoxia. They are comprised of an α and a β subunit. Of the three known HIFs, HIF-1 has been recognized as the main regulator of oxygen homeostasis, and it has been shown to be over-expressed in the several human cancers (Zhong, et al., 1999). Under hypoxic conditions, molecular O2 is unavailable, and HIF-1α is up regulated and dimerizes with HIF-1β. This heterodimer then migrates to the cell nucleus, where it binds to specific DNA sequences and activates the genes necessary for the adaptation of the cell to hypoxia. These genes then regulate pathways linked to cell survival (e.g. GUT-

1 – glucose transporter 1), angiogenesis (e.g. VEGF – vascular endothelial factor) and metastasis (e.g. TGF-α), which are well known to be involved in tumor development and aggressiveness (Melillo, 2006) (Figure 3).

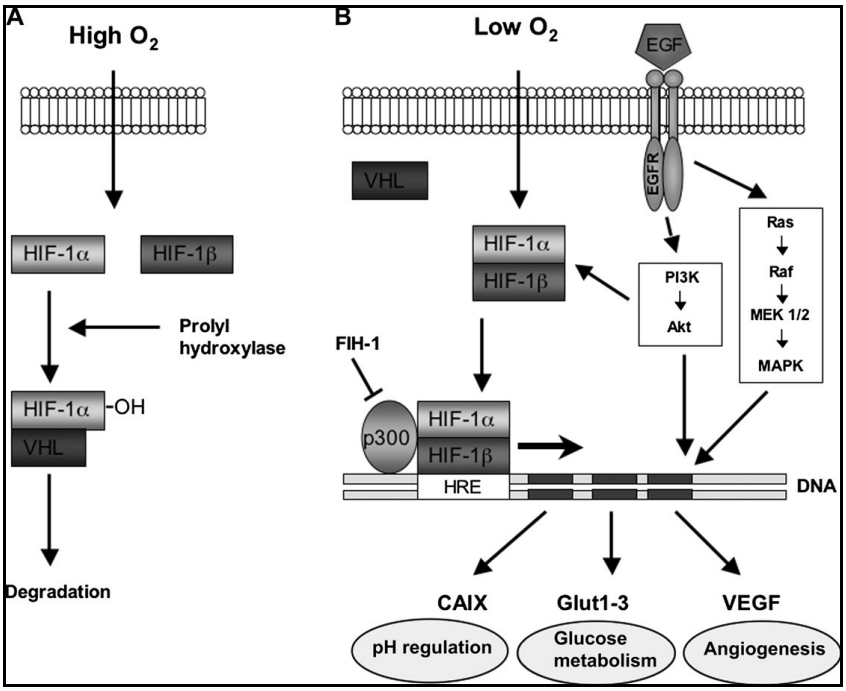

Figure 3 Schematic representation of the HIF-1 pathway. Under normoxic conditions HIF-1α is hydroxilated and rapidly degraded (A). Under hypoxia HIF-1α dimerizes with HIF-1β, becoming a stable complex, which then initiates the transcription of its target genes (adapted from (Rademakers, et al., 2008)).

Ruan and colleagues (2009) wrote a short review where they analyze how hypoxia mediates and regulates each of the hallmarks of cancer (Figure 4). Intra-tumor hypoxia has been demonstrated to be a key factor for the poor prognosis observed in several types of cancer, such as prostate, breast or cervical cancer

(Chan, et al., 2007; Vaupel, et al., 2007). It occurs when cancer cells are located at such a distance from functional blood vessels that they no longer get the necessary oxygen levels. So in order to survive the hypoxic environment, cancer cells must adapt.

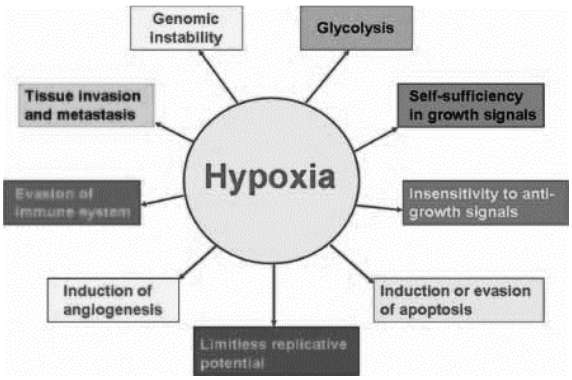

Figure 4 Role of hypoxia in the hallmarks of cancer (adapted from Ruan *et al* l., 2009)

Under hypoxia, cancer cells must first overcome anti-proliferative signals. In order to achieve this, hypoxia induces not only expression of several growth factors that promote cell proliferation (e.g. EGF, insulin, IGFs) (Maxwell, et al., 2001), but can also adapt growth-inhibitory signals. An example is the case of the PTEN gene, which regulates hypoxia and IGF-1 induced angiogenic expression. Knocking-down PTEN promotes tumor growth through the deregulation of Akt activity and HIF-1 regulated gene expression (Zundel, et al., 2000).

Secondly, hypoxic microenvironments induce apoptosis, which at least some cancer cells must avert. Tumor cells have developed several mechanisms to avoid HIF-1 mediated apoptosis. For example, in KHT cells it has been demonstrated that hypoxia up-regulates Mdm2, via the p53 pathway, and this way increases cell resistance to apoptosis (Zhang, et al., 2004). Hypoxia also

mediates the selection of p53 mutant cells which have a reduced apoptotic potential (Graeber, et al., 1996).

Thirdly, hypoxic microenvironments potentiate angiogenesis which is favorable for cancer cell survival. They either directly stimulate the expression of angiogenic factors involved in the development of endothelial cells, or they induce a favorable microenvironment for endothelial cells and consequently for cancer cell survival, migration and invasion (Ruan, et al., 2009).

Hypoxia can thus be considered a very important microenvironmental factor in tumor invasion and metastasis as demonstrated by several studies (Gatenby, et al., 2007; Liao, et al., 2007; Chang, et al., 2006). Furthermore, a strong correlation between hypoxia and HIF-1 production, and consequent prolonged cell survival, has been demonstrated, suggesting the possibility of HIF-1 being targeted for a possible therapeutic strategy (Ruan, et al., 2009; Fraga, et al., 2009).

The role of microRNAs in cancer

Micro RNAs (miRNAs) are small non-coding RNAs, 20-23 nucleotides long, expressed in a tissue- and development-specific manner (Ambros, 2008). They were first identified in *Caenorhabditis elegans* where they were shown to play a role in regulating larval development (Lee, et al., 1993). Since their discovery they have been associated with the regulation of several biological functions, from cellular development, to apoptosis, metabolism, and have more recently been linked to cancer.

MicroRNAs are produced in the cell nucleus. They are excised from a primary harpin precursor RNA structure (pre-miR) which has first been transcribed from a larger Pol II primary transcript (pri-miR). Pre-miRs are then exported to the cytoplasm, where they are processed by Dicer to become the 20-23 nucleotide

mature single-stranded microRNA. Finally the single-stranded miRNA is incorporated into the RISC complex which will bind to their target mRNAs, regulating mRNA degradation and translational repression (Figure 5).

The first association of micro RNAs with cancer was made by Calin *et al* (2002). They showed that miR15 and miR16 were frequently down-regulated in more than half of the patients with B cell chronic lymphocytic leukemia. Later several more studies surfaced linking microRNAs with cancer. The majority of cancer-associated miRs have been located downstream of oncogenes and tumor suppressors that act as transcription factors; as such, miRNAs can either act as oncogenes or as tumor suppressor genes. Micro RNAs play several roles in regulating the hallmarks of cancer (Hanahan, et al., 2000) and their expression in cancer is tissue- and tumor-specific (Santarpia, et al., 2010).

First, some miRs have been shown to help cancer cells to become self-sufficient in growth signals. For example, mir-205 has been demonstrated to act as an onco-suppressor gene in breast cancer, by interfering with the proliferative pathway mediated by HER receptor family (Iorio, et al., 2009). Another study reveals that miR-210 expression progressively increased under hypoxia. This microRNA was necessary and sufficient to down-modulate the expression of Ephrin-A3, which had significant functional consequences: it affected the endothelial cell response to hypoxia, affecting cell survival, migration, and differentiation (Fasanaro, et al., 2008).

Second, cancer cells can become insensitive to antigrowth signals. There are miRs that interfere with the components that manage the transit of the cells through the G1 phase of the cell cycle. For instance, miR-34 induces cell cycle arrest in both primary and tumor-derived cell lines, in response to p53 activation,

mediating G1 arrest by down regulating several cell cycle related transcripts (He, et al., 2007).

Third, some miRs also interfere in cancer cells acquired resistance to PDCD (apoptosis). For example, over-expression of miR-330 in PC-3 cells resulted in cell growth suppression by reducing E2F1-mediated Akt phosphorylation and thus inducing apoptosis (Lee, et al., 2009). In another study, miR-21 has been demonstrated to act as an anti-apoptotic factor in human glioblastoma cells, by blocking the expression of critical apoptosis-related genes (Chan, et al., 2005).

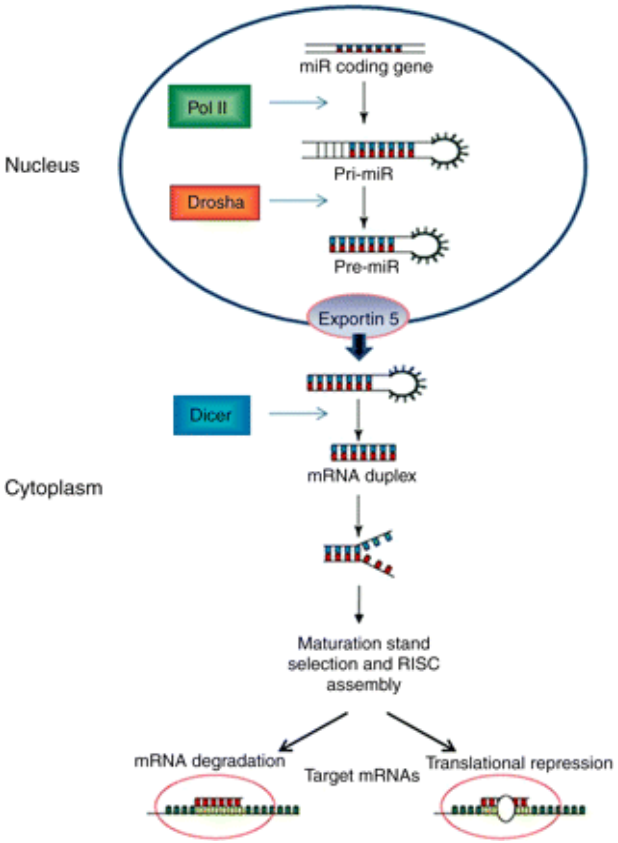

Figure 5 MicroRNA regulatory pathway (adapted from Santarpia *et al, 2010*). In the nucleus, Pol II transcribes the pri-miR from the miR coding gene, and then is processed by Drosha into the pre-miR. The pre-miR is exported from the nucleus to the cytoplasm,

where Dicer processed it into the 20-23 nucleotide single-stranded, mature microRNA. Finally, the mature microRNA is incorporated into the RISC complex. This complex then recognizes the miRs multiple targets.

Fourth, miRs have been validated as playing and important role in diverting oncogene-induced senescence, thus helping cancer cells to gain a limitless replicative potential. Examples are miR-372 and miR-373 which neutralize p53-mediated CDK inhibition through suppression of LATS2, thus allowing tumorigenic growth in the presence of wild-type p53 (Voorhoeve, et al., 2006).

Finally, cancer cells must overcome the hypoxic environment that their rapid expansion causes, and also be able to invade and metastasize to distant tissues. Several miRs have been established as a key factor in tumor hypoxia. Microarray-based expression profiles revealed that there are several microRNAs that are induced in response to low oxygen, some of them via the HIF pathway, and that the vast majority of these hypoxia-induced micro RNAs are also over-expressed in human tumors (Kulshreshtha, et al., 2007). Many of these miRs play a role in the angiogenic process by, for example, regulating VEGF and other angiogenic factors under hypoxic conditions (Hua, et al., 2006). Hua *et al* (2006) used CNE cells to investigate microRNA-directed regulation of VEGF under hypoxia. They found that VEGF could be regulated by different miRs in different cells and also that VEGF was regulated by multiple miRNAs using different combinations. And, at last, numerous studies have demonstrated that the targets of the metastasis associated micro RNAs are proteins that participate in the regulation of cell motility, cell-cell adhesion and cell-matrix interactions (Santarpia, et al., 2010). One such case is micro RNA 10a (miR-10a) that, as described in chapter 4 of this thesis, by down regulating HOXB3 and HOXB4 promotes invasion and metastasis. We demonstrated that miR-10a regulates the expression of proteins of the

cadherin catenin complex (Weiss, et al., 2009). The role of several other microRNAs in tumor invasion and metastasis can be red in the review written by Santarpia *et al* (2010).

Taking into consideration the different roles played by microRNAs in the hallmarks of cancer, it is only natural that miRs are currently being considered as important biomarkers for the prognosis of cancer (for instance, several studies reported that a number of miRs are upregulated in pancreatic cancer and chronic pancreatitis, as it is the case miR-10a (Weiss, et al., 2009), as well as targets for the treatment of the disease. For example, Kota *et al* (2009) established that delivering miR-26 in a mouse model of hepatocellular carcinoma reduced liver tumor size, suggesting that delivery of microRNAs that are highly expressed and tolerated in normal tissue, but absent in cancer cells might provide a strategy for miRNA replacement therapies (Kota, et al., 2009).

Conclusion

Cancer is one of the deadliest diseases of the developed world. Understanding the pathogenesis of a disease is perhaps the most effective way to prevent and treat it. To do so, we must find which genes regulate cancer and its microenvironment.

Although other animal models have a longer tradition in cancer research, the zebrafish as a vertebrate model is becoming ever more relevant for these studies. Its specific characteristics, such has transparency, fast development, gene conservation when compared to humans, makes it an ideal model to study many human diseases, including cancer. We have used this model to better understand hypoxia, a relevant aspect of the microenvironment in which tumors develop. Using also this animal model, we analyzed the role of miR-10a and

developed a hypothetical model for the regulation of miR10a in pancreatic cancer

Thesis outline

Cancer is the epidemic of the 21st century. Every year sees an increase in its global health burden, but developed countries are the most affected by this disease. Stress, diet and tobacco are among the most common causes of cancer. Lifestyles factors together with late diagnosis and hereditary traits are the principle causes of cancer associated deaths. Cancer research now takes a more logical approach focusing on what mutations occur within a cell to make it a cancer cell. Which (onco-)genes are (de-)activated, and how. How are tumor suppressor genes suppressed? How do we prevent these mutations from occurring? Can we reverse these mutations? How does the cell microenvironment affect the development of a cancer? These and many other lines of investigation are nowadays followed on cancer research.

In this thesis we show that an animal model with a relatively recent history, the zebrafish, can be useful in human disease studies, more specifically for hypoxia and cancer research. In the case of hypoxia, understanding how the fish adapts and survives under low oxygen levels may prove very useful to fight human diseases caused by ischemia and low oxygen. In cancer studies the focus of our work was the case of pancreatic cancer, where we demonstrated that the zebrafish can be used as a tool to quickly determine genes involved in formation and development of this type of cancer.

Chapter 2 shows how the zebrafish is a versatile and resilient model. Not many animals can survive for long periods under constant chronic hypoxia. Our research demonstrated that zebrafish under these stressful conditions can adapt

and survive. This results in the up- and down-regulation of several genes, as well as physiological and morphological changes. Understanding how organisms adapt to hypoxia can be fundamental in cancer research. Cancer cells are capable of withstanding and surviving in very stressful conditions, including low oxygen levels. Matching genes that are regulated under hypoxia can be a new strategy to fight and cure cancer.

Chapter 3 demonstrates how the zebrafish can be used as an animal model for cancer studies. Zebrafish embryos can be obtained in large numbers and can be used for a rapid analysis of metastasis. We showed, in this publication, that human cancer cells are capable of invading new tissues and forming micro metastasis in zebrafish embryos. The quick results that we can obtain by using this model can expedite preliminary analysis of metastatic properties of cells, test gene regulation in cancer cells or evaluate potential anti-cancer drugs.

Chapter 4 is a research study on the role of retinoic acid receptors (RAR) and miR-10a in pancreatic cancer. Using the zebrafish as a model and human pancreatic cancer cells and tissue from primary pancreatic tumors we demonstrate the role of retinoic acid receptor (RAR) antagonists in pancreatic cancer. Our model proposes that retinoic acid stimulates the dimerization of retinoic acid receptors, which in turn bind to retinoic acid elements and trigger increased miR-10a expression. This up-regulation of miR-10a, in turn suppresses HoxB1 and HoxB3, promoting invasion and metastasis.

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