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THE ZEBRAFISH AS A MODEL TO ELUCIDATE HUMAN DISEASES AND DEVELOPMENT

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To Papa, Mummy, Jingyi and Ren Jie…
For believing in me…

ABSTRACT

This thesis explores the zebrafish as a model organism to illuminate our understanding of processes that orchestrate the progression of cancer metastasis and myogenesis.

First, I describe the successful establishment of a cancer metastasis model in the zebrafish, specifically to study intravasation - one of the earliest steps in the metastatic cascade. Fluorescently labeled tumour cell are transplanted into pervitelline space of 2 days post fertilisation (dpf) zebrafish embryos before they are exposed to normoxic or hypoxic conditions, allowing us to study the effect of hypoxia on tumour-induced angiogenesis and metastasis. Hypoxia elicited an enhanced angiogenic response and neovascularisation to the transplanted tumour and escalated the extent of metastasis in the living zebrafish embryo. Loss of function experiments such as vascular endothelial growth factor (VEGF) blockade using a clinically available drug – Sunitinib or VEGF morpholino knockdown attenuated tumour-induced angiogenesis and metastasis. The *in vivo* metastasis assay in zebrafish delivers numerous unique advantages over conventional *in vitro* cell-based or biochemical chemotaxis assays. The transparent embryo facilitates the tracking of the entire intravasation process in real time within a living organism, allowing us to explore the dynamic interplay of tumour cells and the environmental cues that drive metastasis. This model can be further employed to discriminate between tumour cells of different metastatic potential and to identify novel factors that present as impetus for the earliest step of the metastatic cascade.

Next, I demonstrate the analysis of one class of motility mutants originally identified in the 1996 Tübingen genetic screen that potentially serve as models for human myopathies and dystrophies. Two of these mutants have previously been cloned and shown to encode proteins involved in muscle fibre attachment whilst a third has been found to encode the molecular chaperone Heat shock protein (HSP) 90. I describe the phenotypic and molecular characterization of another of these zebrafish motility mutants, *frozen (fro)*. I present evidence that the fro^{t027c} mutation disrupts the locus encoding the autophagy pathway component Atg10. This analysis implicates Atg10 in the assembly of both skeletal and cardiac muscle fibers suggesting a previously uncharacterized role for the autophagy pathway in this process.

Together, my findings illustrate the utility of the zebrafish as a model organism that complements established mammalian and invertebrate models. The fecundity and amenability of the zebrafish to genetic manipulation together with the rapid development and translucency of its embryos combine to provide a powerful system with which to unravel and inform the underlying mechanisms that govern fundamental biological processes and shed light on our understanding of human development and diseases.

LIST OF PUBLICATIONS

I. ***Samantha Lin Chiou Lee***, Pegah Rouhi, Lasse Jensen, Danfang Zhang, Hong Ji, Giselbert Hauptmann, Philip Ingham and Yihai Cao, Nov 17 2009, Hypoxia-induced pathological angiogenesis mediates tumor cell dissemination, invasion, and metastasis in a zebrafish tumor model, Proc Natl Acad Sci (PNAS) USA,106, 46, 19485-90.

II. Pegah Rouhi*, ***Samantha Lin Chiou Lee****, Ziquan Cao, Eva Hedlund, Lasse Jensen and Yihai Cao, Mar 1 2010, Pathological angiogenesis facilitates tumor cell dissemination and metastasis, Cell Cycle, 9, 5, 913-7, *Co-first author

III. ***Samantha Lin Chiou Lee***, Sarah Baxendale, Stephen Moore, Ng Chee Peng, Tan Swee Chuan and Philip Ingham, The Autophagy Pathway component, Atg10, is required for skeletal and cardiac muscle fibre assembly in zebrafish (manuscript).

CONTENTS

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LIST OF ABBREVIATIONS

Atg	Autophagy-related gene
DA	Dorsal aorta
dpf	Days post fertilisation
ENU	N-ethyl-N-nitrosourea
FGF	Fibroblast growth factor
GAA	α-glucosidase
GPF	Green fluorescent protein
GR	Glucocorticoid receptor
Hh	Hedgehog
hpf	Hours post fertilisation
ICM	Intermediate cell mass
ISV	Intersegmental vessels
LAMP-2	Lysosomal-associated membrane protein 2
NSAID	Non-steroids anti-inflammatory drugs
PCV	Posterior cardinal vein
PGC	Primordial germ cell
PtdIns	Phosphatidylinositol
SSRI	Selective serotonin reuptake inhibitor
VEGF	Vascular endothelial growth factor
WGD	Whole-genome duplication

INTRODUCTION

1.1   WHY FISH?

1.1.1   What is a zebrafish?!

Figure 1. Hybrid of a zebra and a fish. [1].

"What is a zebrafish?!" friends will probe with amused frowns when I explained that I am performing zebrafish research for my PhD. Yes, the first image that dawned upon many may be a hybrid of a zebra and a fish (Figure 1). Freaking scary?!? Fortunately, this is not the case!

Figure 2. Zebrafish. The zebrafish are tropical freshwater teleosts that are found in northern India, southern Pakistan, Nepal and Bhutan in south Asia. **[2]**

The zebrafish (*Danio rerio*) is a tropical freshwater species named for the five horizontal pigmented stripes that run longitudinally across their bodies (Figure 2). Belonging to the minnow family of Cyprinidae and derived from the genus *Danio*, they originate from the Ganges river in India. The zebrafish used to be known only as ubiquitous inhabitants of home aquariums until Dr. George Streisinger from the University of Oregon adopted this species as an ideal vertebrate in which to perform genetic analysis of behaviour in the late 1960s. Ever since, zebrafish has become a popular animal model for developmental biology studies, especially for investigating mechanisms that orchestrate generation of different germ layers [3], organogenesis [4], locomotion [5], formation of different brain circuits [6] and establishment of vascular architecture [7] (Figure 3). Several illuminating advantages of the zebrafish propelled its popularity to be utilised as the model organism in over 600 laboratories worldwide today. First and foremeost, zebrfish are small (growing up to 4 cm in captivity) and

easy to maintain in large numbers in inexpensive aquariums. They are easy to handle during experimentation, requiring minimally invasive procedures and technically simple manipulations such as microinjection.
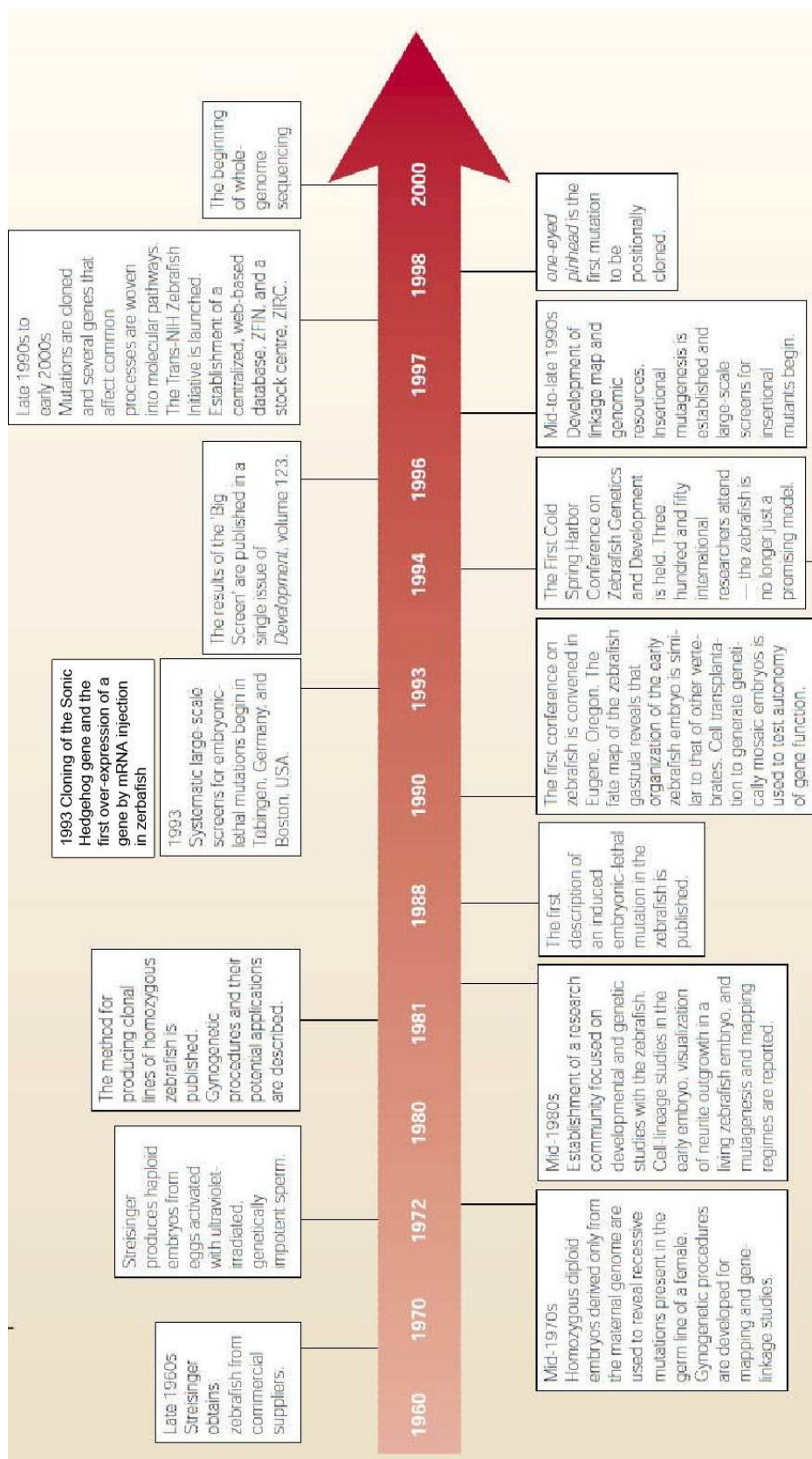

Figure 3. Timeline of landmarks in zebrafish research. [8].

1.1.2 Significant numbers

Problems that many scientist are burdened with when using mouse as animal models include long generation time (2-3 months to reach sexual maturity, each female produces 5-10 litter per year) and limited number of embryos for analysis (each litter will generate at most 6-8 pups). Additionally, mouse embryos develop *in utero* and analysis of embryos requires dissection and sacrificing of pregnant females to allow examination of the pups. Zebrafish on the other hand produce hundreds of eggs in one clutch and more importantly, their embryos develop *ex utero*. This means that females do not need to be exposed to invasive manipulation and the entirety of the developmental process from a fertilized egg to a swimming larva can be observed in a petri dish!

The large numbers of embryos garnered from each mating and their rapid development (heart, vessels and nervous systems are functional within 2-4 days) empower researchers the ability to expedite genetic analysis within a shorter time.

1.1.3 The power of Seeing-Through

Zebrafish embryos are transparent, providing scientists an opportunity to follow the fate of individual cells throughout development as well as to observe processes such as cell migration in real time *in vivo* simply on a dissecting microscope. This is unattainable in other model systems. Research work by Judith Eisen, Brant Weinstein and Leonard Zon's laboratories have demonstrated that with the generation of transgenic lines in these see-through embryos, the zebrafish is an unparallel model for studying neuronal growth [9] and vascular development [10].

The transparent zebrafish is therefore amendable to optogenetics – using optical-sensitve technology to photoactivate proteins, one application of which is to track cell fate in an intact biological organism. The Kaede is a green fluorescent protein (GPF)-like protein that undergoes a permanent green to red conversion upon UV exposure. Transparency of zebrafish embryos merged with photo-convertibility of Kaede has catapulted neural circuitry studies into a new level. For example, neuronal subpopulations can be visualized in real time and extended fate mapping can be performed [11-13].

1.1.4 Genetic Operators

An impressive repertoire of genetic tools such as chemically and transposon induced mutations, binary misexpression systems, antisense morpholino oligonucleotide mediated gene knockdown and zinc finger nuclease mediated targeted mutangenesis, are available to manipulate the zebrafish genome, making it the pre-eminent small animal model for functional genomic studies. Forward genetics using unbiased N-ethyl-N-nitrosourea (ENU) mutagenesis screens permitted the isolating of defective phenotype in mutants, facilitating the rapid identification of novel genes essential for development.

Elegant experiments such as cell transplantation can be performed to investigate if the gene of interest functions autonomously or non-autonomously by transplanting mutant

donor cells into wildtype host embryos and vice versa [14]. Chimera embryos created by transplanting donor cells to targeted host tissues will allow us to investigate how donor cells respond to and influence the host environment, providing insights to numerous questions in developmental biology.

Maternal contribution is frequently required for early embryonic patterning and to a degree compensate for the loss of zygotic gene function [15, 16]. In zebrafish, maternal zygotic mutants can be created by replacing the endogenous primordial germ cell (PGC) with germ cells derived from homozygous mutant donors, therefore developing an excellent tool for unfolding the maternal effects of zygotic lethal mutations.

1.1.5   Have you validated your findings in a vertebrate?

Yes, in case you don't know, zebrafish have backbones and are therefore vertebrates! This means that zebrafish are more closely related to humans than *Drosophila* and *Caenorhabditis elegans* (which are invertebrates).

"The lineages of teleost fish and mammals separated about 500 million years ago, and although fish and people do not look the same, they have many common physiological systems and anatomical features." – Sydney Brenner, False starts [17]

Many important developmental processes are highly conserved between teleosts and human, therefore zebrafish provides a platform for comparative embryological and evolution studies. John Postlethwait's group for instance has used the zebrafish to perform comparative genomics to unveil evolutionary diversification and to investigate the link between conserved non-coding elements and genome contraction and expansion [18].   Teleosts undergo an additional third round of whole-genome duplication (WGD) compared to other vertebrates which go through only two rounds of WGD. The subsequent extra round of WGD that occurred in the zebrafish about 320-400 million years ago [19] is advantageous as it is possible to conduct comparative genomics analysis between mammals and teleosts. Mammals including human contain four clusters of *Hox* genes which are clusters of genes important for determining basic structure and orientation of an organism[20]. Teleosts such as the zebrafish have seven clusters of *Hox* genes - approximately twice that of mammals possibly due to the additional round of WGD [21-24]. The identification of sub-functionalisation of duplicated genes will inform us about genome evolution and more importantly, recognize clusters of genes that are from the same lineage and present information on their predicted functions in mammals.

A robust vertebrate that is transparent, develops rapidly, closely related to humans and is highly amenable for genetic manipulation, the zebrafish is an amazing model organism to bridge the missing gaps between mammalian and invertebrate models.

1.2   HOW TO SEE VESSELS IN THE ZEBRAFISH?

1.2.1   Vasculogenesis & angiogenesis in zebrafish

Vasculogenesis entails the recruitment of circulating endothelial cells differentiated from stem cells (angioblasts) into newly formed blood vessels [25]. The endothelial

cell lattice created by vasculogenesis serves as a scaffold for angiogenesis. The heart is the first organ that is functional in the zebrafish embryo, consisting of two chambers - one atrium and one ventricle - separated by an atrioventricular valve. Angioblasts in the intermediate cell mass (ICM) that are destined for blood vessel development express several transcription factors such as *gata-2, fli1, lmo2* and *scl.* [26, 27]. Vasculature development in zebrafish embryos occurs in two waves. The primary wave of vasculogenesis occurs by 12 hours post fertilisation (hpf) when the vascular cord starts to form by intracellular and intercellular vacuoles fusion. The dorsal aorta (DA) and the posterior cardinal vein (PCV) will be evident by 24 hpf and arterial sprouts will start emanating from the DA and sprout dorsally following the boundaries of the somites. Angiogenesis ensues as sprouting tip cells will extend and retract their filapodia to explore all directions for cues for directional migration. As early as 30 hpf, intersegmental vessels (ISV) will be developed [28]. The robust patterning of the ISV has singled it out as one of the most studied vessels in the zebrafish embryo. Several morphogenic mechanisms have been proposed for the lumenization of the ISV such as budding [29] or cord hallowing [30, 31]. Secondary wave of sprouting emerges from the PCV and specifies the venous fate. Only primary vascular sprouts that are not attacked by the secondary vein-derived cells will retain their arterial fate. Ephrin-B2 marks the arterial cells while Eph-B4 and *flt4* are predominantly expressed in the venous endothelial cells [32, 33].

Numerous studies in the zebrafish have uncovered the molecular control of vessel development which involves Hedgehog (Hh), vascular endothelial growth factor (VEGF) and Notch signaling [34-37]. Hh signalling has been implicated in vascular tube formation in the mouse embryo and for vascular cord formation in cultured mouse endothelial cells [38] as well as in zebrafish. Hh signaling emanating from the notochord stimulates VEGF expression in the somites which further activate Notch signaling to specify arterial fate. There are 4 Notch receptors (1-4). Specifically, Notch 1 and 4 are expressed in the endothelial cells together with their ligands such as Delta-like 1 (Dll1), Delta-like 4 (Dll4), Jagged-1 (Jag1), and Jagged-2 (Jag2). Downstream targets of Notch signaling include Hes-related proteins (Hey/HRT/HERP) [39]. As well as acting indirectly in arterial differentiation through its regulation of VEGF, Hh signaling may also function directly to specific arterial fate as Patched 1 (the downstream receptor of Hh ligand) is expressed in endothelial cells. This suggests that the molecular dissection of arterial differentiation in zebrafish warrant further studies.

The ease of *in vivo* analysis, genetic tractability and amenability to drug administration, makes the zebrafish an excellent model system to dissect molecular mechanisms that orchestrate angiogenesis and metastasis. Furthermore, the availability of transgenic *flk1:EGFP* and *fli1:EGFP* zebrafish lines, in which the endothelial cells are labelled by GFP (Figure 4), allows tracking of neovessel growth with single vessel resolution in real time using a fluorescent dissecting microscope.

1.2.2 Monitoring metastasis

A benign tumour is a mass of high proliferative index cancer cells that are growing rapidly. At this stage the tumours are usually small and therefore attain their supply of nutrients and oxygen via simple diffusion. However, when the tumour reaches a critical mass of 1-2 mm in diameter, the inner core undergoes necrosis and further expansion of the tumour cell population demands higher supply of nutrition to sustain its rapid

growth. Within the confines of a tumour, the availability of nutrients is limited by competition among actively proliferating cells, and diffusion of metabolites is impeded by high interstitial pressure [40]. As a result, tumour cells induce the formation of a new blood supply from the preexisting vasculature, and this offers tumour cells the ability to survive and propagate in a hostile environment [41] by producing multiple growth factors that individually or synergistically stimulate vessel growth. This constitutes the process of pathologically-induced angiogenesis. Similarly, growth of lymphatic vessels into the primary tumour may be induced by angiogenic factors to further support growth of the aggressive cancerous cells [42]. Intravasation occurs when tumour cells protrude through the basement membrane of endothelial cells of blood or lymphatic vessels and invade the hematogenic or lymphatic circulation. Within the circulation, tumour cells may be disseminated to many different organs, including the liver, lungs and brain. Nonetheless, a large proportion of tumour cells will die in such foreign and harsh environment while those that have acquired a higher selective advantage through additional mutations will survive. Upon encountering a favourable environment, these aggressive tumour cells will dislodge into the organ (extravasation) and home onto a secondary site which is distal from the primary tumour, resulting in the process of metastasis (Figure 5). Metastatic spread of cancer indicates that the tumour is malignant and in most cases treatment is usually difficult. At such an advanced stage, palliative treatment is usually given to alleviate patient's suffering. Other therapies such as hormonal therapy, chemotherapy and radiation are given in conjunction only whenever they are deemed suitable. The "soil and seed hypothesis", coined by Stephen Paget as long ago as 1889, suggested that a dynamic interaction between the tumour (seed) and microenvironment (soil) is important for sustained survival and growth of the tumour [43].

To understand mechanisms that govern the metastatic cascade, several classical animal assays have been developed. Orthotopic (tissue of origin) transplantation of tumours into mice to initiate primary tumour growth and subsequent metastasis is used to study the metastatic process. This spontaneous assay method is time-consuming, laborious and results in poor quantification statistically [44]. Injection of tumour cells directly into the circulation via the tail vein in mouse has frequently been employed to investigate steps that facilitate organ selectivity for secondary tumour growth. One disadvantage of this method is that it only uncovers mechanisms that govern that last step of the metastatic cascade – extravasation and not intravasation.

In this thesis, I will present the zebrafish as a model to assay the intravasation process of metastasis. Dissection and elucidation of dynamic interactions of the metastatic cascade will uncover key targets and overcome barriers to clinical development that will ultimately lead to better patient outcome.
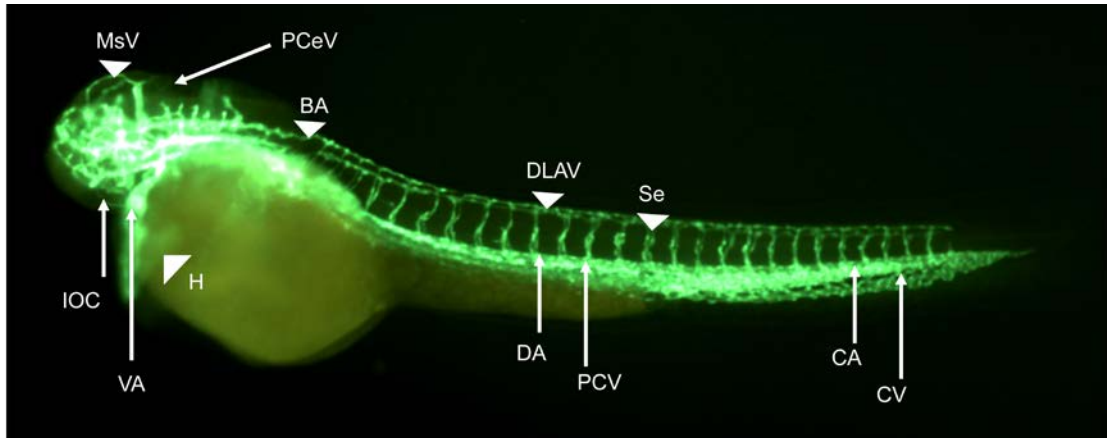
Figure 4. Vasculature of Tg(*flk1:EGFP*) zebrafish at 48 hpf. MsV – Mesencephalic vein; PCeV – Posterior cerebral vein; BA – Basilar artery; DLAL – Dorsal longitudinal anastomotic vessel; Se – Intersegmental vesel; CA – Caudal artery; CV – Caudal vein; DA – Dorsal aorta; PCV – Posterior cardinal vein; H – Heart; IOC – Inner optic circle. (unpublished data)
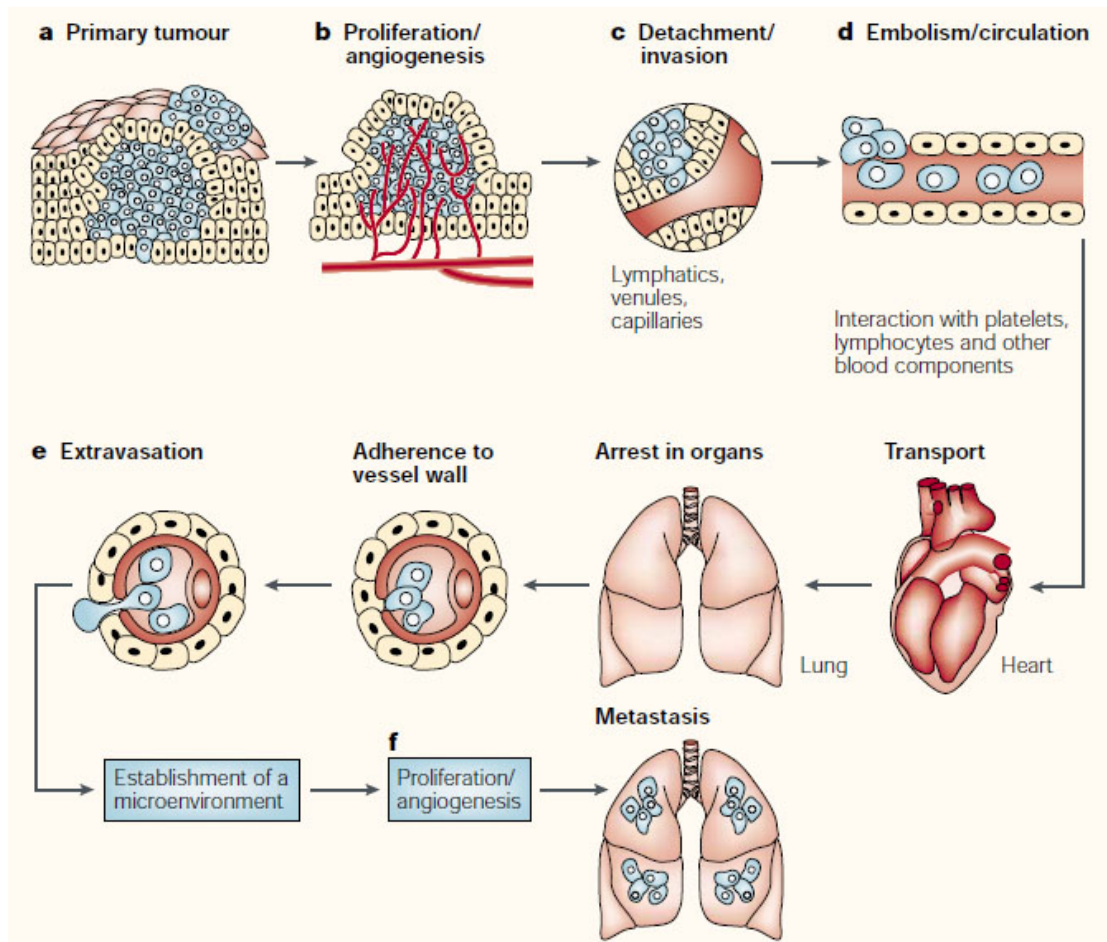
Figure 5. Major steps of the metastatic cascade. (a) Growth of neoplastic cells constitutes the primary tumour. (b) Induction of angiogenesis occurs when tumour growth exceeds 1-2mm in diameter. (c) Intravasation began when local invasion of tumour cells into the stromal tissue and subsequent attack of the lymphatics or blood circulation is initiated. (d) Many tumour cells are destroyed upon entering the circulation. Tumours cells that survive may arrest in capillary beds of a favourable microenvironment in a distal organ. (e) Extravasation occurs when tumour cells home onto a secondary site. (f) The establishment of a secondary tumour away from the primary tumour site signals the spread of the cancer – metastasis. Growth of blood vessels will be induced to further support the proliferation of these micrometastatic tumours. [45]

Figure 6. Atg12 and Atg8 conjugation system in autophagosome formation. [46]

Figure 7. Steps of autophagy. (a) Induction of nucleation. (b) Phagophore formation and vesicle membrane expansion. (c) Autophagosome formation. (d) Autophagosome-lysosome fusion. (e) Degradation of cytosolic components. Cover from [47] and schematic diagram from [48]
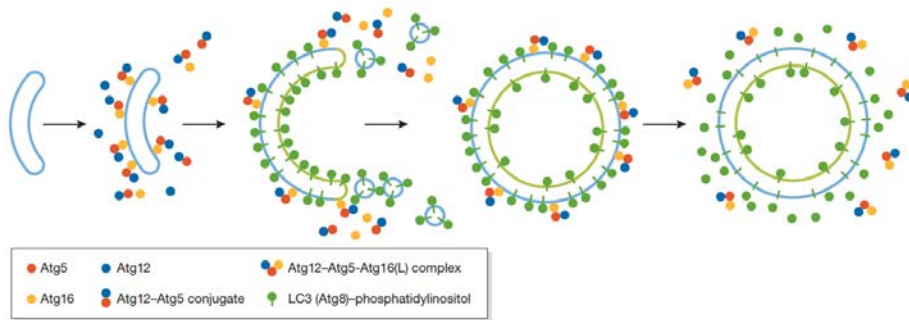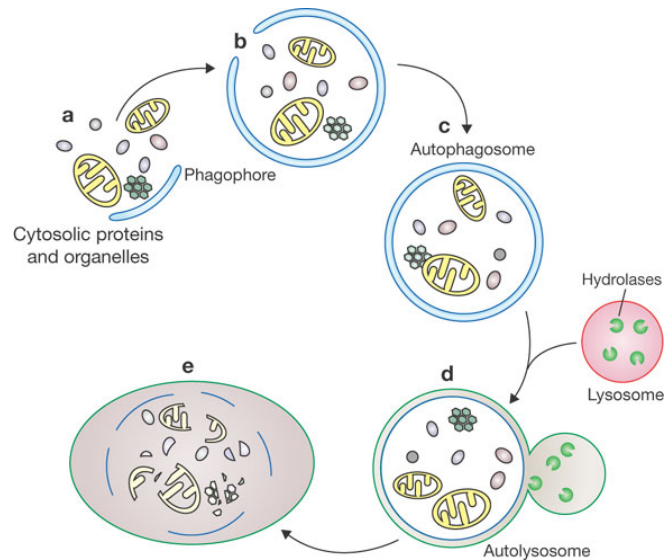
Mouse model	Target tissue/cell	Major phenotype
Atg5 F/F; Nestin-*Cre*, *Atg7 F/F*; Nestin-*Cre*	Neural cell	Neurodegeneration; accumulation of ubiquitin and p62
FIP200 F/F; Nestin-*Cre*	Neural cell	Neurodegeneration; accumulation of ubiquitin, p62 and mitochondria (more severe than *Atg5/7 F/F*; Nestin-*Cre*)
Atg5 F/F; Pcp2-*Cre*, *Atg7 F/F*; Pcp2-*Cre*	Purkinje cell	Axonal degeneration; accumulation of p62
Atg5 F/F; Mx1-*Cre*, *Atg7 F/F*; Mx1-*Cre*, *Atg7 F/F*; Alb-*Cre*	Hepatocyte	Hepatic failure, hepatomegaly; accumulation of ubiquitin, p62 and mitochondria
Atg5 F/F; MLC2v-*Cre*	Cardiomyocyte	Minimal abnormal phenotype (sensitive to pressure overload-cardiac failure)
Atg5 F/F; Mer*Cre*Mer	Cardiomyocyte	Cardiac hypertrophy and dysfunction; accumulation of ubiquitin, p62 and mitochondria
Atg5 F/F; HSA-*Cre*	Skeletal muscle	Atrophy of fast muscle fibres; accumulation of ubiquitin and p62
Atg5 F/F; Mlc1-*Cre*	Skeletal muscle	Muscle atrophy and weakness; accumulation of ubiquitin, p62 and mitochondria
Atg7 F/F; Ap2-*Cre*	Adipocyte	Decreased white adipose tissue mass, resistant to obesity; accumulation of p62 and mitochondria
Atg5 F/F; EL-*Cre*	Pancreatic acinar cell	No abnormal phenotype (resistant to acute pancreatitis)
Atg7 F/F; Rip-*Cre*	Pancreatic β-cell	Impaired β-cell function, reduced β-cell mass; accumulation of ubiquitin, p62 and mitochondria
Atg5 F/F; Zp3-*Cre*	Oocyte	Embryonic lethal at 4–8-cell stage (if fertilized with *Atg5⁻* sperm)
Atg5 F/F; Lck-*Cre*, *Atg7 F/F*; Lck-*Cre*	T cell	Decreased T-cell numbers; accumulation of mitochondria
Atg5 F/F; CD19-*Cre*	B cell	Reduced B-1a B-cell numbers
Atg7 F/F; Vav-*Cre*	Haematopoietic cell	Severe anaemia, lymphopenia (T and B cells); accumulation of mitochondria
Atg5 F/F; CD11c-*Cre*	Dendritic cell	(Succumbed to viral infection due to defects in antigen presentation)
Atg5 F/F; Podocin-*Cre*	Podocyte	Late-onset glomerulosclerosis; accumulation of ubiquitin, p62 and mitochondria

Table 1. Table depicting phenotypes of tissue-specific knockout mice of *ATG*-related genes. *F*, floxed allele; MLC2v, myosin light chain 2v; Mlc1, myosin light chain 1; HAS, human skeletal actin; EL, elastase I; Rip, rat insulin promoter. [49]

1.2.3 Cancer metastasis model in fish

The transparent zebrafish embryo offers unique opportunities for elucidating tumourigenic processes such as metastasis and angiogenesis that are refractory to analysis in mammalian tumour models. The spontaneous metastasis assay and tail-vein metastasis models in mouse suffer from numerous drawbacks such as poor reproducibility, sensitivity to oxygen tension and poor quantification. One of the many attractions of the zebrafish metastasis model is that the progression of a large number of easily observable tumour grafts can be monitored in the optically transparent living zebrafish *in vivo* by utilizing high-resolution confocal imaging. Furthermore with the availability of transgenic *flk1:EGFP* and *fli1:EGFP* zebrafish embryos, with their endothelial cells highlighted, tracking of metastasising cells up to single cell resolution has become possible.

The zebrafish metastasis model presents a number of promising advantages including ease of animal maintenance and experimentation, short assay time; accurate quantitation and amenability to genetic analysis. A number of examples of zebrafish xenograft have recently appeared in the literature, supporting the view that it is a powerful model for understanding human cancer progression such as angiogenesis and metastasis [50].

Xenotransplanting of metastatic melanoma WM-266-4 cells, colorectal cancer SW620 cells and pancreatic cancer FG CAS/Crk cells into hindbrain ventricles of 2 dpf zebrafish embryos supports proliferation, migration, melanin production and cell mass aggregation of these cell lines [51]. Using immunohistochemistry analysis, these authors also show that these xenografts stimulate angiogenesis in zebrafish.

Another group illustrated that implantation of human endometrial adenocarcinoma cells, human ovarian carcinoma A2780 cells, human breast carcinoma MDA-MB-435 cells or murine melanoma B16-BL16 cells into the perivitelline space of 48 hpf zebrafish embryos induced neovascularisation at 72 hpf [52]. Using transgenic VEGFR2:G-RCFP zebrafish embryos to highlight the newly formed vasculature, they also demonstrated that microinjection of cells that overexpress angiogenic factors such as FGF2 (Fibroblast growth factor 2) into the perivitelline cavity elicits neovascularisation from the developing sub-intestinal vessels. More importantly, the same group demonstrated that systematic exposure of the zebrafish xenografts to angiogenic inhibitors such as FGF receptor tyrosine kinase inhibitor SU5402 and VEGFR2/KDR tyrosine kinase inhibitor SU5416 antagonizes neovessel growth without compromising normal vessel development. Antisense morpholino oligonucleotide knockdown of vascular endothelial cadherin (VE-cadherin), an important cell adhesion gene that marks the inter-endothelial cell junction, suppressed tumour-induced angiogenesis. With its well-defined vasculature observed with profound optical clarity within GFP-expressing transgenic fish, the above study illustrated that zebrafish is an ideal model for an intact whole animal *in vivo* angiogenesis assay. Additionally, targeted knock down via antisense morpholino

oligonucleotides and dominant negative inhibition technology available in the zebrafish integrated with its superior imaging qualities offer it as the premier animal to dissect mechanisms that orchestrate tumour-induced angiogenesis and metastasis.

The recent burgeoning literature on zebrafish xenograft models has given us additional insights into how the zebrafish has become a promising *in vivo* intact model for elucidating angiogenesis processes that are unreached by analysis in other mammalian tumour models. It has been demonstrated that the zebrafish xenograft model displays striking similarity in discriminating between highly angiogenic and poorly angiogenic tumour cell lines when compared to the chick chorioallantoic membrane assay and mouse corneal angiogenesis assay [53].

Findings presented in this thesis will illustrate the potential of the zebrafish metastasis model, not only for drug discovery and gene targeting in tumour-induced metastasis but also as an attractive tool for unravelling the many conundrums in tumour biology, cell biology and even tumour immunology.

1.3 FISH MYOGENESIS

1.3.1 Muscle development in the zebrafish

Skeletal muscles empower locomotion and maintain posture, constituting 42% and 36% of the body mass in males and females respectively [54]. A large proportion of the zebrafish body is made up of skeletal muscles (80%), therefore offering it as an attractive vertebrate model to study myogenesis as their muscle fibers are highly accessible for analysis. Muscle fibres are further classified into slow and fast twitch muscles. Slow twitch muscles (Type I) appear red in colour as it is interweaved with blood vessels and undergo oxidative respiration to provide energy for maintenance of normal body functions such as breathing and walking. Fast twitch muscles (Type II) appear white as it is devoid of vasculature and is utilized for burst of activities such as the fight and flight response via non-oxidative respiration. Muscle fibres are composed of bundles of myofibrils which comprises of tandem arrays of sarcomeres – alternating thin and thick myofilaments that form the basic contractile unit of a muscle. The assembly of a sarcomeric unit is a complicated process involving the interdigitation of actin and myosin myofilaments into highly ordered bundles. Precisely how sarcomeres are assembled remains a mystery, although several models have been proposed.

Studies in live chick cardiomyocytes have suggested that premyofibrils initially form at the spreading edge near the cell membrane and act as temporary scaffolds for mature myofibril assembly. Alpha-actinin-rich Z bodies in the premyofibrils fused to form mature Z-bands constituting the Z lines (Zwischenscheibe-lines), giving skeletal muscle its striated appearance [55]. An alternative model is that the thin and thick filaments first form independently before interdigitating to assemble mature myofibrils [56, 57]. Other studies have demonstrated that titin and integrin are essential for the assembly of a highly ordered sarcomeric structures [58-60]. It has also been suggested that calcium influx plays an essential role in the organization and maintenance of sarcomere structures [61]. In the absence of troponin in the developing zebrafish, there is a loss of sarcomeric structures due to a lack of thin filament composition and deregulated of

actin-myosin activity [62]. Several lines of evidence indicated that protein quality control via protein turnover and degradation is critical for maintaining myofibril integrity [63].

In the zebrafish, slow-twitch muscle fibres are superficially located while fast-twitch muscle fibres are medially located. Somites originate from the paraxial mesoderm during gastrulation and by 8-10 somite stage, expression of *MyoD* and *Myf5* (basic helix-loop-helix transcription factors) will drive myogenesis. Hh signaling emanating from the notochord functions in a dose-dependent manner to specify slow cell fate in the zebrafish myotome [64, 65]. Slow muscles migrates to the superficial surface and majority of the medial skeletal muscle in the zebrafish is made up of fast-twitch muscle fibres [66]. Hh activates the Prdm1 (*ubo* gene) in zebrafish embryos, activating slow-twitch fibre differentiation and inhibiting fast-fibre-specific genes, as well as attenuating the repression of slow-fibre-specific genes, therefore specifying the slow-twitch fate [67-73]. Emerging evidence indicates that microRNAs (miRNAs) which are small non-coding RNA may mediate Hh activity for determining slow-twitch muscle fibre fate. miRNA-214 attenuates *Su(fu)* levels – a negative regulator of Hh signaling – and therefore leading to a subtle increase in slow-twitch fate muscle fibres [74]. By 24 hpf, somites will adopt its chevron shape. Dorsal and ventral somites are separated by the horizontal myoseptum and neighbouring somites are divided vertically by the vertical myoseptum.

The ideal platform for investigating the emergent progression of embryonic muscle formation in the teleost is an *in vivo* model to trace how the plethora of sarcomeric proteins dynamically assembles into a functional myotome. The combination of well-defined muscle architecture of the zebrafish together with its unprecedented system for examining genes *in vivo*, the zebrafish have become an emerging and promising model for understanding development of the myotome.

1.3.2 My fish cannot swim – Immotility mutants

Forward genetic analysis offers a powerful approach to dissecting complex biological processes. Mutagenesis is induced in the zebrafish via several methods such as ENU (chemical mutagenesis), radiation or gamma rays or retroviruses insertions [75]. The incredible fecundity of zebrafish [76] expedites genetic mapping and identification of mutations has been made rapid and easy.

Screens by Nobel Prize-winning Christiane Nüsslein-Volhard identified numerous mutants that display immotility phenotypes in zebrafish. Phenotypic studies using birefringency assay identified 18 genes that have a role in muscle development. *frozen* (*fro*), *sloth* (*slo*) and *fibrils unbundled* (*fub*) are three mutants that are classified under the group of immotility mutants which have reduced or no muscle striations. Their offspring are totally paralyzed and unresponsive to tactile stimuli. The *sapje* mutant also isolated from this 1996 large-scale screen was later shown to be defective in the dystrophin gene, disrupting embryonic muscle attachments and resulting in its progressive muscle degeneration phenotype [77-79]. Interestingly, mutations in the same locus in humans causes Duchene and Becker muscular dystrophies in patients, thus offering *sapje* mutant as an ideal for modeling human myopathies and for drug

screening to fish out potential drug targets [80]. These findings illustrated that forward genetic screens has offers us an enticing possibility of uncovering novel genes in disease mechanisms by employing sophisticated genetic manipulation in the zebrafish.

1.3.3 "Eating oneself" – Autophagy

"But while the famous cannibal dined on chunks of his enemies and friends, most people stick to gnawing on themselves at a microscopic level. In fact, the cells of organisms from yeast to humans regularly engage in self-cannibalism. Cells chew on bits of their cytoplasm — the jellylike substance that fills their bellies — and dine on their own internal organs" - *[47]*

Imagine that there is a tiny Hannibal Lecter in the cells among all of us. This may be a macabre thought but in fact, the process of munching on one's own contents is important for every cell to manage stress and for survival. Autophagy (macroautophagy) is a process by which superfluous cytosolic proteins such as macromolecules (abnormal protein aggregates) and organelles (mitochondria) are removed and recycled by the lysosomal degradation pathway. Autophagy can be classified into three different types – autophagy (targeting cytoplasmic components to lysosome via autophagosome), microautophagy (invagination of lysosomal membrane to engulf cytosolic contents) and chaperone-mediated autophagy (translocation directly to the lysosome via the recognition of a specific peptide sequence). Since Christian de Duve first coined the term "autophagy" in 1967, this process has been shown to be essential for many mechanisms such as cellular adaptation and survival during metabolic stress and nutrient deprivation [81-83], neurodegenerative protection for Parkinson's disease [84-86], Huntington's disease [87-89], degradation of intracellular pathogens and viruses in infectious diseases [90-93], protection against muscle diseases such as sporadiac inclusion body myositis, limb girdle muscular dystrophy type 2B and miyoshi myopathies [94, 95]. More interestingly, increasing evidence has suggested autophagy to be a double-edged sword in cancer progression – governing tumour suppression by mediating the Atg6 - Becilin [96] or promoting oncogenesis by supporting survival of metabolically-stressed tumour cells [97, 98].

Autophagy is a complex process consisting of a cascade of steps - induction, cargo packing and selectivity, vesicle nucleation, autophagosome formation, vesicle fusion with lysosome and autophagosome breakdown – each step employing a specific subset of autophagy-related genes (Atg) genes [99]. A repertoire of 32 Atgs is first discovered in budding yeast - *Saccharomyces cerevisiae* by genetic screens [48, 100]. Atg genes are highly conserved among all species as it is a fundamentally important process for controlling protein turnover. Atg13, Atg6 and phosphatidylinositol (PtdIns) 3-kinase complex is essential for induction and nucleation of the phagophore membrane, swallowing up superfluous and abnormal components such as protein aggregates and enlarged mitochondria. Yeast and mammalian studies revealed that Bcl-2 (an anti-apoptotic protein) inhibits Becilin -1 (*atg6*), stalling the nucleation step and inhibiting autophagy [101]. Upon successful nucleation, two conjugation systems are activated.

Firstly, the Atg12-Atg5-Atg16 complex is assembled and this process is facilitated by E1 ubiquitin activating enzyme and E2 ubiquitin conjugating enzyme - Atg7 and Atg10 respectively. Subsequently, formation of a second conjugate - Atg8-phosphatidylethanolamine (PE) is promoted by both Atg7 and Atg3, thereby advancing the autophagome expansion step (Figure 6). Fusion of the autophagosome with the lysosome will initiate the degradation of the cargo by acidic hydrolyases (Figure 7).

Defects in the autophagic pathway have been shown to lead to several myopathies including Danon disease and Pompe disease. Danon disease patients carry mutations in lysosomal-associated membrane protein 2 (LAMP-2), which cause a failure of autophagosome-lysosome fusion and a consequent accumulation of autophagic vacuoles containing cytoplasmic debris or sarcoplasmic membranes [102]. The vacuoles are most prominent in skeletal muscle and ultimately lead to myofibre splitting and degeneration. Danon patients also commonly suffer from hypertrophic cardiomyopathy [103, 104]. Deficiency of glycogen-degrading lysosomal enzyme acid α-glucosidase (GAA) causes Pompe disease, usually characterised by an accumulation of glycogen in the lysosomes due to a reduction in the autophagic clearance [105]. The contractile force of the muscle becomes compromised as the disease progresses. Interestingly, chronic administration of chloroquine – a commonly used anti-malaria drug has been shown to inhibit autophagy, resulting in vacuolar myopathies in both rats and humans that are characterised by the appearance of rimmed vacuoles in the muscle fibres [106].

1.3.4 Autophagy in muscle development

Numerous tissue specific mouse knockouts have been generated to elucidate the function of autophagy in erythrocyte, lymphocyte and adipocyte differentiation (Table 1).

In addition, two Atg tissue specific knockout mouse have been produced to study the role of autophagy in both skeletal and cardiac muscles. Targeted ablation of Atg7 in mouse skeletal muscle leads to atrophy, reduction in muscle mass and age-dependent decrease in muscle contraction force as well as the accumulation of p62 [107] whereas specific cardiac muscle knockout of Atg5 results in cardiac hypertrophy, left ventricle dilation and systolic dysfunction [108]. Both Atg5-/- and Atg7-/- mice suffer from neonatal lethality with reduced amino acids levels. It has also been demonstrated that attenuation of autophagy in cardiomyocytes *in vitro* can lead to dramatically enhanced aggresome accumulation upon excessive cardiac stress and pressure overload [109, 110]. Suppression of autophagy in skeletal muscles of healthy wildtype mouse also increased aggregation of autophagic substrates, such as p67 and polyubiquitinated proteins [111-113]. Armed with the numerous genetic tools previoulsy described, it is enticing to use zebrafish as a model organism to elucidate further the role of autophagy in muscle development.

2 AIMS

The objective of this thesis is to use the transparent zebrafish embryo to uncover some of the molecular and cellular mechanisms underlying the metastatic cascade as well as muscle fibre differentiation.

To achieve these aims, I have conducted two distinct sets of studies:

- Establishment of an *in vivo* metastasis model using the zebrafish embryo (Studies I and II)

- Investigate the role of Atg10 in myofibrillogenesis in a motility zebrafish mutant – *frozen* (Study III)

The ultimate significance of these studies is to hopefully one day:

- Identify subgroup of human tumour cells that are of different metastatic potential using the zebrafish metastasis model. This will lead to better prognosis and treatment of cancer patients.

- Decipher the interplay of Atg10 and muscle fibre assembly during early development and unveil steps that maybe implicated in muscular myopathies.

MATERIALS AND METHODS

Important parameters and limiting factors of key methods to investigate tumour metastasis and myogenesis in the zebrafish will be explained and discussed in this section.

2.1 *IN VITRO* LABELING OF TUMOUR CELLS

Prior to injection, tumour cells were gown in Dulbecco's Modified Eagle Medium (DMEM, Gibco Biocult) supplemented with 10% fetal calf serum (FCS), 1% streptomycin and 1% penicillin until approximately 70-80% confluence. The adherent tumour cells were labeled *in vitro* with 2 µg/ml of 1,1´-Dioctadecyl-3,3,3´,3´-tetramethylindocarbocyanine perchlorate (DiI, Fluka, Germany) for 45 minutes to 60 minutes at 37°C. DiI is a hydrophobic and lipophilic dye that labels cell membranes, therefore labeling the entire tumour cells fluorescent. Instead of labeling adherent cultured cells, suspended cells can also be labeled successfully with DiI. It is important to note that cell suspension of higher density such as $> 1 \times 10^7$ cells / ml would require longer incubation period for uniform staining of cells. Different cells types require varying incubation periods. Optimal staining by DiI cell-labeling solution for different cell type and densities can be quantified by flow cytometry.

DiI was washed away and the labeled cells were rinsed thoroughly three times with PBS, re-plated and incubated for another 24 hours. The tumour cells were checked for ubiquitous and uniform labeling under a fluorescence stereo-miscrocope prior to implantation.

2.2 ZEBRAFISH TUMOUR METASTASIS MODEL

To study the role of mechanisms in promoting early events of the metastatic cascade in relation to angiogenesis, we developed a zebrafish tumour model in which the *Tg(fli1:EGFP)* zebrafish embryos were implanted with mouse or human tumours cells. 24 hpf fish embryos were incubated with water containing 0.2 mM 1-phenyl-2-thiourea (Sigma) to prevent pigmentation. At 48 hpf, zebrafish embryos of the *Tg(fli1:EGFP)* strain were dechorionated with help of a sharp tip forceps and anesthetized with 0.04 mg/ml of tricaine (MS-222, Sigma). Anesthetized embryos were transferred onto a modified agarose gel for microinjection. DiI-labeled murine T241 tumour cells are injected into the perivitelline cavity of 48 hpf-old embryos. Approximately 100-500 DiI-labeled tumour cells were resuspended in serum-free DMEM medium (Sigma) and 5 nl of tumour cell solution were injected into the perivitelline cavity of each embryo using an Eppendorf microinjector (FemtoJet 5247 and Inject Man NI2 microinjection apparatus, Eppendorf, Hamburg Germany). Non-filamentous borosilicate glass capillaries needles were used for the microinjection (1.0 mm in diameter, World Presicion Instruments, Inc. USA). Note that filamentous needles which are utilised for morpholino injections should not be used for transplantation of tumour cells as the filament in the needles will cause sheering of the tumour cells upon injection. Diameter of the non-filamentous needles should be just small enough for single cell transplantation and not too large as this will result in creating a wound in the zebrafish embryos that will cause the transplanted tumour cells

to leak out instead. Wide diameter needles are also unwieldy and bulky for tumour transplantation.

After injection, embryos were immediately transferred into embryo E3-water. Injected embryos were kept at 28°C and were examined every other day for monitoring tumour growth and invasion using a fluorescent microscope (Nikon Eclipse C1, Japan). Mastering of this tumour transplantation technique in zebrafish entails focused attention, perseverance and calm spirit.

Some may argue that cell lines are unable to survive within the zebrafish embryos because established standard incubation temperature of cell lines is 37 °C while in sharp contrast, zebrafish normally require water temperature ranging from 25 °C to 28 °C [114]. Extremely high temperature will lower the oxygen content of the water and will not be healthy for the survival of zebrafish embryos. However, it has been demonstrated that mammalian cells within the zebrafish continue to undergo mitosis and presented no signs of apoptosis during incubation at 28 °C [53]. It has been speculated that aggressive human metastatic melanoma cells transplanted into blastula stage zebrafish embryos retained the ability to metastasize to vascular areas [115]. It has been demonstrated that BrdU is incorporated into the newly synthesized DNA in the human melanoma cell nuclei, indicating that these tumours retain their proliferative potential. Moreover, one group showed that elevation of the incubation temperature to 35 °C did not affect the morphology of various organs such as the heart, liver, pancreas and intestine as these vital organs did not display aberrant differences from those zebrafish incubated at 28 °C [51]. Altogether, these suggest that the zebrafish embryos corroborate the survival of human metastatic melanoma cells and these melanoma cells proliferate and migrate probably by responding to surrounding microenviromental cues.

Besides performing xenograft implantation in zebrafish embryos, recently, one group has also microinjected human tumour cells into juvenile zebrafish. Utilising the transgenic *fli1:EGFP* zebrafish which has green fluorescent-labeled vasculature, they showed that microinjection of human adenocarcinoma MDA-MB-435 (MDA-435), fibrosarcoma (HT1080) and melanoma (B16) cells into the peritoneal cavity of chemically immunosuppressed 1 month old zebrafish leads to formation of microscopic tumours, angiogenesis and intravsation of tumour cells into the vessel [116]. They used their zebrafish xenograft model to investigate whether the human metastatic gene - RhoC and VEGFC work synergistically to promote the initial steps of metastasis by engineering MDA-RhoC cells that secrete human VEGF and implanting these cells into one month old zebrafish. These authors demonstrated that that MDA-435 cells which expressed both RhoC and VEGF formed larger membrane protrusions extending into the vessels in comparison to controls, suggesting that RhoC and VEGFC worked cooperatively to mediate cancer cell intravasation. Zebrafish injected with PBS or 10 µm fluorescent beads did not have any angiogenic or metastatic response, suggesting that the angiogenic response elicited by the tumour cells is indeed caused by tumour-induced angiogenesis and not by wound-induced angiogenesis.

Using juvenile fish as a canvas for studying cancer and blood vessel interaction closely recapitulates tumour-induced remodeling of the vasculature in cancer patients. Another advantage of using embryos over the juvenile zebrafish is that embryos have immature

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immunity, therefore no graft rejection occurs upon xenograft implantation [53]. Moreover, it is important to note that using dexamethasone chemically immunosuppressed 1 month old zebrafish may not be ideal for assaying angiogenic response as it has been reported that dexamethasone inhibits angiogenesis [117] [118] [119] [120], this may render the angiogenesis assay insensitive and underestimate the therapeutic significance of some potential drugs.

2.3   WHOLE MOUNT ZEBRAFISH IMMUNOFLUORESCENCE

Immunohistochemistry was performed as previously described [67]. Embryos were fixed in 4% PFA. Primary antibodies such as mouse monoclonal anti α-Actinin (1:1000, Sigma) and mouse monoclonal anti-light meromyosin (1:500, DSHB) were used. Anti-mouse secondary antibodies Alexa 546 (Molecular Probe) were used at a dilution of 1:2000. 4',6-diamidino-2-phenylindole, dihydrochloride, DAPI (1:2000, Molecular Probe) was used to stain the nuclei. Phalloidin Tetramethylrhodamine B isothiocynate (Sigma) prepared in 2.5 μg/μl stock was used for actin staining at a dilution of 1:300. It is of importance to note that phalloidin-actin staining does not work on methanol-treated embryos. Likewise, phalloidin powder should be dissolved in dimethyl sulfoxide (DMSO) instead of methanol. All embryos should be fixed and stored in phosphate buffered saline (PBS) at 4 °C prior to phalloidin staining. Imaging was performed using a Zeiss LSM 510 confocal microscope.

2.4   WESTERN BLOT ON ZEBRAFISH LYSATE

Whole embryos lysate were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto Immobilon-P Polyvinylidene Fluoride (PVDF) membranes and immunoblotted with specific primary and secondary antibodies conjugated to alkaline phosphatase. Good negative and positive controls should be prepared to test for the specificity of the antibodies. mRNA overexpression in wildtype embryos will produce a band of the desired molecular weight at a higher intensity. While ATG start morpholino or splice morpholino knockdown in wildtype embryos will produce either no band or a weaker band at the same molecular weight. Such controls are essential to validate the specificity of the antibodies used on whole embryo lysate.

2.5   LIVE IMAGING USING CONFOCAL MICROSCOPE

Live embryos were anesthetised with 0.16 mg/ml tricaine (Sigma, A5040) and immersed in 1% low melting agarose gel (Agarose type IX-A, Sigma) on depression slides and observed using a Leica MZ16F stereo-microscope (Germany). To image embryos at a specific lateral surface, zebrafish should be orientated to the desired position immediately upon embedding them in warm low melting agarose. Placing these embedded embryos on ice for 5 minutes will expedite the solidification of the low-melting agarose. A drop of water is placed on the low melting agarose before imaging the embryos under a 40x water immersion lens. High-resolution images were taken using an Olympus FV1000 upright confocal microscope (Japan). Three-dimensional images were assembled and reconstructed using the FV10-ASW software (Olympus, Japan) and processed using Photoshop software (Adobe).

3   RESULTS AND CONCLUSIONS

3.1   THE ZEBRAFISH METASTASIS MODEL (STUDIES I & II)

Mechanisms that orchestrate pathological angiogenesis in relation to hypoxia in tumour invasion and metastasis remain elusive. To date, there is no evidence of an ideal animal model to investigate tumour cell dissemination and metastasis in an entire living organism in real time.

We have developed a zebrafish tumour model that allows us to study the role of tumour-induced angiogenesis under normoxia and hypoxia in arbitrating early events of the metastatic cascade at the single cell resolution. Under normoxia, implantation of a murine T241 fibrosarcoma into the perivitelline cavity of developing embryos of transgenic *fli1:EGFP* zebrafish did not result in significant dissemination, invasion and metastasis. In sharp contrast, significant numbers of tumour cells disseminated from primary sites, invaded into neighboring tissues, and metastasized to distal parts of the fish body under hypoxic conditions. Similarly, overexpression of the hypoxia-regulated angiogenic factor - vascular endothelial growth factor (VEGF) resulted in tumour cell dissemination and metastasis, which is well-correlated with increased tumour neovascularization.

Inhibition of VEGF receptor signaling pathways by sunitinib or VEGFR2 morpholinos abrogated the VEGF-induced tumour cell dissemination and metastasis. Utilizing the transparency of the zebrafish embryo, we demonstrate hypoxia- and VEGF-induced pathological angiogenesis in promoting tumour dissemination, invasion, and metastasis at the single cell level. These findings further provide compelling evidence on the beneficial effects of clinically available anti-VEGF drugs for cancer therapy.

3.2   ATG-10 IN MYOGENESIS (STUDY III)

Autophagy (macroautophagy) is a self-cannabilisation process, which breakdowns long-lived organelles and superfluous harmful misfolded proteins by directing them to the lysosomes for degradation, thereby recycling cellular components to maintain homeostasis and biogenesis within the living cell.

Through the characterization of the zebrafish, frozen (*fro*) mutant identified in the 1996 Tübingen genetic screen as one of a group of immotile mutants [5], a novel role for autophagy in the assembly of myofibrils within muscle fibres has been uncovered. *fro* mutants are easily identified at 48 hpf as they display a significant reduction in birefringency under polarized light, indicative of a defect in myotome patterning, muscle fibre disorganization or muscle volume loss. Meiotic mapping localized *fro* to a genomic interval across which a BAC tiling path was assembled. Injection of individual BACS into mutant embryos identified one BAC, zC219M24, can rescue the mutant phenotype. This BAC contains three transcription units, zgc:123341 - Atg10, ssbp2 and tmem167a.

Injection of Atg10 mRNA and cDNA rescued the sarcomeric structure of muscle fibres in *fro* embryos. Injection of morpholino antisense oligonucleotides targeting Atg10

phenocopied *fro*, confirming *atg10* as the causative locus. Detailed analysis of *fro* mutants using electron microscopy and mitochondria-specific dyes revealed an accumulation of enlarged mitochondria, indicative of a defect in autophagic clearance within the myocytes. In addition, accumulation of large cellular vacuoles, which is morphologically strikingly similar to human vacuolar myopathies, is clearly evident in *fro*. Defects in the autophagic pathway are known to lead to several human myopathies including Danon disease and Pompe disease. Further analysis of this and other autophagy gene mutations in zebrafish will elucidate possible mechanisms for muscular degenerative diseases and human myopathies and hopefully, lead to better therapeutic interventions for patients suffering from these debilitating diseases.

4 DISCUSSION

4.1 WHY VESSELS AND MUSCLES?

My initial motivation to study the mechanisms that govern growth of blood vessels into tumours came from my fascination with the unraveling of teratogenic effects of Thalidomide: this drug that caused devastating malformalities in newborns such as amelia, was found to act by inhibiting integrin leading to abrogation of angiogenesis. Knowledge of the mechanism of action of thalidomide led to its subsequent use as an anti-angiogenic drug for treatment of cancer patients. Amazed by the optical clarity provided by the zebrafish, I proposed to several potential supervisors to study angiogenesis in the zebrafish. More importantly, it dawned upon me that to circumvent a disease, you must first decipher its mode of action and understand at which stage something has gone awry. Philip Ingham cordially accepted my idea of transplanting tumour cells into zebrafish. Mastering the transplantation technique, experimenting with numerous dyes to stain tumour cells and being amazed by how fluorescent tumour cells spread in the transparent zebrafish embryos constituted my first year of PhD work in his lab. Further developing this technique in the Karolinska Institutet, I described how tumour cells metastasized in the zebrafish under hypoxic conditions.

Understanding the cellular and molecular basis of vessel maturation and how metastasis occur is central to delineating the full "life cycle" of blood vessels and lays the foundation for developing effective pro-angiogenic and anti-angiogenic therapies. On an important note, discovering how and which signals initiate the metastatic cascade may create a plethora of novel therapeutic targets. Evidence such as abrogation of the extent of metastasis by VEGF blockade through impairment of tumour-induced angiogenesis in our zebrafish metastasis model has spawned a first glimpse of this therapeutic potential.

Two years later during a discussion with Phil at the EMBO Conference in Amsterdam, he described the frozen mutant phenotype to me and the intriguing finding that it appeared to disrupt a gene involved in the autophagy pathway. Given the rich expertise in Phil's lab in studying muscle development and fascinated by cachexia – a kind of muscle wasting syndrome in cancer patients and enthralled by the implication of autophagy in tumour progression, I was compelled to seize this opportunity and delve into understanding the characterization of this interesting mutation. Intrigued by a documentary which illustrated a young teenager who is diagnosed with muscular dystrophy and how this chronic debilitating disease is slowly rotting away every bit of his muscle, I sought to understand what went wrong in the muscle of this mutant zebrafish.

4.2 DETECTING THE NEEDLE IN A HAYSTACK

When patients are officially diagnosed with cancer after numerous diagnostic tests, usually it is not surprising that these cancers are classified as stage 4 cancer. Stage 4 cancer defines that the tumour is malignant and metastasis has already occurred. In 2011 alone, the American Cancer Society's statistics estimated that 571,950 cancer deaths is expected to occur and approximately 1.5 million new cancer cases are

expected to be diagnosed [121]. With the advances in today's technology, I am astounded to realize that there is a lack of sensitive imaging machines to detect cancerous cells earlier. Why are cancerous cells so hard to detect? Why is it that a primary tumour cannot be first detected before patients are severely riddled with micrometastasis? One explanation is that these cancerous tumour cells evolve and undergo numerous changes in their genetic make-up, therefore acquiring selective advantage over other cells - similar to Darwin's theory of "survival of the fittest" – the theory that supported how finches and tortoises survived on the Galapagos' island. Eventually only the most aggressive tumour cells continue to overproliferate. Constant accumulation of multiple mutations such as activation of membrane transporters that pump out chemotherapeutic drugs, evasion of the immune detection and promotion of blood vessel growth offer these tumour cells a competitive advantage over other cells, rendering chemotherapy treatment challenging and ineffective in patient outcome. Additionally, tumours are not composed of only one type of cell, they are heterogeneous – made up of mosaic cells that are actively acquiring new mutations for survival. Combination treatment using numerous chemotherapeutic drugs may not target and exterminate all cells within the tumour, usually the most resistant and aggressive cells will evade these treatments and continue to proliferate. Only in cases when the cancer is localized, surgery and radiation will be considerably effective.

To combat this "incurable" disease, there are many questions that warrant further study. Which part of the metastatic cascade is most amendable to clinical intervention? Obviously, targeting the intravasation process rather than the extravasation step will theoretically engender better patient outcome as attenuating the very first step of the metastasis cascade before tumour cells invaded the circulation will be the most important step to intervene to abrogate the spread of these aggressive cells. So what are the genomic signature or biomarkers for different kind of cancers before they intravasate into the circulation? Which subset of tumour cells do we target for different kinds of cancer? Given the plethora of different tumours such as breast, lung, ovarian, pancreatic and lung cancers, it seems challenging to scour for good biomarkers for each cancer patient group. Recently five promising candidate markers ascertain for identifying females with ovarian cancers in early studies fell short of expectation after vigorous testing [122, 123]. Likewise, OvaSure - a diagnostic kit selling a panel of six biomarkers which claim to aid early detection in ovarian cancer was pulled out from the market by the US Food and Drug administration (FDA) after erroneous statistics were found in earlier studies which falsely over exemplify the significance of these markers in ovarian cancer detection. Similarly, "breakthrough" biomarkers for early detection of breast and prostate cancers also engender false positive results and failed to go through clinical trials. Evidently, rigorous statistical tests have to be done on potential biomarkers on larger independent groups to deliver better reliable results. Lessons from previous failed biomarkers also suggest that potential biomarkers should be carefully validated before expensive clinical trials are conducted.

Why do some tumours relapse with vengeance? A mass of tumour cells that have acquired a balance between proliferation and cell death resulting in no net increase in tumour volume constitutes dormant tumour cells. Mechanisms supporting tumour dormancy remains elusive to date. Latency period is defined as the time of first treatment and recurrence of the tumour – reflecting relapse of cancer. Mesothelioma

and breast cancers are intractable in nature owing particularly to their long latency period [124-126]. Disseminated tumour cells have been detected in breast cancer patient up to 22 years after successful treatment [127], suggesting that dormant tumour cells can be switched on after very long latency, this can create a lot of uncertainty in patients. Are dormant tumour cells good therapeutic targets? Impetus for re-awakening of dormancy remains largely unknown. Initiation of quiescent dormant cells to active regrowth maybe due to sudden activation of the angiogenic switch, elevation of growth stimulating factors emanating from the microenvironment or weakening of the immunological status of patients. Other external factors such as diet, surgery and unforeseen tissue injury may also reactivate dormant disseminated tumour cells [128, 129]. Though disseminated dormant tumour cells are valid candidates for therapy, without a clear understanding of dormancy biology, specifically targeting dormant tumour cells prove to be challenging. Here, our zebrafish metastasis model can help to understand the genetic signature of the disseminated tumour cells and unravel why some tumour cells choose to specifically home in some organs.

"If you can shrink the tumour 50% or more for 28 days you have got the FDA's definition of an active drug." --- Ralph Moss

Numerous studies use tumour size and volume reduction as measurements to be directly translated to better patient outcome, but this may not be the case! A toxic drug that specifically exterminate tumour cells and shrink tumour volume in a short treatment period may bring along with it several harmful non-specific side effects, overall resulting in doing more harm than benefit to cancer patients. Therefore, overall benefit of any drug should not be based on its ability to shrink tumour volume but should be cautiously validated in the context of benefit versus risk to the patient outcome, thereby prolonging patient survival.

Despite millions of dollars pumped into cancer research, there is still a paucity of good reliable biomarkers, disappointing clinical trial outcomes and lack of understanding of tumour dormancy. Discovering the perfect cure for cancer proved to be challenging like finding a needle in a haystack. Nonetheless, with novel imaging techniques that can detect small dormant glimmers homing in distal organs, harvesting of tumour cells in circulation and investigating genomic signature of metastatic biopsies should allow better validation of putative biological markers.

4.3 FISHING FOR SCIENCE - FOR THE BETTER GOOD OF MANKIND

"the fish is a frog… is a chicken… is a mouse. It did not matter which animal you chose — fundamental processes were fundamentally conserved." [8].

As numerous important mechanisms are highly conserved between zebrafish and human, it is tantalizing to use zebrafish as a model organism to discover novel genes in several human diseases such as neuronal, musculoskeletal, cardiovascular, inflammation diseases and even cancer and obesity.

Besides the above mentioned diseases and *sapje* mutants which are previously described as a model to study human muscular dystrophies, more recently in Nature News, zebrafish became the first fish model for human depression. This "depressed" zebrafish carry a mutation in the glucocorticoid receptor (GR) and when left in isolation in a tank, it stops swimming and hides at the bottom of the tank – an indication of its disability to cope with stress. Glucocorticoids bind to GR and lack of glucocorticoids secretion is known to be associated with the inability to deal with stress. Fear for example is shown to stimulate increase glucocorticoid secretion – an important human cognitive function in order to handle stressful situation. In this fish, there is little or no GR activity and bathing them in anti-depressant drugs such as fluoxetine (Prozac), a selective serotonin reuptake inhibitor (SSRI) rescued their defective behaviour [130].

Other significant advantages of the zebrafish is its tiny size and optical clarity, providing it as a unique promising *in vivo* organism for large scale drug screening over conventional cell based *in vitro* assays.

"Small molecules can be invaluable tools for studying and treating diseases, but discovering novel small molecules is a difficult and risky process. Many of the challenges of small molecule discovery stem from the inability of in vitro assays to model the complex physiology of disease. Consequently, the in vitro assays used in small molecule discovery are poor predictors of the beneficial and adverse effects of the small molecule when translated to an in vivo setting. We are circumventing these problems by performing high throughput small molecule discovery in vivo using zebrafish as a model organism."- Randall Peterson

The tiny size of zebrafish means that drugs only need to be added in minuscule quantities into 96-well plates, allowing screening of candidate drugs to be conducted in a cost-effective manner. Additionally, drug screening done in an *in vivo* living system will provide insights into the benefits of lead drugs as well as its potential drawbacks of non-specific side effective on other non-target organs. One group in Harvard demonstrated that high-throughput behavioral profiling in zebrafish identified promising new psychotropic drugs and these findings can further elucidate pharmacological actions of a myriad of drugs and their targets [131]. For examples, they found that a subset of non-steroid anti-inflammatory drugs (NSAIDs) stimulates waking in daytime and having lesser effect during night, suggesting that NSAIDs have a role in determining daytime activity in addition to inducing sleep during infections [132].

Likewise, the sheer potential of the zebrafish metastasis model poses many important questions about the molecular basis and clinical relevance of this model to human tumourigenesis. How should the results from the zebrafish metastasis assay be quantitated and scrutinized to engender reliable and reproducible information? Are mechanisms deployed by the zebrafish to induce angiogenesis same as those in mammals and humans? Do the leaky angiogenic neovessels in the zebrafish display similar vascular markers to those in humans? These are challenging key issues that should be resolved before the results from the zebrafish metastasis assay can be translated to potential clinical relevance to humans.

The establishment of the zebrafish metastasis assay will illuminate our understanding of how tumour cells intravasate into the circulation by responding to microenvironmental cues. Furthermore with the invention of transgenic lines such as neutrophil reporter zebrafish - *MPO (myeloperoxidase):GFP*, investigating tumour immunology using the zebrafish metastasis model will be easily achievable in the near future.

To me, the humble zebrafish has definitely earned its stripes!

5 ACKNOWLEDGEMENTS

5.1 THE 3 "PHILIP"S

Just to decipher "Philip" a little more, this name is of Greek origin and means "horse lover". My A*STAR graduate scholarship (AGS) senior once commented that I seem to have numerous associations with this name. You may be wondering why?

3 "Philip"s are instrumental in the completion of this thesis.

Professor ***Philip*** Keith Moore – for your tutelage during the undergraduate honours project in NUS and entrusting me the gargantuan task of performing femoral artery cannulation on rats, thereby unveiling my first glimpse into medical research – Thank you!

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5.2 OTHER VIPS

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The zebrafish as a model to elucidate human diseases and development

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ABSTRACT

This thesis explores the zebrafish as a model organism to illuminate our understanding of processes that orchestrate the progression of cancer metastasis and myogenesis.

First, I describe the successful establishment of a cancer metastasis model in the zebrafish, specifically to study intravasation - one of the earliest steps in the metastatic cascade. Fluorescently labeled tumour cell are transplanted into pervitelline space of 2 days post fertilisation (dpf) zebrafish embryos before they are exposed to normoxic or hypoxic conditions, allowing us to study the effect of hypoxia on tumour-induced angiogenesis and metastasis. Hypoxia elicited an enhanced angiogenic response and neovascularisation to the transplanted tumour and escalated the extent of metastasis in the living zebrafish embryo. Loss of function experiments such as vascular endothelial growth factor (VEGF) blockade using a clinically available drug – Sunitinib or VEGF morpholino knockdown attenuated tumour-induced angiogenesis and metastasis. The *in vivo* metastasis assay in zebrafish delivers numerous unique advantages over conventional *in vitro* cell-based or biochemical chemotaxis assays. The transparent embryo facilitates the tracking of the entire intravasation process in real time within a living organism, allowing us to explore the dynamic interplay of tumour cells and the environmental cues that drive metastasis. This model can be further employed to discriminate between tumour cells of different metastatic potential and to identify novel factors that present as impetus for the earliest step of the metastatic cascade.

Next, I demonstrate the analysis of one class of motility mutants originally identified in the 1996 Tübingen genetic screen that potentially serve as models for human myopathies and dystrophies. Two of these mutants have previously been cloned and shown to encode proteins involved in muscle fibre attachment whilst a third has been found to encode the molecular chaperone Heat shock protein (HSP) 90. I describe the phenotypic and molecular characterization of another of these zebrafish motility mutants, *frozen (fro)*. I present evidence that the fro^{t027c} mutation disrupts the locus encoding the autophagy pathway component Atg10. This analysis implicates Atg10 in the assembly of both skeletal and cardiac muscle fibers suggesting a previously uncharacterized role for the autophagy pathway in this process.

Together, my findings illustrate the utility of the zebrafish as a model organism that complements established mammalian and invertebrate models. The fecundity and amenability of the zebrafish to genetic manipulation together with the rapid development and translucency of its embryos combine to provide a powerful system with which to unravel and inform the underlying mechanisms that govern fundamental biological processes and shed light on our understanding of human development and diseases.