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TESE DE DOUTORAMENTO

HCT116 COLORECTAL AND MCF7 BREAST CANCER CELL LINES XENOGRAFTED INTO ZEBRAFISH EMBRYOS GIVE INSIGHT INTO THE IMPORTANCE OF MICROENVIRONMENT IN TUMOR GROWTH AND METASTASIS FOR A FUTURE USE OF THE MODEL IN CLINICAL RESEARCH

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ESCOLA DE DOUTORAMENTO INTERNACIONAL

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DECLARACIÓN DO AUTOR/A DA TESE

HCT116 colorectal and MCF7 breast cancer cell lines xenografted into zebrafish embryos give insight into the importance of microenvironment in tumor growth and metastasis for a future use of the model in clinical research

D./Dna. Pablo Cabezas Sáinz

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COMPETING INTERESTS

The PhD student declare that he has no competing interests in relation with the thesis presented.

'Aut viam inveniam aut faciam'

A mi familia

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Me dispongo a ser sincero, de buen comienzo: tras dos arduos meses relatando sobre papel lo que, con anterioridad a llegar a este apartado está escrito, y lo cual espero hayan podido leer y entender en la medida de lo posible; como comprenderán, no es precisamente de mi agrado seguir con la rutina de la escritura sobre pantalla. Pero a pesar de ello, prometo que intentaré plasmar sobre mi pantalla, la cual me ilumina en medio de la noche del despacho, mi gratitud por todas las personas que, de una u otra manera, han acompañado a este doctorando que escribe estas líneas, hacia su transición a Doctor, una vez la defensa a capa y espada se haya efectuado.

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LIST OF ABBREVIATIONS

5-FU	5-Fluorouracil
ADME-Tox	Absorption, distribution, metabolism, excretion and toxicity
ATCC	American type culture collection
ATP	Adenosine triphosphate
BEC	Blood endothelial cells
BM	Bone marrow
CAF	Cancer associated fibroblast
CHT	Caudal hematopoietic tissue
CSC	Cancer stem cell
CSF-1	Macrophage stimulation factor-1
CTC	Circulating tumor cells
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPF	Days post fertilization
DPI	Days post injection
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGF	Epithelial growth factor

EMT	Epithelial–mesenchymal transition
ENU	N-ethyl-N-nitrosourea
FAP	Fibroblast activating protein
FBS	Fetal bovine serum
FET	Fish embryo acute toxicity test
FGF	Fibroblast growth factor
GFP	Green fluorescent protein
GMV	GFP intensity medium value
HPC	Hematopoietic progenitor cells
HPI	Hours post injection
IFN	Interferon
IGF-1	Insulin-like growth factor-1
IL	Interleukin
IQR	Interquartile range
LEC	Lymphatic endothelial cells
LET	Linear energy transfer
MDSC	Myeloid-derived suppressor cells
MET	Mesenchymal-to-epithelial transition
MMP	Metalloproteinase
MSC	Mesenchymal stem cells
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide colorimetric assay
nGFP	Number of GFP pixels
NK	Natural killer

NO	Nitric oxide
OECD	Organization for Economic Cooperation and Development
OXPHOS	Oxidative phosphorylation
PDGF	Platelet-derived growth factor
PDX	Patient derived xenograft
PI	Proliferation index
POU1F1	Homeobox 1 transcription factor
RB	Retinoblastoma protein
RFP	Red fluorescent protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Radiotherapy
SDF-1	Stroma cell-derived factor-1
SDTW	Salt dechlorinate tap water
sgRNA	Single guide RNA
TAM	Tumor associated macrophages
TEM	Tie2 expressing macrophages
TGF-β	Transforming growth factor beta
TME	Tumor microenvironment
TNF	Tumor necrosis factor
VEGFR	Vascular Endothelial Growth Factor Receptor
WHO	World Health Organization

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ABSTRACT

In the last decades, zebrafish has emerged as one of the best tools for modeling different human diseases due to his genetic similarity (75% of orthologous genes), establishing a viable, fast and low-cost platform to test different strategies with the objective of getting a deeper insight into the mechanisms of action that lays underneath the diseases. One of the most studied diseases nowadays is cancer, a heterogeneous disease that constitutes a big deal for the oncologists all over the world, due to his complex behavior that corresponds with individual differences between patients.

For this reason, zebrafish has been used in cancer research by means of the xenograft technique (injection of human cancer cells in embryos or adults of this model organism) since 2005, when Haldi et al. demonstrated that the injection of human cancer cells inside the yolk of the zebrafish embryo was reliable, being these cells able to proliferate. Since then, the number of publications in which people perform xenografts with human cancer cells lines, patient derived xenografts (PDX) or microenvironment components is constantly rising. The objective of this technique is to simulate a human-like microenvironment inside a model organism in which cancer cells from the patient could be able to proliferate, migrate and metastasize in different places providing a valuable tool for testing chemotherapeutic drugs with the objective of treating cancer patients with a more personalized approach, spending less time and costs and reaching more efficiency than the 'mouse avatars'. This technology could be implemented in the hospitals all over the world to help the oncologists to make a better decision based on more robust data.

As long as the xenograft technique has been implemented in zebrafish, mentioned before, there have been improvements to this technique and its experimental conditions. Nevertheless, there are some bottle necks that should be addressed with the objective of getting a more robust and informative technique in order to establish it in a near future in the personalized medicine field. One of the disadvantages of the xenograft technique is the one related to the incubation temperature of the embryos, trying to get a balance between their normal developmental temperature and the proper temperature of the injected cells. On the other hand, a more accurate and faster software for image analysis is required to track and quantify the injected cells in a more efficient way. Finally, it is important to be able to mimic the tumor microenvironment inside of the embryo by co-injecting different tumor components like macrophages or fibroblasts to determine how they behave against some therapies and, in this way, better reproduce the native state of the tumor inside the human body.

CHAPTER I - GENERAL INTRODUCTION

1. ZEBRAFISH

Zebrafish (*Danio rerio*) (Hamilton, 1822) is a freshwater fish, belonging to the *Danio* genus, which is native from the streams of the southeastern Himalayan region, including India, Pakistan, Bangladesh, Nepal and Myanmar. Zebrafish belongs to the family *Cyprinidae* of the order *Cypriniformes* (McCluskey and Postlethwait, 2015, Stock et al. 2007). Zebrafish owns its name because of its morphology, being fusiform, with horizontal stripes on each side, resembling a zebra. On the one hand, males have torpedo shape, being thinner and smaller; on the other hand, females are larger than the males and have a small white belly, containing the reproductive organs and the eggs (Talwar and Jhingran, 1991). The normal temperature of these fishes ranges between 12ºC and 39ºC in nature, while the best temperature for their development and healthy behavior in controlled conditions is 28'5ºC (Parichy, 2015, Westerfield, 2000).

1.1 TAXONOMY AND DISTRIBUTION IN THE WILD

Zebrafish (*Danio rerio*) belongs to the kingdom *Animalia*, phylum *Chordata*, class *Actinopterygii*, order *Cypriniformes*, family *Cyprinidae*, subfamily *Danionidae*, genus *Danio* and species *D. rerio*. The name of this small fish, *Danio*, derives from the Bengali name '*dhani*', which means 'of the ricefield' (Spence et al., 2008). The genus *Danio*, conforms a wide range of species, being different from zebrafish in pigmentation, size, morphology and behavior. As mentioned before, zebrafish has been described for the first time by Hamilton in 1822, with the name of *Brachydanio rerio*. This name has been used for many

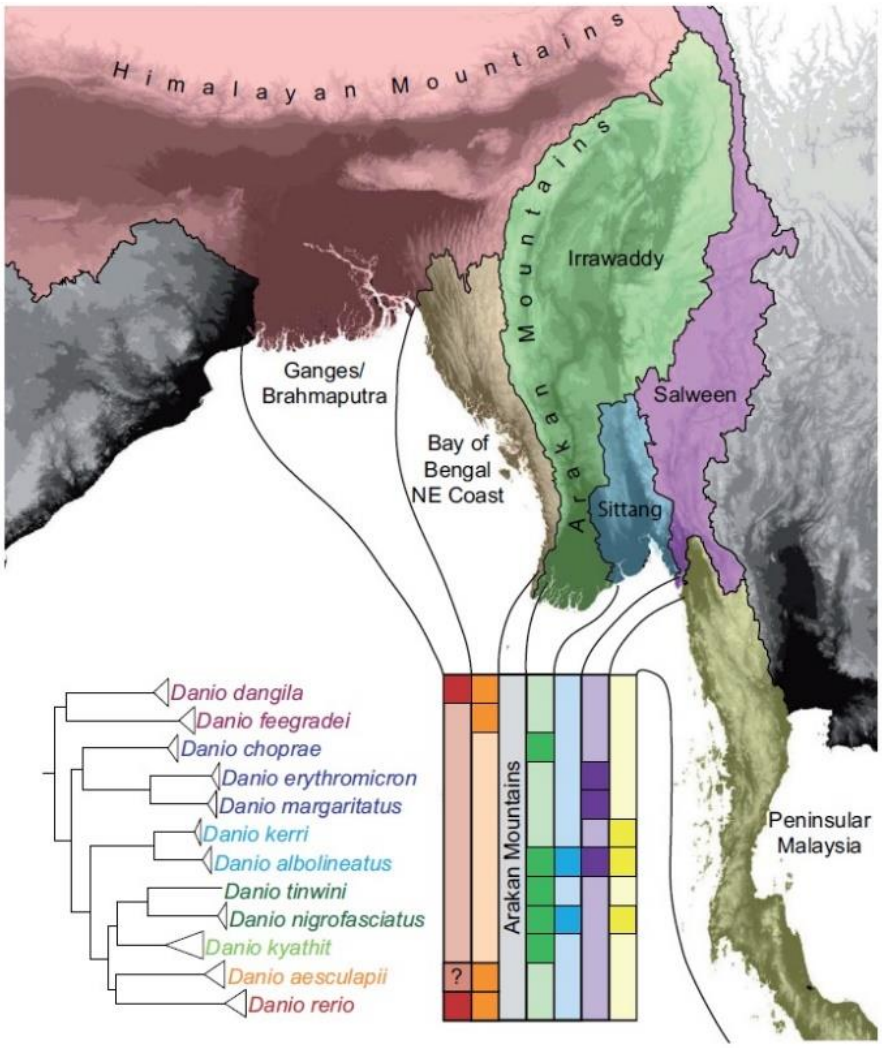

Figure 1. Classification and phylogeography of *Danio* species. *Danio rerio* is mainly located in the west part, south of the Himalayan mountains, in Ganges and Brahmaputra rivers (McCluskey and Postlethwait, 2015. With permission of Oxford University Press).

years and appears in the principal manuals for zebrafish all over the world (Westerfield, 2000). Recently, some studies were published giving the right name to the zebrafish, *Danio rerio*, and conforming a phylogenetic tree where the species most related to the zebrafish is

Danio aesculapii (Fig. 1) (McCluskey and Postlethwait, 2015). The biogeography locations of the *Danio* species are found across southeast Asia, each of the species normally belonging to one specific hydrological base. In the case of *Danio rerio*, the range of locations is wider, distributed all over India and the Ganges/Brahmaputra rivers, in the southern part of the Himalayan mountains (Fig. 1) and covers a range of altitudes from sea level to 1000m (McCluskey and Postlethwait, 2015).

1.2 DESCRIPTION

Zebrafish is a small fish, usually not surpassing the 40mm of length. They have a fusiform and lateral compressed body, with the mouth pointing upwards and the lower jaw being more protruded than the upper one, in order to feed themselves in the water surface. In zebrafish, there are three types of cells responsible for the different color patterns: dark blue melanophores, gold xantophores and iridescent iridophores (Spence et al., 2008).

Figure 2. Zebrafish dimorphisms. (A) Male zebrafish with a yellow coloration around the pectoral and anal fin. (B) Female zebrafish, with rounded body and a white belly (adapted from Parichy, 2015. From eLife, Creative Commons License).

Table 1. Zebrafish International Resource Center (ZIRC) recognized wild-type lines for zebrafish

Name	Description
AB	Derived from two lines purchased by George Streisinger from a pet shop in Albany, Oregon, USA in the late 1970s. The currently used line *AB was derived from the original AB line in 1991–1992 by parthenogenesis
AB/Tuibingen	An 'official' line maintained as a cross but the term is also applied to crosses where the two parental lines are maintained separately
C32	Derived from laboratory strains at Oregon. The current C32bc9 stock is a derivative of Steve Johnson's inbred C32
Cologne	Isolated at the Reugels/Campos-Ortega Laboratory, University of Cologne, Germany
Darjeeling	Collected in Darjeeling in 1987 and sent to Monte Westerfield at Oregon. A much faster swimmer than other wild-type strains. Used extensively for mapping as it contains many polymorphic markers
Ekkwill (EKW)	From Ekkwill breeders in Florida and maintained in Grunwald laboratory, University of Utah, USA
Hong Kong	Stock obtained from a Hong Kong fish dealer
HK/AB	Hybrid of Hong Kong and AB wild-type lines
HK/Sing	Hybrid of Hong Kong and Singapore wild-type lines
India	Stock obtained from expedition to Darjeeling, India (wild isolate)
Indonesia	Stock obtained from Indonesian fish dealer
Nadia	Wild-caught about 40 miles east of Calcutta, India. The fish were collected from stagnant ponds and flood plains. Imported in 1999 by a wholesaler in Oregon. Established in the Oregon laboratory from an initial breeding of about ten individuals
Singapore	Stock obtained from Singapore fish dealer
SjA	sjA is an inbred line of *AB isolated at the Stephen L. Johnson Laboratory, Washington University Medical School, USA. Unlike *AB, which is bred to retain polymorphisms, this subline is bred to reduce polymorphism and is at least 85% monomorphic.
SJD	Isolated at the Stephen L. Johnson Laboratory
Tuibingen (TU)	Wild-type short fins. Strain used by Sanger for the zebrafish sequencing project. It was cleaned up to remove embryonic lethal mutations from the background before being used for mutagenesis and sequencing
Tübingen long fin (TL)	Homozygous for *la*^{tl1}, a recessive mutation causing spotting in adult fish, and *lof*^{tl2} a dominant homozygous viable mutation causing long fins. This is not the line used in the Sanger zebrafish sequencing project. It is genetically different from TU because it was bred differently and not "cleaned up," and therefore retains a lot of polymorphisms
WIK	Derived from wild catch in India and used for genome mapping

These pigments, specially the melanophores can be redistributed in the body due to stimuli from the environment and work as a camouflage for them and, on the other hand, signaling, being darker when they adopt an aggressive behavior. Zebrafish adults have a sex dimorphism between males and females with differential morphologies for each gender (Fig. 2). Zebrafish females has a big white belly, with a more rounded form than the males, which form is thinner and fusiform, without the belly carrying the eggs. Apart from that, regarding the color patterns, the males have larger anal fins with yellow coloration (Spence et al., 2008).

There are different strains of zebrafish in the wild, but the most important ones for researchers are the ones bred in laboratories, cleaned up of deleterious mutations as they try to generate a stable genetic background (Tab. 1). A big number of color patterns exist in zebrafish, regarding the pigmentation and distribution. For example, some zebrafish shows spotted color patters, called 'leopard' phenotype. At the beginning, these zebrafish have been classified in another group, nevertheless, is only a mutation of the same species (Spence et al., 2008).

Zebrafish embryos from embryonic to the larval stage have a different morphology compared to adult individuals. They are transparent during all this period ranging from the 2dpf (days post fertilization) to 14dpf, allowing the researchers to perform different techniques, from developmental genetics and biology, to biomedical assays performing xenograft of human cells and follow them over time inside the embryos (Kirchberguer et al., 2017).

1.3 LABORATORY LIFE CYCLE

Although zebrafish in the wild behaves in a different manner (breeding occurs mostly during summer), under laboratory conditions the spawn of descendants takes place all over the year. At first, males approach the females, attracted by the pheromones secreted in the water. Next, females should lead the male to the ovoposition site and the male starts to quiver against the female belly and tail in order to lay the eggs and fertilize them (Parichy, 2015). Once the breeding takes

place, the eggs get to the ground and start the cell division, passing through the blastula and gastrula stages, overcoming the organogenesis of the embryo and hatching from the chorion at 2dpf.

This is an important development stage of the embryos, because at this stage they are used for most of the xenotransplantation assays (Haldi et al. 2006; Nicoli and Presta, 2007). After hatching from the egg, the embryos will swim as a free larva and they will start feeding themselves between 4-6dpf. Between 12dpf and 14dpf the larva will suffer a metamorphosis with a high mortality rate of the embryos, transforming themselves into a juvenile and becoming adults (Fig. 3) (Willemsen et al. 2011, Howe et al., 2013a).

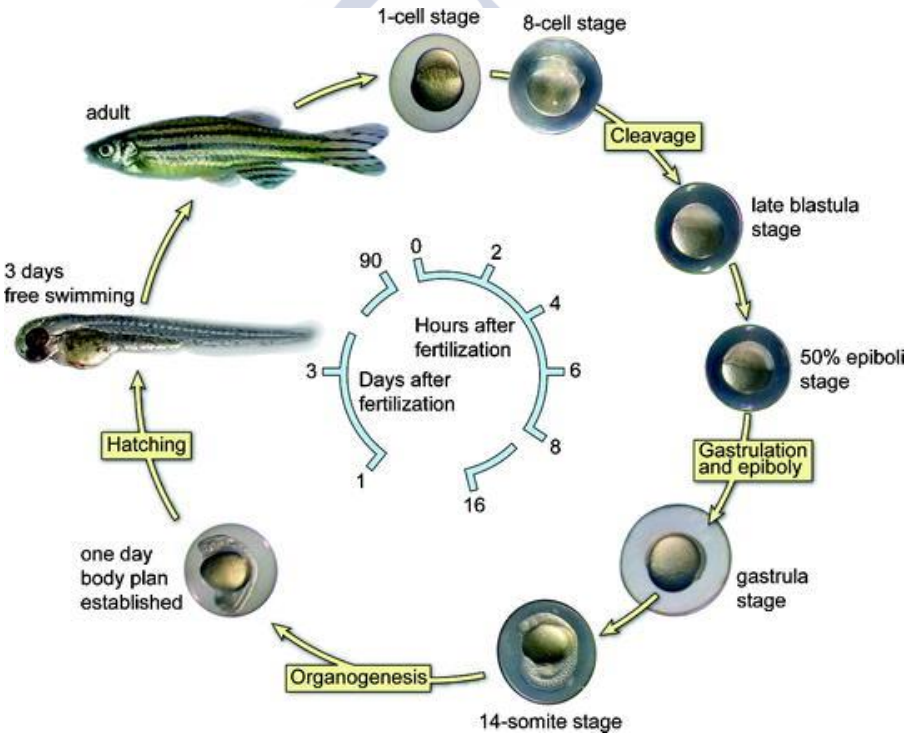

Figure 3. Life cycle of zebrafish (Willemsen et al., 2011. With permission of Springer Nature).

1.4 USE OF ZEBRAFISH FOR BIOMEDICAL STUDIES

Apart from being a very common aquaria fish and, therefore, being sold for domestic use, this fish performs an important role in the laboratories all around the world as an animal model for different types of research, from development to human diseases (Kirchberguer et al., 2017).

Zebrafish have been used since the decade of 1960s for studying developmental genetics. Since then, zebrafish has become a highly used model in developmental biology and vertebrate genetics (Streisinger et al. 1981).

1.4.1 Cancer

Near three decades later, in 2005, the first xenotransplantation assay was performed using de-differentiated human melanoma cells injected in blastula stage of the embryos, demonstrating the capacity of these human tumor cells to survive, migrate and divide inside the embryos (Lee et al., 2005). Besides that, they tested the capacity of human melanocytes to migrate to specific microenvironments, in this case the skin, showing that the human cells can be compatible with the embryos and their niches. Therefore, in this paper the authors established for the first time the xenotransplantation of human cancer cells into a zebrafish, highlighting the capacity of this fish to become a valuable tool in biomedical research of human cancer, apart from its previous use in developmental genetics. These findings were supported in 2006 by another group (Haldi et al., 2006) using also melanoma cells and demonstrating that angiogenesis occurs inside the yolk of the zebrafish embryo towards the implanted cells. The main focus in cancer research, apart from using zebrafish in xenograft approaches, is to use them in genetic approaches as well (Fig. 4).

Even this, the use of zebrafish as a disease model suffered an increase in 2013, due to the fact that the zebrafish genome was published (Howe et al., 2013b), featuring the similarity between human and zebrafish, sharing >80% of disease related genes. Taking this into account, zebrafish has arisen as a model for human diseases in many fields of research, the most important being the following ones:

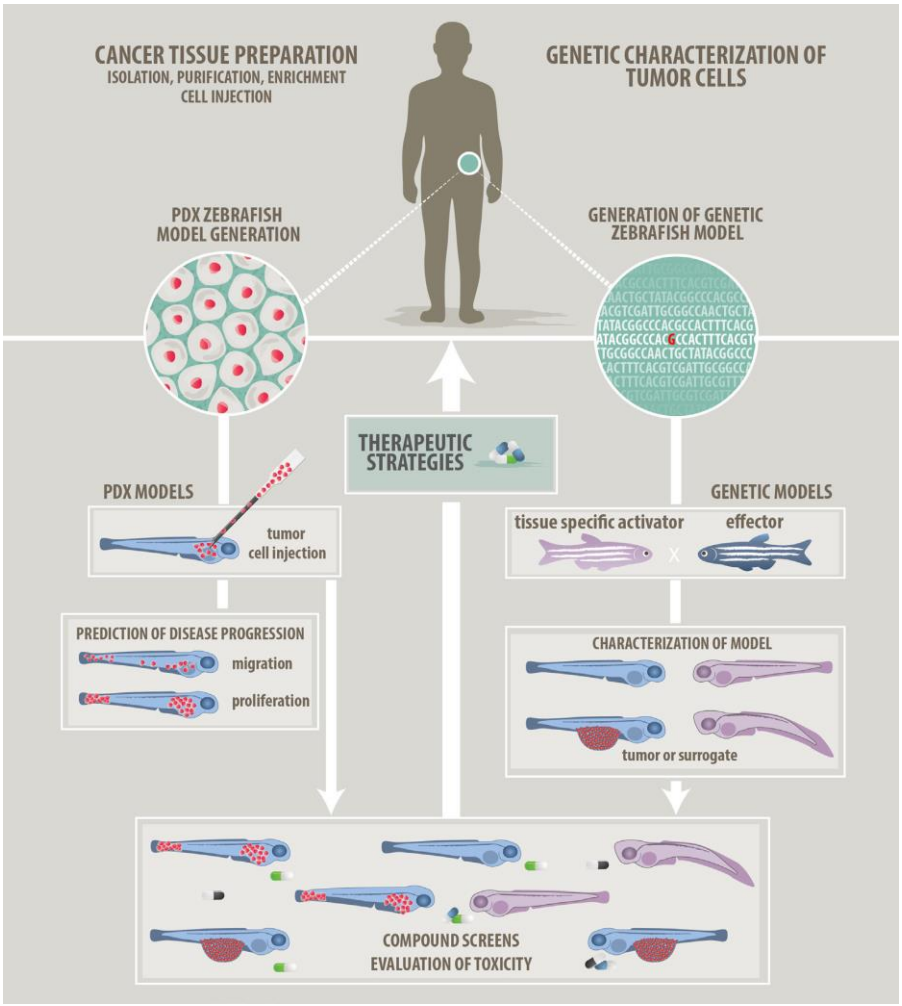

Figure 4. Applications of zebrafish for cancer modeling (Kirchberguer et al., 2017. From Frontiers in Oncology, Open Access).

1.4.2 Drug discovery and toxicity

This field of research, directly related to cancer, includes the analysis of chemic compounds that could be active against specific types of cancer cells and their toxicity in whole organisms like zebrafish embryos. This lead to the possibility of analyzing from the phenotype

of the embryos to organ-specific toxicity or metabolism changes (Fig. 5) (MacRae and Peterson, 2015).

In the last years, drug discovery has been based on target-specific approaches, although the traditional way of measuring and checking toxicity was the phenotypic-based screening (screening with a whole complex organism is necessary, involving different metabolic and genomic pathways, mimicking a native biological context) (Wiley et al., 2017). The problem with the target-based approach is the necessity of the previous identification of the target to develop the drug affecting a specific organ or metabolic pathway, and then test the toxicity with *in vitro* and *in vivo* models. The phenotypic-based screening of compounds has clear advantages against the target-based ones: these assays do not need a validated target to test them, they can identify compounds that are able to produce a desired effect via the action over multiple targets. Assaying compounds in a whole organism gives the researchers the possibility of testing *in vivo* the effects over different biological processes like pain, metastasis, vascular malformations, etc. (MacRae and Peterson, 2015). For all of these reasons, using a phenotypic-based screening of compounds in combination with specific targets yields higher quality results.

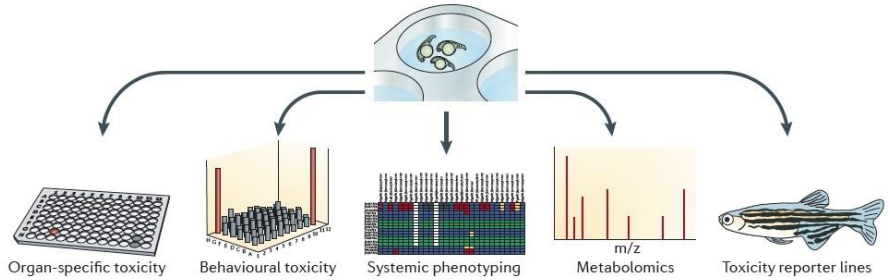

Figure 5. The different uses of zebrafish embryos and adults in drug screening and toxicology (MacRae and Peterson, 2015. With permission of Springer Nature).

In whole organisms like zebrafish embryos; absorption, distribution, metabolism, excretion and toxicity (ADME-Tox) can be tested, whether in cell culture it is not possible. Zebrafish larvae have developed functional organs and the drugs can be absorbed,

metabolized and excreted. Due to this capacity, a high number of compounds can be easily and rapidly tested in zebrafish to follow the next step in mammals (Goldstone et al., 2010; Li et al., 2011).

1.4.3 Modeling human genetic diseases

Zebrafish has greatly evolved on the field of genetic manipulation of specific genes to study the consequences of human diseases in a model organism. On one hand, mutagenesis via ENU (N-ethyl-N-nitrosourea) has been the preferred choice over radiation (Wyatt et al., 2015). Nevertheless, this technique has some drawbacks regarding the identification of the site of mutagenesis and the affected gene, because the mutations generated via this technique of chemical mutagenesis are random. Apart from that, the duplication suffered by the ancestor of zebrafish 200 million years ago, gave these fishes two paralogues for many genes, but in mammals being a single orthologue, so the mutation in one of the paralogues does not need to affect the expression, suffering compensations from the other copies (Santini et al., 2009).

On the other hand, there are different types of techniques that gained relevance on modeling human genetic diseases or alterations by knocking-down these genes responsible for the diseases in zebrafish and depending on the duration of the alteration of gene expression, are classified in transient or permanent (Fig. 6):

-Morpholino oligonucleotides (also called antisense oligos) are a synthetic RNA where the deoxyribose ring is replaced with a morpholine ring, having water solubility, immune to nucleases and low production costs associated (Summerton and Weller, 1997). The main function of the antisense morpholinos is to decrease the gene expression of the gene of interest when we inject them into fertilized zebrafish eggs by either: splice blocking (inhibiting the spliceosome components and impeding pre-mRNA processing) or translational blocking (binding to a complementary RNA sequence near the translational start and blocking the ribosome union) (Nasevicius and Ekker, 2000; Bill et al., 2009). This is an easy technique due to the fact that zebrafish eggs in zygote state are robust, large to microinject them, transparent and as stated in the life cycle section, is easy to obtain a large number of

offspring every day to perform injections at single cell stage. The effect of morpholinos is known because is the most used form of transitory knockdown or an incomplete form of knockdown because they work most efficiently during the first 2 days after injection (Kimmel et al., 2003; Bill et al., 2008).

-Recently, CRISPR technique has emerged providing solutions for fast and more efficient genome editing. The origin of CRISPR is related to bacteria, which use this method as a defense for foreign DNA. Bacteria use RNA strands to guide the CRISPR associated 9 (Cas9) nuclease in order to cut or cleave the foreign DNA (Wyatt et al., 2015). The potential of CRISPR/Cas9 is the customization to target the required sequence, because the specificity is defined by small RNAs. From bacteria, this technique could be adapted to eukaryotic organisms by adding nuclear localization signals for Cas9 and simplifying the guide RNA into a single guide RNA (sgRNA) (Jinek et al., 2012). Although this is a new system, CRISPR has been used in human cells (Cong et al., 2013; Li et al., 2013 a,b,c; Wang et al., 2013) and even *in vivo* with the zebrafish model (Hruscha et al., 2013; Hwang et al., 2013 a,b; Jao et al., 2013), showing that this system is compatible across species.

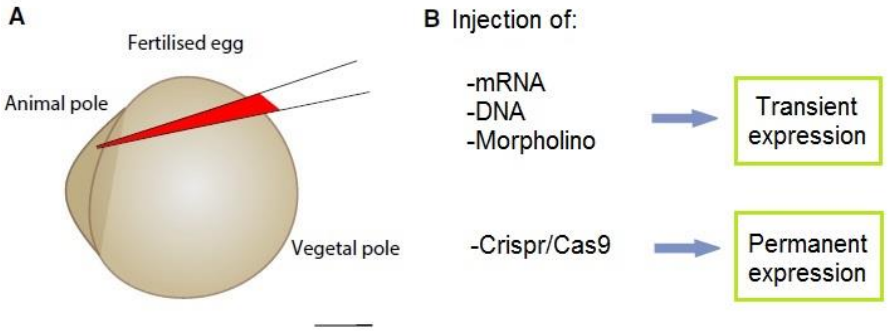

Figure 6. Techniques used for modifying the gene expression via transient or permanent alteration. (A) Representation of microinjection using a glass needle with a capillary inside on the animal pole of a zebrafish zygote. (B) Different molecules injected to achieve a transient (mRNA, DNA and morpholino) or permanent gene expression alteration (CRISPR). Scale bar in A is 250µm (adapted from Wyatt et al., 2015. With permission of John Wiley and Sons).

2. CANCER

Cancer is, by definition, a de-regulation of cell cycle leading to an abnormal cell growth with specific characteristics that provide those cells with the capacity of infiltrate blood torrent and spread to other parts of the host (Munkley and Elliot, 2016). Primary tumors are not the main cause of death of cancer, being the formation of secondary tumor masses in other parts of the individual, called metastasis, the responsible of the deterioration of the patient health, that often leads to death (Liu et al., 2017).

The six main hallmarks of cancer disease could be summarized in: 1) uncontrollable cell growth without proper growth signals, 2) evasion of opposite growth signals, 3) capacity to avoid cell death, 4) limitless divisions, 5) secretion of factors promoting vessel growth in order to provide the tumor with enough nutrients to keep growing, 6) capacity of invading another tissues being transported by the blood torrent inside the body (Fig. 7). In the last years two hallmarks were added to these: 7) reprogram of energy metabolism, 8) evasion of immune system (Hanahan and Weinberg, 2011).

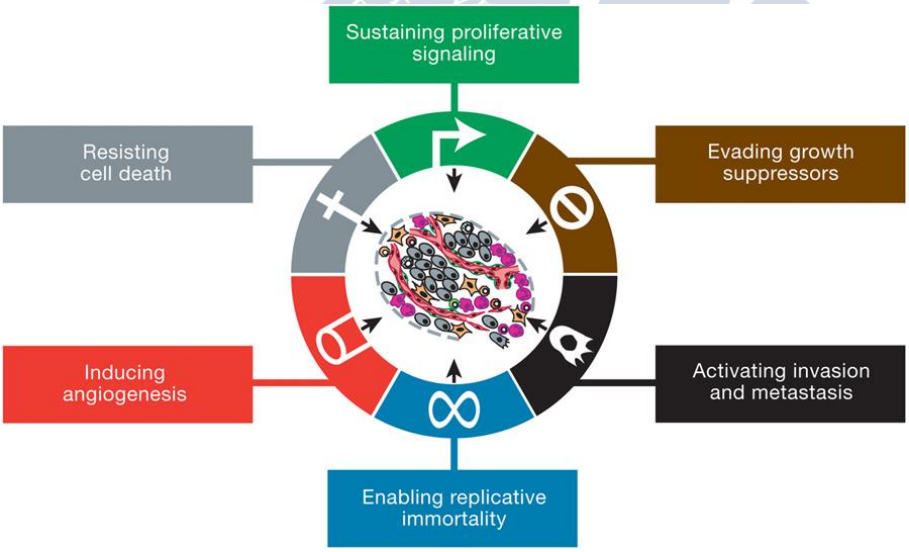

Figure 7. Hallmarks of cancer (Hanahan and Weinberg, 2011. With permission of Elsevier).

Cancer is one of the diseases with highest mortality in the developed countries (Siegel et al., 2016). Most people consider cancer as one disease, being the reality far from this assumption. Every type of cancer is different (e.g. breast and colon cancer), and even among different individual the cancer could be different due to the genetic differences between persons and the different mutations arising in cancer cells in each patient (Özdemir and Dotto, 2017).

2.1 HALLMARKS OF CANCER

Cancer mechanisms of action can be divided into six hallmarks (Fig. 7) and in the last years they have increased to eight hallmarks, adding two new ones due to the contribution of research groups to cancer knowledge and insights:

2.1.1 Sustained cell growth signaling

All of the tissues present in the body are controlled by growth-promoting signals produced by the cells and maintaining the homeostasis and normal architecture. On the contrary, cancer cells are able to avoid this homeostasis and maintain an abnormal growth by de-regulating these growth-promoting signals and achieving a sustained cell growth over time (Munkley and Elliot, 2016). This property of cancer cells could affect other hallmarks of cancer related to cell-biological properties, such as cell viability and energetic metabolism by the uptake of glucose and amino acids, such as glucosamine to support the survival and biosynthesis of new cells (Pavlova and Thompson, 2016). In order to obtain the capacity of de-regulating these growth signals, cells can follow multiple pathways: via autocrine signaling to perform a positive-feedback or modifying the surrounding stroma cells (e.g. fibroblasts) to secrete fibroblast growth factor (FGF), capable of stimulate the growth and proliferation of the tumor cells via paracrine signaling (Cheng et al., 2008; Bhowmick et al., 2004).

2.1.2 Unaffected by anti-proliferation signaling

Besides the capacity of de-regulation of growth factors to obtain the sustained cell growth over time, the cells need to avoid other type of signals: negative cell-proliferation signals (Deshpande et al., 2005).

Most of the negative signals for cell proliferation are sent by tumor suppressor genes, being the most important and studied the retinoblastoma protein (RB) and P53, which regulates the decisions of the cells to proliferate or enter into a senescence/apoptotic state. These genes were discovered by studying different type of tumors and confirming that these genes were inactivated or down-regulated across all tumors (Burkhart and Sage, 2008). Nevertheless, another way of inhibition exists, provided by the surrounding cells contact. Contact-inhibition has been studied *in vitro* by normal cells forming monolayers and inhibiting growth when they reach a full confluence, but not occurring in the same way with cancer cells (Curto et al., 2007).

2.1.3 Cell-death resistance

Cell-death resistance is another characteristic of cancer cells that allows these cells to escape the programmed cell death by apoptosis once they enter the tumorigenic state. These mechanisms of action are triggered when the cells suffer physiologic stress conditions and the cells enter the tumorigenic transition (Hanahan and Weinberg, 2011). The caspases, intracellular proteases, carry out the apoptosis processes in the cell (Adams, 2003). There are two main pathways for apoptosis by the caspases to prevent the cancer cells to progress, being triggered by different factors: A) Stress response, 'mitochondrial' or 'intrinsic' pathway: regulated by Bcl-2 family, which is the result of cellular stress, and factors derived from damaged mitochondria that activate the caspase cascade resulting in the cell apoptosis. B) Death receptors or 'extrinsic' pathway: being the result of tumor necrosis factor (TNF) union in the cell surface with these death receptors, triggering the apoptosis of the targeted cell via cleavage of several cellular proteins (Adams and Cory, 2007; Lowe et al., 2004).

2.1.4 Induction of angiogenesis

In relation with the exposed before, tumor cells need to be provided with oxygen, glucose, amino acids and all type of nutrients in order to sustain the cell growth. Due to this fact, another key point in tumor progression is the capacity of angiogenesis induction towards the tumor and supply it with nutrients, apart from removing metabolic wastes and

carbon dioxide (Hanahan and Weinberg, 2011). The angiogenic switch is turned on under specific situations during adulthood, especially when the body suffers a wound and healing processes are ongoing or female reproductive cycling, otherwise, this switch is not activated. In tumorigenic processes, this switch suffers a de-regulation and is turned on permanently, in order to create new vessels towards the tumor to provide the nutrients needed for its proliferation and expansion. Besides that, this process is able to occur even in avascular regions of the body like the cornea, highlighting the capacity of the tumor cells to secrete angiogenesis inducers (Hanahan and Folkman, 1996) (Fig. 8).

2.1.5 Endless replication

Replication of the cells in the body are limited to a determined number of divisions, and the responsible for that are the telomeres, triggering different mechanisms to stop the proliferation of these cells (Ennour-Idrissi et al., 2017). For that reason, there are two barriers or mechanisms to avoid proliferation of the cells: senescence, which maintains the cells in a quiescent and viable state, but inhibits proliferation; and crisis/apoptosis, involving cell death. Normally, cells suffer the first mechanism to avoid proliferation and they become senescence cells, keeping in a quiescent state. But there are other cells that are able to overcome this barrier, most of them entering the apoptosis process to die. Nevertheless, there are still some cells avoiding cell death by apoptosis in rare occasions, generating immortalized cells with an endless replication potential (Childs et al., 2014). Cancer cells are able to overcome these two barriers, considered the crucial mechanisms for anticancer defense, to avoid unlimited proliferation. Cancer cells achieve this state by protecting the ends of telomeres and stopping the trigger of senescence and apoptosis (Méndez-Pertuz et al., 2017).

2.1.6 Invasion capacity and metastasis

Cancer cells have the capacity of colonize distant sites and perform metastasis in organs located far away from the primary tumor (Pantel et al., 2004). Metastasis is a multistep process consisting in a sequence of steps to colonize distant tissues or organs (Fig. 9): from the primary

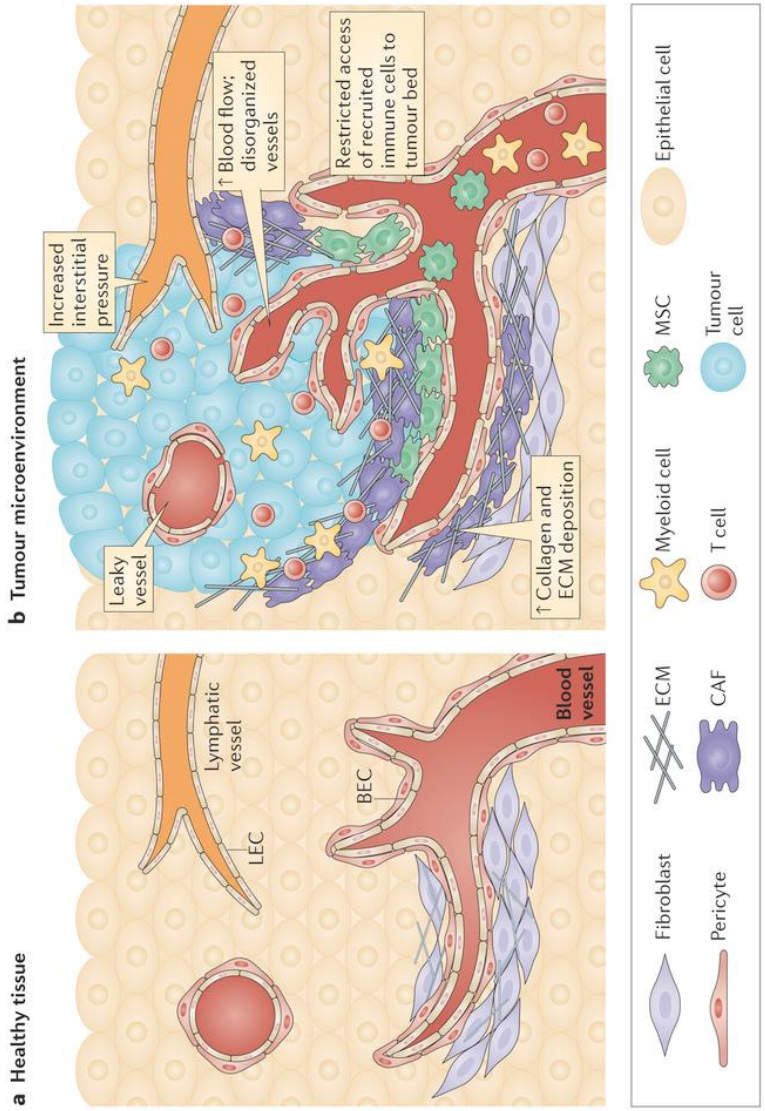

Figure 8. Induced angiogenesis and aberrant vessels. (A) Healthy tissue: epithelial and stromal cells support tissue integrity via growth factors and structural support. (B) Tumor tissue: the overexpression of pro-angiogenic against the anti-angiogenic factors results in a de-regulation and formation of disorganized and leaky vessels in the surroundings of the tumor mass. ECM = extracellular matrix, LEC = lymphatic endothelial cells, BEC = blood endothelial cells, CAF = cancer associated fibroblasts (Turley et al., 2015. With permission of Springer Nature).

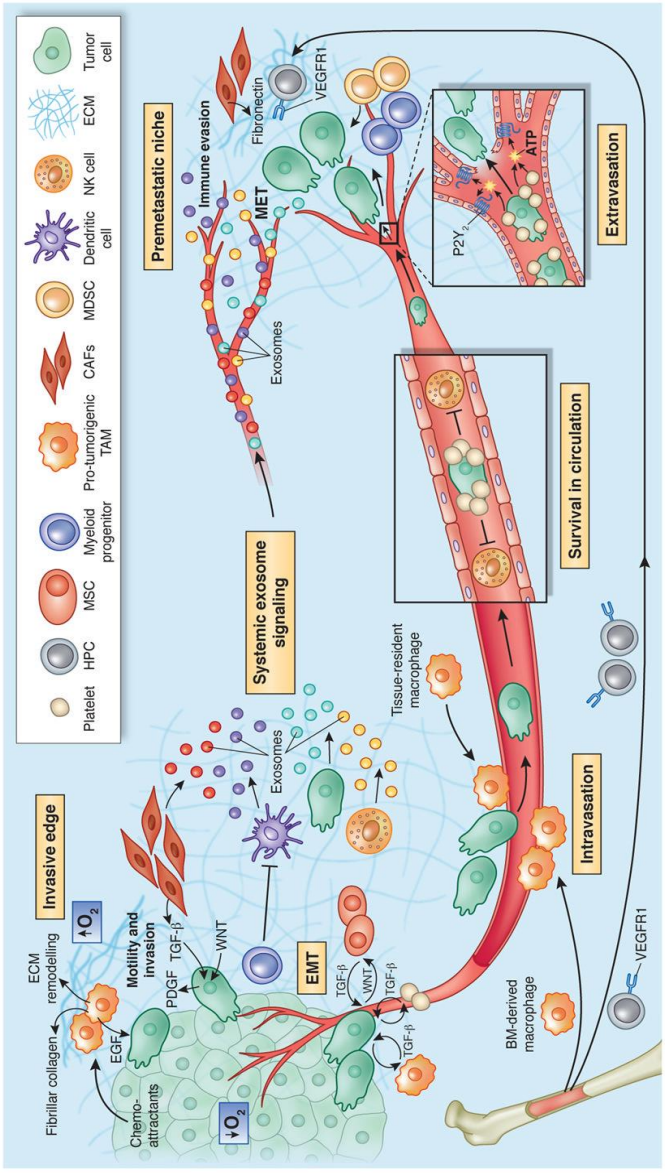

Figure 9. Metastasis process from primary tumor to distant site. ECM = extracellular matrix, EGF = epithelial growth factor, PDGF = platelet-derived growth factor, TGF-β = transforming growth factor beta, EMT = epithelial–mesenchymal transition, BM = bone marrow, VEGFR = vascular endothelial growth factor receptor, MET = mesenchymal-to-epithelial transition (Quail and Joyce, 2013. With permission of Springer Nature).

tumor site, cells need to change their morphology, undergoing epithelial-mesenchymal transition (EMT) process to be able to perform intravasation into the blood torrent or the lymphatic vessels. These cells reach the targeted organ or tissue to suffer an extravasation via mesenchymal-epithelial transition (MET), colonizing and forming micrometastasis (Talmadge and Fidler, 2010). In the last years, this process is getting more attention due to the circulating tumor cells (CTCs) present in the blood that are the main responsible for distant metastasis formation (Massague and Obenauf, 2016).

Apart from these main six hallmarks, another two were added recently (Fig. 10):

2.1.7 Immune evasion

As we have seen in the previous six main hallmarks of cancer, we are still missing one important point, that forms part of the tumor microenvironment: the immune system. In a healthy individual, the immune system is in part responsible of detecting and killing abnormal cells or strange artifacts and represents the main defense barrier against pathogens (Muesnt et al., 2016). Considering this, cancer cells need to be able to avoid the innate and adaptive immune response detection and posterior attack, evading in this way, eradication (Teng et al., 2008). There are some factors that tumors used in their own benefit in order to avoid immune system: regulatory cells (regulatory T cells, called Tregs, and other types of suppressive cells) are one of the main mechanisms of immune suppression in the tumor microenvironment along with immune suppressive mediators (e.g. VEGF, RCAS1); downregulation of tumor antigens that can contribute to tumor proliferation and metastasis because the immune cells are no longer able to recognize the tumor cells by their antigens; and finally they can even evade the immune system by tolerance or immune deviation (shifting from Th1 to Th2) induced by TGF-β and IL-10 (Vinay et al., 2015).

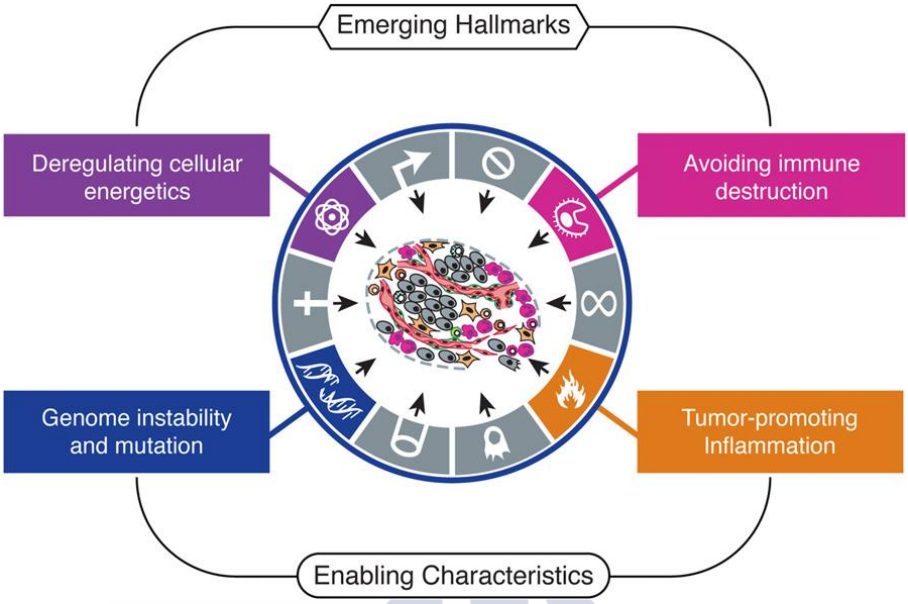

Figure 10. Emerging Hallmarks in cancer (Hanahan and Weinberg, 2011. With permission of Elsevier).

2.1.8 Reprogramming cellular metabolism

Uncontrolled division and proliferation of cancer cells need to be supported by adjusting the energy metabolism of these cells. In normal non-dividing cells of the human body, the oxidative phosphorylation (OXPHOS) strategy is chosen over the glycolysis. Although being counterintuitive, these cancer cells perform the Warburg effect for obtaining energy, and being far less efficient than OXPHOS in the mitochondria, but they compensate this, by upregulating the expression of GLUT1, a glucose transporter to get more glucose inside of the cell. In this way, when there is an excess of glucose in the blood torrent, together with the upregulation of GLUT1 transporters, glycolysis has the potential of producing more ATP in less time than the OXPHOS pathway (Jones and Thompson, 2009; DeBerardinis et al., 2008). In the last years, it has been discovered that the population within a tumor is not homogeneous, indicating that cell subpopulations, with different molecular alterations, exist within the tumor. One of the most important subpopulations favoring the tumor generation and progression are the

cancer stem cells (CSC's). The metabolism of this subpopulation changes and they perform the OXPHOS pathway instead of Warburg effect, showing the plasticity to change the metabolism depending on the circumstances (Sancho et al., 2015).

2.2 CANCER ETIOLOGY

Cancer is a heterogeneous disease that is caused mainly for DNA damage, accumulation of somatic mutations due to the division of the cells, epigenetic events or modifications (Fig. 11) (Luzzatto and Pandolfi, 2015). Events leading to this type of mutations or epigenetic changes produce DNA damage accumulation over time in a cell lineage, being the tumor etiology divided depending on the mutations' origin:

2.2.1 Heredity

Cancer caused by heredity is the less common. Only a small fraction of the cancers nowadays is inheritable. These types of cancer are related to the inheritance of genetic defects that predispose individuals to develop cancer during their lives. Inheritable genetic defects range between defects in the DNA repair genes, oncogenes or genes involved in production of blood vessels (Hodgson, 2008). The most known mutation in heredity cancers are those present in BRCA1 and BRCA2 genes, involved in an increased risk of developing breast cancer of the females carrying these mutations (Roukos, 2009).

2.2.2 Environmental factors

In this area we can find different type of environmental factors, but the most important ones are the following:

2.2.2.1 Chemicals:

The most important chemical or carcinogen that influences cancer developing in first world countries is tobacco. Tobacco is the responsible of 33% of deaths by cancer, producing 85% of lung cancer and a small fraction in passive non-smokers (Warren and Cummings, 2013). Beneath the chemicals, there are materials that can produce cancer such as dioxins, polychlorinated biphenyls and organochlorine pesticides, etc. (Rodgers et al., 2018).

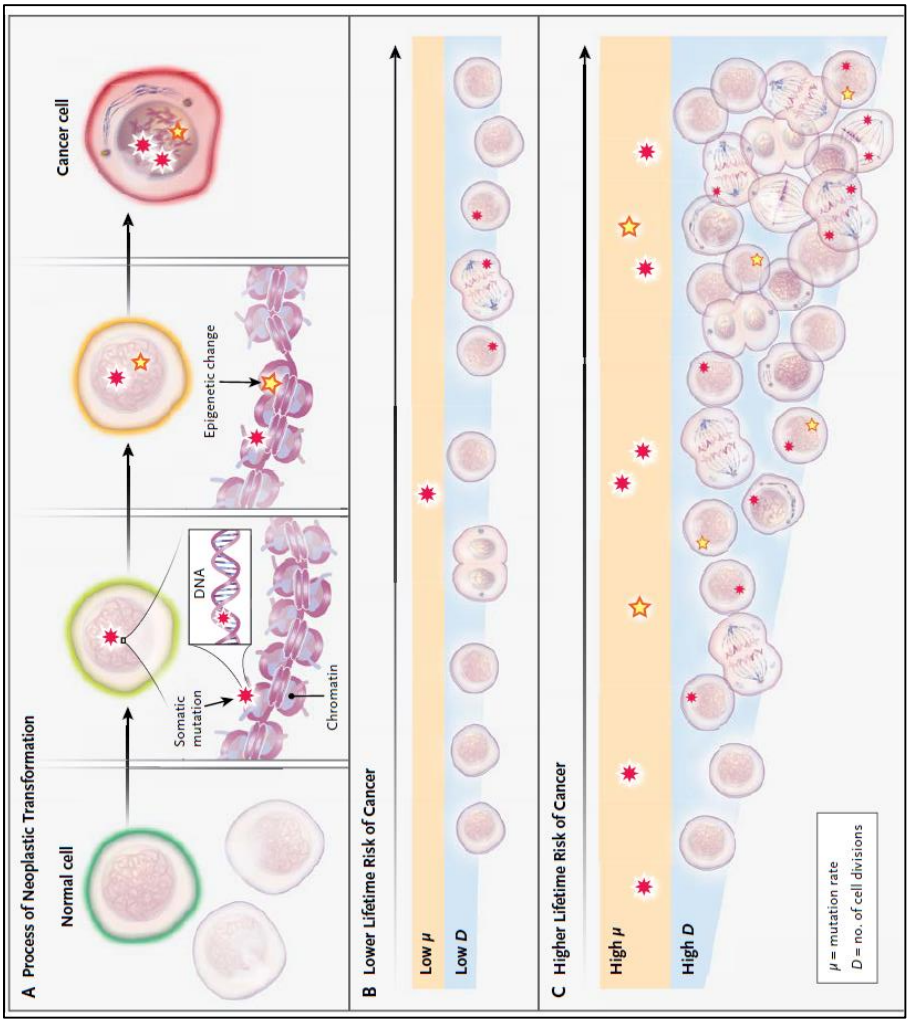

Figure 11. Events that take place during the transformation from a normal cell to a tumor cell. (A) Process of neoplastic transformation. (B) Example of a person in which the mutation rate and the cell division rate are low, leading to a low probability of developing cancer, no accumulating enough mutations in a single cell. (C) Example of a person with a high probability of developing cancer during his life due to the accumulation of mutations or epigenetic changes in the same cell, due to the high mutation and division rate. Red star = somatic mutation. Yellow star = epigenetic event. μ = mutation rate. D = number of cell divisions (Luzzatto and Pandolfi, 2015. Reproduced with permission from Luzzatto and Pandolfi, Copyright Massachusetts Medical Society).

2.2.2.2 Lifestyle

In this section are included some of the main causes of cancer in the developed countries having a direct relationship with the sedentary life of the people. Lack of regular physical exercise and healthy dietary patterns are the main causes directly related with the higher risk of developing cancer due to the increase in obesity (Grosso et al., 2017). Finally, it is important to mention alcohol as one of the underestimated causes of liver cancer in the body (Shi and Zhong, 2017).

2.2.2.3 Infection and inflammation

Some types of cancer have been related to virus infection in certain organs or tissues, representing around the 20% all over the world (Akram et al., 2017). There are several viruses that cause cancer, and they are named 'oncoviruses' (e.g. human papillomavirus, Epstein-Barr virus, hepatitis B and C) (Moore and Chang, 2010). Apart from that, certain bacteria can cause or have been associated to cancer, for example *Helicobacter pylori* (McClain et al., 2017). Finally, chronic inflammation plays an important role in the development and progression of cancer. Chronic inflammation is connected to cancer because of the excessive generation of reactive oxygen species (ROS) that may break DNA strands and incur in DNA damage, apart from silencing DNA repair enzymes. This could lead to a silencing tumor suppressor genes like p53 and activating oncogenes like Kras. Chronic inflammation also plays an important role via infiltration of pro-inflammatory cytokines to attract important factors of tumor microenvironment like tumor associated macrophages (TAMs) (Zhang et al., 2017).

2.2.3 Therapies

Although there are more therapies for cancer treatment, we highlight here the most widely used:

2.2.3.1 Chemotherapy

It has already been proved as a very effective treatment against different types of cancer from the beginning of its use, but with several

secondary effects associated. Chemotherapy helps enormously in the treatment and survival of cancer patients. The development of new chemotherapies started in the 20th century, like the treatment of children with acute leukemia with aminopterin, a folic acid antagonist (Farber and Diamond, 1948). The development of chemotherapeutic compounds or drugs has gone through different phases (Fig. 12):

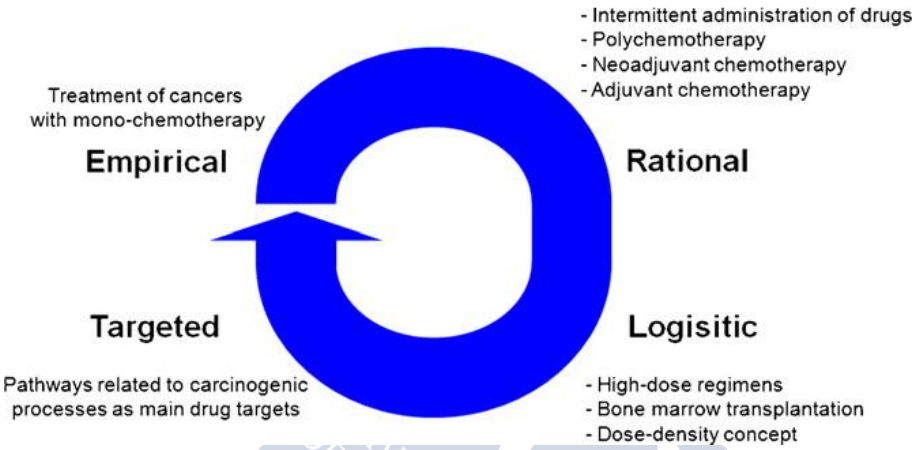

Figure 12. History of anticancer chemotherapy with its phases and milestones (Galmarini et al., 2012. With permission of Elsevier).

2.2.3.1.1 Empirical

At the beginning the attempt on reducing cancer progression was due to coincidences in the treatment of the disease. For example, in the treatment of acute leukemia, folic acid was used and it induces a more rapidly progression of the disease, so researchers decided to try and use antagonist of this folic acid to see if the leukemia progression was reduced, even if the compound itself was very toxic for the human body (Farber and Diamond, 1948).

2.2.3.1.2 Rational

This phase started when in 1953 Watson and Crick published the molecular structure of the nucleic acids (Watson and Crick, 1953). In this way, they gave the opportunity to hypothesize in a rational way the

mechanisms of action of the chemotherapy. Apart from using these antagonists, more compounds were discovered to reduce tumor progression, like antibiotics (bleomycin, anthracyclines…), antimitotics and nucleoside analogs (cytarabine, fluoropyrimidines…). At this phase the main pillars of chemotherapy were assumed, as showed in Fig. 12: intermittent administration of drugs in order for normal tissues to recover from the toxicity of the compounds but not the tumor tissues; use different types of chemotherapy with different mechanisms of action to obtain a synergic effect upon the tumor; and consider the cancer as a systemic disease, combining the radiotherapy and surgery with chemotherapy to obtain better results (Galmarini et al., 2012).

2.2.3.1.3 Logistic

In this phase, another factor was taken into account for the treatment of cancer patients, cell kinetics of the tumor population. In the last phase, chemotherapy was administrated to the patients with a stablished dose, depending on the toxicity of the compounds and in a certain period of time, but in this phase researchers tried to improve the existing chemotherapies and focused on the cytokinetics of the tumors and their growth in mouse models (Watson, 1976).

2.2.3.1.4 Targeted

In this last phase, starting in the late nineties, with the improvements in the knowledge about mechanisms behind cancer generation, chemotherapy focused on a more targeted treatment. The discovery of several oncogenes responsible of tumor progression, dissemination and metastasis, gave rise to a series of compounds that targeted these specific objectives to block certain metabolic pathways and reducing the tumor growth (Galmarini et al., 2012).

2.2.3.2 Radiation

Radiation therapy or radiotherapy (RT), can be classified in radionuclide implants (internal treatment) or linear energy transfer (LET) radiation (external treatment). It consists in radiation of the affected organ or tissue with different sources like gamma or X-rays.

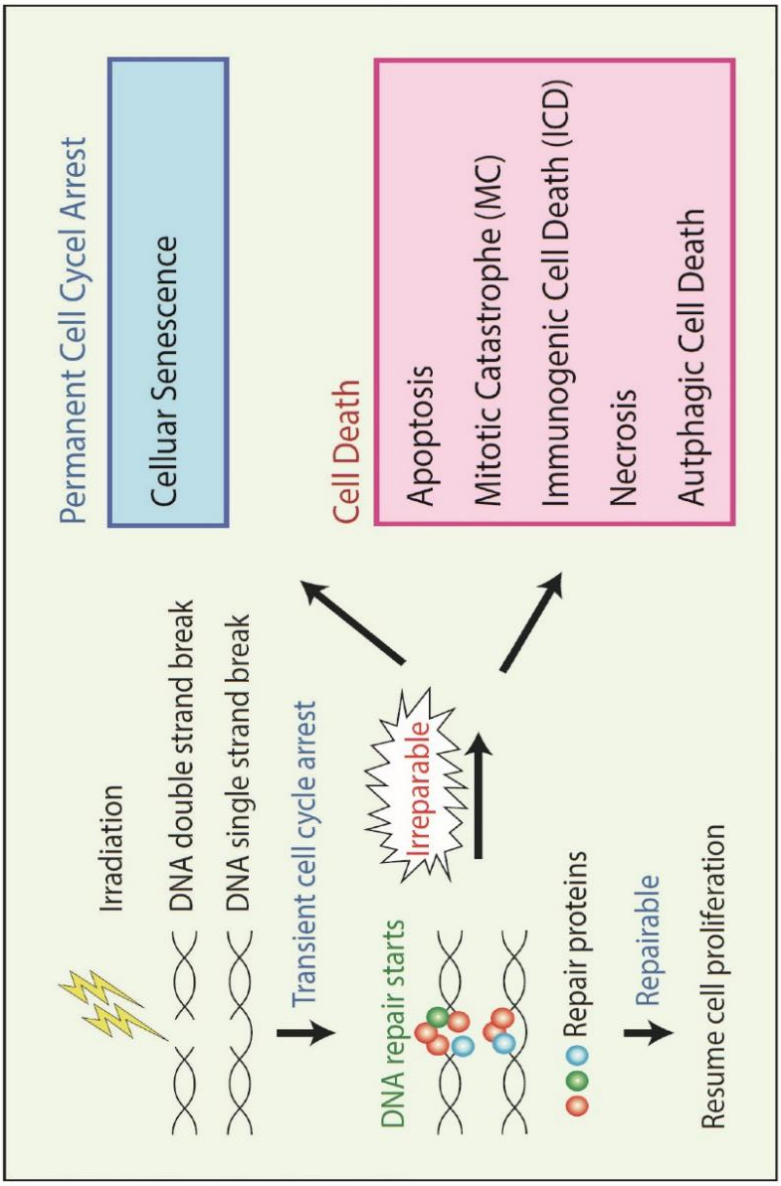

Figure 13. Consequences of radiation upon cells. The irradiation produces a break in double or single strand DNA, leading to a cycle arrest and at this point the repairing mechanisms acts. Cancer cells receiving irreparable DNA damage enter into cell death by different ways (Baskar and Itahana, 2017. From International Journal of Medical Sciences, Creative Commons License).

Charged particles and protons are able to disrupt and break single and double strand DNA to cause an irreparable damage in the cancer cells leading them to death, also affecting tumor microenvironment (Fig. 13).

Because of its low cost, it is one of the therapies most used around the world, with approximately 50% of the cancer patients being treated with radiation (Baskar et al., 2012).

2.3 INCIDENCE OF THE DISEASE AROUND THE WORLD

Cancer is nowadays the second disease responsible of the majority of the deaths worldwide in persons under 70 years old in 91 out of 172 countries in the world according to World Health Organization (WHO) (Fig. 14). The increase in cancer cases around the world and, especially in developed countries, is caused by two main factors: aging of the population and the lifestyle change (Bray et al., 2018). In men, the most common cancer is lung cancer followed by prostate cancer. In women, most common cause of cancer is breast cancer followed by cervical cancer. Besides the risk factors mentioned before, considering the socioeconomic development of some countries and the consequence life expectancy of the population, there are variations between men and women in different countries and even specific incidence of certain types of cancer in specific regions like skin cancers in Australia or New Zealand or colon cancer in Asia. Finally, the mortality due to this disease worldwide is quite different among genders: males have 50% more chance of dying from cancer than women (Bray et al., 2018).

2.4 TUMOR MICROENVIRONMENT

As stated before, cancer is a heterogeneous disease, characterized by the de-regulation of the cells in order to grow without control on the human body and acquiring the capacity of invading distant organs or tissues (Kim, 2005). Since the discover of this abnormal cells, the main focus of the researchers have been the genetic alterations of these cells in order to find the reason related with the de-regulation of the cell cycle. Even this, the acquisition of the capacities to invade, apart from the epigenetic changes that can occur upon the cells and the accumulation of mutations over time are important objectives that needs

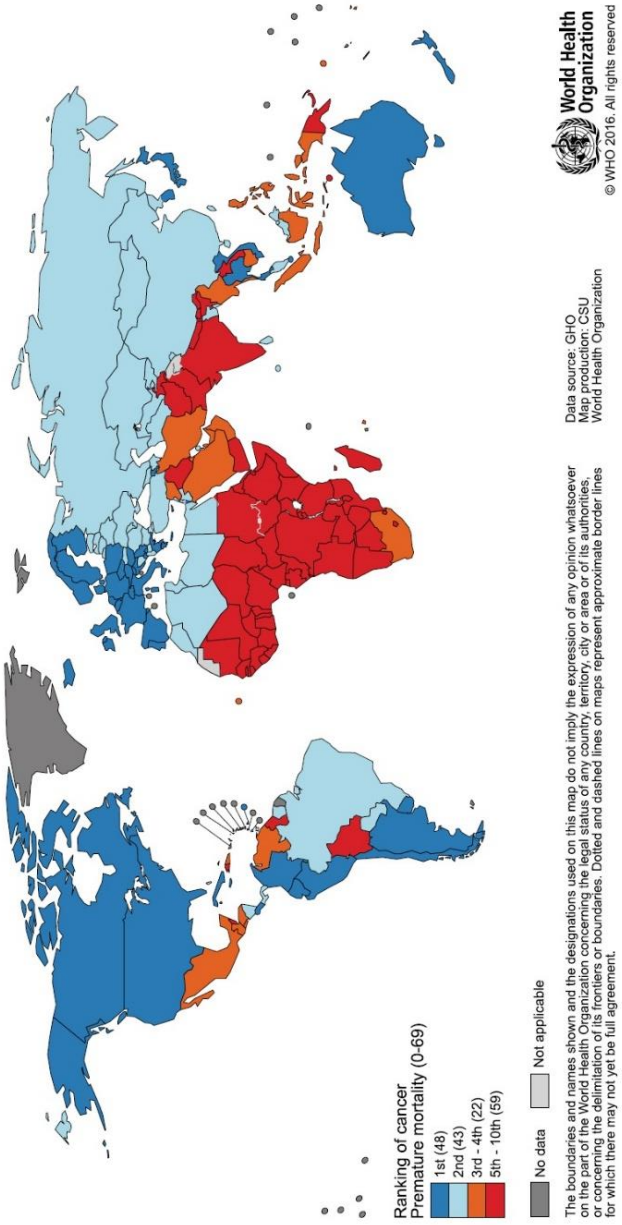

Figure 14. World map presenting cancer as a cause of death in people under 70 years old (Bray et al., 2018. From World Health Organization, Creative Commons).

to be addressed (Stratton et al., 2009).

Nevertheless, nowadays it is known that the surroundings of the tumor, called tumor microenvironment (TME), formed by stroma and different type of cells plays an important role in tumor progression (Fig. 15) (Sounni and Noel, 2013). The host-cell interaction takes places in this tumor microenvironment and modify, in one side, the host cells and, on the other side, the cancer cells. Both cells generate an interaction with cytokines and secrete growth factors, contributing to the control of cell proliferation and migration, facilitating the tumor proliferation in the primary site, dissemination to other parts of the body and their colonization (Kim and Tanner, 2015).

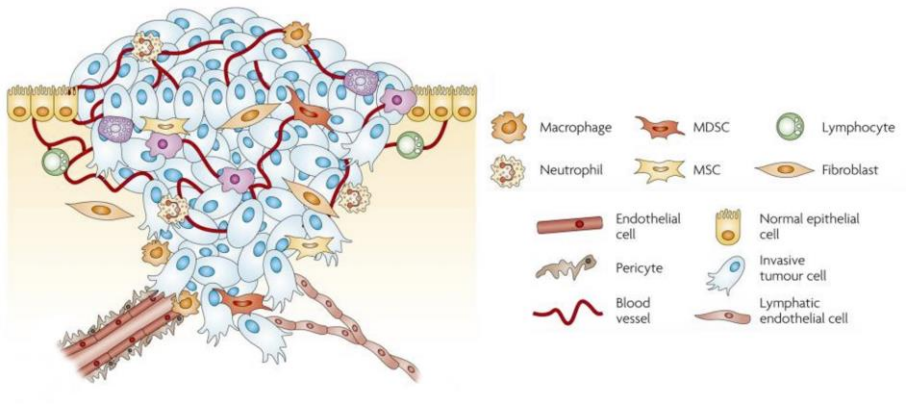

Figure 15. Representation of the tumor microenvironment (TME). Cancer cells of the primary tumor are surrounded by their stroma composed of different cells that can support tumor growth and progression (Adapted from Joyce and Pollard, 2009. With permission of Springer Nature).

2.4.1 Principal cellular components of microenvironment

2.4.1.1 Cancer Associated Fibroblasts (CAFs)

This type of cell is the most abundant in tumor's stroma. When they are activated, expressing different cell surface markers, like fibroblast-activating protein (FAP) (Augsten, 2014; De Veirman et al., 2014), CAFs are able to promote new tumor formation and their proliferation by different ways.

The first mechanism of promoting tumor initiation and proliferation is the secretion of multiple signaling molecules and cytokines: epithelial growth factor (EGF) family, insulin-like growth factor-1 (IGF-1) or stroma cell-derived factor-1 (SDF-1/CXCL12) (Fig. 16) (Orimo et al., 2005; Xing et al., 2010). The second way of contributing to tumor growth is by communicating with cancer cells, modifying the extracellular matrix of the tumor microenvironment to provide support for its constant growth through the production of different types of collagen and fibronectin (Xing et al., 2010; Simian et al., 2001). Besides producing support molecules for tumor growth, another important function is related to the remodeling of the extracellular matrix by degrading enzymes and matrix metalloproteinases (MMPs), contributing to cell migration and invasion (Chaffer and Weinberg, 2011; Cirri and Chiarugi, 2011; Kalluri and Zeisberg, 2006; Pietras and Ostman, 2010). Finally, CAFs can help tumor progression, generating pro-invasive and angiogenic molecules (VEGF and IL-6), increasing the motility and invasiveness of cancer cells (Nagasaki et al., 2014).

2.4.1.2 Endothelial cells and pericytes

Endothelial cells have an important role in the formation of new vasculature from already formed vessels or recruiting bone marrow-derived endothelial progenitor cells (Chouaib et al., 2010). The endothelial cells can produce surface markers that are valuable in the prognosis of cancer such as VEGF factors. All of these factors secreted by endothelial cells play an important role in controlling the leukocyte recruitment, tumor cell behavior, and metastasis (Policastro et al., 2012). On the other hand, pericytes are the responsible for stimulating endothelial cells proliferation, apart from modeling the extracellular matrix and leading the endothelial cells migration (Gee et al., 2003; Sennino et al., 2007; Cooke et al., 2012).

2.4.1.3 Tumor Associated Macrophages (TAMs)

Macrophages are located in almost all organs and they have different capacities and are involved in physiological processes like inflammation and innate immunity, being classified in different

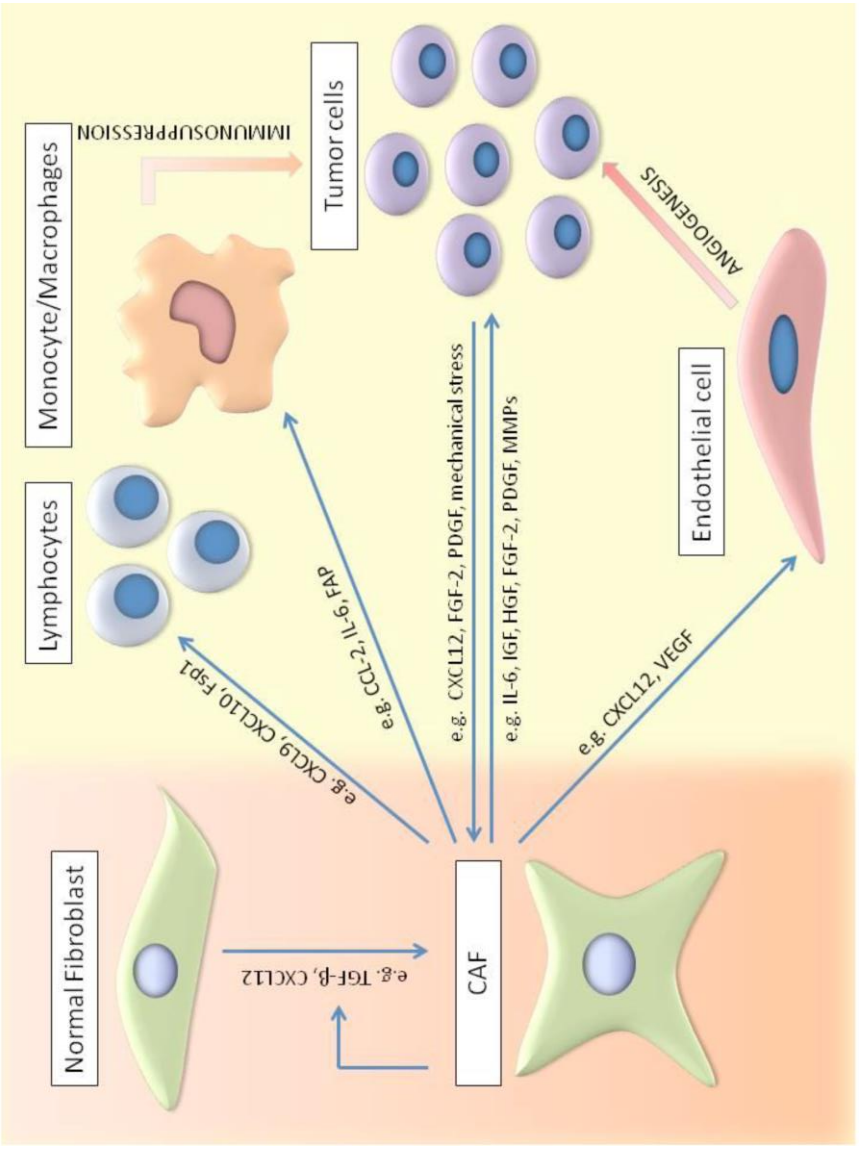

Figure 16. The implication of cancer-associated fibroblasts (CAFs) in tumor growth. Fibroblasts present in the stroma of the host can be stimulated via TGF-ß or cytokines to transform them into CAFs. Tumor cells and CAFs can secrete growth factors and enzymes to promote each other, CAFs modifying the stroma for the progression of tumor cells, and tumor cells converting the normal fibroblasts into CAFs (De Veirman et al., 2014. From MDPI, Creative Commons License).

subpopulations of macrophages (Varol et al., 2015). The classical way of macrophage activation leads to a M1 phenotype with different functions, but there is another way of activation that leads to a M2 phenotype (Fig. 17). Besides this classification, other authors classified the macrophages taking into account their functions: angiogenic, immunosuppressive, invasive, metastasis-associated, perivascular and activated macrophages (Komohara et al., 2016).

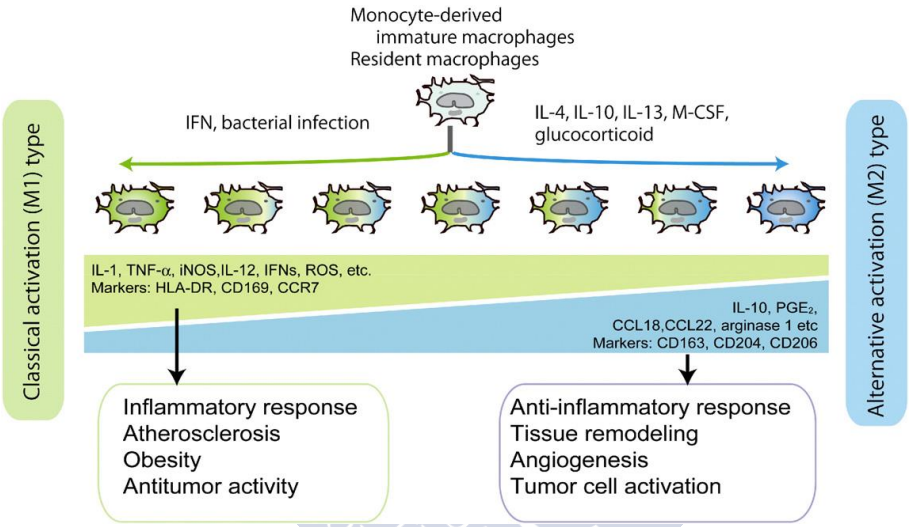

Figure 17. Macrophages heterogeneity. Tumor-associated macrophages (TAMs) are derived from circulating monocytes that are shifted into the M2-like functions and phenotype, in this way contributing to tumor progression by remodeling of tissues, angiogenesis and tumor cells activation (Komohara et al., 2016. With permission of Elsevier).

TAMs derive from circulating monocytes and are the most common cells in tumor microenvironment. The normal function of these M2 macrophages should be the promotion of innate and adaptive immunity and the phagocytosis of death cells. Nevertheless, tumors are able to modulate this activity and shift these M2-like macrophages to help the tumor by promoting cell growth and spread (Brown et al., 2017). Different subsets of TAMs coexist and have a specific activity in this TME: suppression of adaptive immunity, increase the angiogenesis towards the tumor, tumor cell extravasation and invasion via blood vessels (Qian and Pollard, 2010).

Besides, TAMs have different phenotypes to perform different activities within the tumor microenvironment in order to help tumor progression and spreading (Fig. 18), being the following the most important ones:

2.4.1.3.1 Facilitation of metastasis

One of the mechanisms of TAMs to facilitate the invasion of tumor cells involves a paracrine loop. This loop starts with the TAMs producing epidermal growth factor (EGF), increasing the invasiveness of surrounding tumor cells that are able to respond to this factor by the EGF receptor (EGFR). On the other part of the loop, cancer cells express macrophage stimulation factor-1 (CSF-1), being a powerful chemoattractant for the TAMs in possession of the receptor for this signal (CSF1R) (Joyce and Pollard, 2009). In this way, macrophages carry the tumor cells near the blood vessels by remodeling the collagen fibers and once the tumor cell is located near the blood vessel, the TAMs facilitate the intravasation of the tumor cell into the vasculature (Condeelis and Pollard, 2006; Wyckoff et al., 2007).

2.4.1.3.2 Inhibition of the adaptive and innate immunity

TAMs are the responsible for tumor growth by inhibiting immunity with diverse mechanisms. Secretion of molecules like TGF-β, IL-10, arginase-1 or nitric oxide, which act as immune suppressive molecules, blocking T-cell immune response against the tumor antigens (Terabe et al., 2003; Zea et al., 2005; Sica and Bronte, 2007). TGF-β has a direct effect over the conventional CD4+ and CD8+ T-cells that mediates immune response by blocking their stimulation, differentiation, proliferation and effector functions (Sheng et al., 2015; Yoshimura and Muto, 2011). Arginase-1 acts as a blocker for arginine in the conventional T-cells. Those T-cells requires arginine in order to be activated and react against certain antigens, therefore with the arginase-1 acting as a blocker, they lose the capacity of generating immune effector cells (Bronte and Zanovello, 2005; Gallina et al., 2006; Rodriguez and Ochoa, 2008). Nitric oxide (NO) and ROS produced by TAMs, on the other side, has a synergic effect with the arginase-1

against the T-cells, being more efficient than the arginase-1 alone (Brown et al., 2017).

2.4.1.3.3 Pro-angiogenic

The pro-angiogenic capacity of the TAMs is related to specific subpopulations of TAMs in the last side of the M2 spectrum, called the Tie2 expressing macrophages (TEMs). They are located mainly in the perivascular regions of the tumors and it has been demonstrated that they are crucial for vasculature recuperation after treatments (Brown et al., 2017).

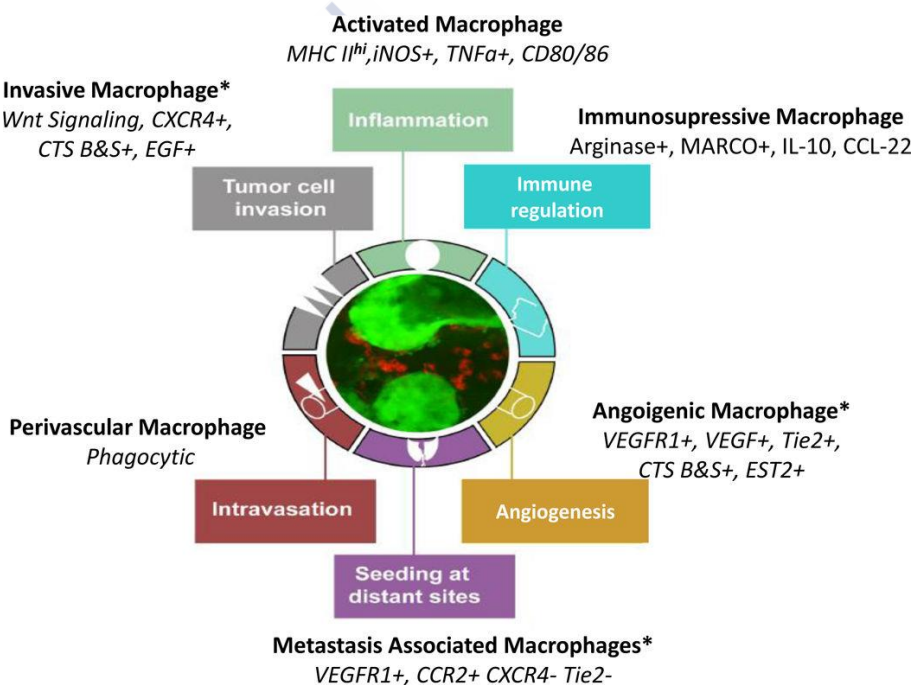

Figure 18. Principal macrophage phenotypes. Classification of macrophages in subpopulations depending on the supporting function provided to the tumor. Each of the subpopulations express different markers in their surface in order to perform their function (Qian and Pollard, 2010. With permission of Elsevier).

3. XENOTRANSPLANTATION TECHNIQUE IN ZEBRAFISH

Xenotransplantation technique in zebrafish, for its use in biomedicine assays, consists on the injection of cancer human cells into different parts of the zebrafish embryos to track their progression, behavior and interaction with the microenvironment of the host (Fig. 19) (Nicoli and Presta, 2007; Nicoli et al., 2007).

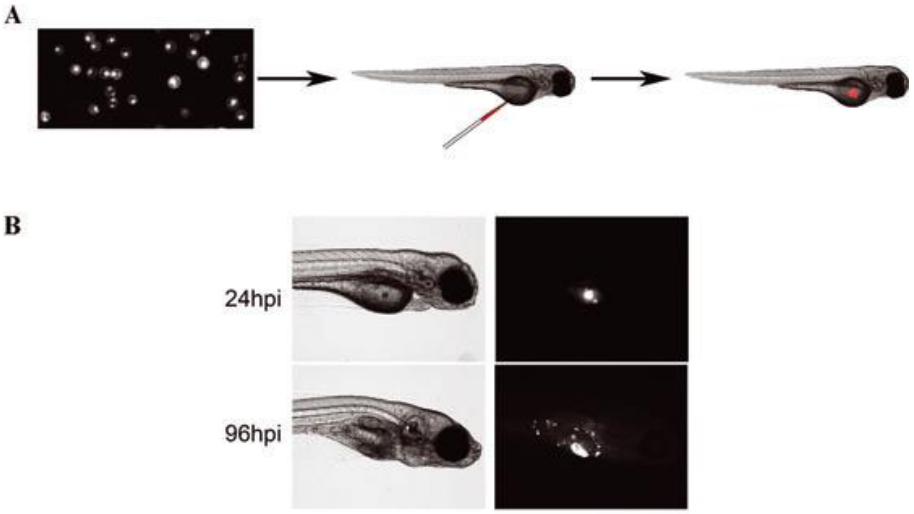

Figure 19. Representation of xenotransplantation procedure in zebrafish embryos. (A) Labelled human cancer cells are loaded in a microneedle to perform the xenograft into the yolk sac of the embryo. (B) Tracking of the cells in real time at 24 hours post injection (hpi) and 96hpi to see their behavior and proliferation (Konantz et al., 2012. With permission of John Wiley and Sons).

The major sites of injection in zebrafish are divided as follows (Fig. 20):

-Yolk sac: the yolk sac of the embryos is an acellular compartment where all the lipids are accumulated to provide the fish with energy for their development until they reach the 5dpf, the moment they are capable of feed themselves (Fraher et al., 2016). Human cancer cells can be injected in the yolk sac of the embryos which provides nutrients for tumor growth. Apart from that, in this compartment of the fish the

cells can be easily visualized post-injection over the days (Veinotte et al., 2014).

-Duct of Cuvier: the injection of the cells directly into the circulation (Duct of Cuvier) allows the researchers to test another stages of the tumor development like mesenchymal-epithelium transition (MET) (Mercatali et al., 2016). Cells injected into the circulation are able to survive, invade and perform extravasation, normally near the caudal hematopoietic tissue (CHT) located in the tail of the embryo and metastasize forming tumors (Tulotta et al., 2016). In this way the cells can be tracked when they are labeled over-time and the proliferation and rate of invasion in the CHT of the embryos can be quantified (Drabsch et al., 2013).

-Intraperitoneal cavity: although all the injections are normally performed in zebrafish embryos due to the lack of adaptive immune system, with the creation of a zebrafish immunocompromised cell line (Rag2 mutant line) (Tang et al., 2014), adult zebrafish can be used as an animal model for xenograft assays without the drawback of the human cancer cells being rejected by the immune system of the fish. Apart from that, other methods for immunosuppression exist like γ-irradiation or dexamethasone without the need of breeding a mutant line, but being more expensive and time consuming (Khan et al., 2019).

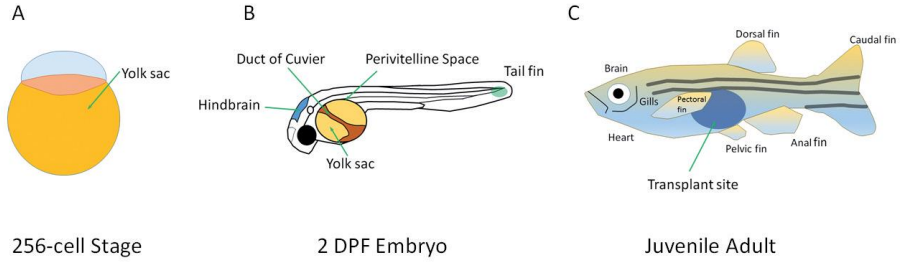

Figure 20. Most common sites of cancer cell injection during different stages of development in zebrafish. (A) The yolk sac (yellow) in the 256-cell stage. (B) 2 days post fertilization (dpf) embryo: yolk sac (yellow) and Duct of Cuvier (orange) being the common sites of injection. (C) Adult zebrafish: intraperitoneal cavity is the chosen place to perform xenograft at this stage (Drabsh et al. 2017. From Sercrisma, Creative Commons license).

3.1 FIRST STEPS IN XENOTRANSPLANTS

The first steps in xenograft with human cancer cells in zebrafish embryos have been carried out in 2005 (Lee et al., 2005). In this study, metastatic human melanoma cells were injected into the blastula stage of the embryos and followed during time in order to see their behavior post-transplantation. Melanoma cells injected in blastula stage were followed over time and authors demonstrate that these cells could survive, proliferate and spread in the embryo. They did not form tumors in this study or showed integration or affinity for specific organs in the host, but instead recapitulated the behavior *in vitro*, being de-differentiated cells. Nevertheless, normal melanoma cells injected were distributed to their optimal environment in the skin, demonstrating that zebrafish embryos, apart from being genetically similar to human, contain the signals for the human cells to integrate in their specific organs. Considering this, the whole study demonstrated that zebrafish could be used as an animal model for studying human cancer and the interaction of the human cells with their microenvironment in the host (Lee et al., 2005).

One year later, another research group refined the technique applied by Lee et al., using human melanoma, colorectal and pancreatic cancer cells but focusing in different parameters like specific site of injection and stage of the embryos, together with zebrafish incubation temperature (Haldi et al., 2006). The results of this research supported the ones obtained by Lee et al., and stablished a standard protocol for xenografts that are being used nowadays (Nicoli and Presta, 2007). This standard protocol includes the stage of the zebrafish embryos in which is most effective the xenograft technique, at 2dpf. The site of injection used by Haldi was the yolk sac of the embryos. Although more sites have been assayed in the posterior years (circulation, periviteline space) (Ikonomopoulou et al., 2018; Fior et al., 2017), yolk sac injection remains as an option to perform the xenograft. Apart from that, they demonstrated that different cell lines including colorectal and pancreatic cancer can proliferate and form tumors inside the yolk sac of the zebrafish embryos. Even that, these tumors could trigger an angiogenesis response of the embryo towards the tumor (Fig. 21),

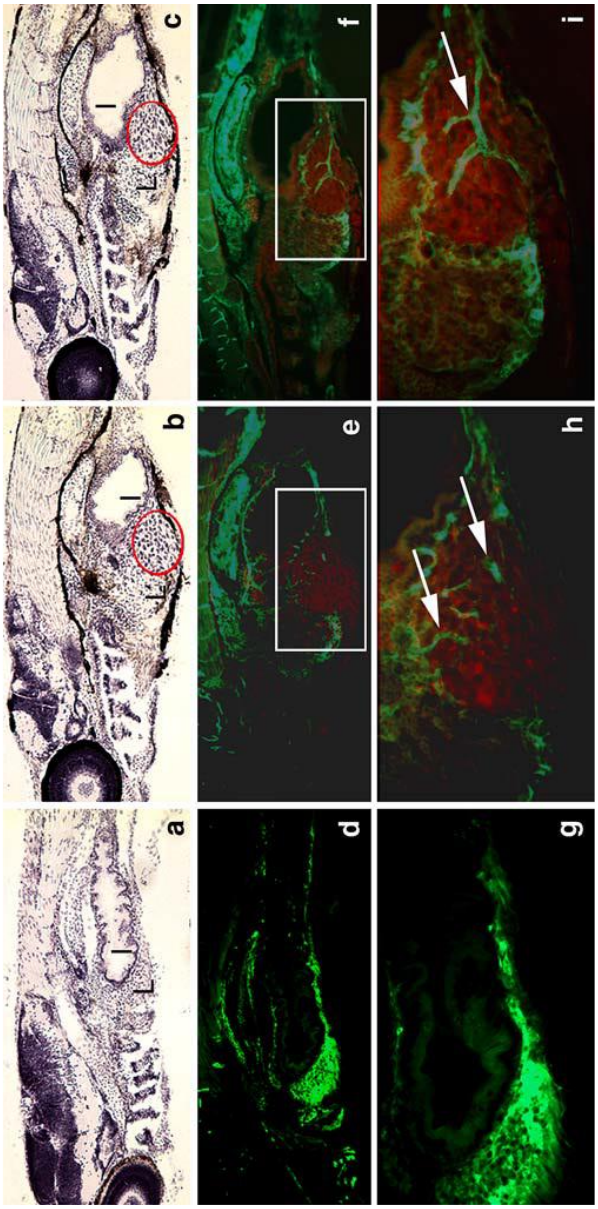

Figure 21. Angiogenesis showed by immunohistology in in a melanoma cell mass xenografted into the yolk of a zebrafish embryo. Bottom panels (g, h, i) show higher magnification (50x) images of the boxed areas in the middle panels; corresponding to cancer cell masses and formed new vessels (h and i, white arrows) (Haldi et al., 2006. With permission of Springer Nature).

stablishing the possibility of performing angiogenesis studies in the zebrafish model (Haldi et al., 2006).

Since then, many researchers around the world started to perform xenograft assays with different cells lines, trying to improve and refine the technique to obtain better and accurate results.

3.2 ADVANTAGES AND LIMITATIONS OF XENOGRAFT ASSAYS IN ZEBRAFISH

Zebrafish has been used for studying development since the early 1980's. Although the main tool for xenograft assays was the mouse model, since 2005 (Lee et al., 2005) the importance of zebrafish model has increased and the research using this animal model has arisen exponentially since 2000 (Kinth et al., 2013).

Although this data is related to all the publications including 'zebrafish', the arisen of studies related to 'zebrafish', in combination with 'cancer' or 'xenograft' suffered a substantial increase since 2005, highlighting the importance of this animal model for cancer research and drug screening.

Zebrafish embryos have some advantages and drawbacks when we compare these two species of animal models (Tab. 2):

Table 2. Advantages and disadvantages of xenotransplantation assays in zebrafish (Konantz et al., 2012. With permission of John Wiley and Sons)

Advantages	Limitations
• Large numbers of offspring • Transplantation at embryonic stages possible • Permeability of zebrafish to small molecules delivered by the water enables drug screens • External development and existence of transparent lines allow visualization and bioluminescence readouts • No immune rejection in early transplantation settings • Small numbers of cells per animal required for xenotransplantation • Visualization (transparency, transgenic lines, measurable bioluminescence) • Fast readout (hours to days)	• Little knowledge about niche structures and microenvironmental cues • Different biological environment in developing zebrafish for transplanted adult cells • Differences in size (small zebrafish organs/vessels—large human cells) • Different maintenance temperatures (overcome at 35 °C) • Absent organs (e.g., breast, lung) (may be overcome using analogous structures, e.g., gills) • Fewer possibilities for orthotopic transplantation • Limited numbers of zebrafish antibodies available • No adult immuno-permissive zebrafish lines available yet

3.2.1 Advantages

As the main advantages of this animal model, we need to mention the large number of offspring each day, the low maintenance cost of the species, small number of cells per animal, and the fact that zebrafish embryos are transparent.

3.2.2 Disadvantages

As the most notable disadvantages, would be the different maintenance temperatures between the injected cells (37ºC) and the optimal temperature for the zebrafish embryos (28ºC), the absent organs (e.g. breast or lung) that disables the possibility of performing a orthotopic transplantation in the embryos.

3.3 ZEBRAFISH, XENOGRAFT ASSAYS AND PERSONALIZED CANCER MEDICINE

Personalized medicine stands for developing therapies for patients where the common available therapies fail. These therapies are needed in case of diseases that are different within each patient, being the genetics and the succession of different mutations in cancer cells of each individual the responsible for the heterogeneity of the disease. Personalized medicine can range from the most complex diseases, having multiple targets for treatment, to monogenic diseases, in which there are no reference of any targets for that disease (Baxendale et al., 2017).

In the case of cancer, being a genetic disease (Hanahan and Weinberg, 2011), most of the researchers aim to understand the genetic and epigenetic basis and their interaction with their microenvironment in order to detect the underlying mechanisms that affect tumor progression in each patient, because response from each patient to chemotherapy is different, and in some cases the tumor cells become resistant to treatments (Drabsch et al., 2017). For this purpose, *in vivo* cancer xenograft models are used, being the mouse the most used due to the similarity between the mouse and human genome (Mouse Genome Sequencing et al., 2002).

Nevertheless, as mentioned before, zebrafish has emerged as new *in vivo* model for xenograft cancer studies applied for personalized medicine. Human cancer cell xenografts in zebrafish are used to investigate cancer cell growth (Marques et al., 2009), angiogenesis (Nicoli et al., 2007), invasion and metastasis (Ren et al., 2017), interaction with the microenvironment (Wang et al., 2015; Roh-Johnson et al., 2018) and the discovering of novel compounds (Roel et al., 2016; Ikonomopoulou et al., 2018). Although we have mentioned different stages of injection, the most commonly used is the 2dpf embryos for the following reasons:

-When cells are injected into a zebrafish embryo, they are able to survive, proliferate and metastasize due to the fact that the adaptive immune system is not developed until 14dpf (Renshaw and Trede, 2012).

-The cell tracking inside the zebrafish embryo is possible due to the transparent phenotype of the embryos and the fluorescent labeling of the cells by constitutively expression of a fluorescent protein (GFP, RFP) or labeling them with a lipophilic dye (DiI, DiD, DiO). Apart from that, the use of transgenic zebrafish lines in order to be capable of imaging specific tissues (e.g. vasculature) under fluorescence is possible (Ignatius and Langenau, 2011).

-Human cancer cells injected in the zebrafish embryo can interact with its host due to the conserved intercommunication between these two species, being the macrophages and the neutrophils of the fish the main components of the microenvironment recruited to the tumor site (Tulotta et al., 2016).

Apart from that, zebrafish has overcome some limitations of the mouse to perform personalized medicine for each patient: low ratio cost/patient, reduced time of the assays (1 week), low number of cells per embryo, and the possibility of high-throughput screening of chemotherapies (Konantz et al., 2012).

3.4 PARAMETERS OF THE XENOTRANSPLANTATION TECHNIQUE

Xenotransplantation technique stablished as a first standard protocol in 2006 (Haldi et al., 2006), have different parameters settled in order to obtain a balance between the human cells injected and the host, in this case, the zebrafish embryos. Zebrafish embryos develop at a temperature of 28ºC in controlled conditions and the human cancer cells at 37ºC (normal physiological temperature in the human body) (Westerfield, 2000). Apart from that, the image analysis was performed via disaggregation of the embryos at the end of the experiment and the cells were visually counted, but the number of cells injected at the beginning were an approximation of the real number (Haldi et al., 2006). Xenotransplantation technique has evolved over the past decade but there were little changes referred to incubation temperature and image analysis of the embryos:

3.4.1 Incubation temperature

As mentioned before, incubation temperature of the zebrafish embryos during the xenograft experiments should be a compromise temperature between the normal development of the embryos under controlled conditions (28ºC) and the optimal temperature of the cells (37ºC). The most used temperature for performing xenograft experiments in the literature is 34ºC and an incubation between 3 and 6 days post injection (dpi) (Eguiara et al., 2011; Ghotra et al., 2012; He et al., 2012; Ban et al., 2014; van der Ent et al., 2015). This temperature is focused on the cell growth at best conditions, without harming the embryos based on mortality and phenotypic studies (Pype et al., 2015), but it is important to take into account the metabolic pathways that could be affected by increasing the incubation temperature of the zebrafish embryos (Long et al., 2012).

3.4.2 Image analysis

The image analysis of the embryos in order to quantify the proliferation of the injected cells inside the embryo has been improving since the first xenograft experiments (Lee et al., 2005; Haldi et al., 2006). Some authors used Photoshop to quantify the intensity of the fluorescent injected cells transfected with GFP (green fluorescent

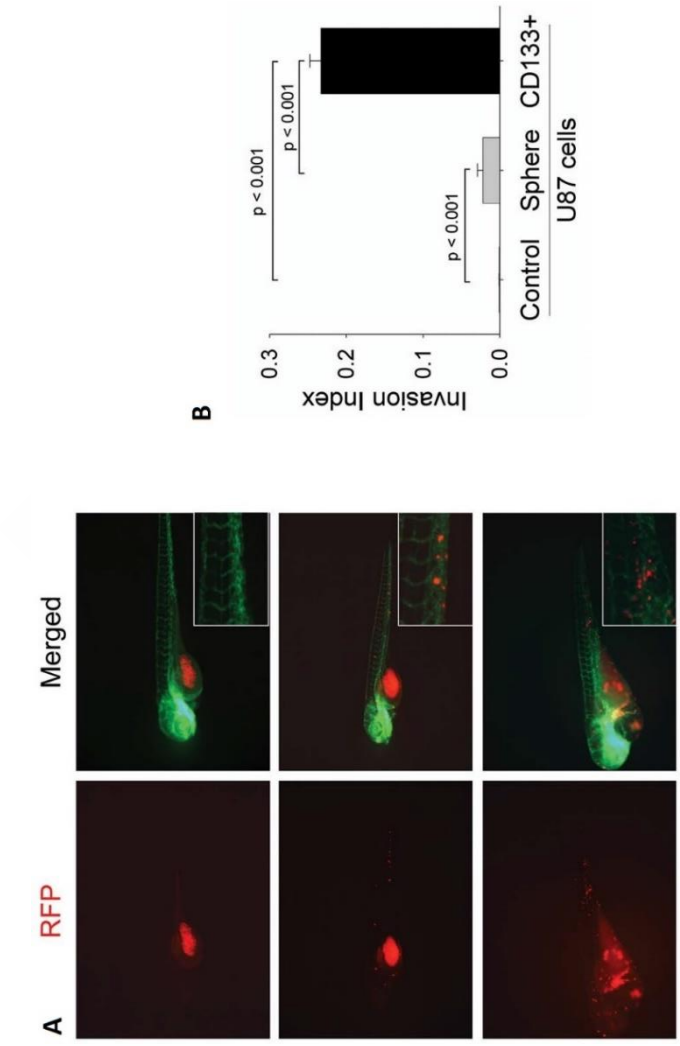

Figure 22. Representative images of the invasion and proliferation of U87 glioma cells in a zebrafish model. (A) Different conditions of the U87 glioma cells yields different proliferation rates and invasion behavior. (B) The percentage of invasive cells within the total injected cells. The images were analyzed with ImageJ software through fluorescence intensity (Adapted from Yang et al., 2013. From Plos One, Creative Commons license).

protein) or cells marked by lipophilic dyes (DiI, DiO, DiD) (Pruvot et al., 2011; Drabsh et al., 2013); although others authors improved the image analysis by using ImageJ or similar software (Fig. 22) (Corkery et al., 2011; Moshal et al., 2011; Yang et al., 2013).

3.5 DRUG SCREENING

The drug screening of novel compounds is one of the advantages of the zebrafish embryos compared to mouse, due to the low concentration of the compounds needed to do a high-throughput screening and the phenotype-based screens of the compounds and their toxicity. In this way the costs are reduced and could be a first step in the *in vivo* research of compounds between *in vitro* studies with cells and rodents (Letrado et al., 2018).

On the other hand, zebrafish has gained acceptance in the last years in order to test the toxicity of synthetic and small molecules (Puerto Galvis and Kouznetsov, 2019). In this sense, it is important to mention that the Organization for Economic Cooperation and Development (OECD) approved a guideline for testing chemicals in the embryos: 'Test Guideline 236: Fish Embryo Acute Toxicity Test' (FET) (OECD Guidelines for the testing of chemicals, 2013).

Finally, more insights into the mechanism of the compound are assayed when we use a complete animal model like zebrafish. The majority of the phenotypic screens are carried out in cell cultures, lacking most of the organs and processes that characterize a whole organism (e.g. pain, tumor metastasis, vascular tone, gut motility) (Fig. 23). Zebrafish embryos can provide information about the absorption, distribution, metabolism, excretion and toxicity (ADME-Tox) because the organs and tissues of the zebrafish embryos are functional (MacRae and Peterson, 2015). But even with all of these advantages presented before, it is important to highlight the relevance of this tool when it comes to compare them to the human biology. Even with mammal models the researchers need to take into consideration all the advantages and limitations of each model, even more in zebrafish, which is more distant to human than rodents. Some of the considerations of zebrafish model relevance are:

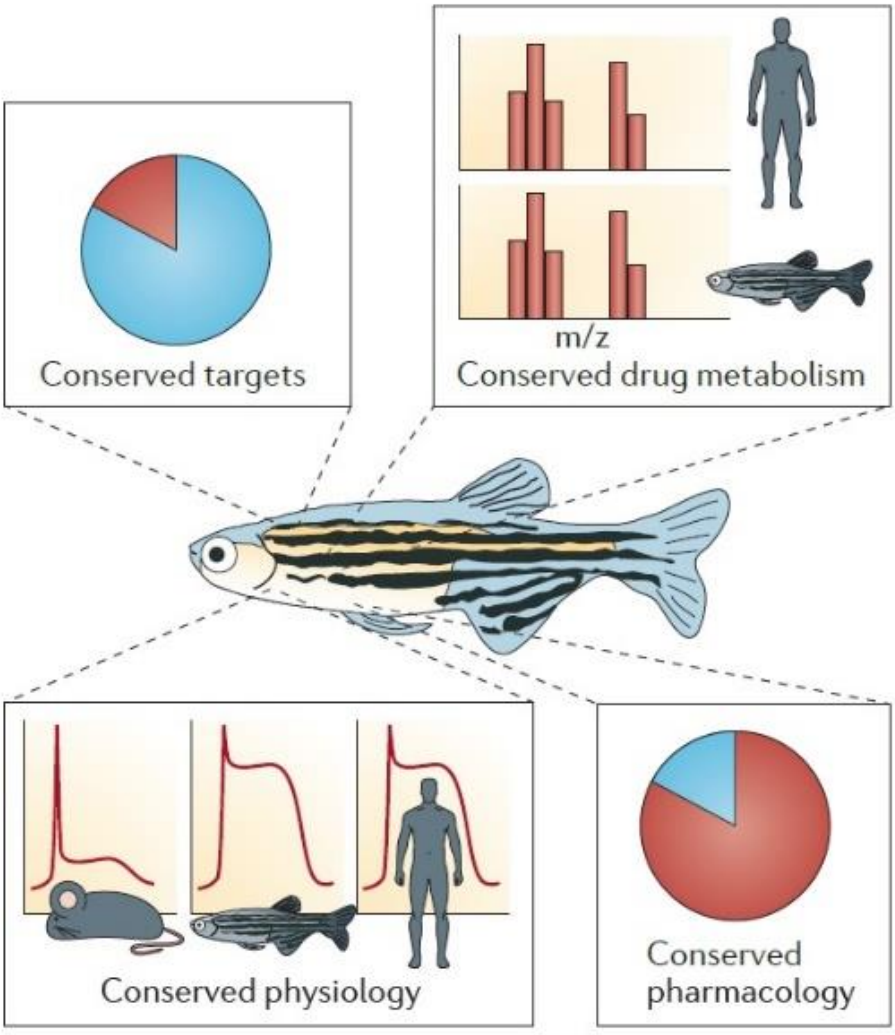

Figure 23. Is zebrafish relevant for discovering new human drugs? Apart from sharing 82% of disease-associated targets with humans, zebrafish has a highly conserved drug metabolism and physiology (e.g. cardiac electrophysiology) (MacRae and Peterson, 2015. With permission of Springer Nature).

3.5.1 Targets

Up to the 82% of proteins that cause human disease have an orthologue in zebrafish. The key point is that even when the sequence divergence between zebrafish and human is big, the active sites of the enzymes, and receptors that are the main target of pharmacological drugs are perfectly conserved between these two species (Howe et al., 2013b).

3.5.1.1 Physiology

In terms of model systems, exist a large number of animal models suitable for drug screening (e.g. yeast, worms or flies) but zebrafish, being a vertebrate, stands over these models due to the highly conserved integrative physiology, except for some organs that differ between the two species like breast or lungs (MacRae and Peterson, 2015; Puerto Galvis and Kouznetsova, 2019)

3.5.1.2 Drug distribution, metabolism and excretion

Apart from the toxicity and the phenotypic defects of individual human drugs, the zebrafish model resembles the drug-drug interaction of some compounds and the distribution in the body of the host and even across active physiological barriers like the blood-brain barrier (Eliceiri et al., 2011).

One important point to highlight, related to the drug screening and toxicity of the compounds in the case of cancer disease, is that temperature conditions are normally stablished around the 31-34ºC to ensure that the cells growth in a normal way and the zebrafish embryos survive (Lee et al., 2016). Different authors already suggested that a temperature closer to 37ºC (physiological temperature or human body) would be desirable in order to test the drugs near the optimal potential of cells (Konantz et al., 2012).

3.6 CELL-HOST INTERACTION

The cell-host interaction is a crucial point in the xenotransplantation technique but almost not well studied by the majority of researchers. Apart from considering the best conditions for

the cells injected and their microenvironment, the host, in this case the zebrafish embryo, plays an important role in the proliferation of the cells.

In xenograft technique, temperature is one of the most important parameters, determining the whole state of the embryos and their survival (Pype et al., 2015). As we mentioned, an incubation temperature of around 34ºC is the most used by researchers in the literature (Eguiara et al., 2011; Ghotra et al., 2012; He et al., 2012; Ban et al., 2014; van der Ent et al., 2014), but some researchers questioned whether this temperature is the optimal for seeing the full proliferation potential of the cells and their resistance to novel drugs (Konantz et al., 2012). In this way, even at 34ºC, some morphological (Fig. 24) and transcriptomic changes of the host could be affecting the proliferation of the cells inside the yolk sac or the circulation of the embryo, related to inflammation or immune response of the host to the xenografted cells (Long et al., 2012).

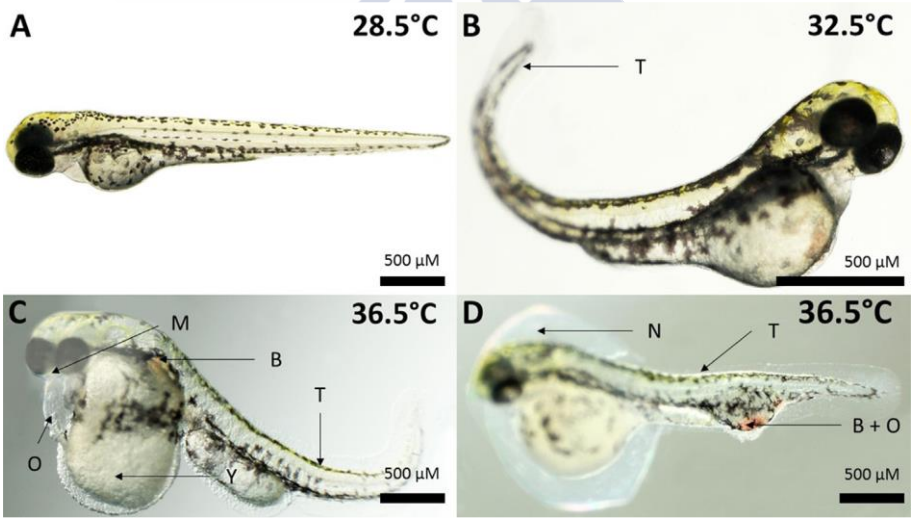

Figure 24. Principal malformations caused by the temperature increment in zebrafish embryos. (A) Normal embryo at 28,5ºC. (B) Tail deviation of the embryo due to increase in temperature. (C, D) Embryos with several malformations due to a high incubation temperature of 36'5ºC. Abbreviations: B= blood accumulation; M= malformation mouth; N= non-hatching; O= edema; T= tail malformation; Y= yolk malformation (Pype et al., 2015. With permission of Elsevier).

3.6.1 Innate immune system of the zebrafish embryos

Zebrafish embryos are a suitable model for xenograft assays because there is no adaptive immune system present until day 12-14 post fertilization, and the complete maturation of the adaptive immune response is achieved between 4 and 6 weeks post fecundation (Lam et al., 2004). Nevertheless, they have an innate immune system provided by the mother, and this system includes macrophages and neutrophils that are distributed all over the embryo. This innate immune system reacts to different stimulus and stress responses of the embryo to the environment (e.g. bacteria, cancer cells, temperature changes) and modifying the inflammation response of the body (Novoa and Figueras, 2012). Following these, the increase in incubation temperature of the zebrafish embryos is modifying these parameters in a molecular basis by the changes in the transcriptional responses and, therefore, influencing the response of the host to the injected cells when the incubation is performed at different temperatures (Long et al., 2012).

CHAPTER II - OBJECTIVES

The general objective of this thesis is the improvement of the xenotransplantation technique in zebrafish embryos to be able to study human cancer cell behavior with a more accurate method based on this model organism.

This general objective can be divided into the following specific objectives:

1. IMPROVEMENT OF THE XENOTRANSPLANTATION TECHNIQUE BY MODIFYING THE INCUBATION TEMPERATURE OF THE EMBRYOS AND DEVELOPING A NEW IMAGE ANALYSIS SOFTWARE

Obtaining a more accurate quantification of the injected cells inside the zebrafish embryos requires the optimal conditions for the injected cells to normally behave and proliferate compared to their natural environment, the human body. For this reason, the present study aims to determine the effect of using an incubation temperature of 34ºC *in vitro* (with different cell lines) and *in vivo* (in zebrafish embryos) in comparison with 36ºC. Apart from that, improvement of embryos image analysis is necessary with the objective of quantifying the cellular mass injected into the yolk of the zebrafish embryos. For this purpose, the development of a novel software based on Matlab for the automation of the image analysis process will be attempted. Finally, the analysis of 5-Fluorouracil (5-FU), a well-known chemotherapeutic drug, will serve us to determine the effect of the increment in the incubation temperature on drugs activity.

2. COMPARISON OF THE DEVELOPMENT DEFECTS, MORTALITY AND METABOLISM OF THE ZEBRAFISH EMBRYOS INCUBATED AT 28ºC, 34ºC AND 36ºC

The comparison between different incubation conditions at high temperatures (34ºC and 36ºC) will be performed in order to find a balance between the survival and cellular proliferation of the injected cells without falling into a high mortality of the injected embryos. The main reason for aiming to a higher incubation temperature, is because the injected cells should be nearly optimal conditions like the ones provided by the human body in terms of temperature. In this way, the cells should be able to provide the researchers with a more accurate proliferation ratio, without incurring in a higher mortality of the zebrafish embryos.

The physiological condition of the zebrafish embryos being the host will be studied with two different approaches:

-Morphological phenotyping: observable morphological defects can be quantified by imaging the embryos at different time points of their development to see the integrity of the host. Apart from that, is important to control the mortality of the embryos at the temperatures mentioned to compare this parameter between the conditions assayed.

-Metabolism profile: obtaining a metabolic profile of different important pathways related to temperature increase (immune system, inflammation, development and general metabolism) will be important to assess how embryos react to higher incubation temperatures.

3. TESTING THE MICROENVIRONMENT EFFECTS: CO-INJECTION OF DIFFERENT CELL LINES WITH CONDITIONED MACROPHAGES

One of the more important components of the human tumor microenvironment are the macrophages. Macrophages are recruited by cancer cells and transformed in tumor associated macrophages (TAMs), being able to help cancer cells with their proliferation and metastasis capacity.

For this reason, with the objective to test if the microenvironment of the human cancer cells will help with the development of the tumor inside the zebrafish embryos, we propose to co-injected MCF7 breast cancer cells with conditioned macrophages inside the yolk of this animal model. In this way, we would be able to analyze the interaction between the macrophages and the cancer cells, and measure the proliferation and spreading of the cells in the presence or absence of macrophages.

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CHAPTER III - IMPROVING ZEBRAFISH EMBRYO XENOTRANSPLANTATION CONDITIONS BY INCREASING INCUBATION TEMPERATURE AND ESTABLISHING A PROLIFERATION INDEX WITH ZFTOOL

1. INTRODUCTION

Model organisms are very important for understanding human diseases (Lieschke and Currie, 2007). Of the current available vertebrate animal models, genetic and experimental zebrafish and mouse models have contributed significantly to advancing our insights into cancer biology and therapy (Liu and Leach, 2011), largely due to the high genomic similarities they share with humans (Howe et al., 2013b). Tumor transplantation in animal models is very informative; not only it can provide data on tumor growth and the metastatic potential of tumor cells, but it also offers the possibility to test drugs in an *in vivo* animal setting, which could be putatively applied to the clinical setting to determine the best treatment for patients (Hidalgo et al., 2011).

While a promising model, several drawbacks need to be considered when using zebrafish for xenotransplantation assays. One of the most important limitations is the temperature (28ºC) (Konantz et al., 2012) at which these fish are routinely maintained, which differs by 9 degrees from that of the human body (37ºC), the latter being the ideal temperature for tumor cell proliferation. To tackle this problem, several groups have described incubation temperatures for xenografts in zebrafish ranging from 31ºC to 34ºC (Tab. 1), as a compromise solution

Table 1. Revision of parameters regarding xenotransplantation conditions

Paper	Temperature	Nº cells	Image Analysis	Xenograft time
Lee *et al.* 2005	31ºC	1-100	Adobe Photoshop	Blastula-5dpf
Haldi *et al.* 2006	35ºC (1h at 28ºC)	50-200	Adobe Photoshop	2dpf-7dpf
Nicoli *et al.* 2007	n/a	1000-2000	Image Pro Plus	2dpf-3dpf
Nicoli & Presta 2007	28ºC (24-48h)	4-10 nanoliter	Image Pro Plus	2dpf
White *et al.* 2008	28ºC adultos	100.000 Kidney Marrow cells (and 200.000 blood cells), 200.000 Melanoma	Image J. Software, NIH, Bethesda, USA	4 weeks
Harfouche *et al.* 2009	n/a	500	NIS-Element software (Nikon Instruments)	n/a
Lee *et al.* 2009	28ºC	100-500	n/a	2dpf-8dpf
Marques *et al.* 2009	35ºC (1h at 31ºC)	n/a	n/a	2dpf-5dpf or 7 dpi
Stoletov *et al.* 2010	35,5ºC	30-100	Confocal	Hours-1 día
Wagner *et al.* 2010	28ºc 1h and 35ºC after	n/a	n/a	Blastula - 5dpf
Corkery *et al.* 2011	35ºC (1h at 28ºC)	25-50	Image J, NIH, Bethesda, MD, USA	2dpf-9dpf (7dpi)
Eguiara *et al.* 2011	34ºC (2h at 28ºC)	500	n/a	2dpf-9dpf (7dpi)
Moshal *et al.* 2011	31ºC 1h and 28,5ºC for treatment	600-800	NIS-Element AR software (Nikon Instruments)	1dpf-3dpf (2dpi)
Pruvot *et al.* 2011	34ºC (1h at 28ºC)	50-200	Adobe Photoshop	2dpf-6dpf (4dpi)
Zhao *et al.* 2011	28ºC	50-100	Axiovision rel. 4.8 software	2dpf-8dpf (6dpi)
Benyumov *et al.* 2012	28.5ºC	100	AxioVision software (Release 4.7.2)	Blastula - 2dpi
Ghotra *et al.* 2012	34ºC	100	Image-Pro Plus-based software from Media Cybernetics	2dpf-6dpi

He *et al.* 2012	34ºC	50-400	Zeiss ZEN2009 software or ImageJ software, Adobe CS4.	2dpf-6dpi (4days)
Jung *et al.* 2012	28, 31 and 35 (31ºC)	25, 100, 200 (better dissemination with 200)	Cell count before injection, disgregation and invert microscopy for counting	2dpf-6dpf (4dpi)
Drabsch *et al.* 2013	33ºC	400	Adobe Photoshop CS4.	2dpf-8dpf (6dpi)
Jo DH *et al.* 2013	n/a	20 y 100	ImageJ Software	2dpf-6dpf (4dpi)
Spaink *et al.* 2013	34ºC	400 minimum (500)	Linutop, COPAS XL cell count + epifluorescence, Perl package software.	256 cells - 5dpf / 2-4hpf - 8dpf (6dpi)
Teppo *et al.* 2013	n/a	n/a	ZebIAT	2dpf-5dpf (3dpi)
Yang *et al.* 2013	35ºC	200-1000 (300)	Image J. Software, NIH, Bethesda, USA. Adobe Photoshop.	2dpf-X
Teng *et al.* 2013	34ºC	300	ImageJ/Fiji.	2dpf-4/6dpf
Ban *et al.* 2014	34ºC	500	Image J	2dpf-6dpf (4dpi)
Bansal *et al.* 2014	33ºC	10-500	n/a	2dpf-14dpf (12dpi)
Bentley *et al.* 2014	35ºC	15-20	n/a	2dpf-6dpf/9dpf (4dpi/7dpi)
Ent *et al.* 2014	34ºC	24-44	Image J / Image analysis (ImagePro Analyzer 7.0)	2dpf-8dpf (6dpi)
Orlova *et al.* 2014	33ºC	100/group	Adobe Photoshop CS4	Early blastula (256/512 cell) / 2dpf-X
Zhang *et al.* 2014 (1)	32ºC	100-200	MetaXpress Software	2dpf-9dpf (7dpi)
Zhao *et al.* 2014	28ºC	n/a	n/a	2dpf-6dpf (4dpi)

between the optimal temperature for human cell proliferation and zebrafish survival.

The analysis of cellular proliferation inside the embryo is another challenge considering the high number of fish that need to be imaged in high resolution, and the short period of time available to test different compounds and examine the effect on the injected cells. Different image analyses can be performed using commercial and free software to estimate the number of cells at the beginning and at the end of the experiment (Spaink et al., 2013; Annila et al., 2013), but these techniques are not accurate enough to reliably measure the proliferation of the cells as they are dependent on user intervention in terms of manually adjusting parameters for each image. In this chapter, we introduce the software ZFtool, which addresses the current problems faced in zebrafish imaging as the features used to extract the proliferation index (area and mean intensity of GFP points) with ZFtool are automatically computed and adapted to the autofluorescent characteristics of each fish. In this way, the measurements are repeatable, reproducible and reliable without user intervention. Performing the necessary computations on a fish-by-fish and stage-by-stage level, and manually adjusting all the parameters results in data that are difficult to compare leading to unreliable results. To provide a solution to this inherent problem, we developed, implemented and validated the automatic ZFtool methodology as described below. At this moment, the software is a Matlab toolbox and the software interface is currently under development.

To significantly improve the technique of assaying different chemotherapeutic agents in an *in vivo* system, at a temperature almost equal to that of the human body, and in a fast and efficient way, in this study we present a zebrafish yolk xenotransplantation assay together with an image analysis software that provides an answer to the main problems currently faced in the zebrafish xenotransplantation community. Tumor cell injection and rearing conditions were established, being the experiments performed at 36ºC, a temperature that to our knowledge has not been reported before for this type of assay. The conditions utilized showed a good overall survival rate of

the embryos, facilitated tumor growth, and together with the automated measurements obtained with the new ad-hoc imaging analysis software ZFtool, we were able to accurately monitor tumor growth with high reproducibility in order to generate reliable results.

2. MATERIAL AND METHODS

2.1 ZEBRAFISH HANDLING

The care, use and treatment of zebrafish were performed in agreement with the Animal Care and Use Committee of the University of Santiago de Compostela and the standard protocols of Spain (Directive 2010-63-UE). The protocol was approved by the Animal Care and Use Committee of the University of Santiago de Compostela and was performed under the experimental project permission MR110250 in the center authorized with REGA code ES270280346401. One-year-old adult zebrafish (*Danio rerio*, wild-type) were maintained at 28.5ºC in 30 L aquaria at a rate of 1 fish per liter of water, with a light-dark cycle of 14:10. Zebrafish embryos were obtained from mating adults according to previously described procedures (Westerfield, 2000).

2.2 REAGENTS AND CELL CULTURE

The human colorectal cancer cell line HCT116 was obtained from American Type Culture Collection (ATCC, Catalog No. CCL-247) and cultured using McCoy's 5A Medium containing 10% FBS (GIBCO, Invitrogen) and 1% Pen/Strep (GIBCO, Invitrogen) at 37ºC with 5% CO_2 in a humidified atmosphere. The HCT116 cell line was transfected to express GFP constitutively.

2.3 FLUORESCENT GFP CELL LABELING

HCT116 cells were transduced using a lentiviral-driven GFP construct (Sigma, Mission TurboGFP, SHC003 V). Cells were placed 72 hours post infection under selective pressure using 10 µg/ml puromycin. The rate of GFP positive cells was tested using flow cytometry (BD FACS Aria I, software FACSDiva 6.0.3).

2.4 CELL PROLIFERATION ASSAYS

Cell proliferation was determined using xCELLigence Real-Time Cell Analyzer; Acea Biosciences (Roche) following the manufacturer instructions. In brief, cells were seeded on E-plates containing electric nodes in their surface that allow the measurement of changes in impedance attributed to cell proliferation. Measurements were performed in quadruplicate, normalizing the initial cell index once the cells were completely adhered.

2.5 CELL INJECTION

Two days post fertilization (dpf), zebrafish embryos were dechorionated (if needed) and anesthetized with 0.003% tricaine (Sigma). Cells were suspended at 10,000-20,000 cells/µl in complete McCoy and maintained at room temperature for no longer than 2 hours before they were injected. The cell suspension was loaded into borosilicate glass capillary needles (1 mm O.D. x 0.78 mm I.D.; Harvard Apparatus), and injections were performed using IM-31 Electric Microinjector (Narishige) with an output pressure of 34 kPa and 30 ms injection time. The injections were performed manually right into the yolk of the embryo. Incorrectly injected embryos without cells inside of the yolk, or showing them in the circulation after xenotransplantation were discarded.

2.6 INCUBATION, IMAGING AND CELL QUANTIFICATION

After injection, 2dpf embryos were incubated at two different conditions (34ºC or 36ºC) in 24-well plates with salt dechlorinate tap water (SDTW, chlorine free water obtained with a reverse osmosis filter system) for 72h to check the proliferation of the cell line by ZFtool. Each plate contained at least 2 negative controls (injected with complete McCoy medium) and 2 blanks (not injected). Apart from those plates, another plate with 12 negative controls and 12 blanks were included in some experiments to test the viability of the embryos. No development abnormalities were observed during incubation at this temperature.

In order to reach a 36ºC incubation temperature without a large amount of embryo mortality, plates were covered with a transparent

sealing tape (PCR Plastics) to prevent evaporation and reduction of dissolved oxygen. After that, plates were placed inside an incubator with minimal contact between the plate and the incubator structure to prevent water overheating. Each embryo was photographed with AZ-100 Nikon fluorescence stereomicroscope at 0 hours post injection (hpi) and 72hpi to be analyzed by ZFtool software. The objective of this software is to automatize and improve the task of measuring the number and mean value of GFP pixels in order to compare them for these two conditions and compute the proliferation index. Finally, this analysis yields the number of GFP pixels in the image (nGFP), which represents the area of the cells inside the yolk sac at two different times and the GFP intensity Medium Value (GMV), which represents the medium intensity of the fluorescence inside the yolk. By multiplying the nGFP number by the GMV of each image, we determined the proliferation ratio between 0hpi and 72hpi to estimate the cell growth. The result obtained at 72hpi was divided by that obtained at 0hpi, yielding a proliferation index value (PI):

$$\frac{nGFP_{72hpi} \cdot GMV_{72hpi}}{nGFP_{0hpi} \cdot GMV_{0hpi}}$$

A PI value =1 means that cells remain stable during incubation, a PI higher than 1 indicates tumor cell proliferation and a PI lower than 1 indicates tumor cell death.

Zebrafish embryos have variable autofluorescence, especially in the yolk area. To accurately quantify the injected cells fluorescence a pre-processing is needed to only count the GFP pixels belonging to injected cells filtering autofluorescence. To achieve this, the software counts the number of GFP pixels with different intensity thresholds, from 0 (no threshold) to 50 and the ZFtool algorithm provides a homogeneous measurement of the GFP area for all fish analyzed comparing nGFP for each threshold analyzed with nGFP for threshold=0, where fish auto fluorescence is mostly present. When the relation between measured nGFP compared to nGFP at threshold=0 surpass a fixed value, we consider the GFP area to be stable and the threshold is fixed at this point. In case there is no autofluorescence in

the embryo, the threshold is established based on a tolerance parameter and a correction is included to assure the accuracy of the measurement in this cases. The ZFtool algorithm automatic thresholding for each analyzed embryo is one of the main automation components of the software, making it efficient in producing reliable fish to fish measurements.

2.7 CELL COUNTING SOFTWARE

The ZFTool extension for cell counting was developed. A drop of cells was placed on a microscope slide and photographed to obtain a fluorescence image. The algorithm detects circular objects of the fluorescence input image with a fixed diameter. The output yields a fluorescence image with nearly every cell or group of cells delimited by a contour and an estimation of the number of cells inside the input image. This algorithm is based on the circular Hough transform and has several parameters fixing the strength of the edge, and a minimum and maximum radius of the circles to detect. As we know the approximate size of the cells, we can fix these parameters in order to obtain an estimation of the number of cells. The method will be more accurate as the cells are more isolated, but as the number of cells injected increases over 400, we do not need the exact number of cells, but only an estimation, so this method still fits our purposes.

2.8 ANTICANCER DRUGS TOXICITY AND TREATMENT

In order to test the toxicity of an anticancer drug (5-Fluorouracil), experiments were performed according to the OECD (Organisation for Economic Co-operation and Development) guideline for the testing of chemicals (OECD Guidelines for the testing of chemicals, 2013). This procedure consists of exposing 0 hours post fecundation (hpf) eggs to dissolved chemicals in 24-well plates, for a period of 96 h. Various indicators (such as coagulation of embryos, lack of somite formation, non-detachment of the tail or lack of heartbeat) were checked every 24 h during the experiment, to test the mortality of the embryos and calculate the LC50 (lethal concentration 50%) at the end of the test. The drug was tested to determine a concentration range that included 0%-100% mortality. Experiments were considered valid when egg

fertilization was ≥70%. At the beginning, the oxygen concentration should have ≥80% saturation, and the water temperature should be 26±1ºC. During the test, the negative control embryos mortality could not be ≥10% at any time of the experiment. Exposure to the positive control resulted in a minimum mortality of 30% at the end, and the hatching rate of the negative control embryos was higher than 80% at 96 h. The concentrations tested were 250 µM, 500 µM, 1000 µM, 1500 µM, 2000 µM, with 1% DMSO (dimethyl sulfoxide). Another analog experiment was conducted changing the treatment starting point from 0hpf to 48hpf in order to evaluate how the toxicity changed with a dechorionated embryo at 36ºC.

2.9 STATISTICAL ANALYSIS

Homoscedasticity and statistical analyses were performed using the SPSS software (IBM). An excel outlier analysis was performed using interquartile range (IQR), while the outliers were discarded. One factor ANOVA for non-parametrical data was applied to non-homoscedastic data with confidence intervals of 95% or 99%, and a Student's t-test was applied to homoscedastic data with confidence intervals of 95% or 99%. Number of embryos analyzed is represented by n_{rep} and n_{total}, being n_{rep} the number of embryos in each replica, and n_{total} the total number of embryos statistically analyzed for the experiment.

3. RESULTS

3.1 FISH VIABILITY AT 34ºC AND 36ºC

Data from all experiments were analysed to determine fish viability between 34ºC and 36ºC for 72 h (experimental time range). Both the control (injected with medium) and blank (not injected) groups had a survival rate higher than 95%. Although the data showed that a difference existed between the survival rate at 34ºC (95.37%) and 36ºC (87.5%), statistical analysis found no significant differences (Tab. 2). At 36,5ºC or above the survival of the embryos is seriously affected and severe deformations were observed (data not shown).

Table 2. Total survival percentage of each set of experiments for the zebrafish embryos at three different conditions tested

HCT116-GFP 34ºC		
Experiments	72 h Injected Alive	Initial embryos injected
1	12	12
2	47	48
3	44	48
TOTALS	103	108
Survival (% ± SD)	95.370 ± 0.043	
HCT116-GFP 36ºC		
Experiments	72 h Injected Alive	Initial embryos injected
1	42	48
2	18	24
3	24	24
TOTALS	84	96
Survival (% ± SD)	87.500 ± 0.125	
HCT116-GFP 36ºC 5-FU		
Experiments	72 h Injected Alive	Initial embryos injected
1	45	48
2	20	24
3	22	24
TOTALS	87	96
Survival (% ± SD)	90.625 ± 0.055	

Despite the differences observed between the two temperatures, experiments at 36ºC show adequate fish viability in terms of cell proliferation, metabolism and behaviour of the injected cells if we are looking to simulate human body conditions.

3.2 *IN VITRO* ANALYSIS OF HCT116 CELL LINE PROLIFERATION

The Xcelligence technology was used to test *in vitro* cell proliferation at 34ºC and 36ºC, starting with different initial cell concentrations per well (2.000 cells, 5.000 cells and 10.000 cells). As

expected, a better proliferation rate was observed at 36ºC, confirming the data obtained *in vivo* (Fig. 1).

3.3 IMAGE ANALYSIS: ZFTOOL SOFTWARE

ZFTool software has been designed to provide specific, intuitive and automated tools for zebrafish xenotransplantation and drug testing assays. This software has two main functionalities: cell counting prior to injection and cell proliferation measurement inside the yolk of the embryo. This can be achieved automatically, without programming knowledge in a very intuitive way. Afterwards, other packages could be implemented to enhance the analysis of the proliferating cells, for example a 3D analysis model. ZFTool software is currently being further developed and tested and for that reason is not available for use outside our group. After being thoroughly tested it will be made available for the scientific community. Image analysis with ZFTool was performed with the parameters established in the code, appropriate to different sets of images taken under different conditions. This tool automatically eliminates fish autofluorescence, as these pixels interfere with the measurement of GFP area (Fig. 2). Usually, the darkest GFP pixels correspond to fish autofluorescence, and these pixels must not be included when measuring GFP area and mean intensity. ZFtool automatically establishes a GFP threshold for each fish, taking into account the decay of the graph representing the GFP area at different thresholds. When the difference is lower than 10%, the threshold is fixed, yielding an image where only the GFP area of the tumor cell mass is highlighted, creating a more accurate result. Different thresholds could be obtained for 0hpi and 72hpi, so the highest is selected to compare the evolution of the GFP area with time. The tolerance parameter establishes the percentage of decay with respect to area for a 0 threshold (Fig. 3).

While performing the experiments, we noticed that cell proliferation at the two temperatures tested varied depending on the initial cell load. To account for this variation, a ZFtool extension was developed to automatically count the number of cells prior to injection. For this, a microinjection with the same conditions of the experiment was performed over a microscope slide with low (100-200 cells) and

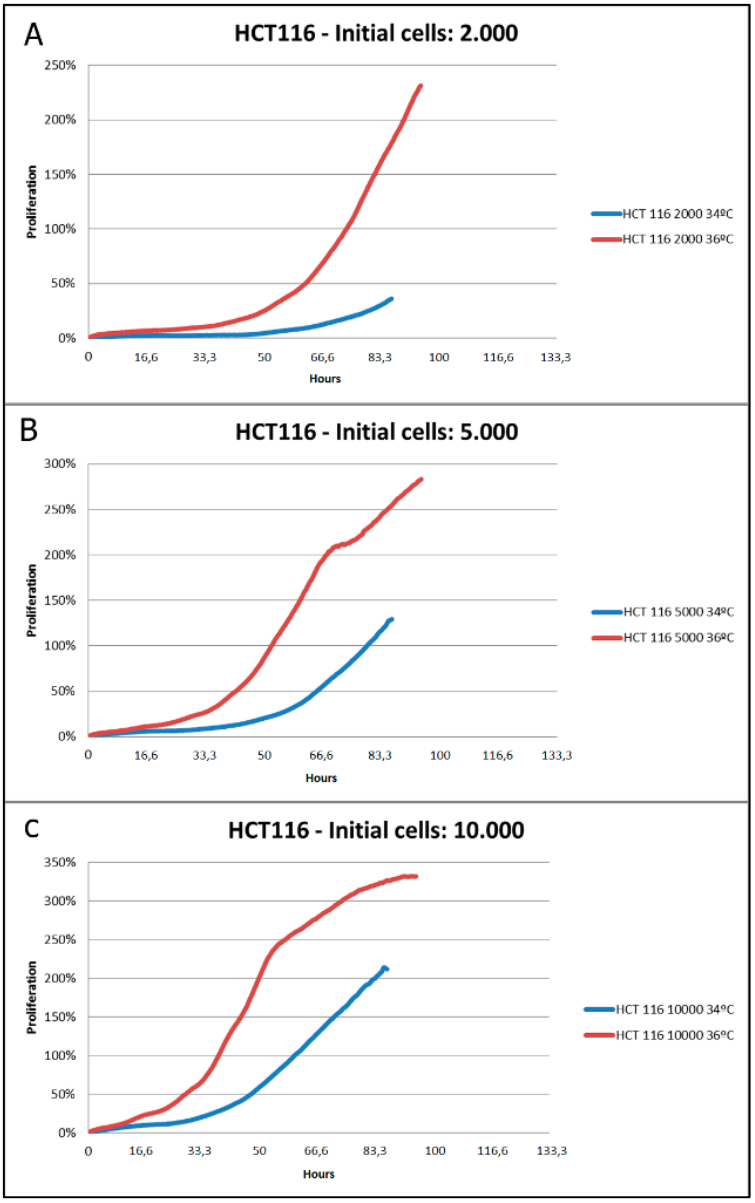

Figure 1. Proliferation of HCT116 cells *in vitro*. XCELLigence technology was used to quantify the proliferation of the HCT116 cell line *in vitro* at 34˚C and 36˚C with different initial number of cells (A: 2.000, B: 5.000 and C: 10.000). A better proliferation rate can be observed at 36˚C. The results shown are the media of 4 independent experiments.

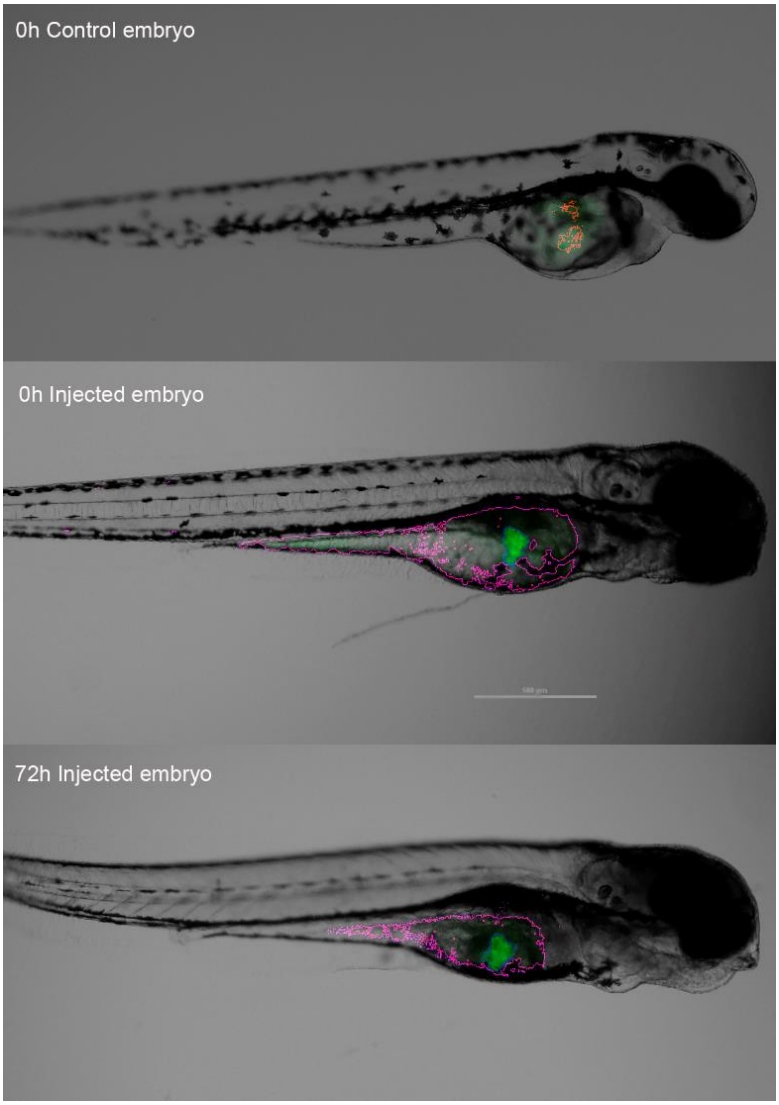

Figure 2. ZFtool automatically elimination of fish autofluorescence. ZFtool software detects all the green pixels in the image (red/pink-line) but eliminates all those pixels corresponding to fish autofluorescence and keeps pixels above an established threshold (blue line).

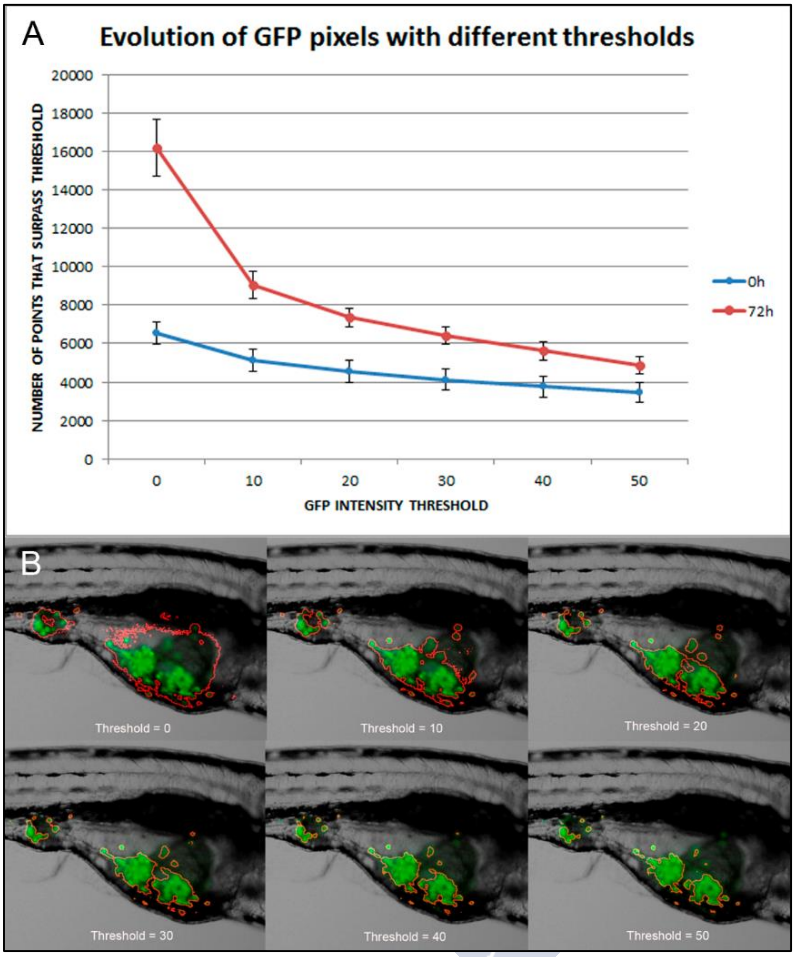

Figure 3. Evolution of the number of GFP pixels based on GFP intensity thresholds for zebrafish embryos and regions of interest of fluorescent zebrafish applied with different thresholds. (A) Graphical representation of average GFP intensity thresholds on the x-axis and mean number of pixels greater than the threshold on the y-axis for the zebrafish embryos tested (n = 6). A progressive decay of the area, more evident at 72hpi (dotted lines), is shown. It can also be observed that as the threshold increases, the area decreases slightly. At a low threshold, auto-fluorescence can represent an important component of GFP intensity. However, as soon as this threshold is raised, auto-fluorescence drastically disappears. Blue line represents 0hpi embryos, and red line represent 72hpi embryos. (B) Example of segmentation in evolution with red outlines over the images with thresholds from 0 to 50. The region inside the red outline is reduced as the threshold increases. This way the brightest pixels with higher fluorescence are selected, eliminating the majority of auto-fluorescence from the zebrafish embryo.

high (400-500) cell numbers, photographed under a fluorescence microscope and analysed, after that, these cells were discarded (Fig. 4). Afterwards, the embryos were injected with the same conditions. When the comparison of initial injected cells is performed between the range of 100-200 and 400-500 cells/injection at 34ºC, the proliferation after 72h remains the same, being not statistically significant when compared to 0h. However, when the injections with 100-200 and 400-500 cells/injection are performed at 36ºC, the proliferation at 72h is statistically significant, resulting in more proliferation of the injected cells when the number of those cells is in the range of 100-200 cells/injection (Fig. 5). This could be due to the space they have in the yolk and the sub estimation of the ZFtool when the number of cells is in the range of 400-500. In any case, the proliferation differences after 72h, despite the initial number of injected cells, are statistically significant between 34ºC and 36ºC.

3.4 *IN VIVO* COMPARATIVE PROLIFERATION ANALYSIS AT TWO DIFFERENT TEMPERATURES

The aim of this experiment was to test whether a better proliferation index exists at a temperature close to the human body (36.5ºC). Embryo post-transplant incubations were performed at two different temperatures (34ºC and 36ºC) to assay tumor cells behaving differently at both temperatures. Cultured HCT116 cells expressing GFP constitutively were microinjected into the embryos at 48hpf. After microinjection, embryos were photographed and placed in an incubator at 34ºC or 36ºC for 72 h. At 72 hours post injection (hpi) embryos were photographed again. The results showed proliferation of HCT116 cells at 36ºC (2.4237). When compared to cells at 34ºC (0.6253), no proliferation was detected in this condition, but on the contrary, cell death appeared as a possibility, based on the lack of fluorescence (Fig. 6). These results are consistent compared with the results obtained *in vitro*.

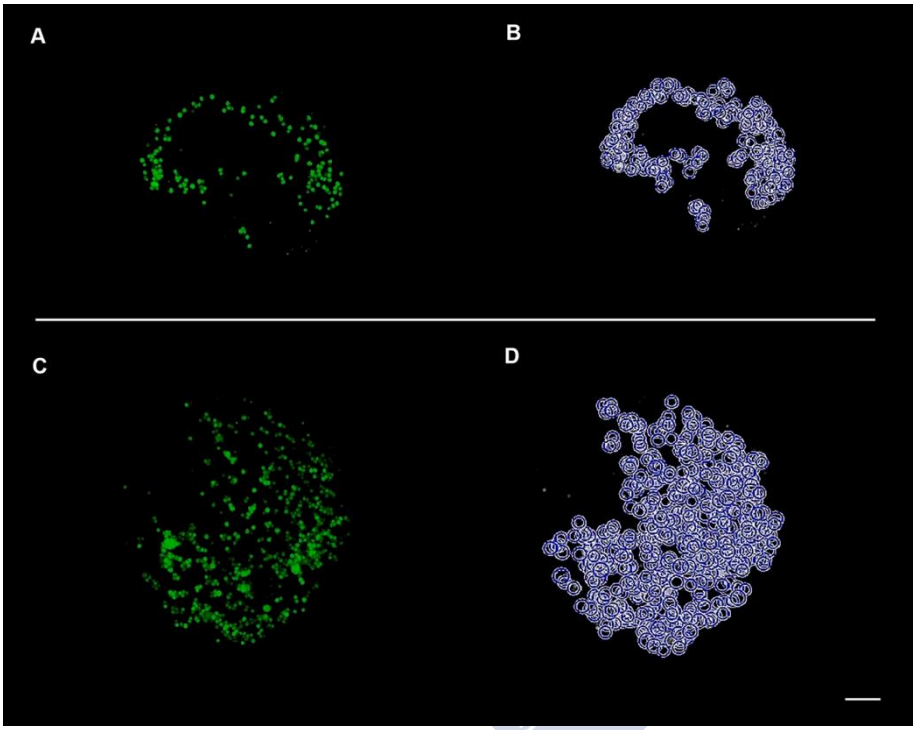

Figure 4. Automated counting of cells. This image shows the process of the software to count the cells on the microscope slide performed before the injection of the zebrafish embryos. (A) Fluorescent image of low cell number. (B) Cells of the A image counted (179). (C) Fluorescent image of high cell number. D: Cells of the C counted (404). Scale bar = 100µm.

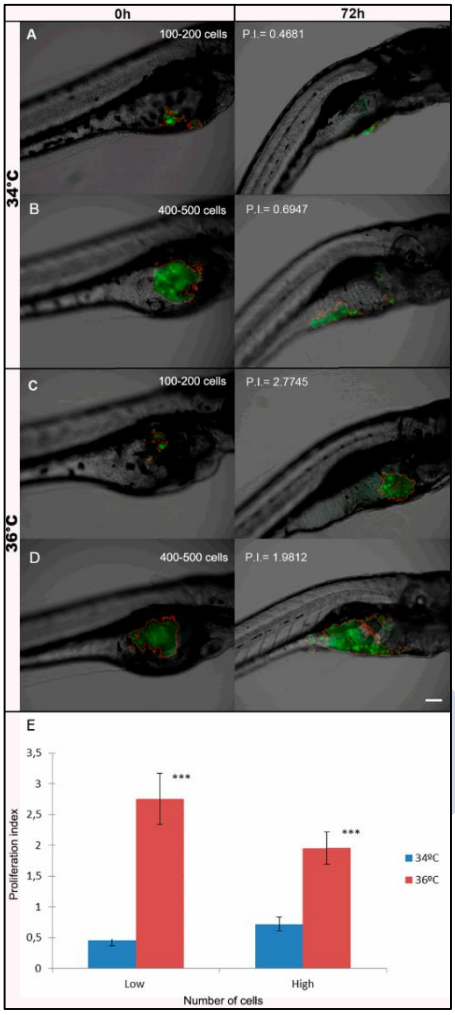

Figure 5. Proliferation assay in zebrafish injected with different cell numbers at 34 ˚C and 36 ˚C. (A, B) Initial injected cells proliferation index at an incubation temperature of 34 ˚C (A: 100-200 cells, P.I. = 0.4603; B: 400-500 cells; P.I. = 0.7196). (C, D) Initial injected cells proliferation index at an incubation temperature of 36 ˚C (C: 100-200 cells, P.I. = 2.7558; D: 400-500 cells, P.I. = 1.9558). Images are representative of each of the conditions assayed. All images are a superposition of a fluorescence field image over a bright field image. In all panels the left image is a 48hpf or 0hpi zebrafish embryo, and the right image is the same zebrafish embryo with 120hpf or 72hpi. Scale bar = 100µm. P.I. = proliferation index. (E) Comparison between the initial number of cells injected (Low: 100-200 or High: 400-500) and their proliferation at two different temperatures tested (n_{rep} = 20-50, n_{total} = 207, ***p < 0.01).

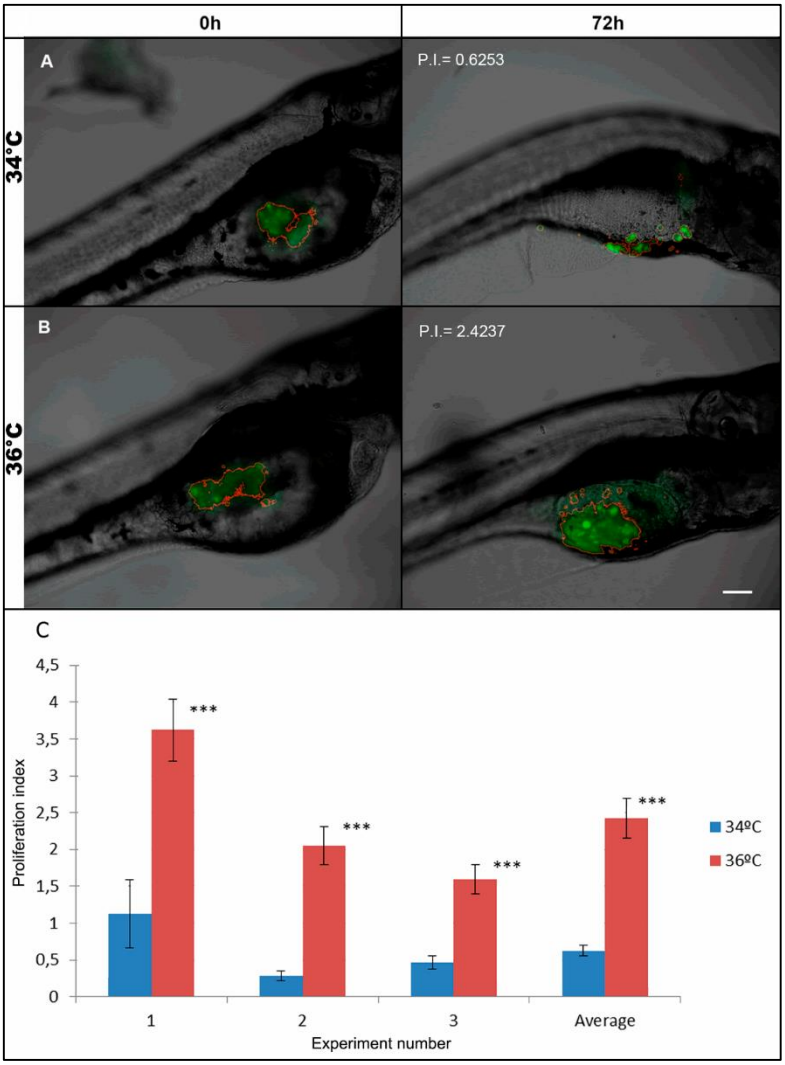

Figure 6. Cell proliferation inside the zebrafish embryos at the two conditions tested. (A) Zebrafish embryo incubation at 34 °C, analyzed with ZFtool, yielded a proliferation index of 0.6253. (B) Zebrafish embryo incubation at 36 °C analyzed with ZFtool yielded a proliferation index of 2.4237. Images are representative of each of the conditions assayed. All images are a superposition of a fluorescence field image over a bright field image. In all panels, the left image is a 48hpf or 0hpi zebrafish embryo, and the right image is the same zebrafish embryo with 120hpf or 72hpi. Scale bar = 100µm. (C) Quantization of cell proliferation inside the embryos at the two temperatures tested in each experiment (34°C-36°C) (n_{rep} = 20-50, n_{total} = 207, ***p< 0.01).

3.5 FLUOROURACIL TOXICITY TEST

A toxicity test was performed using the chemotherapeutic drug 5-Fluorouracil (5-FU) to establish a suitable concentration with the lowest toxicity possible at an effective therapeutic concentration for later use in our experiments.

For this purpose, the OECD zebrafish toxicity protocol was performed using 0hpf embryos and exposing them to different concentrations of 5-FU for 96 h at 26ºC (OECD Guidelines for the testing of chemicals, 2013). Mortality was then determined in order to find the lowest toxicity of the compound over the embryos. Recent studies indicated that the LC50 for the 5-FU following the OECD protocol at 26ºC for 120hpf exposed embryos is 2222 mg/L or 17082 µM (Kovacs et al., 2016).

The results of the OECD protocol at 26ºC showed that the concentrations tested were not sufficiently toxic enough to calculate the LC50. The aim of this toxicity test was not the calculation of the LC50 of the 5-FU, but to find the concentration at which the mortality of the fish was acceptable for our experiments and also effective against the injected cells (HCT116). The concentration with the greatest embryo survival (500µM), could be determined at 26ºC (Tab. 3).

Due to the lack of toxicity of 5-FU at 26ºC with the concentrations tested, the same experiment was performed with the final conditions of our experiments: 48hpf embryos, instead of 0 h, for a period of 96 h at 36ºC. The same concentrations were tested, and the ideal concentration to assure the survival of the embryos was again at 500 µM. At 36ºC and 48hpf, the mortality of the embryos using this compound was higher than at 26ºC and 0hpf (Tab. 4).

Table 3. OECD protocol toxicity results

24 h	R1		R2		R3	
Control -	2		2		0	
Conc.	5-FU	IC	5-FU	IC	5-FU	IC
250 µM	0	1	0	0	3	0
500 µM	0	0	1	0	0	1
1000 µM	0	0	1	2	1	0
1500 µM	0	1	3	0	2	0
2000 µM	1	1	2	0	2	1

48 h	R1		R2		R3	
Control -	6		2		2	
Conc.	5-FU	IC	5-FU	IC	5-FU	IC
250 µM	0	1	0	1	3	0
500 µM	0	0	1	0	0	2
1000 µM	0	0	2	2	1	1
1500 µM	0	2	3	0	2	0
2000 µM	1	1	2	0	2	1

72 h	R1		R2		R3	
Control -	6		3		2	
Conc.	5-FU	IC	5-FU	IC	5-FU	IC
250 µM	0	1	0	1	3	2
500 µM	0	0	1	0	0	2
1000 µM	0	0	2	2	1	1
1500 µM	0	2	3	0	2	0
2000 µM	1	1	2	0	2	1

96 h	R1		R2		R3	
Control -	6		3		2	
Conc.	5-FU	IC	5-FU	IC	5-FU	IC
250 µM	0	1	0	1	3	2
500 µM	0	0	1	0	0	2
1000 µM	1	0	2	2	1	1
1500 µM	0	2	3	0	2	0
2000 µM	1	1	3	0	2	1

**The number of dead embryos is shown in the table for each concentration of the compound.*

R1, R2, R3, Replica 1, 2, 3; Conc = concentration; IC = internal control.

3.6 ZFTOOL ANALYSIS OF ANTI-TUMOR DRUG EFFECTIVENESS

To determine the effect of the anti-tumor drug 5-FU on the injected cells at two different temperatures, an experiment was performed with the HCT116 colorectal cancer cell line. For this, 48hpf HCT116 injected embryos were photographed and placed individually in 24-well plates with 2 mL SDTW/well and incubated at 34ºC and 36ºC for 24 h. After the end of the incubation, embryos were photographed again to check the injected cells, and embryos without any cells were discarded.

At this time, the embryos were transferred to 24-well plates containing: 2 mL SDTW/well and 500 µM 5-FU (DMSO final concentration 1%) or 2mL SDTW/well with 1% DMSO (control fish). Final concentration of DMSO was used to dissolve the 5-FU, with no toxic effects as previously reported (Maes et al., 2012) and assayed in our laboratory to test the conditions in our embryos (data not shown). The fish were returned to the incubator at 34ºC or 36ºC for another 48 h. At 72hpi, the embryos were photographed again and analysed with ZFtool (Fig. 7 and 8). The results obtained showed a reduction in the injected tumor cells at 36ºC compared to the controls, nevertheless no reduction was observed at 34ºC. At 34ºC the control group showed a proliferation index of 0.4748, while the 5-FU treated fish had a proliferation ratio of 0.5415, being the difference not statistically significant between control and treated embryos.

Table 4. Toxicity test and mortality rates at 36ºC from 48hpf to 144hpf

24 h	34ºC		36ºC	
Control -	0/24		0/24	
Conc.*	5-FU	IC^	5-FU	IC^
250 µM	0/20	0/4	0/20	0/4
500 µM	0/20	0/4	0/20	0/4
1000 µM	0/20	0/4	0/20	0/4
1500 µM	0/20	0/4	1/20	0/4
2000 µM	1/20	0/4	2/20	0/4

48 h	34ºC		36ºC	
Control -	0/24		0/24	
Conc.*	5-FU	IC^	5-FU	IC^
250 µM	0/20	0/4	1/20	0/4
500 µM	0/20	0/4	0/20	0/4
1000 µM	0/20	0/4	3/20	0/4
1500 µM	1/20	0/4	3/20	0/4
2000 µM	1/20	0/4	10/20	0/4

72 h	34ºC		36ºC	
Control -	0/24		0/24	
Conc.*	5-FU	IC^	5-FU	IC^
250 µM	0/20	0/4	2/20	0/4
500 µM	0/20	0/4	0/20	0/4
1000 µM	0/20	0/4	5/20	0/4
1500 µM	2/20	0/4	5/20	0/4
2000 µM	2/20	0/4	11/20	0/4

96 h	34ºC		36ºC	
Control -	0/24		1/24	
Conc.*	5-FU	IC^	5-FU	IC^
250 µM	0/20	1/4	2/20	0/4
500 µM	0/20	0/4	0/20	0/4
1000 µM	0/20	1/4	5/20	0/4
1500 µM	2/20	0/4	6/20	1/4
2000 µM	3/20	0/4	14/20	0/4

*Conc, concentration; ^IC, internal control. Negative control embryos were assayed in a separate 24 well plate. Additionally, four negative internal controls were placed in 4 of the 24 wells in each 5-FU treated plate being the other 20 wells 5-FU treatment.

At 36ºC the control group showed a proliferation ratio of 2.6653, while the 5-FU treated fish had a proliferation ratio of 1.9592. Again, the proliferation index performing this analysis was statistical significant. The statistical analysis demonstrated significant differences between the control and the treated group at this temperature (Fig. 9).

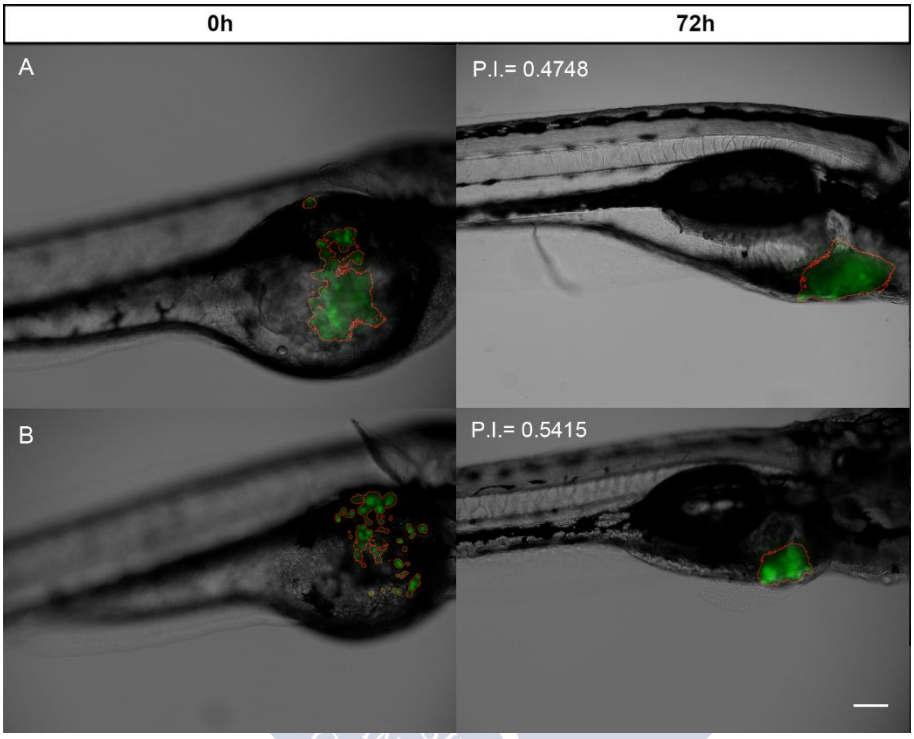

Figure 7. Cell proliferation inside the zebrafish embryos at 34ºC and 34ºC with 5-FU. (A) Zebrafish embryo incubation at 34ºC analyzed with ZFtool yielding a proliferation index of 0.4748. (B) Zebrafish embryo incubation at 34ºC, with 5-FU analyzed with the ZFtool yielding a proliferation index of 0.5415. All images are a superposition of a fluorescence field image over a bright field image. In all panels, the left image is a 48hpf or 0hpi zebrafish embryo, and the right image is the same zebrafish embryo with 120hpf or 72hpi. Scale bar = 100µm.

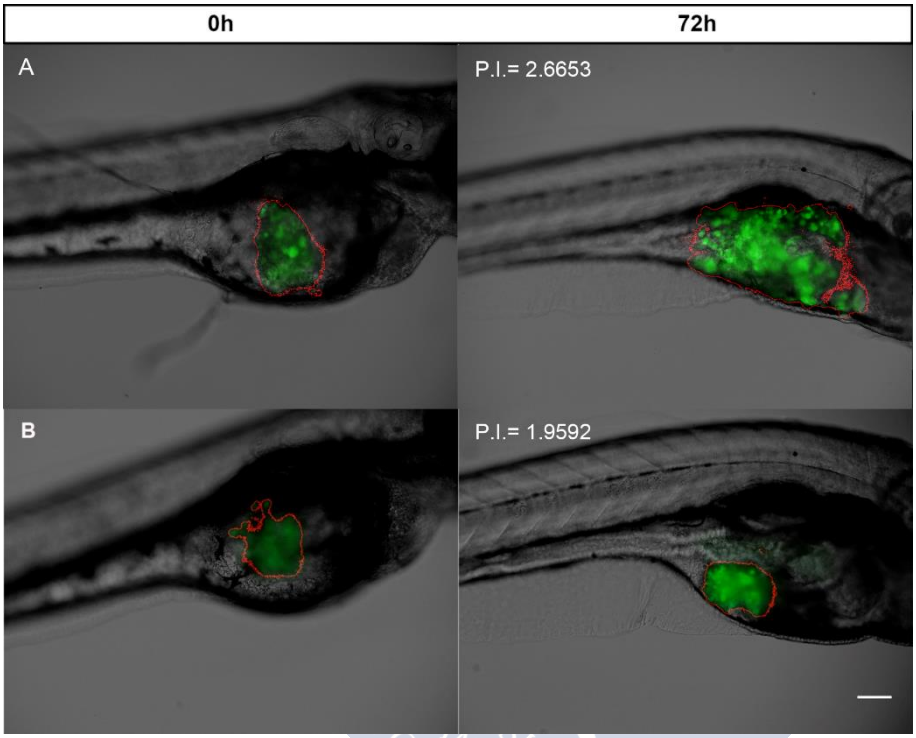

Figure 8. Cell proliferation inside the zebrafish embryos at 36ºC and 36ºC with 5-FU. (A) Zebrafish embryo incubation at 36ºC analyzed with ZFtool yielding a proliferation index of 2.6653. (B) Zebrafish embryo incubation at 36ºC, with 5-FU analyzed with the ZFtool yielding a proliferation index of 1.9592. All images are a superposition of a fluorescence field image over a bright field image. In all panels, the left image is a 48hpf or 0hpi zebrafish embryo, and the right image is the same zebrafish embryo with 120hpf or 72hpi. Scale bar = 100µm.

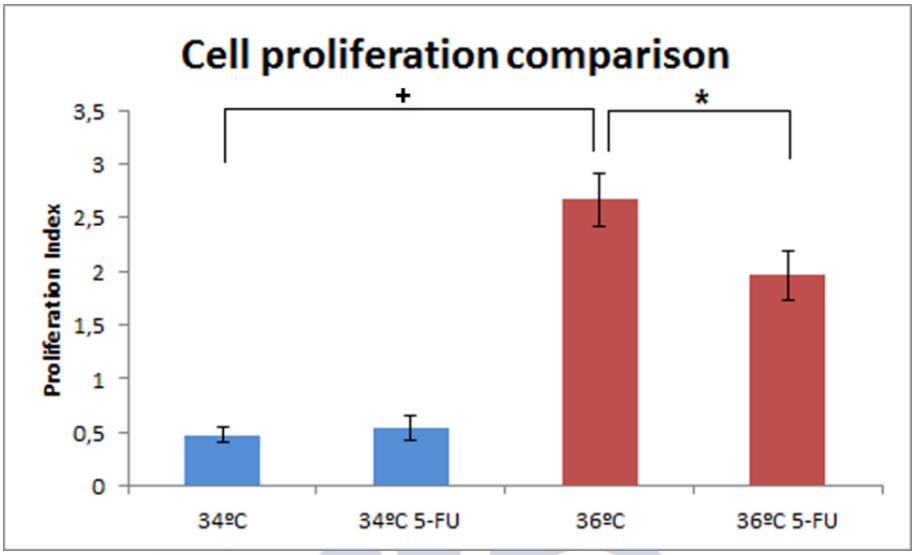

Figure 9. Cell proliferation inside the zebrafish embryos between 34˚C/34˚C 5-FU and 36˚C/36˚C 5-FU. Xenografted tumor cell proliferation at 34˚C/34˚C 5-FU and 36˚C/36˚C 5-FU. Proliferation at 34˚C=0.4748; 34˚C 5-FU=0.5415; 36˚C=2.6652; 36˚C 5-FU=1.9592. Each column is an average representation of four independent experiments (n_{rep}=81-102, n_{total}=300, *p<0.01; +p<0.01).

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CHAPTER IV - QUANTIFICATION OF MORPHOLOGICAL ABNORMALITIES AND METABOLISM RELATED CHANGES IN XENOGRAFTED ZEBRAFISH EMBRYOS AT DIFFERENT INCUBATION TEMPERATURES

1. INTRODUCTION

Zebrafish embryos have been widely used as a tool for cancer research since 2006 (Haldi et al., 2006) by means of the xenograft technique. This involves the injection of human cancer cells inside the yolk (Tonon et al., 2016) or in the circulation of the zebrafish embryo at 48 hours post fertilization (hpf) (Ikonomopoulou et al., 2018).

Although many aspects of the technique had been taken into account in the last decade (site of injection, number of cells, image analysis, etc) to develop the most similar model to mimic the human body (Cabezas-Sainz et al., 2018), the temperature have not received much attention. Due to this, most of the studies focus their efforts on the proliferation/invasion of the cells without taking into account the effect of the incubation temperature on the host, and the cell-host interaction underlying this process. The zebrafish embryos carrying the cells have an optimal developmental temperature of 28ºC, and it is necessary to find a compromise temperature between this temperature and that ideal for the human injected cells (37ºC). This temperature should be high enough for xenografted cells to growth properly while allowing the embryos to stay alive and without malformations that compromises the survival of the embryos during the incubation to promote a normal cell-host interaction. Apart from that, gene expression changes are expected depending on the embryos incubation

temperature at different levels including stress response, immune response and development (Long et al., 2012).

As mentioned, temperature plays an important role for the proliferation and metastatic potential of the injected cells, being optimal the human body physiological temperature (36-37ºC). In this context, a wide range of incubation temperatures (31°C to 36°C) have been assayed in xenografted zebrafish embryos to understand injected cancer cells behavior, metabolic phenotype, or invasion; always keeping a balance between mortality and malformations of the embryos and the full proliferative potential of the human cells (Lee et al., 2005; Yang et al., 2013; Zhang et al., 2014; Bansal et al., 2014). Based on the *in vitro* behavior (proliferation, migration, invasion) of tumor cells at different temperatures (31ºC-37ºC), it has been suggested that a higher temperature would be a better approach to deeply understand the fate of the injected cells and propose working at a temperature range closer to that of the human body (Konantz et al., 2012).

Increasing the incubation temperature of the zebrafish embryos could lead to heat induced teratogenic effects, previously described in other species (Ornsrud, et al., 2004) and should be quantified to determine the effect of different temperatures in the xenografted embryos. For this purpose, we selected specific malformations of the embryos to quantify the teratogenic effects at different time points of the developmental stages based on previous studies (Pype et al., 2015). While most of the xenograft studies in the literature perform the incubation at 34ºC, our precious studies (Cabezas-Sainz et al., 2018) showed the possibility of performing this technique raising the temperature to 36ºC and reducing the incubation time of the embryos, obtaining different proliferation rates of the injected cells without incurring in excessive mortality. Considering this, we focused in the quantification of malformations due to the teratogenic effects of the temperature and tested if they were enough to cause mortality of the embryos.

Apart from malformations, temperature modifies metabolic pathways in the zebrafish embryos involved in immune/stress response, inflammation, metabolism, and development interfering with the

overall state of the host in xenograft experiments (Long et al., 2012; Laux et al., 2017). While lacking of an adaptive immune system, zebrafish embryos still have an innate immune system when the xenotransplantation of the cells takes place at 48hpf (Meijer, 2016; van der Ent et al., 2015). Therefore, immune and stress response could be interfering in the behavior of the innate immune system of the embryo and the reaction to the human injected cells inside the embryo depending on the incubation temperature and activation of these metabolic pathways. So, to better characterize the effect of zebrafish incubation at 36ºC and determine if differences exist respect to that observed at 34ºC, we selected genes, previously shown to be affected by temperature (Pype et al., 2015), involved in development (Lft2, Mmp9, Haus3, Junb-a and Lum), immune response (Socs3a, Junb-a), stress response (Apex1, Hspa9), and metabolism (Per2, Wisp) and quantified their expression at 28, 34, and 36ºC.

2. MATERIALS AND METHODS

2.1 ZEBRAFISH HANDLING

One-year-old adult zebrafish (*Danio rerio*, wild-type, strain AB) were maintained at 28ºC in 30 L aquaria at a rate of 1 fish per liter of water, with a light-dark cycle of 14:10 hours. Zebrafish embryos were obtained from mating adults according to previously described procedures (Westerfield, 2000). Zebrafish care, use and treatment were performed in agreement with the Animal Care and Use Committee of the University of Santiago de Compostela and the standard protocols of Spain (Directive 2010-63-UE) and was performed under the experimental project permission MR110250 in the center authorized with REGA code ES270280346401.

2.2 CELL CULTURE AND GFP LABELING

The MCF7 human breast cancer cell line was obtained from the American Type Culture Collection (ATCC) and cultured using RPMI medium (GIBCO, Invitrogen) containing 10% FBS (GIBCO, Invitrogen) and 1% Pen/Strep (GIBCO, Invitrogen) at 37ºC with 5%

CO_2 in a humidified atmosphere. MCF7 cells were transduced using a lentiviral-driven GFP construct (Sigma, Mission TurboGFP, SHC003 V). GFP positive cells were selected 72 hours post infection using 10 µg/ml puromycin.

2.3 CELL INJECTION IN ZEBRAFISH EMBRYOS

Embryos were collected and placed in Petri dishes at a ratio of 50 embryos/plate at 28ºC. Two days post fertilization (dpf) embryos were dechorionized (if needed) and anesthetized with 0.003% tricaine (Sigma). MCF7 breast cancer cells (10.000-20.000 cells/µl) were loaded into borosilicate glass capillary needles (1 mm O.D. x 0.75 mm I.D.; World Precision Instruments), and injected into embryos yolk sac using IM-31 Electric Microinjector (Narishige). Embryos showing cells outside the yolk were discarded.

2.4 INCUBATION AND ASSAYS CONDITIONS

The incubation of the zebrafish embryos was performed in incubators without CO_2 at different temperatures depending on the experiment. Embryos were incubated at 28ºC, 34ºC and/or 36ºC in 140mm x 20mm Petri dishes (DeltaLab) for the duration of the experiments at the ratio of 50 embryos/plate, preventing the plates from touching the metal parts of the incubator to avoid water overheating. Every 24h, the egg water (salt dechlorinate tap water, SDTW) was refreshed to account for evaporation, oxygen reduction or accumulation of substances from the embryos. Each assay was performed in triplicate. To determine and compare the effects produced by higher temperatures (34ºC and 36ºC) in different zebrafish embryos developmental stages (0hpf and 48hpf) two types of assays were performed:

2.4.1 Assay starting at 0hpf

Embryos were collected at 0hpf and screened after 1hpf to guarantee a normal cell division. Eggs showing abnormal or asymmetric cell division were discarded and replaced by normal ones in order to reach the selected number of embryos for each treatment. Embryos were placed before 2hpf in Petri dishes at two different temperatures (28ºC and 36°C) and incubated for 48hpf, screening the

embryos for different malformations and mortality at different critical developmental time points (5h, 10h, 24h, 48h) as reported before (Pype et al., 2015). When the 48hs has elapsed, the embryos were placed at 28ºC and the mortality was quantified at different time points up to 336hpf (Fig. 1).

2.4.2 Assay starting at 48hpf

In this case embryos were maintained at 28ºC until 48hpf. The embryos showing a normal developmental pattern were divided in four groups. One was injected with the breast cancer cell line MCF7 labeled in GFP, a second group was injected with complete RPMI medium (vehicle), a third group was a blank group (no injection) at 34ºC or 36ºC, and last group was another blank group incubated at 28ºC. Each treatment was then incubated until the 4dpf at 28ºC, 34ºC and 36ºC and finally incubated at 28ºC until 14dpf. Embryos were screened for mortality on a daily basis, and morphological abnormalities were screened at 0, 2, 6, and 12dpi (Fig. 1).

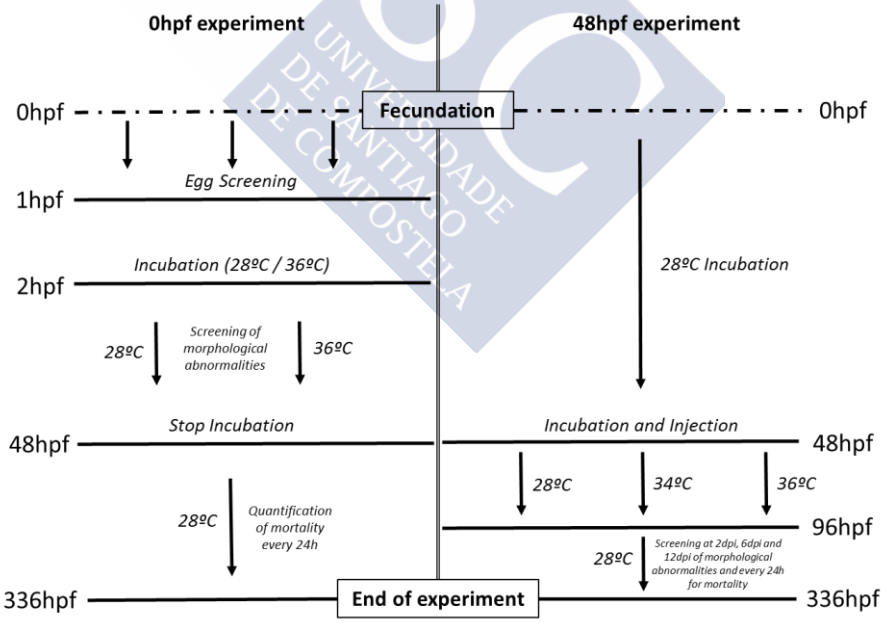

Figure 1. Overview of the two assays starting at two different start points. Hpf = hours post fecundation. Dpi = days post injection.

2.5 EMBRYO IMAGING

Embryos were photographed using an AZ-100 Nikon fluorescence stereomicroscope (bright field and GFP fluorescence). Embryo morphological malformations and mortality for the different time points assayed, together with the proliferation of the xenografted cells inside the yolk when present were quantified. Most relevant abnormalities quantified were (Fig. 2): spinal deviation, edema, hatching rate and head deformation. Malformations are expressed in percentage of the total embryos alive at the time point analyzed.

2.6 RNA ISOLATION, CDNA SYNTHESIS AND QPCR

A total of 10 embryos were euthanized and disaggregated for each condition (28ºC, 34ºC and 36ºC) at 0hpi, 2dpi and 6dpi in triplicate. Zebrafish embryo RNA was then purified from cell suspensions using the Qiagen RNeasy Mini Kit (Qiajen) according to manufacturer instructions.

After DNAase treatment, cDNA synthesis was performed using the AffinityScript Multi Temperature cDNA Synthesis Kit (Agilent Technologies) following the manufacturer instructions. Gene expression was then assayed using the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies) in a Stratagene Mx3005P quantitative qPCR. Relative fold changes of gene expression were calculated using the ΔΔCt method (Livak and Schmittgen, 2001).

2.7 STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California USA). Unpaired student t-test was performed to analyze the mortality and morphological defects from 0hpf to 48hpf. One-way ANOVA was performed to analyze the morphological abnormalities experiment beginning at 48h and the qPCR experiments. Differences were considered significant when $*p<0.05$.

3. RESULTS

3.1 MORTALITY AND MORPHOLOGICAL EFFECTS COMPARISON BETWEEN 28ºC AND 36ºC FROM 0HPF TO 48HPF

Zebrafish embryos were harvested at 0hpf, incubated at 28ºC and 36ºC for 48h. Embryos incubated at 36ºC were returned to a normal incubation temperature of 28ºC and incubation continued until 336hpf.

Different morphological abnormalities were observed at 36ºC but not evident at 28ºC. These included spinal deviation, edema, and head deformation (Fig. 2). The spinal deviation, could be due to the higher developmental speed and increases with temperature (Pype et al., 2015). Apart from that, the hatching rate of the embryos increased drastically at 36ºC (Fig. 3).

Besides that, no statistical difference in mortality was observed for the embryos incubated up to 48hpf at 28ºC compared to embryos incubated at 36ºC. Although the mortality of the embryos stabilizes from 24hpf onwards, in the first 24h of development the mortality at 36ºC occurs earlier than at 28ºC (Fig. 4A). To test if the incubation at 36ºC from 0hpf to 48hpf produces long term effects, the embryos were incubated at 28ºC for the rest of the experiment until 336hpf, checking the mortality on a daily basis (Fig. 4B).

3.2 QUANTIFICATION AND COMPARISON OF MORTALITY AND MORPHOLOGICAL EFFECTS BETWEEN DIFFERENT INCUBATION TEMPERATURES IN 48HPF EMBRYOS

In this assay, zebrafish embryos were collected at 48hpf and treated as described in the methods section to obtain four experimental conditions (blank, control, medium injected and cells injected). Each condition was then incubated at 28ºC, 34ºC and 36ºC for 48h, and finally returned to 28ºC for the remaining of the experiments.

3.2.1 Mortality

No statistical difference in mortality was observed after the incubation at 28ºC, 34ºC and 36ºC for 48h. Nevertheless, a higher but not significant mortality was observed at 34ºC and 36ºC in zebrafish

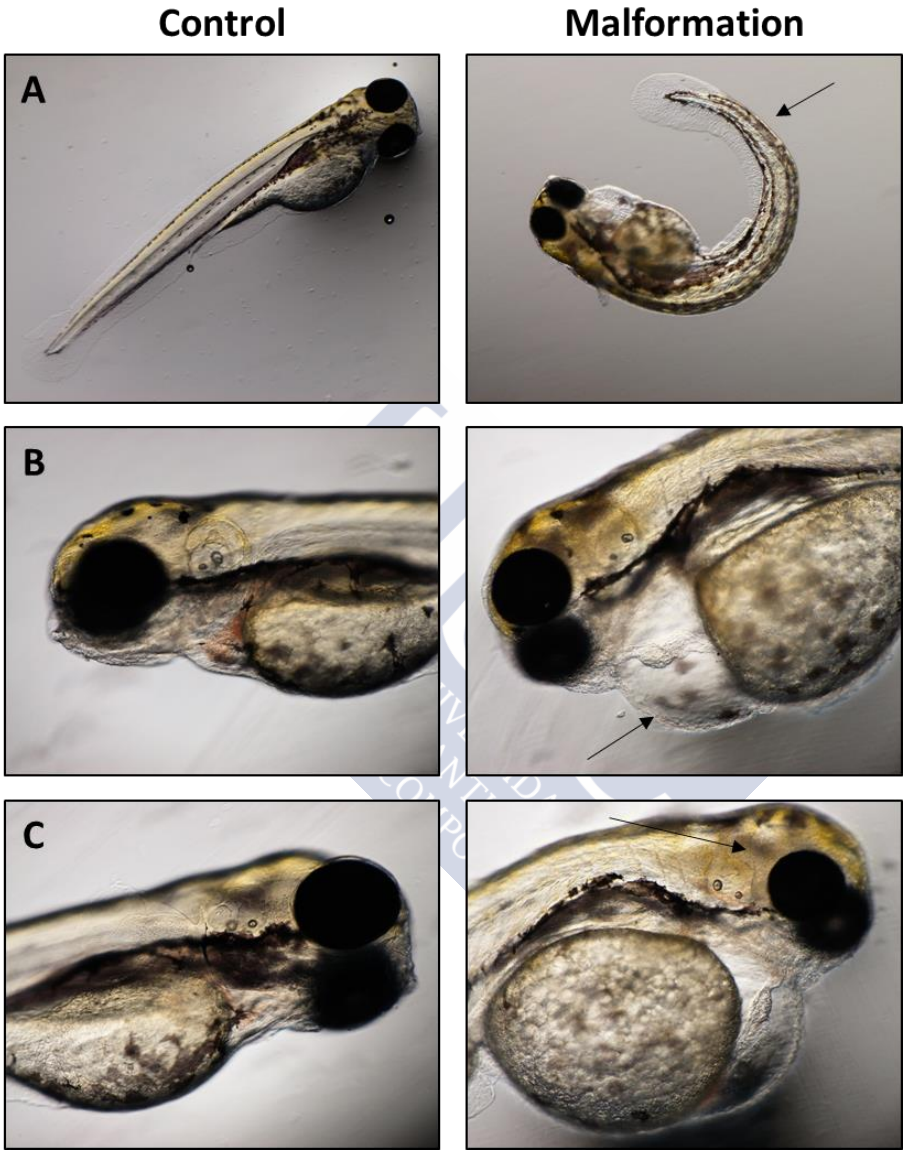

Figure 2. Representative images of morphological defects of the zebrafish embryos. (A) Spinal deviation. (B) Edema. (C) Head deformation. The images were taken at 96hpf.

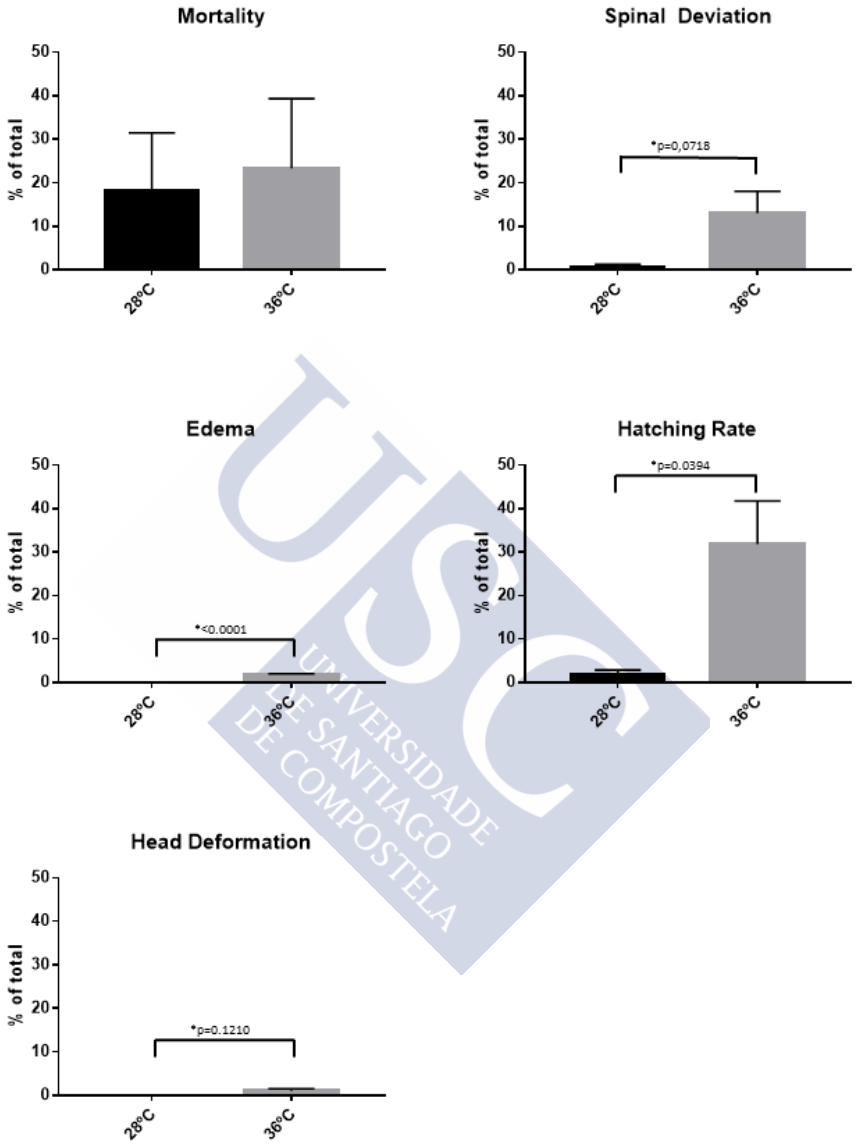

Figure 3. Effect of temperature on development of 48hpf zebrafish embryos. Parameters are expressed as percentages of the total number of embryos assayed after 48hpf at 28ºC and 36ºC. Unpaired student t-test was performed and differences were considered significant when *p<0.05.

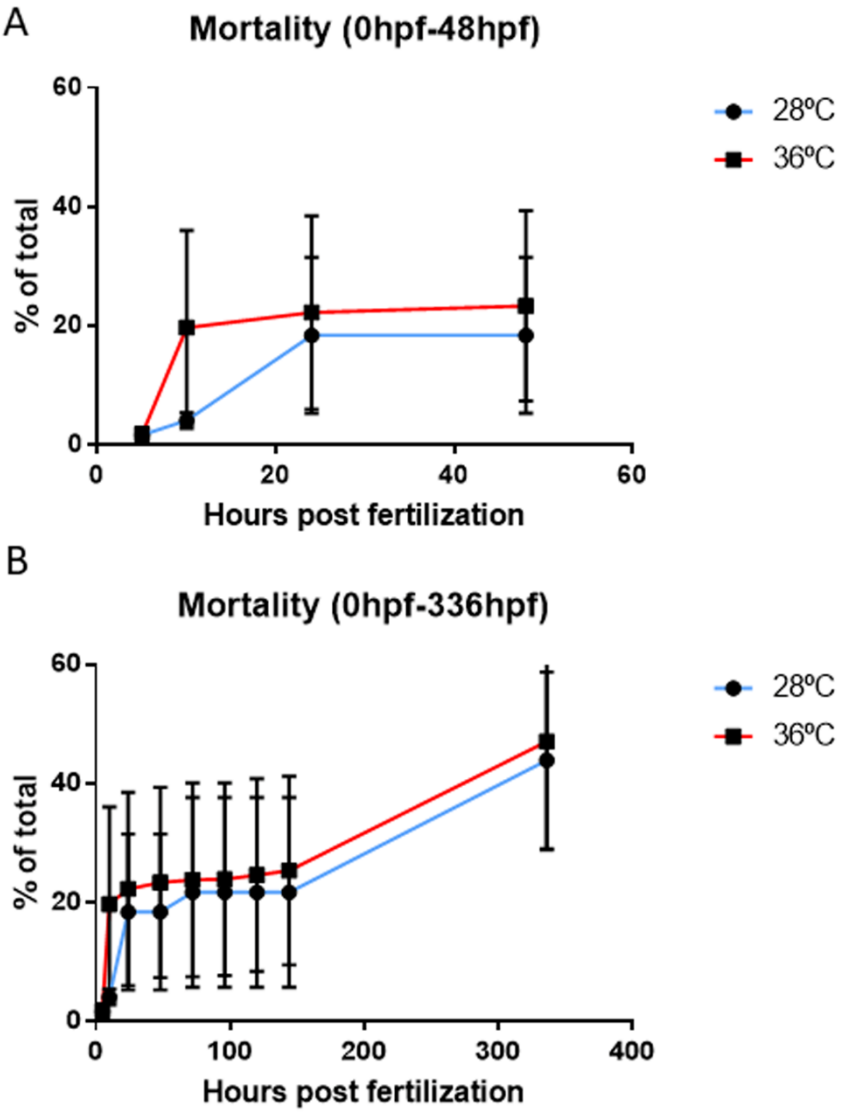

Figure 4. Effect of temperature on viability of 48hpf zebrafish embryos. (A) Mortality of the embryos from 5hpf to 48hpf in closer detail. (B) Mortality of the embryos up to 336h comparing 28ºC and 36ºC ($n_{28ºC}$= 270, $n_{36ºC}$= 700).

embryo injected with medium or MCF7 cells in the yolk (Fig. 5A).

No significant differences were observed between embryos at 34ºC and 36ºC. These results imply that xenotransplantation assays are possible at 36ºC without higher mortality than that observed at 34ºC for 48h of incubation.

When the embryos returned to a normal incubation temperature of 28ºC for the next 4 days, the mortality of the embryos was different between controls at 34ºC and 36ºC. Apart from this, there was an increase in mortality of the embryos previously incubated at 34ºC and 36ºC for 48h (Fig. 5B). Finally, after 10 days of recovery at a normal temperature, zebrafish embryos reach a critical point of the development and most of them die, even in the blank condition (Fig. 5C).

3.2.2 Morphological abnormalities of the embryos

The morphological changes (Fig. 2) suffered by the embryos in the same conditions described previously for mortality, were only statistically significant for edema at 6dpi when comparing embryos injected with cells and incubated at 36ºC with those injected with medium and not injected at all incubated at 36ºC (*$p<0.05$) (Fig. 6B). Therefore, related to xenograft assays there are no differences between cell injection at 34ºC and 36ºC in terms of morphological abnormalities of the embryos. The most important abnormality of the zebrafish embryos at 2dpi is the spinal deviation of the tail, showing an increasing tendency in all the embryos incubated at 36ºC compared to 34ºC and 28ºC (Fig. 6A). There was a slight increase in edemas and head deformations at 36ºC without a higher mortality rate, as previously analyzed (Fig. 6B-C). As observed in Fig. 6 almost all the morphological defects increase with time, except for un-injected embryos at 28ºC (blank).

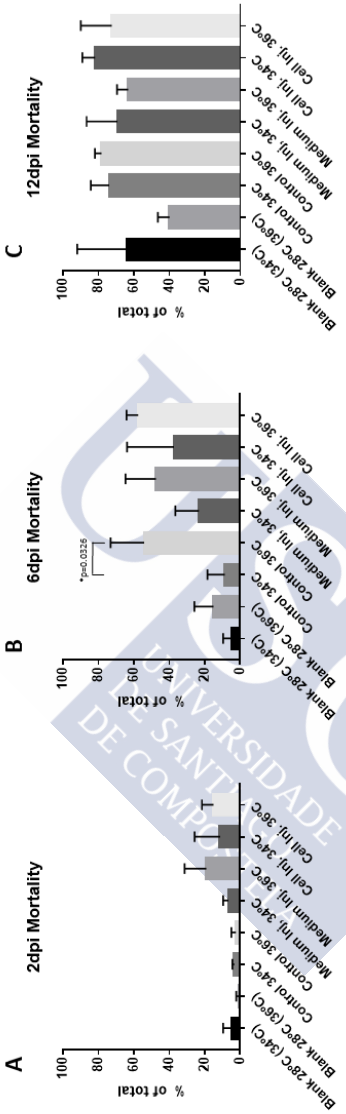

Figure 5. Mortality quantification at different time points comparing the different incubation conditions of the zebrafish embryos. (A) Mortality quantified at 2dpi. (B) Mortality quantified at 6dpi. (C) Mortality quantified at 12dpi. Parameters are expressed as percentages from the total number of embryos assayed for each condition comparing the incubation of the embryos from 2dpi to 12dpi at 28ºC, 34ºC and 36ºC. One-way ANOVA was performed and differences were considered significant when *p<0.05.

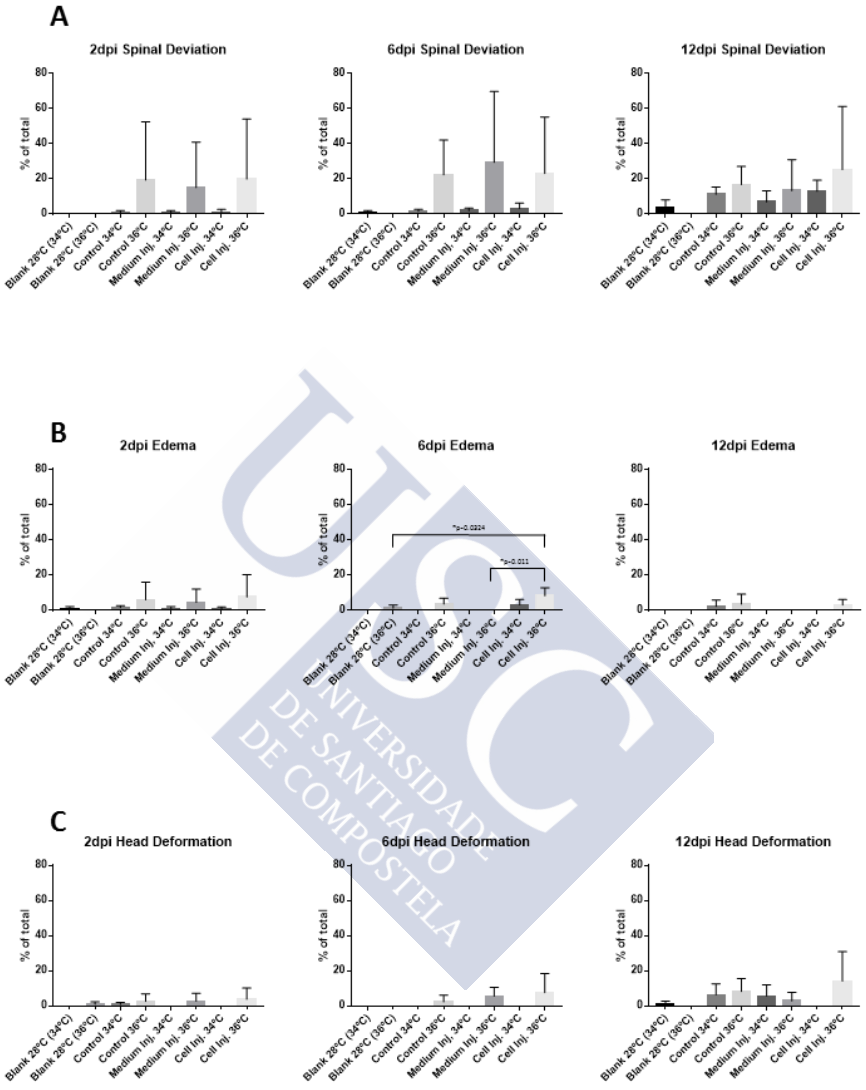

Figure 6. Morphological abnormalities quantification comparing the different time points and incubation temperatures. (A) Spinal deviation comparison at different time points. (B) Edema comparison at different time points. (C) Head deformation comparison at different time points. Parameters are expressed in percentage of the total number of embryos assayed for each condition comparing the incubation of the embryos for 48h at 28ºC, 34ºC and 36ºC and the posterior recovery at 28ºC until the end of the experiment. One-way ANOVA was performed and differences were considered significant when *p<0.05.

3.3 GENE EXPRESSION QUANTIFICATION

Embryos were incubated at 34ºC and 36ºC for 48h (from 48hpf to 96hpf) and then returned to an optimal temperature of 28ºC for another 72h, with the control group incubated at 28ºC permanently. Gene expression varied for genes analyzed (Tab. 1) in embryos incubated at 34ºC and 36ºC when compared to those at 28ºC after 48h. Some genes did not return to normal expression levels in embryos incubated at 28ºC for another 72h.

3.3.1 Gene expression after 48h of incubation

After 48h of incubation at 28ºC, 34ºC and 36ºC the expression of developmental genes (Lft2, Mmp9, Junba and Haus3) and a stress response gene (Hspa9) showed no significant differences. On the contrary, we observed upregulation of Socs3a related to immune response and inflammation, and downregulation of genes involved in stress response (Apex1), metabolism (Wisp and Per2) and structural function (Lum). Of these only Per2 showed a significant difference in embryos grown at 34ºC and 36ºC (Fig. 7A). These results show that except for one gene, there are no significant expression differences in selected genes between 34ºC and 36ºC after 2 days of incubation.

3.3.2 Gene expression after 72h of recovery

The expression of the same genes was analyzed in fish incubated at 34ºC and 36ºC and then returned at 28ºC for another 72h. Most of the genes return to normal expression levels except for Socs3a and Per2, this last showing an inverted expression pattern compared to that observed after 48h for the 36ºC condition (Fig. 7B). Apart from that, the expression of Hspa9 is down-regulated probably due to the recovery at a normal temperature for the embryos.

Table 1. qPCR primers sequence

Name	Short name	Forward Primer	Reverse Primer	Amplicon Size
Apurinic/Apyrimidinic Endodeoxyribonuclease 1	**Apex1**	5'- AAAAGGGGAAAGAGCCCGAG-3'	5'- GTTCTTTTTGACCCACGCCC-3'	137bp
HAUS Augmin Like Complex Subunit 3	**Haus3**	5'- CCGCTCTGCATTACGAGACT -3'	5'- AAGCTGGTCACGAACCTCAG -3'	124bp
Left-Right Determination Factor 2	**Lft2**	5'- GATGGCCGAACTGAAGCTCT -3'	5'- ACTCTGGCGTGGTTTATCGG -3'	90bp
Lumican	**Lum**	5'-GCCATGTACTGCAATGAGCG-3'	5'- GGTCGGTGGCATTGTCAAAC -3'	127bp
WNT1 Inducible Signaling Pathway Protein	**Wisp3**	5'- AGGGAAAACCTGCAAACCCA -3'	5'- TCTTCCAGCGCACATTGGAT -3'	194bp
Period Circadian Regulator 2	**Per2**	5'- TGGGTTTGACCAAGCAGGTT -3'	5'- CTGCTTCAGACGTGACCTGT -3'	153bp
Matrix Metallopeptidase 9	**Mmp9**	5'- AGACGATGCCTGCCAAATCA -3'	5'- GAGATCTTCCAGTAGCGCCC -3'	93bp
Heat Shock Protein Family A (Hsp70) Member 9	**Hspa9**	5'- CGGTGCATGTCAAAGCACAA -3'	5'- TCTCTTTCGCTGGTTTCGCT -3'	193bp
Suppressor Of Cytokine Signaling 3	**Socs3a**	5'- AGTTCGGACAATCGGCACTT -3'	5'- GGAGTCCGTCTGCAGGAAAA -3'	108bp
JunB Proto-Oncogene, AP-1 Transcription Factor Subunit	**Junb-a**	5'- ACGCCGAAGATCAAGTCGTT -3'	5'- TCCGTTCGGCTGAAAGTACC -3'	198bp
Beta Actin	**β-Actin (housekeeping)**	5'-CATCAGCATGGCTTCTGCTCTGTATGG-3'	5'-GACTTGTCAGTGTACAGAGACACCCT-3'	

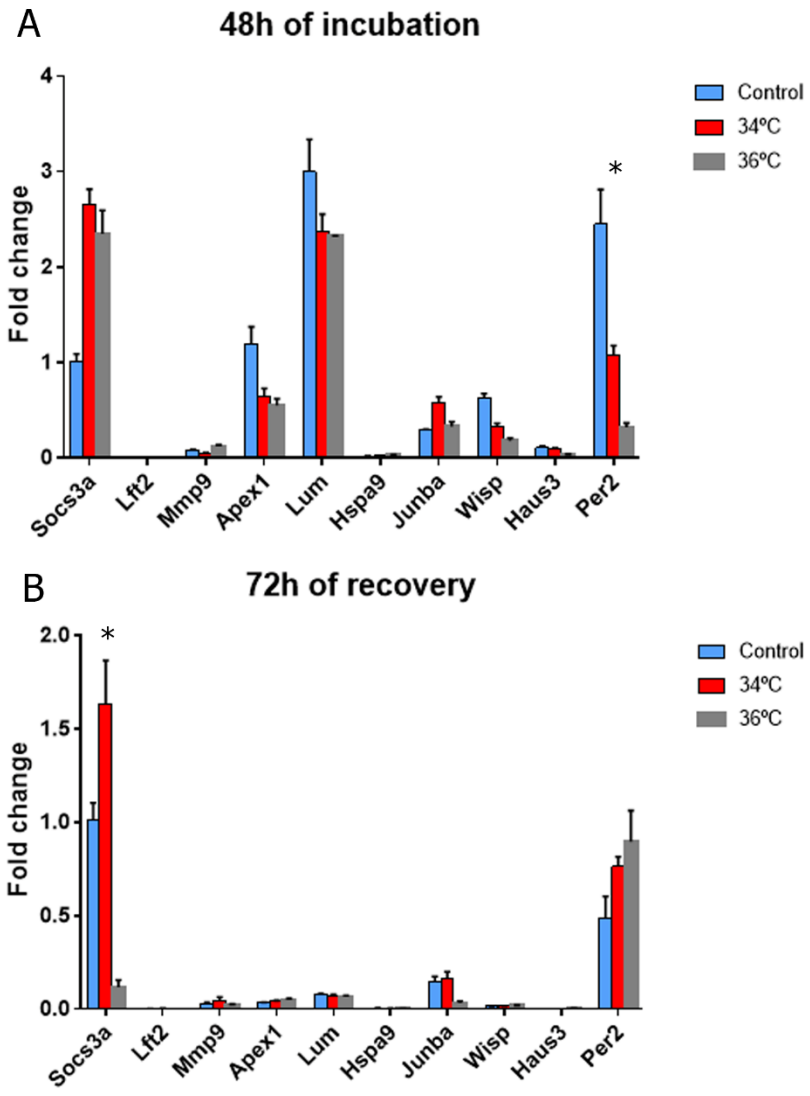

Figure 7. Gene expression analyzed by qPCR after incubation at different temperatures for 48h or 48h+72h of recovery. (A) Results obtained after incubation of the embryos at 28ºC, 34ºC and 36ºC during 48h. (B) Results obtained after 72h of recovery at 28ºC when the incubation at higher temperatures for 48h had finished.

CHAPTER V - MICROENVIRONMENT: CO-INJECTION OF DIFFERENT CELL LINES WITH CONDITIONED MACROPHAGES

1. INTRODUCTION

The majority of the breast cancer-related deaths occurs due to metastases, which correlate with a poor clinical outcome (Lambert et al., 2017). Metastases are mainly produced in lung, bone, liver and brain, and are produced by spreading cells from the initial site of the primary tumor through the bloodstream or lymph system to the secondary sites (Nguyen et al., 2009).

Tumor microenvironment plays a crucial role in facilitating and enhancing the metastasis processes and the communication between the tumor cells with the surrounding cells (endothelial cells, fibroblast, adipocytes and immune cells). One of the most important cells in tumor microenvironment are the macrophages, being the largest population of infiltrating inflammatory cells (Pollard, 2004; Lewis and Pollard, 2006). Macrophages are divided into different categories attending to the effect produced in the tumor microenvironment: activated M1 macrophages, also known as 'good macrophages', are able to kill tumor cells by producing pro-inflammatory cytokines and reactive oxygen/nitrogen species; and activated M2 macrophages, known as 'bad macrophages', being the responsible for the promotion of tumor progression, producing anti-inflammatory cytokines (Mills, 2012; Mantovani et al., 2017). In tumor microenvironment, another type of macrophages appears, called tumor-associated macrophages or TAMs, exhibiting a behavior closer to the M2 or 'bad macrophages'. As a result, these macrophages promote key steps in tumor progression,

including angiogenesis, tumor cell migration/invasion and metastasis (Noy and Pollard, 2014; Williams et al., 2016).

Apart from that, and related to breast cancer progression, the homeobox 1 transcription factor (POU1F1), known as Pit-1, apart from the regulation of the growth hormone and the prolactin gene transcription in the human pituitary gland, is also expressed in human mammary gland (Lefevre et al., 1987; Nelson et al., 1988). Its expression is higher in tumor than in normal breast (Gil-Puig et al., 2005). This Pit-1 overexpression in the mammary gland when a tumor is present, can be considered as a tumor promoting factor, increasing cell proliferation, reducing apoptosis and up-regulating certain factors like Snail, metalloproteases, and CXCR4/CXCL12 (Ben-Batalla et al., 2010; Sendon-Lago et al., 2014; Martinez-Ordoñez et al., 2018), all factors involved in breast cancer progression (Vizoso et al., 2007; Nieto et al., 2016; Müller et al., 2001). Taking this into account, studies performed correlates the Pit-1 overexpression with spreading of the cells, distant metastasis and poor prognosis of patients with breast cancer (Ben-Batalla et al., 2010; Martinez-Ordoñez et al., 2018; Gao et al., 2016). However, some mediators of Pit-1 actions are still unknown like CXCR4 chemokine receptor and its ligand CXCL12 that could play a critical role in the pro-tumoral process induced by Pit-1.

Zebrafish has emerged as an ideal model to get a broader view of the behavior of human cancer injected cells inside the embryos and the development of the tumor in an *in vivo* situation (Lee et al., 2005; Nicoli and Presta, 2007; Fior et al., 2017; Ikonomopoulou et al., 2018). The cells can be labeled with fluorescent proteins like GFP and the macrophages can be easily dyed with commercial lipophilic dyes (DiI, DiO, DiD), in order to track the progression of the proliferation of the cells and the distribution of the macrophages inside the yolk sac of the fish (Pruvot et al., 2011; Drabsh et al., 2013).

Here, we propose to evaluate, through the co-injection of the Pit-1 breast cancer tumor cells and the tumor-associated macrophages (TAMs) inside the yolk sac of the embryo, the interaction between the

injected cells and the microenvironment; and try to know how conditioned TAMs may affect breast cancer progression. Apart from that, the spread of the cells supported by the macrophages were measured to test if this important component of the tumor stroma is helping the cells in their process of dispersion and metastasis. Finally, we evaluated the role of the CXCR4 chemokine receptor and its ligand CXCL12 by knocking-down the CXCR4 chemokine receptor in MCF7-Pit-1 cells and injecting them into the yolk sac of zebrafish embryos.

2. MATERIALS AND METHODS

2.1 CELL LINES AND CULTURES

The human breast adenocarcinoma MCF7-GFP cell line was purchased from Cell Biolabs (San Diego, USA). The human monocyte U937 cell line was obtained from the European Collection of Cell Culture (ECACC; Porton Down, UK). MCF7-GFP cell line was cultured using DMEM Medium (GIBCO, Invitrogen) containing 10% FBS (GIBCO, Invitrogen) and 1% Pen/Strep (GIBCO, Invitrogen) at 37°C with 5% CO_2 in a humidified atmosphere.

2.2 ZEBRAFISH CARE AND BREEDING

Adult zebrafish (*Danio rerio*) were maintained in 30L aquaria with a ratio of 1 fish per liter of water, with 14:10 day/night cycle and a temperature of 28,5ºC according to the standard procedures (Westerfield, 2000). Zebrafish embryos were obtained mating adult zebrafish in a proportion of 2 females / 1 male. All the procedures used in the experiments, fish care and treatment were performed in agreement with the Animal Care and Use Committee of the University of Santiago de Compostela and the standard protocols of Spain (Directive 2010-63-UE) and was performed under the experimental project permission MR110250 in the center authorized with REGA code ES270280346401. At the final point of the experiments, zebrafish embryos were euthanized by tricaine overdose.

2.3 ZEBRAFISH TUMOR XENOGRAFT ASSAY AND COMPUTERIZED IMAGE ANALYSIS

In order to perform the xenografts, 48 hours post fecundation (hpf) zebrafish embryos were anesthetized with 0.003% tricaine (Sigma). MCF7, MCF7-Pit-1 and MCF7-Pit-1shCXCR4 cells expressing GFP were incubated at 37ºC and 5% CO_2 before injection until they reach 80% confluence. Macrophages were conditioned to each cell line medium, $TAM^{lowCD163}$ or $TAM^{highCD163}$ corresponding to MCF7-GFP and MCF7-Pit-1-GFP respectively; collected before injection and dyed with Vybrant DiI (red) cell-tracker dye (Thermo Scientific) following manufacturer protocol. Afterwards, cells and macrophages were collected and resuspended at 10,000-20,000 cells/µl and with a proportion of 3:1 cells/macrophages in complete DMEM and maintained at room temperature for no longer than two hours before they were injected. Borosilicate glass capillary needles (1 mm O.D. x 0.78 mm I.D.; Harvard Apparatus) were used in order to inject cells into the yolk manually using IM-31 Electric Microinjector (Narishige) with an output pressure of 34 kPa and 30 ms injection time. Poorly or non-injected embryos were discarded.

After the injection, zebrafish embryos were incubated for 48hpi at 36ºC in 24-well plates with salt de-chlorinated tap water (SDTW) and a PCR-tape covering the plate to avoid evaporation. Each of the embryos was photographed at 0hpi and 72hpi with AZ-100 Nikon fluorescence stereomicroscope in order to track the proliferation of the injected GFP cells and DiI macrophages. The analysis was performed with ZFtool software yielding the number of GFP pixel in each image, which represents the area of the cells inside the yolk sac of the embryo and the GFP Intensity Medium Value, which represents the medium intensity of the GFP inside the embryo. This analysis is executed at 0hpi and 48hpi to obtain a comparison between them. The results were processed to obtain a proliferation ratio (1: the number at which the cells are maintained during incubation, >1: tumor cell proliferation during incubation, and <1: tumor cell death during incubation).

2.4 STATISTICS

Statistical analyses were performed using SPSS Software (IBM). Homoscedasticity was tested for all the data and then an excel outlier analysis was carry out using interquartile range (IQR) to discard possible outliers. U Mann-Whitney test for non-parametrical data was applied to non-homoscedastic data with confidence intervals of 95%.

3. RESULTS

Based on a collaboration with another research group (Román Perez, CIMUS), we assayed the co-injection of human breast cancer cells (MCF7-GFP) with conditioned macrophages in order to test if the addition of a principal component of the human stroma is crucial for the progression and spreading of tumor cancer cells *in vivo*. Besides that, another assay was carried out to confirm that the axis CXCL12 chemokine and its CXCR4 receptor mediates tumor growth and migration *in vivo*.

As we stated in the introduction of this chapter, the Pit-1 gene overexpression in the cells is related with tumor environment in the breast of the patients and it is considered as a tumor promoting factor. The overexpression of this gene causes a poor prognosis and we wanted to assay *in vivo* with the zebrafish as a model organism the relation between the overexpression of this gene in the human tumor cells and the TAMs that arises when a tumor is present. In this way, normal monocytes were exposed to the medium of MCF7-Pit-1 human breast cancer cells and isolated to perform the co-injection of MCF7-Pit-1 cancer cells and the macrophages conditioned to a normal MCF7 cell line against macrophages conditioned with MCF7-Pit-1 cell line, in order to see the differences between them. The CXCR4 chemokine receptor could be influencing the tumor growth and spreading of the cells. To test this hypothesis, MCF7-GFP cells were knocked-down for this CRCR4 receptor, in order to test whether this receptor could be interfering in the cell spread.

3.1 TAM^{HIGHCD163} INFLUENCES TUMOR CELLS WITH PIT-1 OVEREXPRESSION TO INCREASE TUMOR GROWTH AND SPREADING *IN VIVO*

MCF7-Pit-1 cells were collected and combined together with macrophages to test two conditions: MCF7-Pit-1 cells/ TAM^{lowCD163} and MCF7-Pit-1 cells/ TAM^{highCD163} conditioned macrophages. The proportion used to perform the injections was 3:1 ratio cells/macrophages. Experiments were carried out at 36ºC incubation temperature to favor normal tumor cell growth according to the results obtained in Chapter I and II. To evaluate if tumor growth is influenced by the Pit-1-TAMs relationship *in vivo*, TAM^{lowCD163} and TAM^{highCD163} were stained with DiI (red) and injected with MCF7-Pit-1-GFP into the yolk sac of zebrafish (Fig. 1A). 48 hours later, MCF7-Pit-1-GFP had significantly more growth when cultured with TAM^{highCD163}. Notably, the ratio of overall tumor growth in zebrafish injected with TAM^{highCD163} was higher than in those injected with TAM^{lowCD163} ($p<0.05$) (Fig. 1B-C). Tumor cell spreading was also measured; MCF7-Pit-1-GFP co-injected with TAM^{highCD163} showed a significant ($p=0.023$) increase in cell spreading as compared to those co-injected with TAM^{lowCD163} (Fig. 2A-B).

3.2 PIT-1 REGULATES THE CXCL12 CHEMOKINE AND ITS CXCR4 RECEPTOR AND MEDIATES TUMOR GROWTH AND MIGRATION *IN VIVO*

As stated in the introduction, they found that Pit-1 increases mRNA and protein in both CXCR4 and CXCL12. Knock-down of CXCR4 chemokine receptor reduced tumor growth and spread of Pit-1 overexpressing cells *in vitro*. In order to corroborate the *in vitro* effects *in vivo*, a zebrafish xenograft model was carried out to evaluate tumor growth and spread. MCF-7-GFP cells either (a) infected with control lentiviral activation particles + transfected with the pLKO control vector (MCF-7-GFP- control), (b) infected with Pit-1 lentiviral activation particles + transfected with the pLKO control vector,

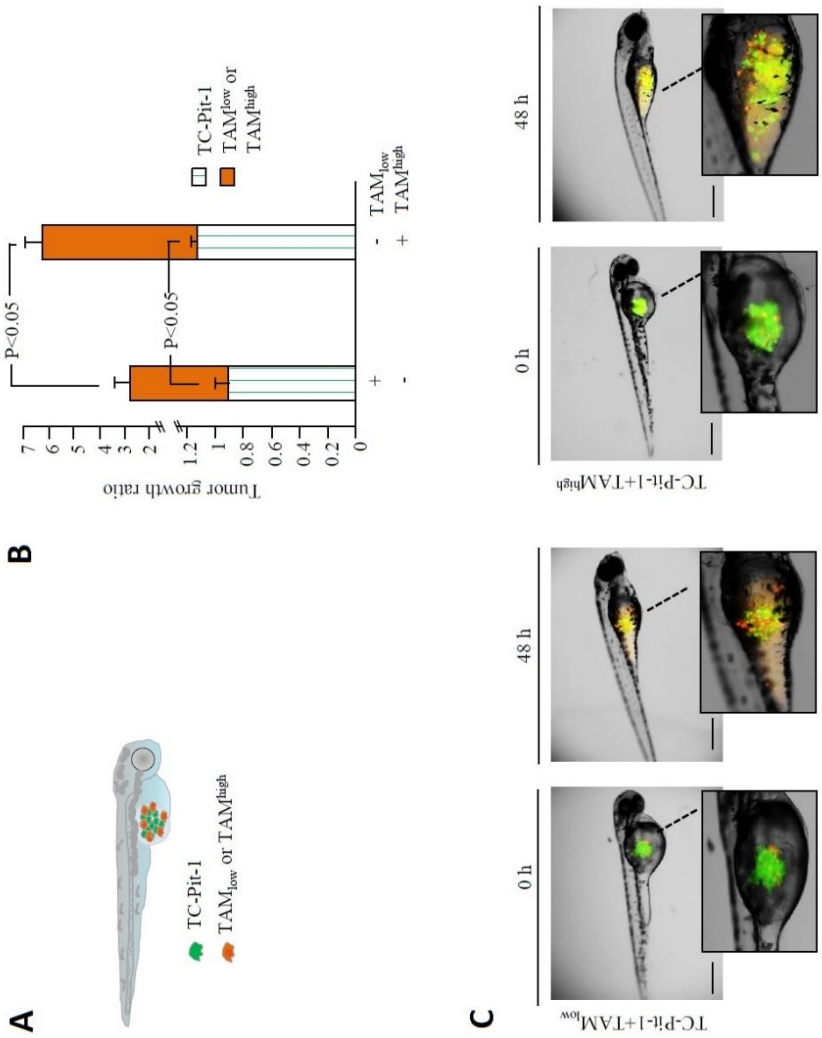

Figure 1. Tumor cells and macrophages cooperate to increase tumor growth. (A) Zebrafish were injected in the yolk sac with either TAM^{lowCD163} (macrophages cultured for 6 days with CM from MCF7) or TAM^{highCD163} (macrophages cultured for 6 days with CM from MCF7-Pit-1) and MCF7-Pit-1-GFP. Tumor growth was measured at 0 and 48 h post-injection. (B) Tumor growth increases significantly when MCF7-Pit-1 are injected with TAM^{highCD163}. Data were obtained using the ZFtool software. (C) Representative images of tumor growth in zebrafish embryos (n=50 per group) injected in yolk sac with MCF7-Pit-1 (green) + TAM^{lowCD163} (red) and MCF7-Pit-1 (green) + TAM^{highCD163} (red) at 0hpi and 48hpi. Scale bar: 300 µm. Bars in B represent mean ± SD.

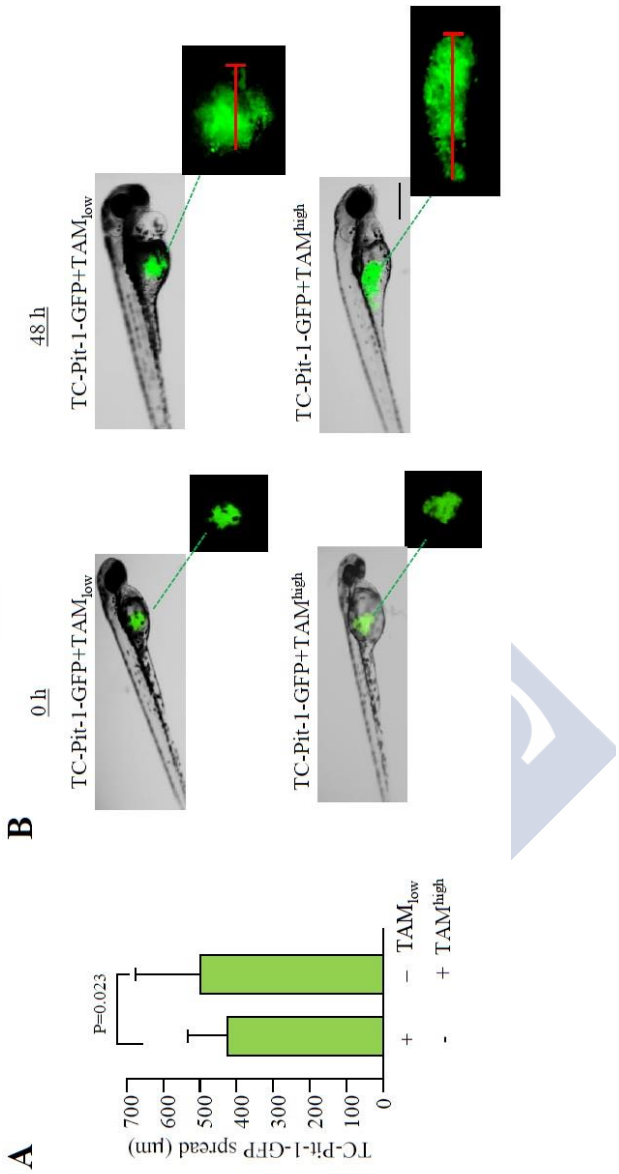

Figure 2. Tumor cells and macrophages cooperate to increase spreading. (A) MCF7-Pit-1-GFP spreading in zebrafish embryos (n=50 per group) injected in yolk sac with $TAM^{lowCD163}$ and $TAM^{highCD163}$ after 48 h. (B) Representative images of MCF7-Pit-1-GFP spreading. Red line indicates cell spread. Data were obtained using the ZFtool software. Scale bar: 300 µm. Bars in B and D represent mean ± SD.

(MCF-7-GFP-Pit-1), or (c) infected with Pit-1 lentiviral activation particles + transfected with the pLKO-CXCR4 shRNA vector that knocked-down endogenous CXCR4 (MCF-7-GFP-Pit-1-shCXCR4) were injected into the yolk sac of zebrafish embryos and fluorescence intensity and area were measured 48 h later. Our data indicate a significant ($P = 0.021$) increase in tumor growth as well as in cell spread ($P = 0.003$) after Pit-1 overexpression with respect to MCF-7-GFP control cells. However, CXCR4 knock-down significantly reduces tumor growth ($P = 0.002$) and cell spread ($P < 0.001$) in relation to MCF-7-GFP-Pit- 1 cells (Fig. 3A–C).

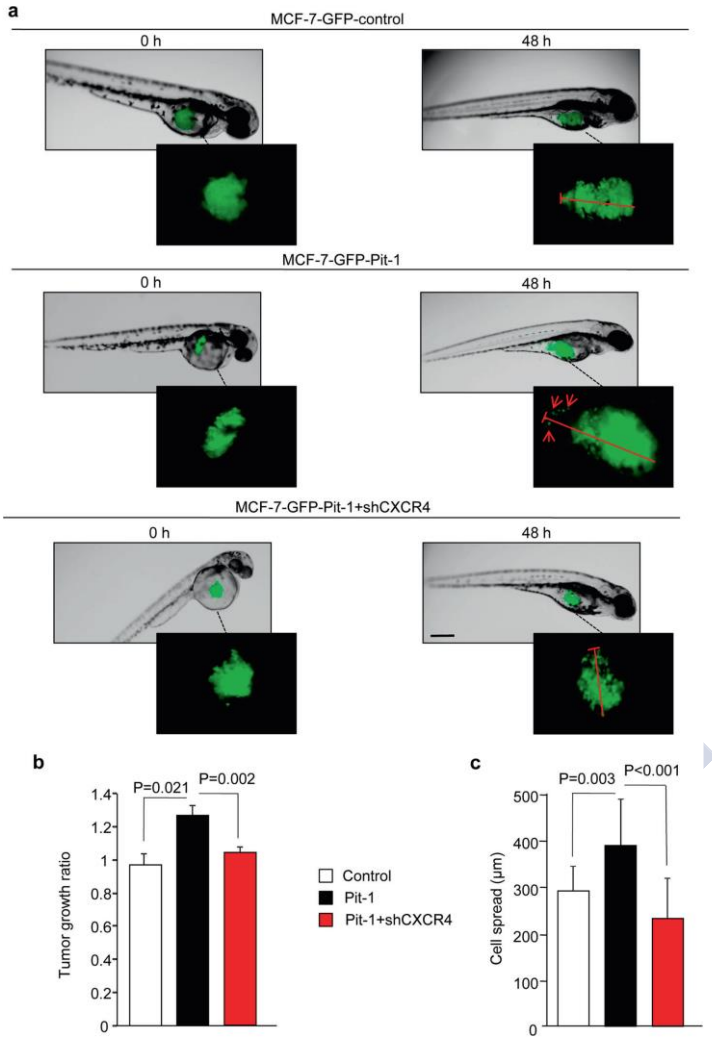

Figure 3. Fig. 3 CXCR4 knock-down in Pit-1 overexpressed MCF-7 cells reduces tumor growth and spreading in zebrafish embryos. (A) Representative images of tumor growth and cell spreading in zebrafish embryos (n = 40 per group) injected in yolk sac with control cells (MCF-7-GFP-control), Pit-1 overexpressing cells (MCF-7-GFP-Pit-1), and Pit-1 overexpressing + CXCR4 knocked-down cells (MCF-7- GFP-Pit-1 + shCXCR4) at 0 hours post injection (hpi) and 48hpi. Red line indicates cell spread, and red arrows in MCF-7-GFP-Pit-1 cells indicate early metastasis. Fish images are a superposition of a fluorescence field image over a bright field image. Scale bar: 250µm. (B) Tumor growth ratio in zebrafish embryos injected with the cells described in A. Data were obtained using the ZFtool software. (C) Cell spread (in µm) in zebrafish embryos injected with the cells described in A.

CHAPTER VI - DISCUSSION

Zebrafish has been known as a model organism from the 1960's since George Streisinger was the first researcher using this model in the field of developmental biology and vertebrate genetics (Streisinger et al., 1981). In 2005, Lee et al. performed the first xenograft in the embryos of this animal model, injecting melanoma human cancer cells into the yolk sac of the embryo and demonstrating that the cells actually survive, divide and migrate in the host (Lee et al., 2005). Besides that, in 2006, another research group supported these finding performed by Lee, demonstrating that, apart from dividing and proliferate, the angiogenesis occurs inside the yolk sac of the embryo towards the implanted tumor (Haldi et al., 2006). With these findings the zebrafish emerged as a new model for biomedical studies related to cancer, with the possibility of being a complement of the murine model in xenograft assays, with some advantages compared to mice like small size, large number of offspring, cost-effective ratio and the possibility of testing multiple chemotherapeutic drugs with more robust and accurate results (Konantz et al., 2012).

Cancer is a cell cycle de-regulation, causing abnormal cell growth with the capacity of infiltrate the blood torrent and spreading to other parts of the human body (Munkley and Elliot, 2016), producing another neoplasm or tumor mass, called metastasis, that often leads to death (Liu et al., 2017). Cancer cells have some principal characteristics: unregulated cell growth; they can avoid cell death; they can divide without limit; they secrete factors promoting the formation of blood vessels and they can invade another tissues or organs forming metastasis (Hanahan and Weinberg, 2011). Importantly, cancer is considered one of the three main death causes in the developed countries all around the globe (Siegel et al., 2016). Although it can be

considered as one disease, the reality is far from that, due to the fact that each case of cancer has their own peculiarities even in the same type of cancer (e.g. breast cancer) (Özdemir and Dotto, 2017).

Considering the differences that exists between types of cancer, and on the other hand the differences between individuals, the necessity of animal modeling for personalized medicine has increased in the last years. Being mice nowadays the main avatar model for cancer personalized medicine due to the genome similarity with the human (Mouse Genome Sequencing et al., 2002), zebrafish can offer a more affordable alternative to mice, being able to perform in this small embryos high-throughput screening of different combinations of chemotherapeutic drugs and reducing the time needed to obtain an accurate result (MacRae and Peterson, 2015; Letrado et al., 2018).

In the present thesis, we tested overall conditions of the xenograft technique improving image analysis and temperature, demonstrating that the temperature of 36ºC is an important factor for obtaining more accurate and precise results (Cabezas-Sainz et al., 2018). Besides that, these results were complemented by testing how this temperature affects the zebrafish embryos at different levels (malformations and metabolism related changes) and highlighting that this temperature is, in terms of abnormalities, close to the most used temperature in the bibliography, 34ºC (Eguiara et al., 2011; Ghotra et al., 2012; He et al., 2012; Ban et al., 2014; Ent et al., 2014). Finally, we tested this improved technique, and advance another step in the way of the model for reaching the personalized medicine, by measuring the proliferation and spreading of the MCF7 breast cancer cells injected in the zebrafish embryo with another component of the human stroma present on all of the tumors: the macrophages (Brown et al., 2017).

1. IMPROVEMENT OF THE XENOTRANSPLANTATION TECHNIQUE BY MODIFYING THE INCUBATION TEMPERATURE OF THE EMBRYOS AND DEVELOPING A NEW IMAGE ANALYSIS SOFTWARE

An enhancement of the xenotransplantation technique is required, together with accurate imaging analysis software to verify the behavior of the cells inside the zebrafish embryo. This study describes an

improvement in the xenotransplantation conditions in relation to temperature and the establishment of a proliferation index of the injected cells in combination with the new image analysis ZFtool software.

Different authors reported normal development of zebrafish embryos up to 35.5ºC (Nicoli and Presta, 2007; Stoletov et al., 2010), but a range of temperatures was tested in order to reduce the mortality of the embryos. Some authors noted that more assays would be needed to check the proliferation, migration, and response of the cells to drugs at higher temperatures despite the potential increase in mortality (Konantz et al., 2012).

We have set the temperature of the cells xenotransplanted into the zebrafish embryos closer to human temperature by raising the temperature from 28ºC (normal temperature at which zebrafish embryos develop) to 36ºC, with no significant change in mortality and no developmental defects on the surviving embryos at three days post injection. Embryo incubation temperature is important to test the effects of anticancer drugs (Jung et al., 2012; Bentley et al., 2015), otherwise the temperature could affect the proliferation rate of the injected cells, and the drug effect is underestimated. The results in this study clearly showed that the proliferation of injected cells and their response to anticancer drugs is better at 36ºC than at 34ºC. Importantly, 36ºC is more close to the cell optimal growth temperature of 37ºC in the human body.

The number of injected cells is very relevant in terms of the proliferation and migration of these cells and should be considered for improving xenotransplantation and anticancer drug proliferation assays. The growth rate of the cells injected inside the embryos decays when the number of initial cells is insufficient at 34ºC. This may be due to cell-cell interactions: the cells injected appear to be isolated and cannot interact among themselves in order to proliferate properly. Nevertheless, even if we reduce the number of injected cells at the initial time point of 0hpi, when the embryos are incubated at 36ºC, a higher proliferation rate exists, when compared to 34ºC and low number of cells injected. This previous point was assayed *in vivo*, demonstrating

that, despite the number of injected cells and mortality, 36ºC is an optimal temperature for cell growth. On the other hand, cell migration can also be modified, depending on the number of cells injected. Cells will not be able to migrate when the number of injected cells is insufficient. It is reported that 400 cells are the optimal number of injected cells to study these behaviors. The injection of different numbers of the colorectal cancer cell line HCT116 remained in the yolk of the embryo from 0hpi to 72hpi, consistent with other authors that used the HCT116 cell line. In fact, this cell line has a low dissemination ratio (Jung et al., 2012; Spaink et al., 2013).

Using *in vitro* studies, other authors have performed proliferation assays with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide colorimetric assay (MTT). The initial cell density seeded on the plates was the same for each experiment, therefore, there was no assessment of how the proliferation could change with different concentrations of the initial cells seeded (Jung et al., 2012; Zhao et al., 2011). In this study, we show that, at least for the cell line HCT116, the temperature and number of initially seeded cells are critical factors for the proliferation of injected cells.

Together with the work done for the improvement of incubation temperature in zebrafish xenotransplantation, a new software (ZFTool) was developed to measure the cell proliferation inside the yolk of the embryo, that could be extrapolated to other sites of injection. This method was designed to fill the gap present in the current methodology that does not adequately quantify cell proliferation at different times *in vivo*. For example, flow cytometry is not sensitive enough to quantify the number of cells in dispersed embryos (Pruvot et al., 2011), and software used by other authors, such as ImageJ or Photoshop, do not automatically quantify proliferation in an accurate, reliable or reproducible way enough to compare different injected embryos (Corkery et al., 2011; Moshal et al., 2011; Yang et al., 2013). This software establishes in each pair of images analyzed an automatic threshold in order to measure the fluorescence of the cells, discarding the autofluorescence of the embryo, yielding more accurate results.

In summary, we demonstrated that at 36ºC, a better proliferation rate exists for the injected cells inside the embryos, with no significant mortality changes compared with 34ºC. Our results also revealed a correlation between the number of initially injected cells and the proliferation ratio when comparing the two different temperatures. In addition, we used a new image analysis software, the ZFtool, which improves tumor cell quantification *in vivo* with accuracy and speed. One of the future challenges will be the quantification of these cells with a 3D method with much greater accuracy, reaching the count of each cell individually.

2. COMPARISON OF THE DEVELOPMENT DEFECTS, MORTALITY AND METABOLISM OF THE ZEBRAFISH EMBRYOS INCUBATED AT 28ºC, 34ºC AND 36ºC

One of the most important parameters of the xenograft affecting the proliferation rate of the cells and the integrity of the host, is temperature. Higher incubation temperatures result is a boost of cells proliferation in detriment of integrity of the host (Konantz et al., 2012), but it is important in order to obtain accurate results, even if a treatment is applied to the injected cells. This last point is highlighted in recent works assaying 5-Fluorouracil in xenografted colorectal cancer cells at different temperatures and obtaining different inhibition ratios (Cabezas-Sainz et al., 2018).

We have previously shown that incubating embryos at 36ºC for up to three days is feasible and no higher mortality is observed when compared to embryos incubated a 34ºC (Cabezas-Sainz et al., 2018). Temperature induced malformations is less clear, while it was recently reported that incubation of zebrafish embryos at 32,5ºC and above causes malformations on the fishes (Pype et al., 2015), other authors reported normal development up to 35,5ºC (Nicoli and Presta, 2007; Stoletov et al., 2010). In this work, we aimed to study the effect of the temperature range used in xenograft experiments on the host and further explore the possibility of increasing the temperature of xenograft assays up to 36ºC.

In a first experiment, embryos were incubated at 36ºC from 0hpf to 48hpf to confirm if they could be more sensitive in this stage of development to temperature. While mortality was not different at 48hpf when compared to previous reports (Pype et al., 2015), malformations were present in most of the embryos incubated at 36ºC, being significant the spinal deviation and the edema. Apart from that, at this point (48hpf), the hatching rate of the embryos was higher at 36ºC due to the increase in development speed produced by temperature. Interestingly, the hatching rate of 36'5ºC is 0% (Pype et al., 2015) pointing to an inflexion point in the tolerated temperature between 36ºC and 36'5ºC. No differences in mortality were observed between treatments at 336hpf. Based on this data, the most sensitive stage in terms of temperature for the zebrafish embryos development appears to be between 5hpf and 24hpf.

In a second experiment, we incubated xenografted and no-xenografted embryos during 2 days at 34ºC (this is the normal temperature for this type of experiment (Eguiara et al., 2011, He et al., 2012, Spaink et al., 2013)) and 36ºC to compare mortality, morphological effects, and metabolism. Compared to the first stage of development (0hpf - 48hpf, previous experiment), the incubation at 36ºC for 48h (48hpf to 96hpf, range we stablished for xenograft assays at 36ºC (Cabezas-Sainz et al., 2018)) and the posterior recovery of the embryos showed different results. At this stage of development and under different conditions (control, injected with medium or injected with cells), the embryos are less sensitive to temperature changes than in the first 48h. This could be related to a higher incidence of temperature on development between 0hpf and 48hpf yielding more abnormalities and mortality of the embryos. Hatched embryos in larval stage of development incubated at 36ºC and injected with medium or cells could lead to higher mortality compared to 34ºC. For example, when the embryos are injected and incubated at 36ºC, there is an increased tendency to spinal deviation and edema. The absence of differences in mortality and malformations in injected embryos (medium and cells) between 34ºC and 36ºC at 2dpi open up to the possibility of reducing the incubation time to 2dpi to see xenografted cells proliferation, invasion or drug effects. The optimal temperature

would be 36ºC to assure a nearly optimal temperature for the cells, allowing more consistent and realistic results, taking into account the state of the host and the cell-host interaction. Otherwise, lower temperatures could lead to lower tumor cell proliferation (Fior et al., 2017; Wu et al., 2017), ending up in an overestimation of the chemotherapeutic effect for assayed drugs (Cabezas-Sainz et al., 2018).

A gene expression analysis of different genes related to development, immune system, stress, and metabolism based on a previous work (Long et al., 2012) was performed to assess the effect of different incubation temperatures and xenograft conditions on zebrafish embryos. Despite the incubation start point between our work and Long's work (Long et al., 2012) (48hpf *vs* 96hpf) and the incubation period (48h *vs* 2h/48h), the results for the negatively regulated genes (Apex1, Haus3, Lft2, Lum and Wisp3) was concordant. This was also the case for the up-regulated genes (Hspa9, Junb-a and Socs3a), except for Per2 and Mmp9. Mmp9 appeared repressed when the incubation was performed at 34ºC while up-regulated at 36ºC. After 72h of recovery these two values switched, but without significant differences between temperatures.

After the 48h incubation at 34ºC and 36ºC, Per2, involved in the circadian cycle regulation (Kim et al., 2018) and metabolism, was affected, being repressed to a higher degree at 36ºC than at 34ºC. After the 72h recovery period the gene differentially expressed according to the temperature was Socs3a (pro-inflammatory and immune response) (Yee et al., 2011), with up-regulation at 34ºC and down-regulation at 36ºC, highlighting an unusual behavior when we raise the temperature from 34ºC to 36ºC. While there is a recovery of this component of the immune system for embryos incubated at 34ºC no such thing happens for embryos incubated at 36ºC.

In relation with Per2, down-regulation of this gene induces the expression of pro-inflammatory cytokines in zebrafish. Generally, these pro-inflammatory cytokines show an expression pattern related to the light-dark cycle showing a peak during the dark period. So one explanation for the expression profile of Per2 could be that when the zebrafish embryos are in dark as occurs in this study (embryos are

incubated in the dark at 34ºC and 36ºC), the down-regulation of this gene probably induces the expression of pro-inflammatory cytokines (Ren et al., 2018). Also, the strong down-regulation of Per2 after 48h at 34ºC and even more at 36ºC could be due an effect of the temperature upon the expression of this gene. This makes sense if the embryos are subject to increased temperatures: genes related to the immune and inflammatory response (as for example Socs3a) are up-regulated, highlighting that the embryo is reacting to thermal stress. At this point, Per2, related to pro-inflammatory cytokines, is down-regulated to promote the inflammatory response of the embryo to high temperatures.

Socs3a, apart from being involved in immune response and inflammatory pathways, is related to tissue regeneration, acting as a negative regulator of the STAT3 signaling pathway. This pathway is involved in regeneration of the liver, skin, fin, retinas, and the sensory epithelium hair cells of the inner ear of zebrafish embryos, apart from being involved in cell proliferation, migration, and survival (Khaliq et al., 2018; Liang et al., 2012). Socs3a is differentially regulated after the 72h of embryo recovery, being slightly up-regulated at 34ºC and down-regulated at 36ºC. The down-regulation we observed in this gene after the 72h of recovery at 28ºC after incubation at 36ºC could be a consequence of the embryo tissue damage after incubation at 36ºC during 48h that needs to be compensated by continued activation the STAT3 pathway. At 34ºC tissue damage could be lower and/or be already repaired by the time we assayed the Socs3a expression.

In conclusion, no significant differences exist in mortality or malformations in control and xenografted zebrafish embryos incubated at 34ºC and 36ºC for 48h. Gene expression changes between these two temperatures may affect the inflammatory response and regeneration differentially. Considering this, 36ºC should be the temperature of choice in experiments with a duration of 48h in order to get robust and accurate results in terms of tumor cell proliferation and/or invasion, and drug testing *in vivo*.

3. TESTING THE MICROENVIRONMENT EFFECTS: CO-INJECTION OF DIFFERENT CELL LINES WITH CONDITIONED MACROPHAGES

In this study, we performed the *in vivo* part with zebrafish embryos as a model organism in order to measure and test the different conditions of MCF7-Pit-1-GFP cell line with different conditioned macrophages acting as a microenvironment inside the yolk sac of the embryos.

The communication that takes place between the tumor and the surrounding microenvironment is crucial for the cancer progression inside the human body (Lazennec and Richmond, 2010). Pit-1 overexpression in MCF7 breast cancer cells produce an increase in secretion of different factors like cytokines and chemokines (Lazennec and Richmond, 2010) that are relevant for the mobilization of macrophages to tumor area and transforming them into tumor associated macrophages (TAM's) (Quail and Joice, 2013; Bin-Zhi and Pollard, 2010). Besides that, this overexpression is related to more tumor growth, angiogenesis and cell spreading (Lewis et al., 2000). The most important consequence of cell spreading is the metastasis, mostly occurring in lung in this case. Considering this, patients with high Pit-1 expression have poor prognosis and a high risk of lung metastasis.

In this study we applied our own developed methodology to test the involvement of $TAM^{highCD163}$ and the $TAM^{lowCD163}$ in the processes underlying the interaction between these conditioned macrophages and the MCF7-Pit-1-GFP tumor cells inside the yolk sac of the embryo in terms of proliferation and the spreading of the tumor cells. Interestingly, our data agree with a recent study performed in zebrafish using tumor cells co-injected with TAMs isolated from metastatic human primary tumors that exhibit high invasion and metastasis capacity compared to TAMs isolated from non-metastatic tumors, demonstrating that tumor cells are highly dependent on macrophages for their dissemination (Wang et al., 2015).

Zebrafish as an animal model, compared to the murine model, still has some drawbacks in terms of metastatic potential of tumors and the spreading of the cells due to the fact that the organs of the zebrafish are

pre-formed at 2 days post fertilization (Howe et al., 2013a), but the mouse model provides more information about the metastatic potential of the cells and the preferred site of implantation when they spread from the primary tumor site (Paschall and Liu, 2016; Jäger et al., 2018). The tropism of the cells injected into zebrafish embryos is difficult to evaluate, as previously mentioned, and in this case, because zebrafish lacks some analog organs compared to human in the case of breast cancer. Even more, the organ where breast cancer metastasizes with higher probability, the lung, is missing in zebrafish. Considering this, there are some experiments easily performed in zebrafish that could be worth before testing them in mice, being the two animal models complementary for different situations.

Finally, some authors highlighted another problem regarding the innate immune system of the embryos. Zebrafish embryos develop the adaptive immune system from day 12-14 of life and afterwards the only immune system is the innate immune system that the female provides (Renshaw and Trede, 2012). This innate system includes mostly macrophages and neutrophils and it has been demonstrated that this innate immune system interacts with the xenografted cells inside the embryos (Tulotta et al., 2016). Considering this, the innate immune system could be playing a role in the proliferation and spread of the primary tumor of the fish.

In summary, the data obtained indicates that the zebrafish is a valid model to test the microenvironment of human tumor cells with the co-injection of conditioned macrophages. Being Pit-1 overexpression the cause of recruiting and differentiating normal macrophages into TAM's with high expression of CD163. The cells where co-injected with different conditions of macrophages and the results showed an increase in proliferation of the cells injected with $TAM^{highCD163}$, supporting the results obtained *in vitro* prior to the *in vivo* assays. Apart from that, it could be possible to measure the spreading of the cells inside the yolk sac of the embryos within different conditions due to the influence of the $TAM^{highCD163}$ macrophages.

CHAPTER VII - CONCLUSIONS

The work summarized in these three chapters aimed to improve the xenotransplantation technique in zebrafish embryos through raising the incubation temperature to 36ºC and analyzing the phenotypic and metabolism effects of this temperature in the zebrafish embryos, to finally add the effect of the microenvironment and reach closest conditions to the human body.

The results allowed us to withdraw the following conclusions:

1.- ZFtool software allows the researcher for an accurate and automatized analysis of the images of the zebrafish embryos and, on the other hand, human colorectal cancer cell line HCT116 injected into zebrafish embryos has a better proliferation index at 36ºC rather than at 34ºC. Furthermore, 36ºC is the most suitable temperature for testing chemotherapeutic drugs like the 5-Fluorouracil.

2.- No significant differences exist in mortality or malformations in control and xenografted zebrafish embryos incubated at 34ºC and 36ºC for 48h. Gene expression changes between these temperatures may affect the inflammatory response and regeneration during the incubation through Per2 down-regulation and afterwards, after the recovery period through Socs3a of the embryos at 36ºC. Taking this into account, experiments with a duration of 48h and an incubation temperature of 36ºC should be the choice in order to get robust and accurate results in terms of tumor cell proliferation and/or invasion, and drug testing *in vivo*.

3.- Co-injection of conditioned macrophages with human MCF7 breast cancer cell line demonstrates that the microenvironment components are important to reach the full potential of the injected human cancer cells inside the yolk of the embryos, giving support and enhancing the proliferation and spreading of the cells. This study

supports the possibility of co-injecting the human cancer cells with other cell types of the humor matrix to mimic the human microenvironment.

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USC
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RESUMEN

1. INTRODUCCIÓN

1.1 PEZ CEBRA

El pez cebra (*Danio rerio*) (Hamilton, 1822) es un pez de agua dulce, perteneciente al género *Danio*, el cual es originario de los ríos de la parte sudeste de la región del Himalaya, incluyendo India, Pakistán, Bangladesh, Nepal y Myanmar. El pez cebra pertenece a la familia *Cyprinidae* del orden *Cypriniformes* (McCluskey et al., 2014, Stock et al. 2007), y debe su nombre a su característica morfología, siendo un pez fusiforme con rayas horizontales a cada lado del cuerpo, recordando a una cebra. Los machos tienen forma de torpedo y suelen ser más alargados y pequeños que las hembras; y por otra parte las hembras suelen ser más grandes, y poseen un vientre blanco en el cual se alojan los órganos reproductores y huevos (Talwar et al. 1991). Aunque la temperatura de estos peces pueda variar en la naturaleza entre los 12ºC y los 39ºC dependiendo de la estación del año, su temperatura ideal de crecimiento en condiciones de laboratorio es de 28’5ºC (Parichy, 2015, Westerfield, 2000).

En la naturaleza existen diferentes tipos de líneas salvajes de pez cebra, pero las más importantes para los investigadores son las que han crecido en los laboratorios, de manera que se ha intentado eliminar mutaciones deletéreas y tratar de generar un fondo genético estable. A mayores, existen una gran cantidad de patrones de pigmentación de los peces cebra, relacionados con la distribución (Spence et al., 2008).

Aunque en estado salvaje viven a distintas temperaturas y se aparean principalmente en verano, en condiciones de laboratorio el

ciclo de vida del pez cebra ocurre durante todo el año. El proceso de apareamiento ocurre cuándo los machos se acercan a las hembras atraídos por las feromonas, de manera que las hembras los conducen al lugar de ovoposición para después ser agitadas por los machos para que los huevos se desprendan y de esta manera poder fertilizarlos (Parichy, 2015). Una vez fertilizados, los huevos comienzan sus primeras divisiones pasando por los estadios de blástula, gástrula y sufriendo una organogénesis temprana para eclosionar del huevo a los 2 días post fertilización (hpf). Esta edad es importante puesto que es el momento en el que se realizan la mayoría de estudios de xenotrasplante (He et al., 2012; Ban et al., 2014; Ent et al., 2014). Una vez eclosionan, siguen creciendo hasta alimentarse por sí mismos a los 5-6dpf y sufriendo una metamorfosis a los 12-14dpf, la cual conlleva una gran mortalidad, alcanzando los supervivientes la fase de juvenil y posteriormente la fase adulta (Willemsen et al. 2011, Howe et al., 2013a).

Los embriones de pez cebra, desde la fase embrionaria hasta la fase larvaria, tienen diferentes morfologías comparados con el pez cebra adulto. Son casi totalmente transparentes desde los 2dpf hasta los 14dpf, permitiendo a los investigadores realizar diferentes técnicas sobre los embriones: desde genética del desarrollo, hasta ensayos de xenotrasplantes, inyectando células de cáncer humanas (Kirchberguer et al., 2017).

En la década de los 80, el pez cebra se utilizó como modelo en el estudio de biología del desarrollo y genética de vertebrados (Streisinger et al. 1981). Pero aparte de este uso primigenio que se le dio a este organismo modelo, los usos actuales se extienden a enfermedades humanas como cáncer, toxicidad y descubrimiento de nuevos fármacos o el modelado de enfermedades humanas:

1.1.1 Cáncer

En el año 2005 se realizó el primer xenotrasplante satisfactorio de células de cáncer humano de piel (melanoma) en el vitelo de un embrión de pez cebra, teniendo las células la capacidad de proliferar y formar un tumor dentro de este organismo modelo (Lee et al., 2005). Con ello, se consiguió demostrar que el pez cebra podría ser un modelo para utilizar

en la investigación de cáncer humano. Al año siguiente, se volvió a demostrar que las células podían proliferar, y que las señales de las células humanas también afectaban al embrión del pez cebra al atraer mediante quimiotaxis los vasos sanguíneos del pez hacia el tumor y producir el proceso de angiogénesis hacia el tumor (Haldi et al., 2006). Estos hallazgos cobraron aún más relevancia en el año 2013, cuándo se publicó el genoma completamente secuenciado del pez cebra, demostrando que compartimos más de un 80% de genes relacionados con enfermedades con este modelo (Howe et al., 2013b).

1.1.2 Descubrimiento de fármacos y toxicidad

El uso que tiene este modelo en el testado de nuevos fármacos y su toxicidad es muy importante. Esto incluye todo tipo de drogas que se puedan disolver en agua, incluyendo compuestos no relacionados previamente con cáncer, para analizar desde la toxicidad órgano-específica, hasta la alteración metabólica y fenotípica (MacRae and Peterson, 2015). En este organismo modelo, es muy importante tener en cuenta que puede recrear todo el proceso de absorción, distribución, metabolismo, excreción y toxicidad (ADME-Tox, por sus siglas en inglés) de distintas sustancias, mientras que, *in vitro*, utilizando cultivos celulares, no sería posible puesto que las células no conforman un organismo completo. Por ello, se considera al pez cebra como el primer nivel de organismo completo con utilidad para el testaje de distintas sustancias previamente a su análisis en mamíferos (Goldstone et al., 2010; Li et al., 2011).

1.1.3 Modelo de enfermedades genéticas humanas

La manipulación genética ha evolucionado enormemente en los últimos años con el objetivo de estudiar las consecuencias de la modificación de ciertos genes con relevancia en enfermedades humanas. Existen diversas técnicas actualmente con aplicabilidad en el pez cebra, que clasificaremos en función de la duración del silenciamiento del gen en transitorios o permanentes. La forma de silenciamiento transitoria más habitual son los morfolinos. Los morfolinos, también llamados oligonucleótidos anti-sentido son ADN sintético, en los cuales el anillo de desoxirribosa es sustituido por un

anillo de morfolino, tienen la ventaja de que son solubles en agua, inmunes a las nucleasas y tienen un bajo coste asociado (Summerton and Weller, 1997). La vía de actuación de estos morfolinos consiste en la reducción de la expresión del gen de interés impidiendo el procesamiento del pre-ARN o bien la unión con el ribosoma, y de esta manera inhibe su traducción (Nasevicius and Ekker, 2000; Bill et al., 2009). Otra manera de provocar un silenciamiento permanente de ciertos genes es la técnica CRISPR/Cas9. El origen de la técnica está relacionado con las bacterias, las cuales usan este método como defensa contra un DNA extraño, de manera que cuándo es detectado, mediante el uso de hebras de ARN guían a la nucleasa Cas9 para cortar el ADN externo de manera que quede inutilizado (Wyatt et al., 2015). La principal ventaja de esta técnica es la de poder definir específicamente cuál es la secuencia que nos interesa cortar para realizar el silenciamiento.

Aparte de los campos de investigación principales que hemos mencionado, el pez cebra también se usa como herramienta en un amplio abanico de temáticas como pueden ser la neurociencia o el desarrollo nervioso, etc. (Stewart et al., 2014; Wyatt et al., 2015; Vornanen and Hassinen, 2016).

1.2 CÁNCER

El cáncer consiste por definición en la pérdida de regulación del ciclo celular que lleva a un crecimiento incontrolado de las células y les concede ciertas características como la capacidad de infiltrarse en el torrente sanguíneo y poder colonizar otras partes del cuerpo (Munkley and Elliot, 2016). La principal causa de muerte por esta enfermedad no suelen ser los tumores primarios, sino la formación de tumores secundarios (metástasis) en otras partes del cuerpo, especialmente órganos, lo que lleva al paciente a la muerte (Liu et al., 2017).

El cáncer tiene varias etiquetas distintivas que se pueden resumir en:

1.2.1 Crecimiento incontrolado sin la necesidad de señales de crecimiento

Todos los tejidos que están presentes en el cuerpo humano están controlados por señales de crecimiento producidas por las células y que mantienen su homeostasis y arquitectura correcta. Por el contrario, las células tumorales tienen la capacidad de evadir esa homeostasis y mantener un crecimiento anormal y fuera de control mediante la pérdida de regulación por parte de estos factores secretados (Munkley and Elliot, 2016). Esta propiedad característica de las células tumorales podría también afectar a otras de las marcas distintivas de la enfermedad como podría ser la viabilidad celular y el metabolismo (Pavlova and Thompson, 2016).

1.2.2 Crecimiento con señales de inhibición del crecimiento

Aparte de tener un crecimiento incontrolado sin la necesidad de las señales apropiadas para ello, estas células también son insensibles a las señales opuestas, que controlan el crecimiento celular de todas las células del cuerpo en el caso de que pierdan la homeostasis, las señales negativas de proliferación (Deshpande et al., 2005). La mayoría de las señales negativas de proliferación celular son enviadas por genes supresores de tumores, como podrían ser el gen P53, el cual regula la la entrada en proliferación o en estado de senescencia o apoptosis (Burkhart and Sage, 2008).

1.2.3 Capacidad de escapar de la muerte celular

La resistencia a la muerte celular programada es otra de las características que tienen estas células que les permite, tras la pérdida de la homeostasis evitar la entrada en apoptosis, una vez que la célula sufre condiciones de estrés y se convierte en una célula tumoral (Hanahan and Weinberg, 2011).

1.2.4 División ilimitada

La división de las células en el cuerpo está limitado a un número concreto de divisiones, y los responsables de que esto se mantenga así, son los telómeros, los cuales se encargan de que estas células detengan

su proliferación (Ennour-Idrissi et al., 2017). Por este motivo existen dos barreras para evitar que esto ocurra: la primera sería la senescencia, de manera que las células quedarían en un estado viable pero quiescente, que inhibe la proliferación y la muerte celular. Pero algunas células que consiguen saltarse esta barrera, entran en un estado de apoptosis que las lleva a la muerte celular programada. Aun así, existen ciertos tipos de células que consiguen sobrepasar estas dos barreras y se convierten en un subtipo celular inmortal con la capacidad de proliferar indefinidamente (Childs et al., 2014).

1.2.5 Angiogénesis

Las células tumorales necesitan ciertos nutrientes como oxígeno, glucosa, aminoácidos, etc; con el objetivo de mantener esa proliferación continuada a lo largo del tiempo, ya que sin estos nutrientes no sería posible crecer y dividirse a ese ritmo. Debido a esto, otro punto importante en la progresión tumoral es la capacidad de las células de inducir angiogénesis, de manera que los vasos sanguíneos lleguen al tumor para poder aportar nutrientes y retirar las sustancias de deshecho de las células tumorales (Hanahan and Weinberg, 2011).

1.2.6 Invasión de otros tejidos a través del torrente sanguíneo

Las células tumorales tienen también la capacidad añadida de poder intravasar a la circulación sanguínea desde el tumor primario, y colonizar tejidos u órganos distales en donde pueden formar un nuevo tumor (Pantel et al., 2004). La formación de metástasis es un proceso complejo que consiste en una secuencia de eventos que facilitan la diseminación y colonización de localizaciones distales: desde el tumor primario, las células tienen que cambiar su morfología y conformación, pasando por un proceso de transición epitelio-mesénquima (EMT) para adquirir mayor capacidad de invadir tejidos adyacentes e intravasar a la circulación o a las vías linfáticas. De la misma manera, una vez llegan a su tejido u órgano diana, tras un proceso de extravasación, y allí las células sufren un proceso inverso de transición mesénquima-epitelio (MET), adquiriendo la capacidad de colonizar y formar metástasis en nuevos tejidos (Talmadge and Fidler, 2010).

Y en los últimos se han añadido años dos características más, distintitas de esta enfermedad a las anteriormente mencionadas:

1.2.7 Reprogramación del metabolismo celular

La división incontrolada de las células tumorales necesita ser sustentada por el metabolismo celular, ya que sin estos nutrientes no podrían proliferar por falta de recursos. Las células sanas y normales obtienen su energía mediante la fosforilación oxidativa (OXPHOS) de la glucosa de una manera muy efectiva y con un rendimiento alto de ATP. Sin embargo, las células tumorales, aunque parezca contradictorio, obtienen la energía mediante el efecto Warburg, aun siendo menos eficiente que la fosforilación oxidativa llevada a cabo por las células normales. A pesar de ello, lo que hacen estas células ante la gran demanda de glucosa es aumentar la expresión de los receptores GLUT1, los cuales son los responsables de mediar la entrada de glucosa al interior celular (Jones and Thompson, 2009; DeBerardinis et al., 2008).

1.2.8 Evasión del sistema inmune

De entre las principales características de las células tumorales expuestas anteriormente, faltaría un punto importante que forma parte del microambiente tumoral: el sistema inmune. En un individuo sano, el sistema inmune es una parte responsable de detectar y eliminar células que se escapen fuera del control y la homeostasis, y es la principal barrera también contra patógenos (Muesnt et al., 2016). Las células tumorales, tienen la capacidad de no ser reconocidas como células extrañas o con comportamiento proliferativo anómalo, de manera que el sistema inmune no las detecta, y lo pueden evadir (Teng et al., 2008).

La principal causa de cáncer es el daño en el ADN, y la acumulación de mutaciones somáticas a lo largo de la vida de las células, así como eventos epigenéticos y modificaciones que podrían afectar al ADN celular (Luzzatto and Pandolfi, 2015). Estos eventos que pueden llevar a que se dañe el material genético de las células y acumular mutaciones a lo largo del tiempo. Estas alteraciones se pueden dividir en hereditarias o por factores ambientales.

-Hereditarios: el origen hereditario de los tumores es el menos frecuente. Sólo una pequeña fracción de los casos de cáncer en el mundo se deben a factores hereditarios, pero predisponen a los pacientes que tienen estas modificaciones genéticas a padecer cáncer a lo largo de su vida. Estos defectos pueden abarcar desde problemas en los genes de reparación de ADN a oncogenes o genes relacionados con la producción de vasos sanguíneos (Hodgson, 2008).

-Factores ambientales: entre los factores ambientales más importantes que pueden llevar a una persona a padecer cáncer, se podrían incluir los químicos, el estilo de vida y la infección e inflamación. Entre los factores químicos, aparte de algunos materiales que se pueden bio-acumular en cuerpo humano, dando lugar a daños en el ADN celular, está a la cabeza el tabaco, responsable del 33% de las muertes por cáncer y causante del 85% de los cánceres de pulmón a nivel mundial (Warren and Cummings, 2013). En otro de los apartados incluimos el estilo de vida sedentario, que se atribuye normalmente a los países más desarrollados. La falta de ejercicio físico, produce un aumento en la obesidad, y el cuerpo ante la imposibilidad de mantener ese metabolismo corporal acaba teniendo fallos en la replicación celular o su reparación, dando lugar al cáncer (Ramirez et al., 2017; Grosso et al., 2017). Por último, las infecciones mediante virus o bacterias tienen la capacidad conocida en algunos casos de causar cáncer. Virus conocidos como 'oncovirus' (por ejemplo, el virus del papiloma humano, virus de Epstein-Barr, o hepatitis B y C) (Moore and Chang, 2010) y bacterias como la Helicobacter pylori (Kmieć et al., 2017) tienen esta capacidad. La inflamación crónica de un tejido u órgano también es importante mencionarla, ya que mediante la inflamación se produce la generación en exceso de especies reactivas de oxígeno (ROS) que podrían acabar rompiendo las cadenas de ADN e incurrir en el daño de este ADN, aparte de silenciar las enzimas encargadas de su reparación, dando lugar a una falta de regulación o fallos, que ocasionalmente llevaría a la aparición de cáncer (Zhang et al., 2017).

Por otra parte, también mencionaremos las dos terapias más usadas en la lucha contra esta enfermedad:

-Quimioterapia: este tratamiento se empezó a utilizar hace tiempo y ha sido una de las armas más efectivas en el tratamiento contra el cáncer a lo largo del siglo XX (Farber and Diamond, 1948), aunque con grandes efectos secundarios asociados. En los últimos tiempos las quimioterapias se desarrollaron enormemente en torno al descubrimiento de ciertos oncogenes y dianas terapéuticas responsables de la progresión tumoral, la diseminación y la metástasis, dando lugar a compuestos que bloquean de manera efectiva ciertas rutas intracelulares importantes para el desarrollo y evolución del tumor (Galmarini et al., 2012).

-Radiación: la terapia con radiación o radioterapia (RT), puede ser clasificada como tratamiento interno y tratamiento externo, dependiendo de la metodología y del tipo de radiación. Esta técnica consiste en la radiación del órgano o tejido afectado con diferentes fuentes de rayos gamma o rayos X, provocando una disrupción y daño permanente al ADN de las células tumorales, obligándolas entrar en apoptosis, y también afectando al microambiente del tumor (Baskar et al., 2012).

1.3 TÉCNICA DE XENOTRASPLANTE EN PEZ CEBRA

La técnica de xenotrasplante en embriones de pez cebra se descubrió por primera vez en el 2005 (Lee et al., 2005) y ha evolucionado a lo largo de la pasada década para incrementar la precisión de la metodología y tratar de establecer el modelo de pez cebra como complementario al ratón en estudios de medicina personalizada. La técnica del xenotrasplante en pez cebra, consiste en la inyección de células tumorales de origen humano en diferentes partes del embrión para seguir su progresión a lo largo del tiempo y su interacción con su microambiente dentro del pez (Nicoli and Presta, 2007; X).

Para realizar dichas inyecciones, existen distintos sitios óptimos dentro del embrión del pez cebra para realizarlas:

1.3.1 Saco vitelino

El saco vitelino de los embriones es un compartimento acelular donde se acumulan lípidos con el objetivo de proveer al embrión con

energía durante sus primeras fases de desarrollo hasta que este se pueda alimentar autónomamente cuándo alcanzan los 5-6dpf (Fraher et al., 2016). Las células tumorales pueden ser inyectadas en este saco vitelino el cual les proporciona nutrientes para el crecimiento tumoral. Aparte, en el vitelo las células pueden ser fácilmente visualizadas mediante microscopia de fluorescencia para realizar un seguimiento de las mismas a lo largo de los días (Veinotte et al., 2014).

1.3.2 Ducto de Cuvier

La inyección de las células directamente en la circulación del embrión (Ducto de Cuvier) permite a los investigadores realizar pruebas en otras etapas del desarrollo tumoral como por ejemplo el proceso de transición mesénquima-epitelio (MET) (Mercatali et al., 2016). Las células inyectadas en circulación son capaces de sobrevivir, invadir y extravasar, normalmente cerca del tejido hematopoyético caudal (CHT) localizado en la cola del embrión, y metastatizar formando nuevos focos tumorales (Tulotta et al., 2016). De esta manera las células pueden ser seguidas a lo largo del tiempo mediante el marcaje de fluorescencia y cuantificar la proliferación y la ratio de invasión en el CHT de los embriones (Drabsch et al., 2013).

1.3.3 Cavidad intraperitoneal

A pesar de que casi todas las inyecciones se realizan normalmente en embriones de pez cebra por la ausencia de sistema inmune hasta el día 11-12hpf, con la creación de la nueva línea inmunocomprometida mutante de pez cebra (Rag2) (Tang et al., 2014), los peces adultos pueden ser usados como animal modelo para los estudios de xenotrasplante sin el problema del rechazo de las células humanas por el sistema inmune del pez, y esta cavidad intraperitoneal es la elegida para realizar dichos estudios.

La técnica de xenotrasplante en pez cebra tiene ciertas ventajas e inconvenientes cuándo la comparamos con el ratón:

-Las principales ventajas que encontramos serían por una parte la gran cantidad de descendientes que se pueden obtener cada día por cada pareja de peces en al acuario, proporcionando de esta manera la

posibilidad de realizar experimentos de '*high-throughput screening*' de compuestos. Por otra parte, presenta un coste muy bajo de mantenimiento de la especie modelo en el laboratorio debido al poco espacio que ocupan. Asimismo, dos de las ventajas más importantes de este modelo animal para los estudios de xenotrasplante consiste en que no desarrollan su sistema inmune hasta el día 11-12hpf, como ya se ha mencionado, por lo que no es necesario tratarlos para que no exista rechazo de las células implantadas; y por otra parte, el hecho de que son transparentes hasta que alcanzan la fase de juvenil, permitiendo el seguimiento de las células tumorales inyectadas *in vivo* a lo largo del tiempo (Konantz et al., 2012).

-Entre las desventajas que tenemos en esta especie modelo destaca sobre todo el no ser un mamífero, lo cual nos lleva a que la temperatura a la que están adaptados estos peces en condiciones de laboratorio son aproximadamente 28'5ºC mientras que las células humanas inyectadas necesitarían una temperatura cercana a la fisiológica del cuerpo humano (37ºC) para poder desarrollar todo su potencial proliferativo e invasivo. Por otra parte, hay ciertos órganos que no están presentes en el pez como las mamas o los pulmones, por lo que no se pueden hacer xenotrasplantes ortotópicos (Konantz et al., 2012).

La técnica de xenotrasplante estableció su primer protocolo estandarizado en 2006 (Haldi et al., 2006) y se determinaron ciertos parámetros para intentar conseguir un equilibrio entre las células humanas inyectadas y el hospedador, en este caso, el pez cebra. Mientras que los embriones de pez cebra se desarrollan a una temperatura de alrededor de 28'5ºC en condiciones de laboratorio, tal y como ya mencionamos (Westerfield, 2000), las células humanas necesitan de unos 37ºC para sobrevivir y estar en condiciones óptimas. Por ello, la temperatura elegida de incubación se estableció en torno a los 34ºC para los ensayos de xenotrasplantes a lo largo de la última década, con una incubación desde los 3 a los 6 días post inyección (dpi) (Eguiara et al., 2011; Ghotra et al., 2012; He et al., 2012; Ban et al., 2014; Ent et al., 2014). En este manuscrito de Haldi y colaboradores, la cuantificación de las células se realizaba mediante la disgregación final del embrión y el contaje de las células, mientras que las células

inyectadas inicialmente eran estimadas. A pesar de que esta técnica ha evolucionado a lo largo de los años no se han modificado en gran medida los parámetros relativos a la temperatura de incubación ni al análisis de imagen que se realiza sobre las fotografías de los embriones en cada experimento, ni su automatización (Corkery et al., 2011; Moshal et al., 2011; Yang et al., 2013). Por último, hay que mencionar un apartado que últimamente se está teniendo muy en cuenta a la hora de realizar la técnica de xenotrasplante, la interacción que se da entre las células inyectadas y el hospedador. Tradicionalmente, se ha considerado que las células tumorales tienen que estar en un ambiente lo más óptimo posible para observar su proliferación y comportamiento biológico dentro del organismo modelo, pero también es importante ver si el estado general del embrión puede afectar a la hora de obtener unos resultados precisos y robustos. De los principales parámetros, el único parámetro que podría alterar totalmente el embrión sería la temperatura de incubación anteriormente mencionada (Pype et al., 2015). A pesar de que la temperatura más utilizada es 34ºC, hay algunos investigadores que se han cuestionado si esta sería la temperatura ideal para medir la proliferación celular y el efecto de ciertos fármacos (Konantz et al., 2012). En este sentido, incluso a 34ºC, algunos cambios morfológicos y metabólicos en el hospedador podrían estar afectando a la proliferación y comportamiento óptimo de las células inyectadas en el vitelo o la circulación del embrión, sobre todo cambios relacionados con la inflamación y la respuesta inmune (Long et al., 2012).

2. OBJETIVOS

El objetivo general de esta tesis es la implementación de la técnica de xenotrasplante en embriones de pez cebra con el objetivo de ser capaz de estudiar el comportamiento de las células tumorales humanas de manera más precisa y así poder contribuir a la investigación en oncología con este organismo modelo.

Este objetivo principal puede ser subdividido en distintos objetivos más específicos:

1. IMPLEMENTACIÓN DE LA TÉCNICA DE XENOTRASPLANTE MEDIANTE EL INCREMENTO DE LA TEMPERATURA DE INCUBACIÓN DE LOS EMBRIONES DE PEZ CEBRA Y EL DESARROLLO DE UN NUEVO SOFTWARE DE ANÁLISIS DE IMAGEN

La obtención de una cuantificación más precisa de las células tumorales inyectadas en el interior del vitelo del pez cebra, requiere de un ambiente óptimo para que las células inyectadas puedan proliferar y comportarse de forma similar a lo que hacen en a su ambiente original, el cuerpo humano. Por esta razón, uno de los objetivos del presente estudio fue analizar el efecto de utilizar una temperatura de incubación de 36ºC, comparándola con una temperatura de 34ºC, *in vitro* (con diferentes líneas celulares) e *in vivo* (en embriones de pez cebra). Por otra parte, la mejora y automatización del análisis de imagen de los propios embriones es uno de los objetivos, con el fin de cuantificar la masa tumoral inyectada en el vitelo de los embriones de pez cebra de una manera mucho más robusta estadísticamente. Con este propósito, el desarrollo de un nuevo software de análisis de imagen basado en Matlab es necesario para mejorar la precisión y automatización del proceso de análisis de imagen. Finalmente, el análisis del efecto del 5-fluorouracilo (5-FU), un quimioterápico de uso extenso, nos servirá para determinar el efecto del incremento de temperatura en la actividad del fármaco.

2. COMPARACIÓN BASADA EN LOS DEFECTOS DE DESARROLLO, MORTALIDAD Y METABOLISMO DE LOS EMBRIONES DE PEZ CEBRA INCUBADOS A 28ºC, 34ºC Y 36ºC

La comparación entre las diferentes condiciones de incubación a altas temperaturas (34ºC y 36ºC) se realizará con el objetivo de encontrar un equilibrio entre la supervivencia y proliferación de las células inyectadas sin incurrir en una alta mortalidad de los embriones inyectados. La razón por la cual se pretende incrementar la temperatura de incubación es que las células inyectadas se encontrarían, de esta manera, a una temperatura más cercana a la del cuerpo humano, dando lugar a una proliferación más precisa sin inducir una alta mortalidad de los embriones de pez cebra. Las condiciones fisiológicas de los

embriones de pez cebra como hospedador, se estudiarán con dos aproximaciones distintas:

-Fenotípica: los defectos morfológicos observables pueden ser cuantificados mediante la observación y fotografía de los embriones en distintos puntos de su desarrollo para ver la integridad del propio hospedador en respuesta a estas temperaturas. Por otra parte, es importante cuantificar la mortalidad de los embriones a las diferentes temperaturas testadas, con el objetivo de comprobar si existe alguna diferencia en términos de mortalidad entre ellas.

-Metabólica: la obtención de información sobre diferentes rutas metabólicas que están relacionadas con los xenotrasplantes sería importante para comprobar cómo los embriones responden a nivel fisiológico a altas temperaturas de incubación. Las rutas metabólicas estudiadas serían el sistema inmune, la inflamación, el desarrollo y el metabolismo en respuesta a la temperatura.

3. MICROAMBIENTE: CO-INYECCIÓN DE DIFERENTES LÍNEAS CELULARES CON MACRÓFAGOS CONDICIONADOS

La adición de parámetros específicos relacionados con el microambiente tumoral humano es la siguiente etapa para poder proporcionar resultados mucho más precisos en cuánto a la proliferación de las células. De esta manera, uno de los componentes más importantes del microambiente tumoral humano son los macrófagos. Los macrófagos son reclutados por las células tumorales y transformados en macrófagos asociados a tumor (TAMs), siendo capaces de ayudar a las células tumorales con su proliferación y capacidad metastásica. Por esta razón, con el objetivo de probar si el microambiente que acompaña a las células de cáncer humanas pudiese ayudar o no al desarrollo del tumor, se propone la co-inyección de células de cáncer de mama humano MCF7 con macrófagos condicionados en el interior del vitelo de los embriones de pez cebra. De esta manera, se podría analizar la interacción entre los macrófagos y las células tumorales, y medir la proliferación y la diseminación de las células en presencia o ausencia de macrófagos.

2. RESULTADOS

En el conjunto de capítulos, se ha comprobado y testado la mejora de la técnica de xenotrasplante habiéndose mejorado tanto el análisis de imagen mediante el software de nuevo desarrollo ZFtool, aumentando la temperatura de incubación y a su vez demostrando que dicha temperatura es la más adecuada para la obtención de resultados con mayor robustez y precisión (Cabezas-Sainz et al., 2018). A mayores, estos resultados se han visto complementados mediante la comprobación para ver si esta temperatura de 36ºC afecta a los embriones de pez cebra a diferentes niveles (malformaciones fenotípicas y cambios a nivel de metabolismo), poniendo de manifiesto que esta temperatura a nivel de malformaciones y metabolismo es similar a la temperatura más usada en la bibliografía, 34ºC (Eguiara et al., 2011; Ghotra et al., 2012; He et al., 2012; Ban et al., 2014; Ent et al., 2014). Finalmente, se probó que esta técnica mejorada, con la nueva temperatura de incubación y el análisis de imagen automatizado, se puede utilizar para estudios en los cuales, además de las células, se añadió un componente del microambiente tumoral, los macrófagos. De esta manera, se da un paso más hacia la medicina personalizada al ver que los macrófagos ayudaron a la línea de cáncer de mama humana, MCF7, a proliferar en mayor medida y diseminarse más al estar presentes los macrófagos condicionados en el vitelo del pez cebra.

A continuación, se analizarán de manera más pormenorizada cada uno de los resultados obtenidos en cada capítulo correspondientes a los objetivos planteados:

1.- Implementación de la técnica de xenotrasplante mediante el incremento de la temperatura de incubación de los embriones de pez cebra y el desarrollo de un nuevo software de análisis de imagen

Una mejora de la técnica de xenotrasplante era necesaria, junto con un mejor análisis de imagen que fuese más preciso y robusto para poder comprender de una manera más profunda el comportamiento celular dentro del vitelo del pez cebra, donde las células de cáncer humano son inyectadas.

Diferentes autores han comprobado que existe un desarrollo normal del pez cebra hasta los 35.5ºC (Nicoli and Presta, 2007; Stoletov et al., 2010), pero aun así se han probado diferentes rangos de temperaturas para maximizar la supervivencia de los embriones. A pesar de que la temperatura elegida después de diversos estudios ha sido 34ºC, algunos autores sugirieron que una temperatura más alta sería beneficiosa a la hora de ver el comportamiento real de las células en el embrión del pez cebra, aunque fuese en detrimento de la supervivencia de los propios embriones, puesto que la temperatura normal de desarrollo de esas células en el cuerpo humano sería de 37ºC (Konantz et al., 2012).

En primer lugar, se desarrolló mediante una colaboración un software que permitiese cuantificar de una manera más precisa y automatizada las células inyectadas en el vitelo de los embriones de pez cebra, dando lugar a ZFtool. Este software analiza diferentes tipos de imágenes en las cuales es capaz de cuantificar de manera automática el número de píxeles que superen cierto umbral, calculado automáticamente en función de cada par de imágenes que se le proporciona para comparar, y por otra parte la intensidad de cada pixel, dando lugar a un parámetro llamado densidad integrada, la cual no es más que la multiplicación del número de píxeles por la intensidad de media de fluorescencia de la imagen. Con la densidad integrada, posteriormente se puede establecer un índice de proliferación al dividir el valor de un tiempo entre el inicial.

En estos experimentos, se ha incrementado la temperatura de desarrollo normal de los embriones de pez cebra desde los 28ºC hasta los 36ºC, sin un cambio significativo en la mortalidad de los embriones ni en los defectos fenotípicos de dichas larvas, durante el periodo que dura la incubación, que sería de 3 días. Los resultados obtenidos muestran claramente que la proliferación de las células de cáncer colorrectal humanas inyectadas de la línea HCT116 crecen en mayor medida cuándo la temperatura de incubación fue de 36ºC comparando dicha temperatura con la más usada en la bibliografía actual, 34ºC. Por otra parte, cuándo a las mencionadas células se les aplica un quimioterápico (5-fluorouracil), se ha encontrado una diferencia entre

las células incubadas a 36ºC y a 34ºC, puesto que dicho compuesto mata muchas más células cuándo se aplica a una temperatura de 34ºC, en la cual las células no están comportándose de una manera óptima, por lo tanto, se podría plantear la hipótesis de que a mayor temperatura (36ºC) tendríamos un efecto más real de los compuestos a testar que a 34ºC.

A mayores, se descubrió que el número de células inyectadas influyen en el grado de proliferación de la línea de cáncer humana HCT116. Esto se comprobó mediante la inyección de una cantidad de células pequeña, y otra cantidad mayor de células a las diferentes temperaturas de incubación, 34ºC y 36ºC. Se demostró que cuándo se inyectaba una cantidad de células pequeña a 34ºC, el crecimiento de las mismas se veía afectado en gran medida, mientras que a 36ºC el crecimiento se incrementaba, en vez de mermar. Esto puede deberse a la interacción célula-célula, el cuál al inyectarse una menor cantidad de células en el vitelo pueden quedar semi-aisladas unas de otras con la consiguiente falta estímulos y quimiotaxis entre ellas dando lugar a una gran mortalidad a 34ºC, no obstante, este fenómeno no ocurre a 36ºC. Con esto también se demuestra que la temperatura de 36ºC sería mucho mejor en el caso de no disponer de tanto material celular para realizar los xenotrasplantes.

2.- Comparación basada en los defectos de desarrollo, mortalidad y metabolismo de los embriones de pez cebra incubados a 28ºC, 34ºC y 36ºC

Uno de los parámetros más importantes de la técnica de xenograft que afecta a la proliferación celular y a la vez a la integridad del hospedador, en este caso el embrión de pez cebra, es la temperatura. Altas temperaturas de incubación favorecen la proliferación celular en detrimento del estado del embrión (Konantz et al., 2012).

Se ha comprobado previamente que la incubación de los embriones de pez cebra a 36ºC durante 3 días es factible sin tener una mayor mortalidad comparada con 34ºC (Cabezas-Sainz et al., 2018). Las malformaciones inducidas por los efectos teratogénicos de la temperatura son poco claros, aunque recientemente ha sido publicado un estudio en el cual se postula que a partir de 32.5ºC existen

malformaciones en los embriones de pez cebra (Pype et al., 2015). A pesar de ello, otros estudios avalan que las malformaciones no empiezan a formarse hasta los 35.5ºC (Nicoli and Presta, 2007; Stoletov et al., 2010).

Los resultados obtenidos se clasifican según las distintas metodologías empleadas:

-En una primera aproximación, los embriones fueron incubados a 36ºC desde las 0 horas post-fecundación hasta las 48 horas post-fecundación con el objetivo de corroborar, tal y como muestra estudios anteriores (Pype et al., 2015) que en esta etapa los embriones son mucho más sensibles a la temperatura, incrementándose su mortalidad y malformaciones. Al contrario que en el estudio de Pype et al., la proporción de embriones eclosionados fue de 100%, puesto que en el estudio anteriormente citado la temperatura máxima de incubación fueron 36'5ºC, poniendo de manifiesto que podría haber un punto de tolerancia entre los 36ºC ensayados y los 36.5ºC. En el resto de parámetros y malformaciones, los resultados se corresponden en mayor medida con el estudio mencionado.

-En una segunda aproximación, se incubaron los embriones de pez cebra de 48h post-fecundación bajo diferentes condiciones (controles, inyectados con medio o inyectados con células) durante un periodo de 2 días a 34ºC y 36ºC, con una posterior recuperación a 28ºC hasta los 14 días. De esta manera los resultados indicaron que, comparado con la anterior aproximación, efectivamente a partir de que eclosionan a las 48h de vida, los embriones de pez cebra son menos sensibles a la temperatura, a pesar de que muestran tendencia a algunos defectos morfológicos como son la desviación espinal y el edema a una temperatura de incubación de 36ºC comparado con 34ºC. Aun así, y a pesar de la tendencia anteriormente descrita, la ausencia de diferencias estadísticamente significativas entre ambas temperaturas nos abre la posibilidad a poder realizar experimentos de xenotrasplante con estos embriones reduciendo su tiempo de incubación a 2 días post inyección, con el objetivo de reducir la mortalidad/malformaciones de los mismos y a la vez obtener unos resultados más realistas del comportamiento de

las células inyectadas de cáncer, sobre todo cuándo se aplica algún compuesto para testar su efecto sobre esas células *in vivo*.

A mayores de estos resultados, un análisis de expresión génica se llevó a cabo usando qPCR para poder estudiar a nivel metabólico los cambios que se producían por el efecto de las diferentes temperaturas de incubación, basado en un trabajo anteriormente publicado (Long et al., 2012), relacionado sobre todo con genes reguladores de la inflamación, el ciclo circadiano, metabolismo general y sistema inmune. Los resultados obtenidos muestran que existe una correlación en la mayoría de los genes ensayados tanto los infra-expresados (Apex1, Haus3, Lft2, Lum and Wisp3) como con los sobre-expresados (Hspa9 and Junb-a). Pero a pesar de esto, existen algunos genes que varían su expresión en función de la temperatura de incubación y del tiempo en el que las muestras fueron tomadas.

Tanto después de las 48h de incubación, como de las 72h de recuperación a 34ºC y 36ºC, el gen Per2, relacionado con la regulación del ciclo circadiano y metabolismo, se vio afectado, estando infra-expresado en mayor medida a 36ºC que a 34ºC. La infra-expresión de este gen induce la expresión de citoquinas pro-inflamatorias en el pez cebra, lo cual tiene relación con que el embrión esté sometido a un estrés térmico continuado durante las primeras 48h y posteriormente en la recuperación puede que no se recupere a nivel metabólico. Por otra parte, el otro gen diferencial en cuestión de temperatura es Socs3a. Dicho gen está relacionado con el sistema inmune y la inflamación, aunque aparte de esto se relaciona también con la regeneración tisular (Khaliq et al., 2018; Liang et al., 2012). Socs3a está diferencialmente expresado 72h después de devolver a los embriones una temperatura de incubación normal, estando sobre-expresado a 34ºC, mientras que a 36ºC está ligeramente infra-expresado. Esto pone de manifiesto que a nivel metabólico el embrión ha sufrido daños relacionados con la temperatura en tejidos cuándo la incubación se hace a 36ºC, a mayores de tener que activarse la respuesta inflamatoria y del sistema inmune debido al estrés térmico que supone.

3.- Microambiente: co-inyección de diferentes líneas celulares con macrófagos condicionados

La comunicación que tiene lugar entre el tumor y el microambiente tumoral que lo rodea es crucial para la progresión de esta enfermedad dentro del cuerpo humano (Lazennec and Richmond, 2010). La sobre-expresión del gen Pit-1 en la línea de cáncer de mama humana MCF7 produce un incremento en la secreción de diferentes factores como citoquinas y quimioquinas (Lazennec and Richmond, 2010), las cuales son relevantes a la hora de la movilización de los macrófagos al área tumoral con el objetivo de transformarlos en macrófagos asociados a tumor (TAM's) (Quail and Joice, 2013; Bin-Zhi and Pollard, 2010). A mayores de esto, esta sobre-expresión de Pit-1 está relacionada con un mayor crecimiento tumoral, angiogénesis e invasión tumoral (Lewis et al., 2000). La consecuencia más importante de todo esto es la dispersión que puede llegar a tener el tumor y la metástasis del mismo. Considerando lo anteriormente mencionado, pacientes que tengan una elevada expresión del gen Pit-1, tendrán un mayor riesgo de metástasis, en este caso en el pulmón.

En este apartado se ha aplicado la metodología desarrollada previamente para poder testar la implicación de $TAM^{highCD163}$ y la de $TAM^{lowCD163}$ en los procesos que subyacen a la interacción entre los macrófagos condicionados del microambiente tumoral y las células MCF7-Pit1-GFP inyectadas en el vitelo de los embriones de pez cebra, en todo lo relacionado con la proliferación y la diseminación de las mismas. Los datos obtenidos de estos experimentos corroboraron lo que ya se había publicado anteriormente (Wang et al., 2015), los componentes del microambiente tumoral son importantes y favorecen el soporte y la proliferación de las propias células cancerosas inyectadas en este animal modelo. Se ha podido ver tanto una mayor proliferación de esta línea celular como una mayor diseminación de nivel del vitelo del pez cebra cuándo se realizó la co-inyección con los macrófagos $TAM^{highCD163}$.

2. CONCLUSIONES

El trabajo que se resume en estos tres capítulos pretendió mejorar la técnica de xenotrasplante en embriones de pez cebra mediante el incremento de la temperatura de incubación hasta los 36ºC y analizar tanto los efectos fenotípicos como metabólicos que esta temperatura pueda tener sobre los embriones, para finalmente, poder añadir el efecto del microambiente con el objetivo de alcanzar unas condiciones lo más cercanas posibles a las del cuerpo humano.

Los resultados anteriormente mencionados nos han permitido extraer las siguientes conclusiones:

1.- Se ha demostrado que el software ZFtool permite a los investigadores obtener un resultado mucho más preciso y automatizado de las imágenes analizadas de los embriones de pez cebra. Por otra parte, la línea de cáncer colorrectal humana HCT116 que ha sido inyectada en los embriones tiene un índice de proliferación mucho mayor cuándo la temperatura de incubación se establece en 36ºC en vez de 34ºC. A mayores, 36ºC es la mejor temperatura de incubación de estos embriones si lo que se pretende es testar quimioterápicos como el 5-fluorouracil.

2.- No ha habido diferencias significativas en la mortalidad o en las malformaciones cuándo se comparan peces cebra control frente a los inyectados con células a temperaturas de incubación de 34ºC y de 36ºC durante 48h. Los cambios de expresión génica entre estas temperaturas ensayadas podrían estar afectando a la respuesta inflamatoria y la regeneración durante la incubación a través de la infra-regulación del gen Per2 y después de ello, posterior al periodo de recuperación a través del gen Socs3a de los embriones a 36ºC. Teniendo todo esto en cuenta, los experimentos con una duración de 48h y una incubación de 36ºC deberían de ser la elección cuándo queramos obtener resultados más precisos y robustos en términos de proliferación tumoral y/o invasión, a mayores del testeo de compuestos *in vivo*.

3.- La co-inyección de macrófagos condicionados con la línea de cáncer de mama humana MCF7 ha demostrado que los componentes del

microambiente celular del tumor son importantes para conseguir la totalidad del potencial de estas células inyectadas en el vitelo de los embriones, aportando tanto soporte como mejorando la proliferación y capacidad de invasión de las células. Este estudio avala la posibilidad de realizar la co-inyección de células de cáncer humanas con otro tipo de células de la matriz tumoral para poder mimetizar el microambiente humano con mayor eficacia en este organismo modelo.