Using the Cre/loxP recombination system to study the role of the PI3K/AKT signalling transduction pathway and the embryonic cellular origin of adult haematopoietic stem cells in the zebrafish.

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List of Abbreviations:

BSA bovine serum albumin

CHT caudal haematopoietic tissue

DAB diaminobenzidine

DMSO dimethyl sulfoxide

dpf days post fertilization

EDTA ethylenediamine tetra acetate

GFP green fluorescent protein

hpf hours post fertilisation

HSC haematopoietic stem cell

OCT optimal cutting temperature compound

PBS paraformaldehyde

PFA phosphate buffered saline

PI3K phosphotidylinositol 3-kinase

Runx1 runt-related transcription factor 1

TAE tris acetate EDTA buffer

TE tris EDTA buffer

vDA vascular endothelial growth factor

VEGF ventral wall of the dorsal aorta

4-OHT 4-hydroxy tamoxifen

Abstract:

Haematopoietic stem cells (HSCs) are present in the bone marrow (BM) and maintain our blood system throughout life. These cells have clinical importance and their transplantation constitutes the most common type of stem cell therapy available. Lack of BM donors and our inability to expand HSCs *ex vivo*; make it desirable to be able to generate patient specific HSCs from pluripotent stem cells. Using the zebrafish as model organism we have two parallel long-term projects, one to address the cellular origin of HSCs and the other to understand an aspect of the molecular programming of HSCs. Establishing a CreERT2 system we have labelled cells by inducing with 4-OHT at different time points during embryonic development. Since lineage tracing experiment are long term experiments, only a part of this was done. During this project, however methods to isolate, section and stain the adult zebrafish kidney, which is the site of adult haematopoiesis in the fish, have been established which would later be used to look at the kidneys of the fish that have been recombined in order to trace the origin of the cells that seed the kidney.

The Cre/loxP system is used to force expression of a dominant negative AKT and a constitutively active AKT in endothelial cells. Characterisation of these lines was carried out in order to see whether these lines could be used in the future to study the effect of the loss and gain of function of the PI3K/AKT signal transduction pathway on vessel formation and HSC specification.

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Chapter I: General Introduction

1.1. Haematopoiesis

Haematopoiesis is the process by which mature cells of all blood lineages are formed in an organism. This process is sustained throughout life by haematopoietic stem cells (HSCs) (Weissman et al., 2000). HSCs are immature, multipotent (can give rise to cells of all blood lineages) progenitors that are capable of self-renewal. These cells reside in the bone marrow (BM) of adult mammals.

1.2. Clinical importance of HSCs

In a healthy human adult an average of 10^{10}-10^{11} new blood cells are produced everyday to maintain homeostasis. HSCs are extremely potent and in case of an injury and thereby complete loss of both peripheral blood and bone marrow cells, it takes only a few HSCs to revive the blood system back to normal Bordignon (2006). This is what makes HSCs of enormous clinical relevance today.

HSCs are being widely used in bone marrow (BM) transplants to re-establish the loss of the blood system in patients with BM failure or due to the therapy administered to treat leukemia or a solid cancer. Therapies usually involve autologous (using the patient's own BM) bone marrow transplants and allogeneic (BM taken from an antigen-matched donor, usually a parent or sibling). Autologous bone marrow transplants are used depending on whether functional, disease-free HSCs can be procured from the patient. Autologous BM transplants are preferred to allogenic transplants, because the latter bares the risk of causing graft versus host disease or graft rejection. The lack of BM donors and our inability to expand HSCs *ex vivo* require generation of patient specific HSCs from pluripotent

stem cells (Murry and Keller, 2008).

1.3. Haematopoiesis occurs in waves in the mammalian conceptus

In the vertebrate conceptus haematopoiesis occurs in 2 successive waves:

A primitive wave in which primitive red blood cells and myeloid cells are formed, and a permanent or definitive wave, which occurs later in development to form mature cells of erythroid, myeloid and lymphoid lineages (T and B cells). HSCs are formed during this second or definitive wave (Medvinsky et al., 2011).

1.3.1. Primitive haematopoiesis in the mouse

In the 1970s in a sophisticated study, using chimeric experiments in which quail embryos were transplanted into the yolk sac of a chick showed that cells coming from the yolk sac did not make a significant contribution to adult haematopoiesis. This would mean that HSCs found in the adult came from the embryo itself (Dieterlenlievre, 1975; Medvinsky et al., 2011). The relevance of the data generated in the avian system to all vertebrates became clear only when these findings were confirmed in the murine system.

In mammals and birds the primitive wave takes place in the mesodermal layer of the extraembryonic yolk sac (YS).

In the mouse YS, at around embryonic day 7.5 (E7.5) blood cells emerge from a population of cells known as haemangioblasts. These cells leave the posterior primitive streak to undergo haematopoietic differentiation in which they differentiate to form blood and endothelial cells. This differentiation takes place in the YS (Huber et al., 2004).

Colony forming units CFU-C are described as progenitor cells that form haematopoietic cell colonies on solid media when supplied with the right nutrients and cytokines. These cells do not have the potential to self-renew, although some may be able to form colonies when re-plated (Medvinsky et al., 2011). It has been seen that cells isolated from the YS are myeloid progenitors that are capable of forming CFU-Cs.

Another term, colony forming unit-splenic (CFU-S) is used for cells that are undifferentiated, immature progenitor cells capable of forming colonies in spleens of irradiated mice. CFU-S is the term used to describe adult type or definitive haematopoietic stem cells (dHSC) (Medvinsky et al., 2011).

1.3.2. Definitive haematopoiesis in the mouse

It has been seen that the aorta of the E10.5 murine embryo displays HSC clusters and these clusters were responsible for establishing definitive haematopoiesis (Adamo and García-Cardeña, 2012)

Definitive haematopoiesis occurs from E11.5 in the aorta-gonad-mesonephros (AGM) of the murine embryo and this region has been shown to be the hub of HSC formation. At the pre-liver stage of murine development the 11.5E embryo contains at least a single HSC in the AGM, placenta and yolk sac and hence subsequent HSCs are generated.

The fetal liver begins to get colonised by HSCs by E12.5. Simultaneously HSCs are seen to be in circulation. HSCs further multiply in the fetal liver and this multiplication is mediated by Sox17 a transcription factor and molecules such as angiopoietin-like factors. β1 integrins also play a pivotal role in colonizing the fetal liver with HSCs(Medvinsky et al., 2011)

HSCs in the developing embryo do not have a marker specific to them. Cells migrate from the dorsolateral plate (DLP) to the midline to form the dorsal aorta. DLP derived cells form endothelial cells that express flk1 and fli1. Hematopoietic cells are marked by Scl, Gata2 and lmo2.

Once the cells of the DLP reach the midline, it has be seen that some of these cells express crucial haematopoietic transcription factors such as Runx1 (Runt-related transcription factor 1) that has been shown to be crucial in the formation of haematopoietic clusters. Runx1 is expressed in the HSCs that are in the AGM.

In the murine conceptus the bone morphogenetic protein4 (Bmp4) and the sonic hedgehog (shh) have been found to be pivotal in expansion of HSCs within the AGM.

In Dr. Martin Gering's lab we are studying haematopoiesis during vertebrate embryogenesis using the zebrafish (Danio rerio) as a model organism.

1.4. Zebrafish as a model organism to study vertebrate haematopoiesis

- The transparent nature of its embryo makes the zebrafish extremely favourable for *in vivo* imaging thus making them an ideal model to study haematopoiesis.
- Especially for HSC development, zebrafish have a circulatory system similar to that of humans, thus making it a good model to understand and study haematopoiesis.
- Zebrafish can easily be genetically modified hence making it easy to carry out transgenic studies.

1.4.1. Haematopoiesis in zebrafish

Like with all other vertebrates haematopoiesis in zebrafish occurs in 2 successive waves;

A primitive wave and a secondary or definitive wave.

1.4.1.1. Primitive haematopoiesis in zebrafish

In the zebrafish this wave takes place in the intermediate cell mass (ICM) (Davidson and Zon, 0000). The ICM is a chord of cells located in the trunk midline between the notochord, the endoderm and the somites. These cells then differentiate into

primitive red blood cells and the ICM further differentiates to form the two major trunk vessels, the dorsal aorta (DA) and the posterior cardinal vein (PCV)

Erythroid progenitors express the gata1 transcription factor and definitive erythropoiesis is dependent on gata1(Ellett and Lieschke, 2010). Myeloid progenitors however express spi1 (also known as pu.1). Both gata1 and spi1 seem to interact with each other (Rhodes et al., 2005) but play no significant role in the next wave of haematopoiesis which is the definitive wave.

Dividing haematopoiesis into waves is not straightforward, because the waves could be distinguished on the basis of a number of factors such as the time or region in which they occur.

Another wave is said to occur in the caudal haematopoietic tissue (CHT) of the developing embryo just before the definitive wave is believed to begin. This wave occurs from 26-48 hpf, is not dependent on notch and forms erthythromyloid progenitors (EMPs) from lmo2+ precursors (Bertrand et al., 2010). Recent lineage tracing experiments by Warga et al (2009) suggest that EMP formation takes place at the start of gastrulation at around 5hpf, differentiating these cells from unipotent progenitors that seed the rostral blood island (RBI) and the RBCs that are formed during primitive haematopoiesis (Warga et al., 2009).

1.4.1.2. Definitive haematopoiesis in zebrafish

In the zebrafish the definitive wave begins at about 23-24hpf. At this time HSCs begin to emerge from the ventral aspect of the dorsal aorta (vDA), also referred to as the hemogenic endothelium. The vDA in the zebrafish is analogous of the AGM

region in the mouse. The HSCs begin to appear with the advent of circulation, when the first primitive blood cells enter circulation at around 24hpf.

HSCs are identified by the expression of the transcription factors runx1 and c-myb, and by their intimate association to the vDA (Gering and Patient, 2005). In runx1 morphants there is a loss of c-myb expressing definitive progenitors as well as a loss of T-cells in the thymus (Gering and Patient, 2005; Kalev-Zylinska et al., 2002). T-cells are progeny of HSCs in the vertebrate conceptus and the loss of T-cells in the runx1 morpholino experiments suggests that the runx1 positive cells in the ICM are HSCs or their precursors.

Cells leave the vDA in a process called 'endothelial to haematopoietic transition' (ETH) where elongated endothelial cells leave the vDA to assume a more rounded morphology before leaving the lumen. During ETH, which is optimum at 30-60 hpf, endothelial cells transition to become blood cells. This process is runx1 dependent and in embryos lacking runx1 it has been seen that when cell a tries to exit does not survive (Kissa and Herbomel, 2010).

Studies carried out on zebrafish mutants such as the mindbomb, smoothened (smo) and embryos treated with a VEGF (vascular endothelial growth factor) receptor inhibitor showed that notch, hedgehog and vegf are required for definitive haematopoiesis, but not for primitive erythropoiesis (Gering and Patient, 2005).

Chapter 2: Materials and Methods

2.1. Zebrafish husbandry

All zebrafish lines, both adult and embryos were raised, bred and maintained according to guidelines in The Zebrafish Book (Westerfield, 2000).

2.1.1. Lines that were used and generated for the flk1Cre/flk1CreERT2 experiments

A number of lines were used in our experiments and were as follows; F0 generation fish, these are the injected fish and they were SN9 Tg(flk1CreERT2) HA.M date of birth (DOB) of these was 28.4.11 was the founder of qmc104. F1 generation fish which were SN51 Tg(flk1CreERT2;Cre reporter) DOB of these fish was 27.7.11 and are the qmc104s. Other lines used were SN41 Tg(ef1α-loxP-GFP-terminator-loxP-dsRed) are heterozygous carriers for the transgene and the DOB for these fish is 12.7.11. These fish will be referred to as Cre reporter. SN903 (flk1Cre) (founder of qmc101) carries the transgene (flk1CreXERT2) and 'X' referes to a frameshift between the Cre and the ERT2, making the Cre in this line constitutively active and will be referred to as flk1Cre throughout the text. The SN55 (flk1Cre) are the F1 generation of the qmc101 and their DOB is 12.9.11 these fish will be referred to as qmc101. SN907 (qmc101; Cre reporter) and the DOB for these dish is 30.9.10. Wild type (wt) fish used were the SN19 and SN26.

2.1.2. Lines used and generated for the PI3K/AKT study

F0 fish SN44 Tg(ef1α-loxP-Cerulean-loxP-HA-AKT1K179M) DOB of these was 20.7.11 and these fish will be referred to as KDAKT for convenience.

SN47 Tg(ef1α-cerulean-terminator-myrAKT) the DOB of this stock is 22.7.11 and these fish will be referred to as myrAKT.

F1 Fish SN69 Tg(ef1α-cerulean-terminator-myrAKT)^{qmc162} DOB 8.11.11.
SN75 Tg(ef1α-loxP-Cerulean-loxP-HA-AKT1K179M)^{qmc171} SN76 Tg(ef1α-loxP-Cerulean-loxP-HA-AKT1K179M)^{qmc172} SN77 Tg(ef1α-loxP-Cerulean-loxP-HA-AKT1K179M)^{qmc173} SN81 Tg(ef1α-loxP-Cerulean-loxP-HA-AKT1K179M)^{qmc174}
SN56 wild type (wt) DOB 7.10.11

All fish and their embryos were maintained at 28.5°C. All work carried out on zebrafish including culling were according to the standard Home office regulations.

2.2. Isolation of genomic DNA (gDNA) from embryos

Genomic DNA was to be isolated from embryos that were first dechorionated. The number of embryos used to extract DNA from fish is depicted in Table1 Chapter 4.

We then treated the embryos with extraction buffer which was to be added in a volume ten times the number of embryos present in the eppendorf tube. The tubes were left overnight at 55°C.

2.3. Phenol/chloroform extraction

In order to remove any remaining proteins and other impurities from the tubes it is important to carry out phenol/chloroform extraction. Around 650µl of phenol: chloroform:isoamyl (25:24:1) alcohol was added The tubes were first centrifuged at 13000rpm for 5minutes. After spinning there were two layers formed, an aqueous layer and

a yellow phenol layer. The aqueous layer was transferred to a new set of tubes and the phenol layer discarded. This step was done under a fume hood. In order to get rid of any traces of phenol in the aqueous layer it was treated with equal volumes of a 24:1 chloroform:isoamyl alcohol solution. Each tube was vortexed for 1 minute before centrifuging them at 13000rpm for 5 minutes. Two layers were obtained an aqueous layer (top layer) and chloroform: isoamyl layer. The aqueous layer was transferred to a new set of tubes. To the aqueous layer 1/10th the volume of 3M pH5.2 potassium acetate was added followed by 2.5 volumes of 100% ethanol in order to precipitate out the DNA. Tubes were then centrifuged at 13000rpm/15min. The supernatant was discarded, making sure not to disturb the pellet. The pellet was washed with 70% ethanol to remove any salts and centrifuged if required. The tubes were inverted to get rid of the alcohol and left inverted to dry completely as any remnants of ethanol would interfere with the enzyme Taq polymerase in the PCR. Once the pellet was completely dry, it was reconstituted in tris EDTA (TE) buffer. TE contains EDTA that is a chelating agent and prevents nucleases from degrading DNA.

The centrifuge used was a 'Fisher Scientific accuspin Micro' and the vortex used was 'Grant bio pv-1'.

2.4. Polymerase chain reaction (PCR)

In order to carry out a PCR, depending on the number of samples a master mix was prepared. We prepared a master mix for 15 samples, which was a total of 22.5µl. The master mix included 37.5µl of 10X buffer, 262.5µl of sterile distilled water, 7.5µlof 10mM dNTPs and 15µl of each primer #143 (Forward Primer) 5'-CTGAATAAGTAGATAGCCTATC-3' which

anneals in the flk1 promoter fragment and Primer #144 (Reverse Primer) 5'-CTCATCACTCGTTGCATCG-3' which anneals in the Cre. Both the primers were in a concentration of 10pmol/µl; 10uM. Once the master mix was ready in PCR tubes, to 22.5µl of master mix 2µl of DNA template and 0.5µl of Taq polymerase was added. The total volume in each PCR tube was 25µl. Once all the tubes were ready they were placed in the PCR machine 'Techne TC-512' and the PCR conditions were set to 1cycle at 90°C for 2minutes, 40 cycles of denaturation at 94°C for 20 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 50 seconds, 1cycle at 72°C for 5 minutes was done to check all the DNA has been replicated and finally 1cycle at 4°C was on hold indefinitely.

2.5. Agarose gel electrophoresis

The PCR products were separated on a 1.5% agarose gel prepared in 0.5X TAE buffer to which 4µl of ethidium bromide was added. 10µl of the samples was mixed with 2µl of loading dye. The gel was run in a Gel Exel Ultra V2 electrophoresis chamber at 100v for 30 minutes.

2.6. *In vivo* fluorescent imaging

Fluorescence analysis in embryos was done on a Nikon SMZ1500 microscope fitted with filters to detect fluorescence. These microscopes were either attached to a Nikon DXM1200F or Nikon Digital Sight DS-U1 that took coloured images. A more sensitive camera was the Hamamatsu ORCA-ER C4742-80 digital camera. Although extremely sensitive it took only black and white images that could be pseudocoloured. The Hamamatsu was used with the Nikon Eclipse 80i microscope. GFP and dsRed expression were viewed with the help of a

mercury light source and a FITC filter set and a TRITC filter set respectively. Cerulean expression of myrAKT and KDAKT fish was observed with the help of a mercury light source and a Cyan-GFP filter set.

All embryos were anesthetised before observing and imaging by adding 750µl of 4g/l pH 7-7.5 tricaine methanesulfonate (MS222) into the petri plate containing fish water and the embryos, in order immobilize them. This was necessary especially when the embryos were out of their chorions and were mobile.

2.7. Culling of fish

Since the KDAKT lines qmc171, 172 and 173 did not express cerulean very convincingly we decided to terminate these lines. Fish were put into MS222 (4g/l pH 7-7.5). The fish were allowed to stay in the MS222 for about 10 minutes. According to home office regulations, the brain of the fish must be pithed to confirm death.

2.8. Adult zebrafish dissection and kidney isolation

The method for dissection of the adult zebrafish and isolation of the kidney was adapted from the method described by Gerlach et al (Gerlach et al., 2011).

Briefly, the fish that were to be dissected were euthanized by placing the fish in (MS222) (4g/L pH 7-7.5) for about 10 minutes. The fish were then taken out of the MS222 and placed on an absorbent paper towel after which they were ready for dissection. A fish was then placed on a dissecting tray and the dissection was carried out under a dissecting microscope. The head of the fish was cut from under the operculum at an angle of approximately 45°, to avoid rupturing the heart which if ruptured causes blood to cover other tissues making in difficult to view the kidney. Once the head was detached from the body of the fish the body of the fish was cut open making sure not to cut too deep which could damage the kidney. The fish was pinned down to the dissection tray and the internal organs such as the gonads, intestine, swim bladder were removed. The kidney was identified. The body of the fish with the kidney was placed in a container containing 4% paraformaldehyde (PFA), which was prepared in a 50ml falcon tube, by dissolving 2g of PFA in 40ml of sterile water and 20µl of 1M NaOH. This was left at 65°C for about 2 hours and inverting the tube from time to time to allow the PFA to dissolve completely. Once the PFA had dissolved completely it was cooled to 4°C on ice. When cool 5ml of 10X phosphate buffered saline (PBS) pH 7.5 was added and the volume in the tube was brought up to 50 ml with distilled water. The tube was kept on ice again and 50µl of DMSO was added. The fish were left in this 4% PFA solution at 4°C overnight in order to fix the kidney.

The next day using 2 tweezers the kidney was carefully detached from the connective tissue and placed into a 1.5ml eppendorf tube containing 1ml of 1X PBST (1X PBS with 0.1% (v/v) Tween 20). This was left overnight at 4°C. The next day the kidney was washed with 5%, 15% and 30% sucrose solution (in water) for 30 minutes each on the rocker. The kidney was finally suspended in a 30% solution of sucrose which was left at 4°C.

2.9. Optimal cutting temperature (OCT) compound embedding

In order to carry out embedding of the kidney, it was first removed from the 30% sucrose solution and was moved into OCT compound for 30 minutes. After 30 minutes the kidney was removed from the OCT and placed in a mould using a fish pipette, however making sure not to introduce air bubbles. The kidney was orientated as desired and was covered with fresh OCT. The mould containing the kidney was left on dry ice for 30 minutes for quick freezing of the tissue, and was then moved into -80°C overnight.

2.10. Cryostat sectioning

Before sectioning the kidney the Leica cryostat was switched on and the temperature set to -24°C, it takes a few hours for this temperature to be reached. Once the cryostat has attained the desired temperature, the mould containing the frozen kidney was removed from the -80°C freezer. The OCT block in which the kidney embedded was detached from the mould and placed on the chuck in the cryostat machine and sectioned at 10 micron (μ) sections. Once cut the sections were placed on superfrost (++) slides. The slides were left on a heat block at 37°C for 30 minutes, in order to dry them.

After 30 minutes the slides were placed in a slide box and could be stored at -20°C indefinitely.

2.11. Immunohistochemistry on kidney sections to detect GFP and dsRed

2.11.1. Treating sections with the primary antibody for detection of GFP and dsRed positive cells

The day slides were to be stained they were removed from the -20°C freezer and were allowed to thaw for 5 minutes before they were washed in a Coplin jar with incubation solution (1XPBS; 1%DMSO and 0.5%Tween20) for 30 minutes. This was followed by washing the slides with 3% H_2O_2 (in methanol). This was done in a Coplin jar and left for 30 minutes on the rocker. After 30 minutes the slides were removed and the 3% H_2O_2 was replaced with tap water in which the slides were rinsed, another wash of 5 minutes in incubation solution was followed before the slides were treated with blocking solution. Blocking solution was made using PBS (1X), DMSO (1%), Tween 20 (0.5%), Bovine serum albumin (BSA) (1%) and goat serum (10%), 500µl of blocking solution was added to each slide and the slides were incubated in a humidified chamber for 2 hours.

After 2 hours the slides were drained on a paper towel and the primary antibody was added which was diluted 1:250 in a solution consisting of PBS (1X), DMSO (1%), Tween20 (0.5%), Goat serum (2%) for the GFP the antibody used was an anti-GFP goat polyclonal antibody (biotin) from abcam (ab6658). The primary antibody used for detecting dsRed was a polyclonal antibody rabbit antisera raised against dsRed-express from Clontech, this antibody is not biotinylated. After

the addition of the primary antibody the slides were incubated in a humidified chamber at 4°C overnight.

2.11.2. Washing of slides

The next day the humidified chamber containing the slides was brought out and allowed to attain room temperature, The slides were then drained and washed using a 'high-salt wash solution' which was done in order to reduce non-specific binding of the antibody which could cause background. This solution was prepared using PBS (1X), DMSO (1%), Tween20 (0.5%), Goat serum (2%) and 5M NaCl (0.18%). The slides were washed in this solution for 30 minutes in a Coplin jar kept on the rocker. The 'high-salt wash solution' was followed by 3X30 minute washes with incubation solution.

2.11.3. Detection of GFP^+ cells using vectastainABC kit followed by DAB staining

The VectastainABC kit was used in order to detect GFP^+ cells in the kidney. The Elite PK-6100 standard vectastainABC kit was used. This commercially available kit consists of 2 solutions, solution A is an avidin solution and solution B is a biotinylated enzyme. The two solutions are added in equal amounts in an eppendorf tube containing 1XPBS/Tween. We prepared a total of 1.5ml of this solution in which to 1.5ml of 1XPBS/Tween 15µl of each of the solutions A and B were added. The tube was incubated at room temperature on the rocker for 30minutes, which allows the formation of an avidin/biotinylated enzyme complex (ABC). After 30 minutes this solution was applied to the slides that were incubated in a humidified chamber for 1 hour in the dark. After an hour the slides were drained and rinsed 3X5 minutess with incubation solution. After the last wash DAB solution A and B were mixed

we made 1.5 ml of the solution, hence to 1.5ml of solution A 45µl of solution B was added. The DAB was purchase from Sigma-Aldrich. The slides were left in a humidified chamber for with the DAB for approximately 30 seconds. The avidin/biotinylated enzyme complex (ABC) which attaches to the GFP^+ cells is detected by the DAB stain, hence showing a brown colour in GFP^+ cells. Drain the slides and rinse with incubation solution 3X5 minutes.

2.11.4. Detection of $dsRed^+$ cells in kidney sections using secondary antibody followed by DAB staining

We found that dsRed cells were detected better using a secondary antibody rather than the avidin/biotinylated enzyme complex (ABC). After the washing steps (2.10.2.) a secondary antibody which was an anti-rabbit IgG peroxidase conjugate (Sigma Aldrich) was added which was first diluted 1:300 in a solution containing PBS (1X), DMSO (1%), Tween20 (0.5%), goat serum (2%). After the addition of the secondary antibody, the slides were incubated in a humidified chamber for 2hours in the dark. After 2hours the slides were drained and washed 3X30 minutes in incubation solution. DAB was prepared and added as done above (2.10.3.). However the incubation time required for staining $dsRed^+$ cells with DAB stain was longer around 5 minutes. After 5 minutes the DAB was drained from the slides and the slides were rinsed 3X5 minutes with incubation solution.

2.12. Observation and documentation of sections on slides

Before the slides were observed a few drops of 80% glycerol was added to the slides, a coverslip was put on the slides and the slides were observed under the bright field compound

microscope Nikon Eclipse 80i using the DIC optics. Images were taken on the Nikon camera digital sight DS-SMC.

Chapter 3. Introduction to lineage tracing of haematopoietic stem cells (HSCs)

3.1. Lineage tracing of cells

A single cell can give rise to a number of cells. Lineage tracing would be the identification of the progeny of a single cell (Kretzschmar and Watt, 2012). Lineage tracing or fate mapping has become a major part of stem cell biology studies and helps biologists gain insights into properties of tissues. This process provides a means of studying the cellular origin of cells.

Lineage tracing can be done in a number of ways both in mice as well as in fish. Strategies of lineage tracing could involve both short term as well as long-term tracing. For a more detailed review of different lineage tracing methods refer to (Kretzschmar and Watt, 2012).

3.1.1. Short-term lineage tracing of cells

Short-term lineage tracing includes the use of laser-activated dyes to label cells. Short-term experiments using such dyes were used in zebrafish to show that cells leaving the vDA so indeed migrate to the CHT and eventually seed the thymus as we as the kidney (Jin et al., 2007; Murayama et al., 2006).

3.1.2. Long-term lineage tracing of cells

Methods used for long term lineage tracing both in murine and fish systems include tracing by genetic recombination. The Cre loxP recombination system has been used for genetic recombination and has proven extremely efficient in mouse. Hans et al (2009) have shown that the Cre/loxP system is indeed a good tool to carry out recombination even in the fish (Hans et al., 2009).

Cre recombinase catalyses the deletion (usually) of the floxed region to express a protein downstream. A floxed region is the region that is flanked by two loxP sites (Fig. 1). Bertrand et al. (2010) have used a constitutively active Cre protein to trace cells from the early embryo to the adult kidney marrow (Bertrand et al. 2010).

Fig. 1: Illustrates the recombination event: Where Cre recombinase excises the region between two lox P sites (GFP is excised) to express the protein (dsRed). Before the recombination event the EGFP reporter is under the control of the promoter (ef1α promoter). After recombination the EGFP that is floxed gets excised thus expressing the second reporter (dsRed). The expression of dsRed is under the control of the ef1α promoter.

The Cre/loxP recombination system could either be constitutively active or inducible. A constitutively active Cre protein carries out recombination throughout and can be only spatially controlled by using a tissue specific promoter. However, temporal and special control of Cre activity can be controlled with an inducible system. Spatial control can be included by using a tissue specific promoter, for example flk1 promoter to drive expression in endothelial cells. Temporal control can be given by fusing the Cre protein to the human eastrogen receptors (ERT2), which in the absence of a ligand, like the drug tamoxifen or its active metabolite-hydroxytamoxifen (4-OHT) is kept in the cytoplasm by the (heat shock promoter) hsp90. In the presence of the ligand (tamoxifen or 4-OHT) ERT2 binds to the ligand hence causing a conformational change and activation of the ERT2 which now bound to the ligand translocates to the nucleus, leaving the hsp protein in the cytoplasm. In the nucleus Cre thus recombines the loxP sites (Kretzschmar and Watt, 2012) (Fig.

6I) We have used this system in our experiments and will look at the Cre/lox P system with respect to our experiments.

3.2. Migratoy route of HSCs during definitive haematopoiesis

In the developing zebrafish embryo, during the second or definitive wave of haematopoiesis, cells appear from the ventral wall of the dorsal aorta (vDA) that are putative HSCs and are identified by the expression of the transcription factors runx-1 and c-myb. These cells first emerge in the vDA just before the primitive erythrocytes enter circulation around 24 hours post-fertilisation (hpf) (Gering and Patient, 2005). They then migrate to the caudal haematopoietic tissue (CHT) at day 1 post fertilization (1 dpf) to 7 dpf (Ellett and Lieschke, 2010), here they expand and differentiate before migrating to seed the kidney and the thymus (Fig. 2.I) The thymus is colonised by T and B cells (Willett et al, 1999, Lieshke et al 2001, Lin et al 2005) and seems to be the seeding and maturation site for lymphocytes (Menke et al 2011) while the HSCs from the CHT seed the kidney from 4 dpf (Ellett and Lieschke, 2010). The kidney and the thymus are the sites of larval and adult haematopoiesis and lymhpopoiesis (Ellett and Lieschke, 2010 Fig. 2.II).

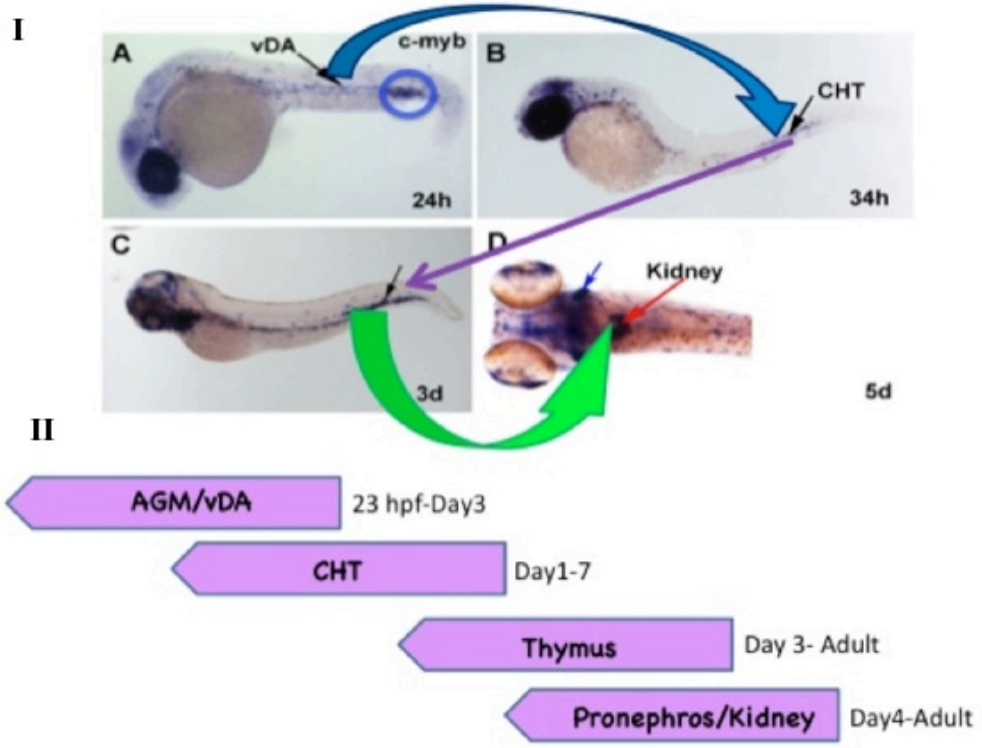


Fig. 2. I: Migration of cells that express the transcription factor c-myb. *In-situ* hybridisation done on the developing zebrafish embryo for c-myb positive cells that are formed in the (A) vDA at 24 hpf, also observed are a population of c-myb positive cells which are most likely to be erythromyloid progenitors and primitive red blood cells in the posterior blood islands (PBI) (circled purple) (B) the c-myb positive cells migrate from the vDA to the caudal haematopoietic tissue (CHT) (green arrow; *in-situ* experiments carried out on 34 hpf embryo) (C) In a 3 dpf embryo, cells in the CHT multiply and migrate to (D) the thymus (small blue arrows) and the kidney (red arrow; *in situ* carried out on a 5 dpf embryo). (Fig.1 I Supplied by Dr. Martin Gering and was modified)

II: Schematic representation of the timeline of migration of putative HSCs from the vDA to the kidney

3.3. Generation of the Tg(flk1CreERT2)qmc104 line.

Previous work done in the lab included the cloning of the Flk1/kdrl:eGFP transgene into a Tol2 vector fragment (Fig. 3 A). This construct carries a 0.8kb flk1/kdrl enhancer and a 1.5kb flk1/kdrl promoter that drives expression of an enhanced green fluorescent (EGFP, more commonly known as GFP) reporter. The expression pattern of flk1:GFP is depicted in (Fig.3 B and C) where flk1:GFP labels the endothelium and the endocardium in the developing embryo.

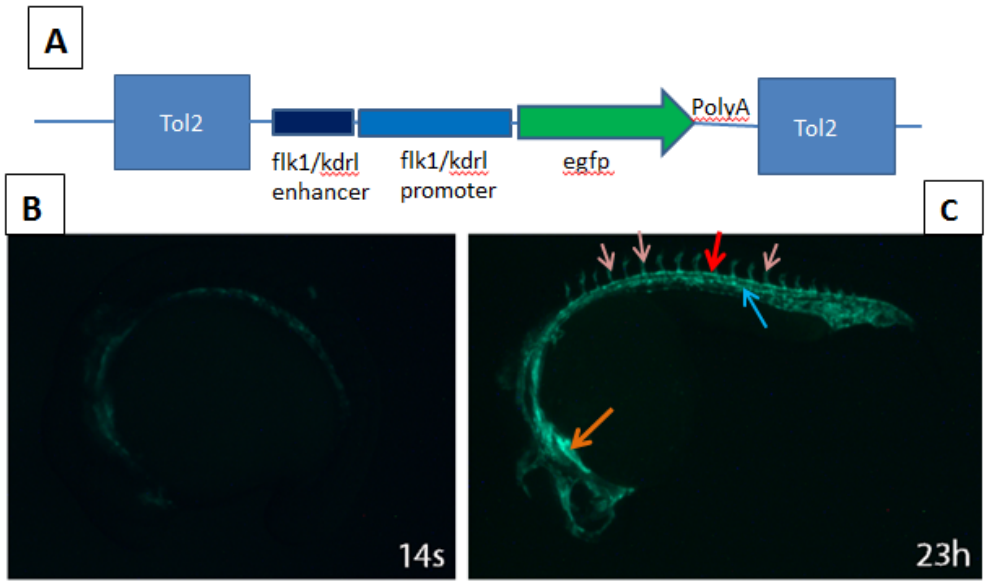


Fig. 3: Expression pattern of flk1/kdrl GFP in qmc 67 which is governed by the flk1 promoter and enhancer.

(A) The flk1:GFP construct that shows the 0.8 kb enhancer and 1.5 kb promoter fragment

(B and C) GFP as seen at 14s (somite stage) (16 hpf) and 23 hpf in the developing zebrafish embryo. (C) flk1:GFP is expressed in the dorsal aorta (red arrow), in the vein (blue arrow), in intersomatic vessels (pink arrows) and in the endocardium (orange arrow).

The Cre repoter line was received from Bally-Cuif's lab in Paris. Fish that carry only the Cre reporter transgene express GFP ubiquitously and not dsRed (Fig. 4). GFP expression is under the control of the ef1α promoter. In the event of recombination of the loxP sites, in this construct there will be expression of dsRed, which again would be under the control of the ef1α promoter.

Two constructs were generated that made use of the same flk1 promoter and enhancer fragment (Fig. 3A) to drive Cre (Fig. 5 A and B). We expect the flk1 promoter and enhancer to drive Cre in the similar manner as it drives GFP in endothelial and endocardial cells (Fig. 3B and C). These two constructs were then injected into wt embryos. The first was a flk1Cre line, also called as the qmc101 that has a frame shift between Cre and ERT2 and hence is constitutively active and not inducible. As we progress through the text this line would be referred to as flk1Cre(qmc101) (Fig. 5A). The second is an inducible flk1CreERT2 line. This line is an inducible line and hence would be dependent on the presence of the ligand 4-OHT to carry out recombination (Fig. 5B).

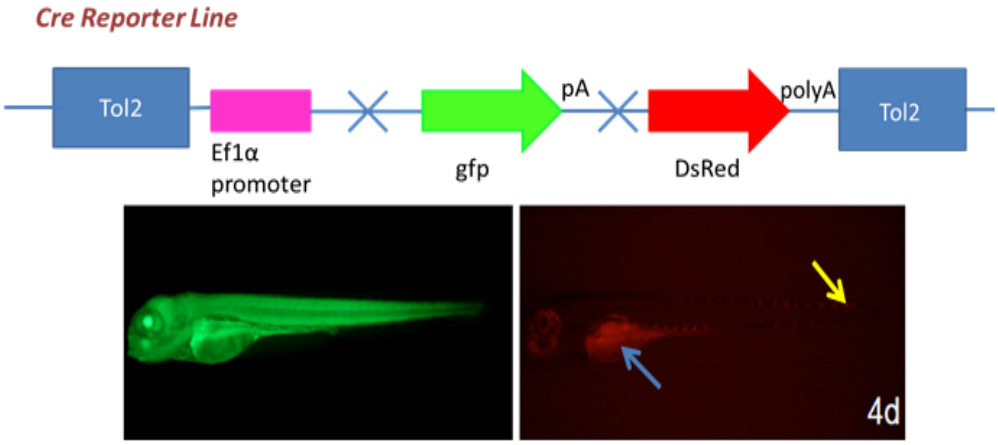
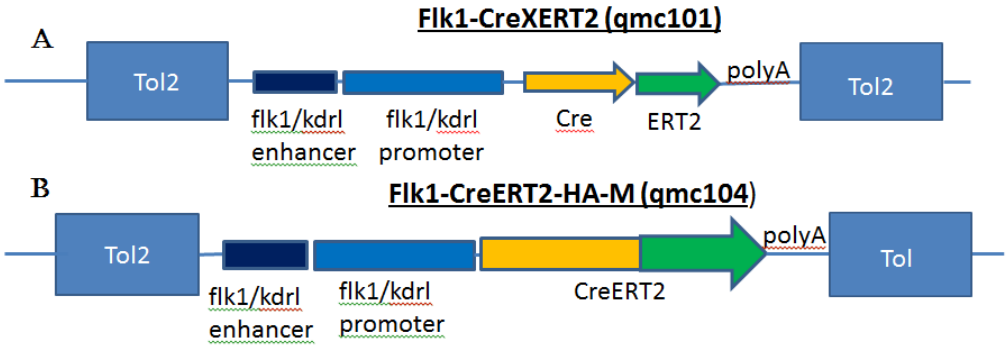


Fig. 4: Cre reporter embryos express GFP ubiquitously and do not express dsRed.

Embryos express only GFP and no dsRed expression is seen in these embryos. Auto-fluorescence seen in the yolk sac (blue arrow) and in some pigment cells (yellow arrow). (Picture of the Cre reporter embryos was supplied by Dr. Martin Gering)


A
Flk1-CreXERT2 (qmc101)
Tol2
flk1/kdrl enhancer
flk1/kdrl promoter
Cre
ERT2
polyA
Tol2
B
Flk1-CreERT2-HA-M (qmc104)
Tol2
flk1/kdrl enhancer
flk1/kdrl promoter
CreERT2
polyA
Tol

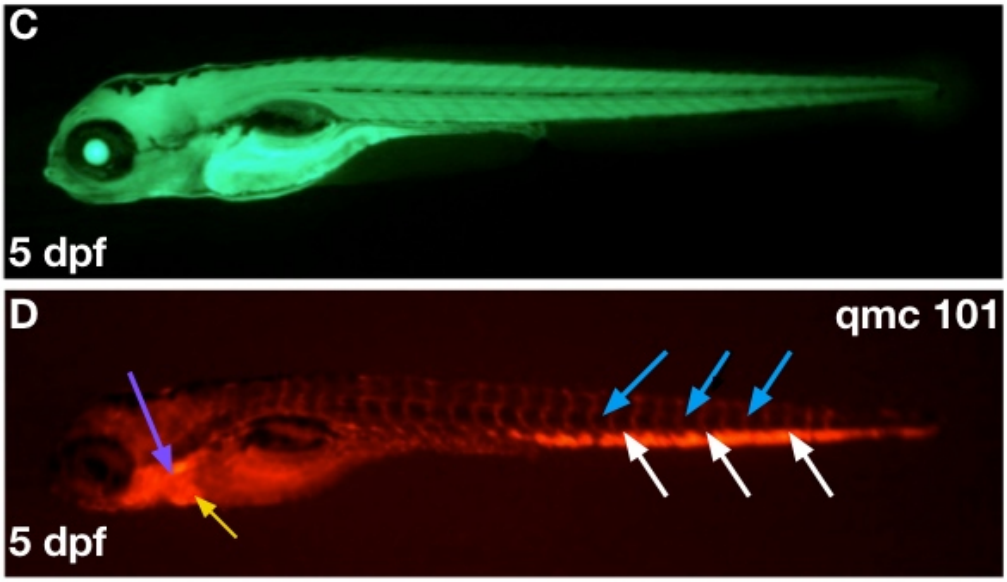

C
5 dpf
D
qmc 101
5 dpf

Fig. 5: Different lines generated using the flk1 enhancer and promoter.

A) The flk1CreXERT2 qmc101 there is a frame shift between Cre and ERT2 and hence the Cre is constitutively active and not inducible. 'X' indicates the frame shift between Cre and ERT2.

B) The qmc 104 line an inducible Cre fused is to the human oestrogen receptor (ERT2). Hence in order to carry out recombination the Cre is dependent on tamoxifen or its active metabolite 4-hydroxy tamoxifen.

C-D) when the flk1Cre transgenics were crossed with Cre reporters some of the progeny that were GFP positive (C) also expressed dsRed on 5 dpf, in the CHT (white arrows), in intersomitic vessels (blue arrows), in the thymus (purple arrow) and in the endocardium (yellow arrow). This is the qmc 101 line (Fig. 5 C and D was supplied by Dr. Martin Gering)

As explained earlier recombination of the loxP sites takes place under the control of the flk1 promoter which is a tissue specific promoter, specific to endothelial cells, hence provides the spacial control in our lines. In the absence of 4-OHT the CreERT2 is retained in the cytoplasm by the hsp90 protein, which is bound to CreERT2. In the presence of the drug 4-OHT, which is a ligand for the CreERT2 fusion protein ERT2 binds to the ligand and translocates from the cytoplasm to the nucleus where Cre catalyses recombination (Fig. 6 I).

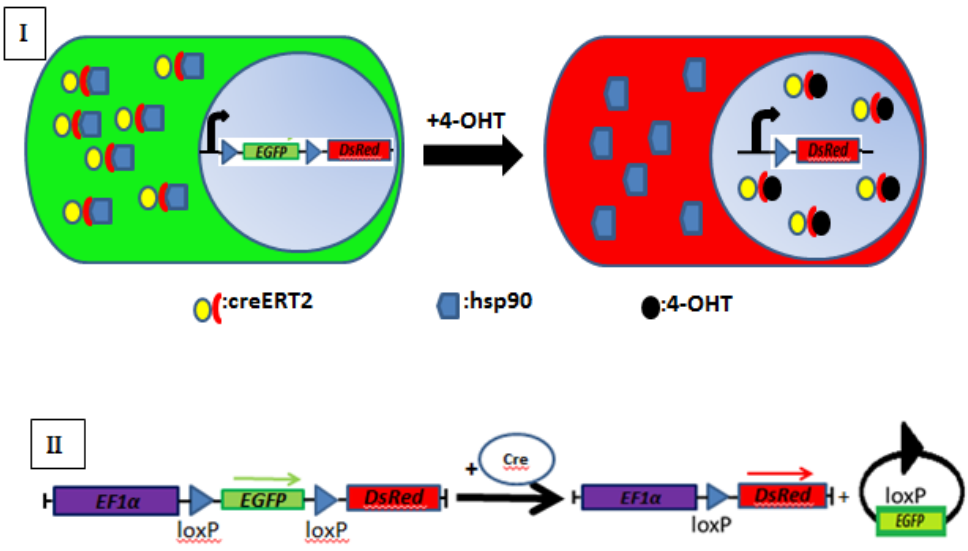
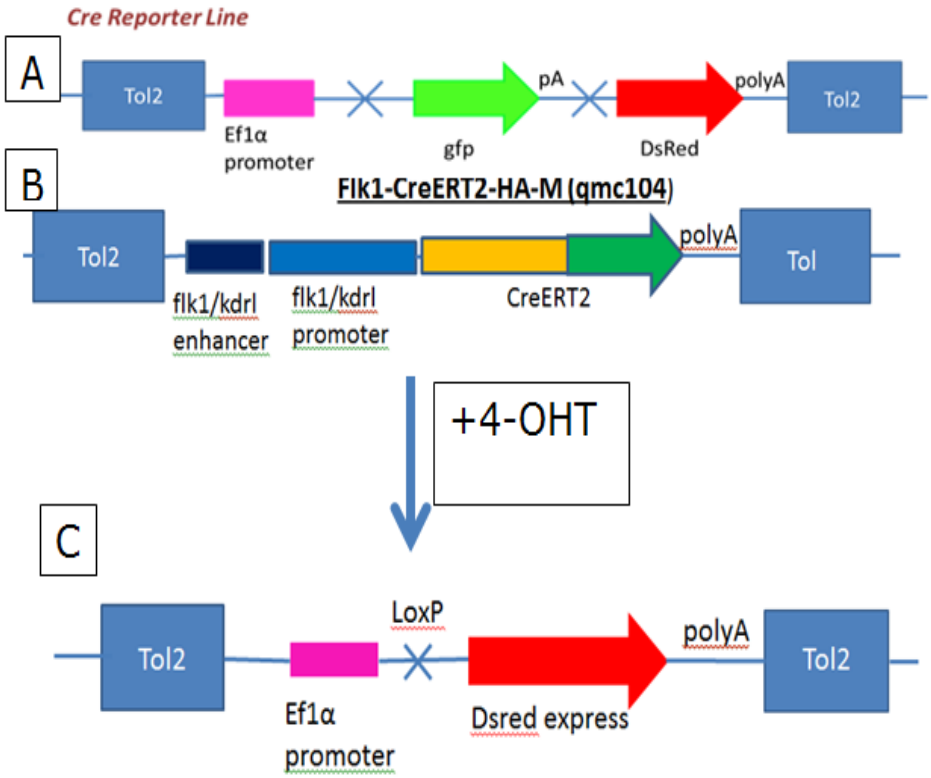


Fig. 6: CreERT2 recombination system: I Schematic representation of the mechanism of temporal control of recombination by the ligand 4-OHT in cells of the green-to-red reporter line. II) Schematic diagram of the recombination event. In the absence of Cre the ef1α promoter drives GFP expression that changes to dsRed after a successful Cre mediated recombination of the loxP sites (figure modified from Hans et al, 2009).

When the flk1CreERT2 fish were crossed to the Cre reporter line, after a successful recombination event in the presence of 4-OHT (Fig. 7A, B and C), dsRed is expressed which is under the control of the ef1α promoter.



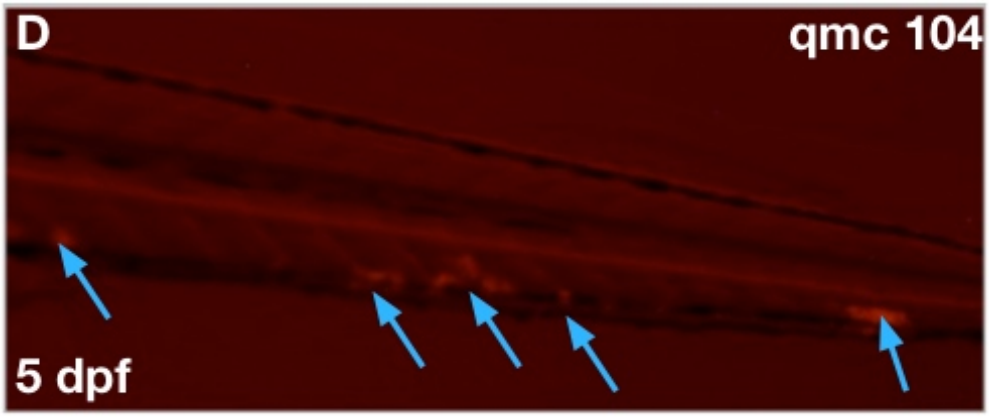


Fig. 7: Schematic representation of recombination of the loxP sites in the presence of 4-OHT in the Flk1CreERT2 line

A) The Cre reporter construct

In order to recombine the flk1CreERT2 the SN9 fish were to be crossed with the Cre reporter (SN41) that have the transgene (ef1α-loxP-GFP-terminator-loxP-dsRed). The embryos from this cross were then treated with 4-hydroxy tamoxifen (4-OHT), an active metabolite of the drug Tamoxifen

D) dsRed expression seen on 5 dpf embryos that were the 4-OHT treated progeny of the flk1CreERT2 transgenics that were crossed to the Cre reporters. This is the qmc 104 line. Expression in the CHT (blue arrows) was weak and only in a few cells, expression in the intersomitic vessels was particularly weak and are hence not annotated.

Dr. Martin Gering had injected wt embryos with the flkiCreERT2-HA.M transgene, coinjected with the transposase mRNA (Fig. 8) in April 2011. These embryos were grown up under the stock number SN9. After 3 months when the embryos were adults and were ready to be bred, they were crossed to the Cre reporters. This led to the identification of an F0 SN9 fish ♀2L that carried the flk1CreERT2 transgene. This SN9 ♀2L was crossed with a Cre reporter fish. The embryos were then treated with 4-OHT at 10 hpf to induce recombination in the endothelium and HSCs. After treatment with 4-OHT, on 5 dpf 10% of the GFP positive embryos showed dsRed expression in the vasculature confirming recombination of the flk1CreERT2 transgene in the endothelium and in HSCs. These embryos that expressed both gfp and dsRed were grown up in the nursery under the stock number SN51 and this new line generated was called qmc104 (Fig. 8).

The SN numbers are the stock numbers that define a batch of embryos that have the same date of birth and same genetic modification (genotype). This number is unique to every batch of embryos. A qmc number is assigned to a batch of embryos that carry the same transgene in an identical genomic location.

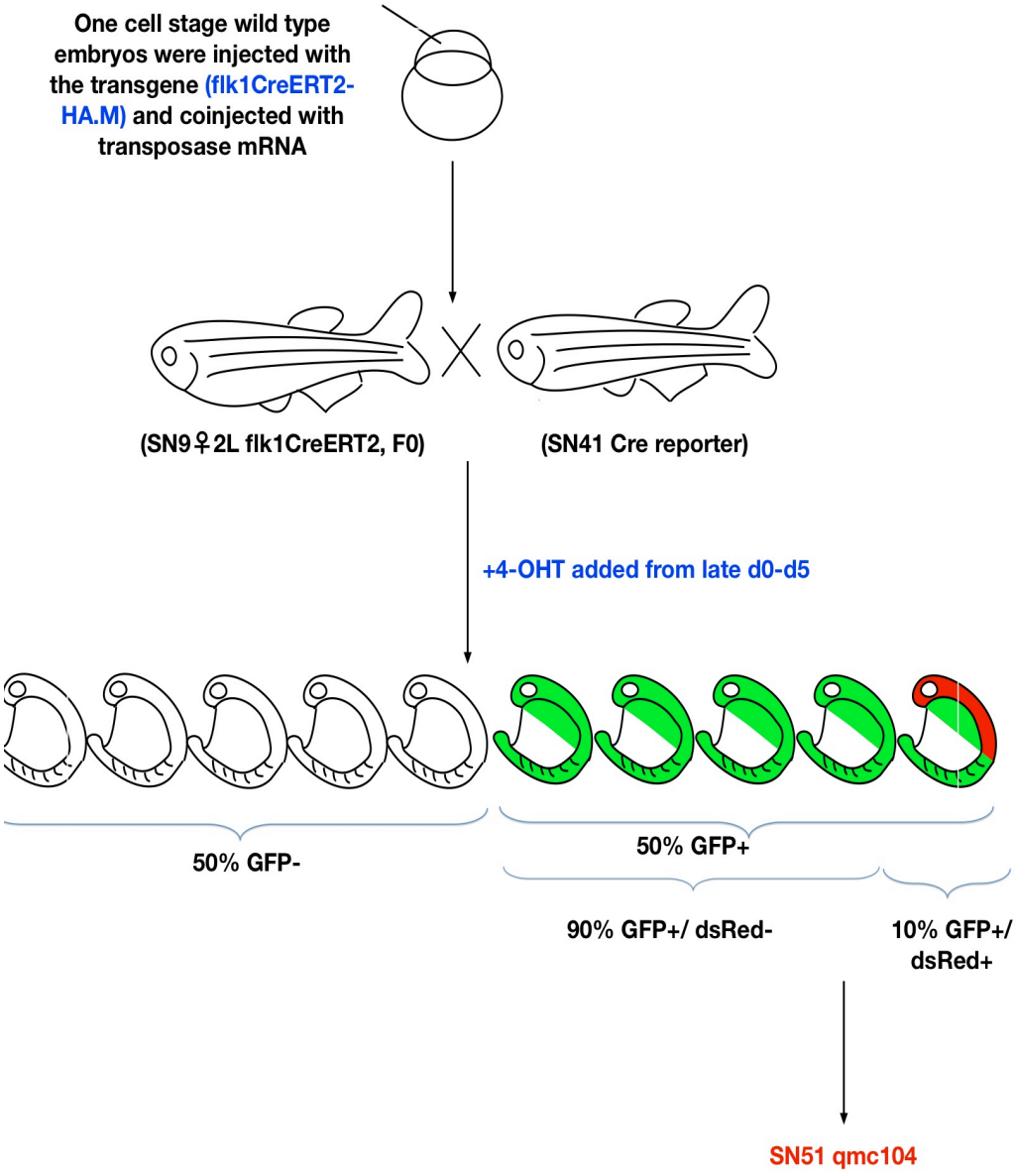


Fig. 8: Schematic illustration of the SN9 F0 fish (♀2L) that was the founder of the qmc104 (flk1CreERT2) line.

Murayama et al in 2006 injected a caged dye, used a laser to uncage the dye, thus tracking the migratory route of the cells that emerged from the vDA to the definitive haematopoietic organs, the thymus and the kidney. However the method used by them, had 2 drawbacks,

a) They were able to track cells only until 5 dpf of the developing embryo and not until adulthood.

b) During uncaging of the tracking dye while trying to target cells only of the endothelium, neighbouring cells from other tissues could also get activated and uncaged, making it difficult to distinguish been different cells.

Bertrand et al (2010), using a constitutively active flk1Cre, lineage traced dsRed positive cells from an endothelial origin, i.e. cells that expressed flk1, in the developing zebrafish embryo and showed that these cells made up all of the kidney marrow cells in the adult fish. However using a constitutively active Cre protein (Fig. 9) Bertrand et al could not claim that all cells that seed the adult kidney came from the early embryo alone. Their experiments raised further questions of whether or not there were HSCs that formed at a later time point other than the expected EHT time? From their experiments they could not rule out it that if flk1 was expressed at some other time point during development in some mersodermal cell (Fig. 10 circled orange), or if an HSC at any time during development switched 'on' flk1, then in fact, they could not claim that all cells that seed the adult kidney came from the early embryo alone.

3.4. Aims and objectives

For the characterisation of CreERT2 founder lines we have labelled cells by inducing with 4-OHT at different time points

during embryonic development. Since lineage tracing experiments are long term experiments, only a part of this was done. During this project, we aim to establish methods to isolate, section and stain the adult zebrafish kidney.

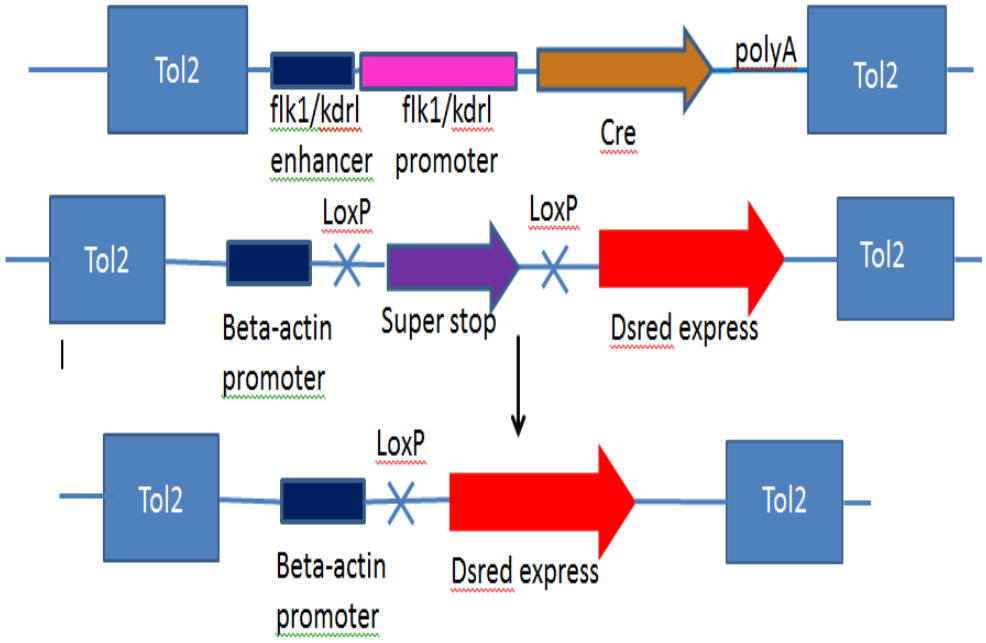


Fig. 9: Recombination system described by Bertrand et al (2010): Where the constitutively active Cre protein is under the control of the flk1 promoter. Their Cre reporter has a β-actin promoter up front and a super stop cassette is floxed. In the event of recombination the loxP sites are recombined and the superstop cassette is excised hence giving expression of the dsRed which is under the control of the β-actin promoter.

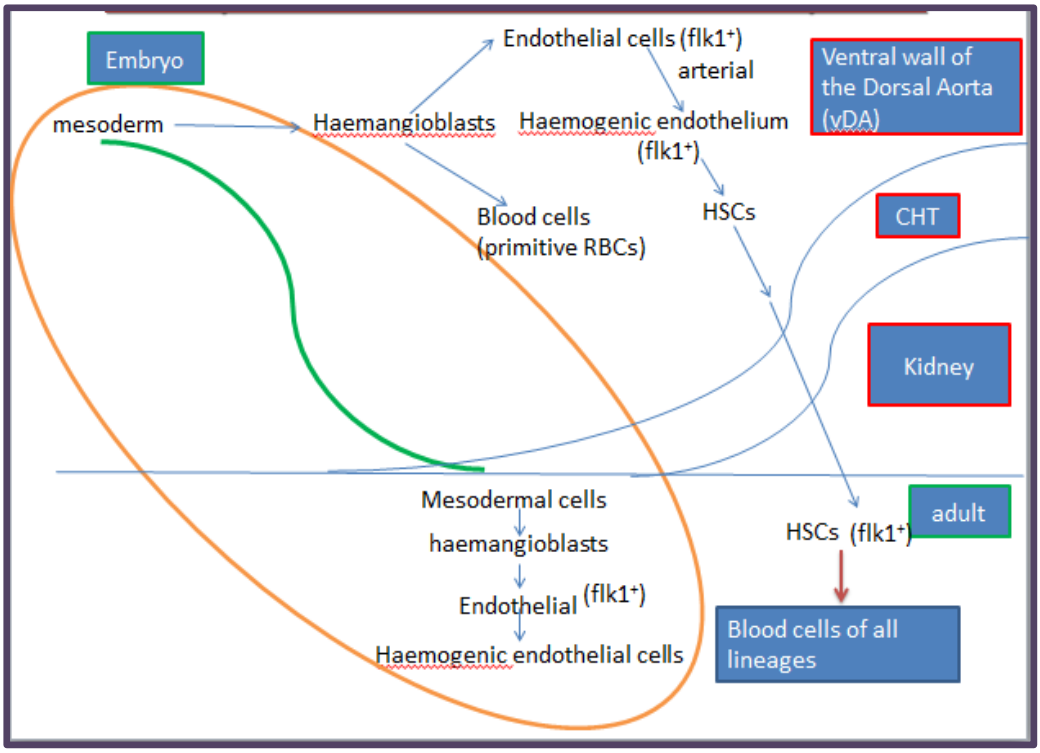


Fig. 10: Illustrates the development of HSCs in Definitive Haematopoiesis

So far it has been shown that the route of HSC development and formation is initiated in the mesoderm in the early embryo which gives rise to hemangioblasts. Hemangioblasts then give rise to primitive RBCs and flk1 positive arterial endothelial cells. HSCs emerge from the flk1 positive hemogenic endothelium of the ventral wall of the dorsal aorta (vDA). These HSCs that emerge from the vDA migrate to the caudal haematopoietic tissue (CHT) and then to seed the kidney marrow which is the site of adult haematopoiesis.

Could there be the possibility of an HSC being formed from a mesodermal cell? (Fig. 10 circled orange).

Chapter 4: Results

4.1. Identification of SN9 F0 parental fish that carry the flk1CreERT2 transgene in their germline and generation of flk1CreERT2 transgenic lines

Previously in the lab an inducible flk1CreERT2 construct (Fig. 5b) was made and injected into wt embryos which were grown up under the stock number SN9. The flk1 promoter and enhancer have been shown to drive expression of a reporter protein; green fluorescent protein (GFP) in the endothelium and the endocardium (Fig. 3). We therefore expected flk1 to drive recombination in a similar manner in our flk1CreERT2 line. Recombination of the loxP sites should occur in endothelial as well as in endocardial cells only in the presence of the drug Tamoxifen or its active metabolite 4-hydroxytamoxifen (4-OHT).

Identification of the SN9 fish was carried out, by checking for the presence of the flk1CreERT2 transgene. It might be worth mentioning here, that the SN9 fish, if at all, would carry only the flk1CreERT2 transgene, and not the Cre reporter. Hence in order to identify carriers of the flk1CreERT2 transgene amongst the SN9 fish, these were crossed to wt fish and genomic DNA was isolated (Table 1) from the embryos obtained. Using primer number 143 (forward primer) and 144 (reverse primer) the 592bp fragment (flk1CreERT2) was identified, which thus confirmed the presence of the transgene in potential carriers among the SN9 fish. Undergraduate students, Helen Swan, Chandni Modha, Margaritta Ruivo and Vicki King who I supervised for their 3rd year projects were involved in setting up some crosses. Mohammad Al Khamees a 1st year PhD student was also involved in setting up crosses.

2 positive controls were also set up (Fig. 11 lane 1 and lane 2), along with the test samples. Positive controls included DNA isolated from embryos from known flk1CreERT2 carriers. Both controls showed bands at the 592bp position (Fig. 11). The control in Lane 1 (Fig. 11) contained DNA isolated from embryos obtained from the founder (SN9♀2L) of the qmc104 line, hence we already know that this fish carries the flk1CreERT2 transgene, however only 10% of its germline is transgenic, due to which 10% of embryos from this fish would be transgenic (Fig. 8). This would explain the lower band intensity level in lane 1 as compared to lane 2 (Fig. 11). Lane 2 (Fig. 11) comprised of DNA that was isolated from embryos from the qmc 101 line (Fig. 5A) that carried a constitutively active Cre protein. The qmc101 fish used to obtain embryos for DNA isolation were F1 fish that gave 50% transgenic embryos. Hence the higher percentage of transgenic embryos used could explain why the band in Lane2 was stronger than the band in Lane1 (Table 1 and Fig. 11).

Lanes 12 and 13 were negative controls (Fig. 2 lane12 and lane 13). Lane 12 contained DNA from flk1GFP embryos and Lane13 contained no DNA, it was a water control, which was done, in order to rule out any contamination introduced while pipetting or through other reagents and/or from the water that was used to make dilutions.

Table 1: Depicts the SN9 fish that were identified as potential carriers of the flk1CreERT2 transgene by PCR, the number of embryos used from each fish to isolate genomic DNA and the PCR result.

Lane/Well number	Fish and line numbers	Number of embryos used for genomic DNA isolation	PCR Results
1	qmc104 (10% transgenic) positive control	80	PCR+
2	qmc101 (50% transgenic) positive control	77	PCR+
3	SN9♀1	73	very weak
4	SN9♂1	54	very weak
5	SN9♂2	45	weak
6	SN9♂4	65	weak
8	SN9♂5	61	bright band/ PCR+
9	SN9♂6	78	bright band/ PCR+
10	SN9♂7	60	no band
11	SN9♂8	77	bright band/ PCR+
12	*flk1:GFP* (negative control)	-	no band
13	water control (negative control)	-	no band

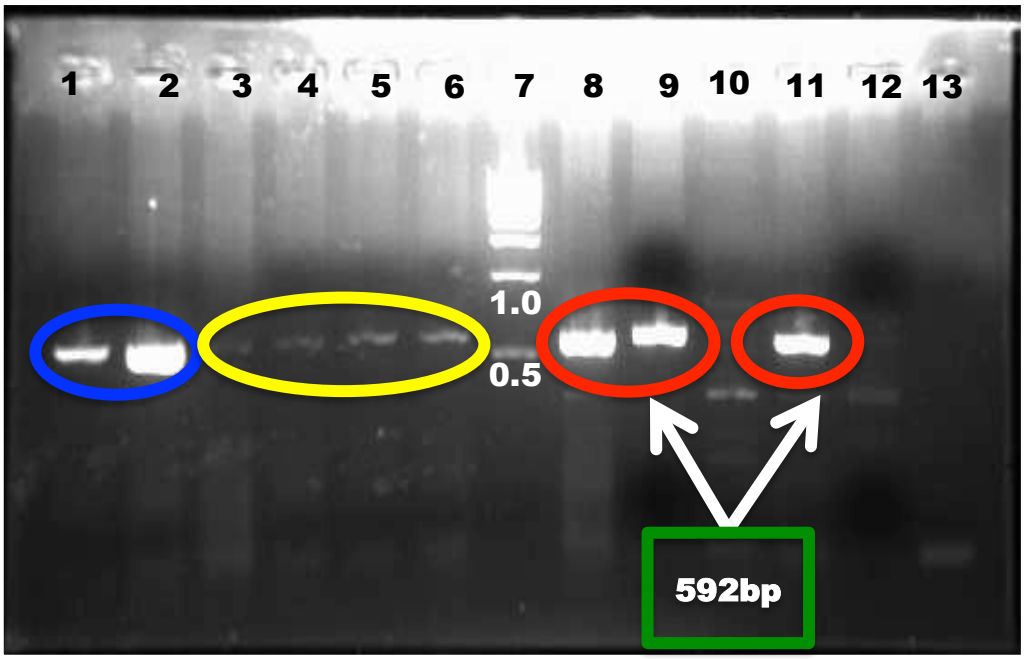


Fig. 11: Primary identification of SN9 fish by PCR. Agarose gel showing lanes 1 to 13. Lanes 1 and 2 had the positive controls (circled blue) and show bright bands on the gel at the 592bp position. Lanes 3, 4, 5 and 6 showed weak bands (circled yellow). Lane7 displays the 1kb ladder, where the 0.5kb and 1kb bands have been labelled. Lanes 8, 9 and 11 contained DNA from SN9 fish ♂5, SN9 ♂6 and SN9 ♂8 respectively, that gave bright bands (circled red) at the 592bp position. Lane 10 showed no band at the 592bp position; hence SN9♂7 did not carry the flk1CreERT2 transgene. Lanes 12 and 13 were negative controls and showed no bands at the 592bp position.

From Fig. 11 we observed that 3 fish are potential carriers of the flk1CreERT2 transgene among the SN9 fish that were tested and they were SN9 ♂5, SN9 ♂6 and SN9 ♂8, this excludes the SN9♀2L that had already been identified as a flk1CreERT2 carrier and was the founder of qmc104. A pedigree of the SN9 fish that have been identified as potential carriers for the flk1CreERT2 transgene has been illustrated in Fig. 12 It was observed that genomic DNA isolated from the embryos of these fish gave the brightest bands at the 592bp position. When these bands are compared to a 1kb DNA ladder, it was observed that they lie between the 0.5kb and 1kb fragments, hence suggesting that these fish carried the flk1CreERT2 transgene. SN9♂2 and ♂4 showed slightly weak bands, however SN9♂7 showed no band (Fig. 11) at the 592bp position, hence indicating ♂7 was not a carrier of the flk1CreERT2 transgene (Table 1).

Once we had identified 3 potential carriers of the flk1CreERT2 transgene among the SN9 fish, namely SN9 ♂5, ♂6 and ♂8, we then crossed them to the Cre reporter line (SN41) in order to be able to detect the activity of the Cre. The SN41 fish were heterozygous carriers for the transgene ef1α-loxP-GFP-terminator-loxP-dsRed therefore we expect 50% of the progeny to be GFP positive. Depending on what percentage of the germline of these SN9 F0 fish was transgenic (had the flk1CreERT2 transgene), we would expect a percentage of the embryos to carry out recombination in the presence of 4-OHT and express dsRed. The embryos were treated with 5µl of 2.5mM 4-OH from tailbud stage (10hpf), in order to induce recombination.

We treated all of the embryos, from each cross with 4-OHT and left no embryos untreated from any batch. We did this

since we were not sure about what percentage of the germline of each of these fish was transgenic. Hence, what percentage of embryos obtained from these fish would be transgenic (carry the flk1CreERT2 transgene) considering the fact that these fish were F0 fish. Thus if a fish had a very small percentage of its germline to be transgenic, treating all the embryos with 4-OHT increased our chances of identifying a transgenic fish. GFP expression was ubiquitously seen in embryos on 1 dpf. Choi et al. (2007) have reported that GFP expression driven by flk1 can be detected by the 8 somite stage of embryonic development (Choi et al., 2007).

Ubiquitous expression of GFP is seen on 1dpf and is driven by the ef1α promoter. Recombination of the loxP sites takes place under the control of the flk1 promoter, which is a tissue specific promoter, specific to endothelial and endocardial cells. Therefore, we expect recombination to occur in endothelial cells and endocardial cells and any cell that would express flk1 at that time point during treatment with 4-OHT (in our case from day0 to day5). Recombination results in dsRed expression from late 4 dpf to 5 dpf in the endothelium and endocardium of the developing embryo. dsRed expression is under the control of the ef1α promoter. The reason for the delay in dsRed expression is because a lot of dsRed mRNA needs to be translated in order to make a sufficient amount of the fluorescent protein that can be visualised (Drew, MSci 2010) under the microscope. Therefore even though recombination takes place much earlier on in the developing embryo the dsRed fluorescence is only seen after 4 dpf (Drew, MSci 2010).

Table 2: Represents the SN9 fish that gave weak or strong band on PCR analysis and were crossed to the Cre reporter line (SN41).

SN9 fish	Band on gel (592bp fragment)	Total number of embryos treated with 4-OHT (n)	GFP-	GFP + only	GFP+ and dsRed+	qmc line generated		Percentage of the germline that is transgenic/ flk1CreERT2
♂2	Weak band	334	163	171	0	None		<1
♂4	Weak band	28	16	12	0	none		<1
♂5	Strong band PCR+	221	83	138	1	qmc 110		0.7%
♂6	Strong band PCR+	303	163	140	Ectopic expression	10	qmc 107	13%
					No ectopic expression	11	qmc 109	
♂8	Strong band PCR+	69	37	30	2	qmc105		6.3%

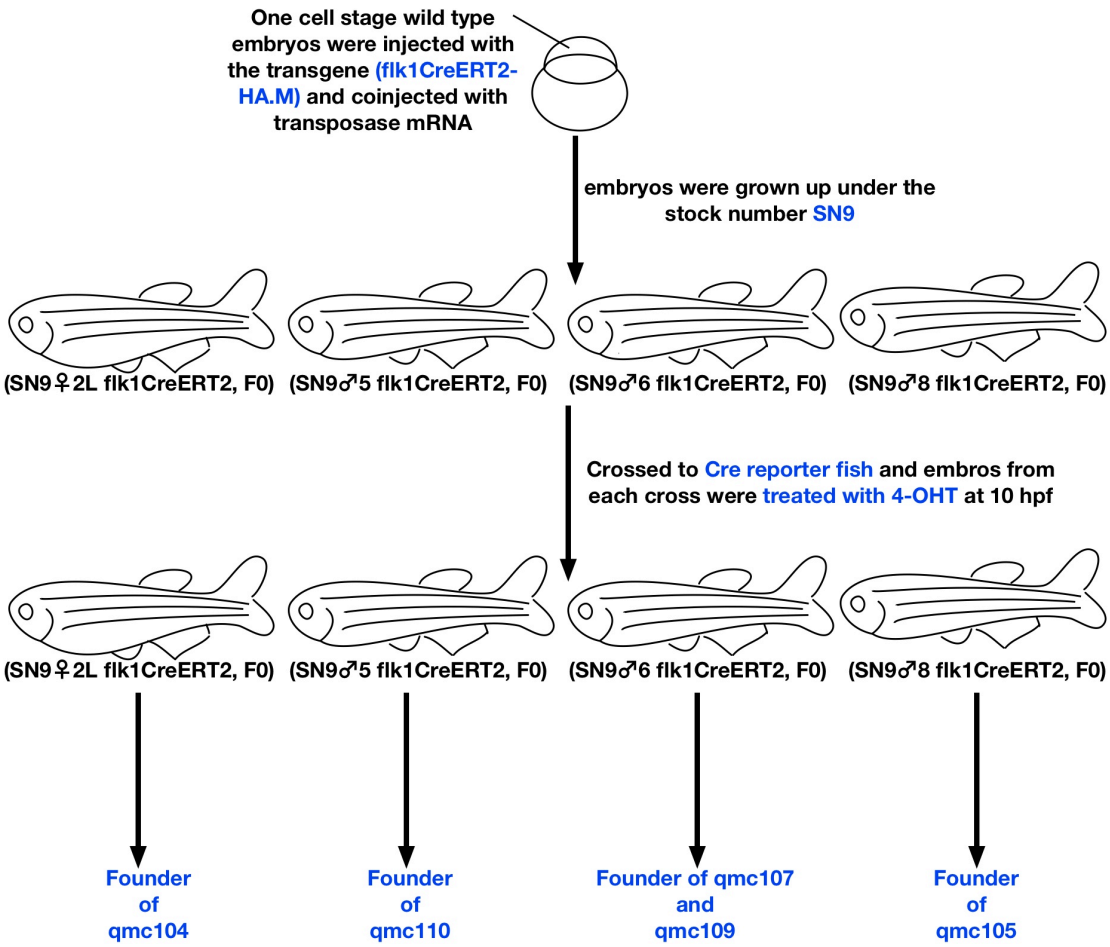


Fig. 12: Illustrates the pedigree of the SN9 F0 fish that were carriers of the flk1CreERT2 transgene and hence were founders of the various qmc lines

SN9♂8 is the founder of qmc105. All 69 embryos were treated with 4-OHT (Table 2). 6.3% of the germline of this fish was transgenic (Table 2). We know this because 2 out of 32 GFP positive embryos (Fig. 13) carried both the transgenes, the flk1CreERT2 and the Cre reporter, and in the presence of 4-OHT carried out site specific recombination in the endothelium and in the endocardium to express the red fluorescent reporter (Fig. 13).

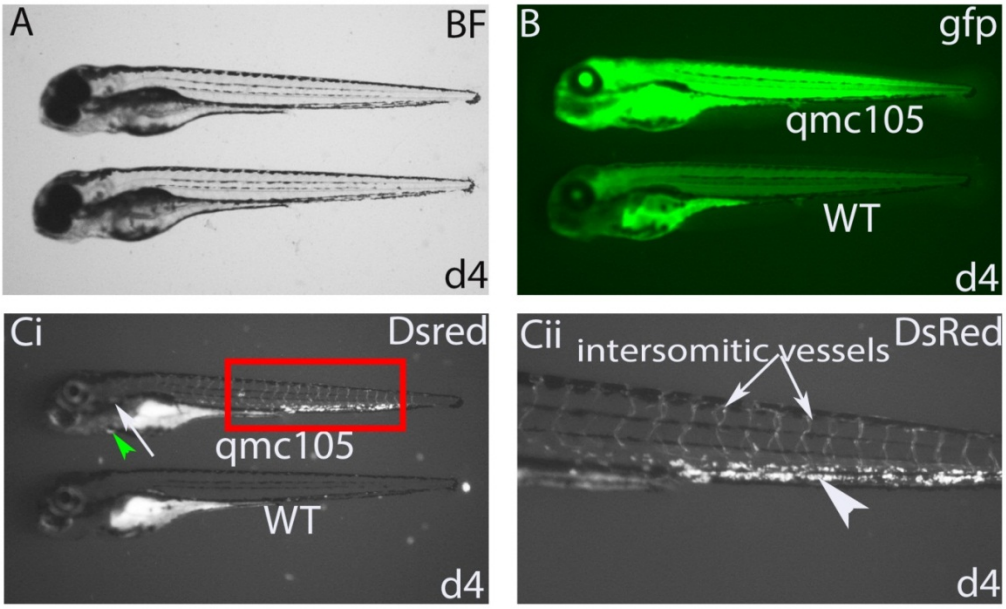


Fig. 13: Progeny of SN9♂8, qmc105. Pictures were taken with a wild type (wt) embryo from the same batch that were treated with 4-OHT.All pictures were taken at 4 dpf

A) Bright field (BF) image of a qmc105 embryo with a wt embryo,

B) the image was taken in the green channel with a black and white camera and was later pseudocoloured green. The GFP positive embryo is qmc105.

Ci and Cii) were taken in the red channel and were photographed using a black and white camera. The red pseudocoloured image was not of a very good and convincing quality hence were left in black and white which looks much better. **Ci)** shows a qmc105 embryo and a wt embryo the heart (green arrowhead) showed dsRed positive cells, thymus (white arrow) expressed dsRed. Caudal haematopoietic tissue (CHT) (red boxed area) magnified in Cii. **Cii** shows the CHT containing dsRed positive cells (white arrowhead) and also shows intersomitic vessels that expressed the red fluorescent protein.

From the cross SN9♂6 with the Cre reporter line a total of 303 embryos were treated with 4-OHT. 140 of these were only GFP positive embryos and 21 were double positive embryos. 13% of the germline of SN9♂6 was transgenic (Table 2). This means that these 21 embryos had done the recombination in the endothelium and hence expressed both GFP as well as dsRed. However, 10/140 embryos showed ectopic expression (Table 2) in the brain, somatic muscle cells and neural crest (Fig. 14 aiii, aiv, av and avi) in addition to the normal expression pattern seen in endothelial cells which includes CHT, thymus, intersomitic vessels and the heart (Fig. 14 aiii, aiv, av and avi). Ectopic expression is seen in an abnormal place in the embryo. This could be because, when SN9♂6 was injected as an embryo, the transposon might have jumped into the vicinity of an enhancer that drives expression in the brain and somatic muscle cells. These 10 embryos were given a new line number, qmc107, but this line was not maintained due to the dsRed expression that was seen ectopically.

The same cross, SN9♂6 to the SN41 fish also had 11/140 embryos (Table 2) that did the recombination only in the endothelium, and gave the normal expression pattern of dsRed, which was seen in the CHT, thymus, intersomitic vessels and the heart (Fig. 15 Biii and Biv). These 11 embryos were grown up under a new line number, qmc 109. Therefore SN9♂6 is the founder of 2 lines, qmc 107 and qmc109, and the expression pattern differences can be observed in (Fig. 16 Aiii).

The fact that the progeny of SN9♂6 displayed different expression patterns could suggest that this is due to the presence of multiple copies of the flk1CreERT2 transgene.

Fig. 14: Progeny of SN9♂6, qmc107. Pictures were taken with a wild type (wt) embryo from the same batch that were treated with 4-OHT. All pictures were taken on 4 dpf

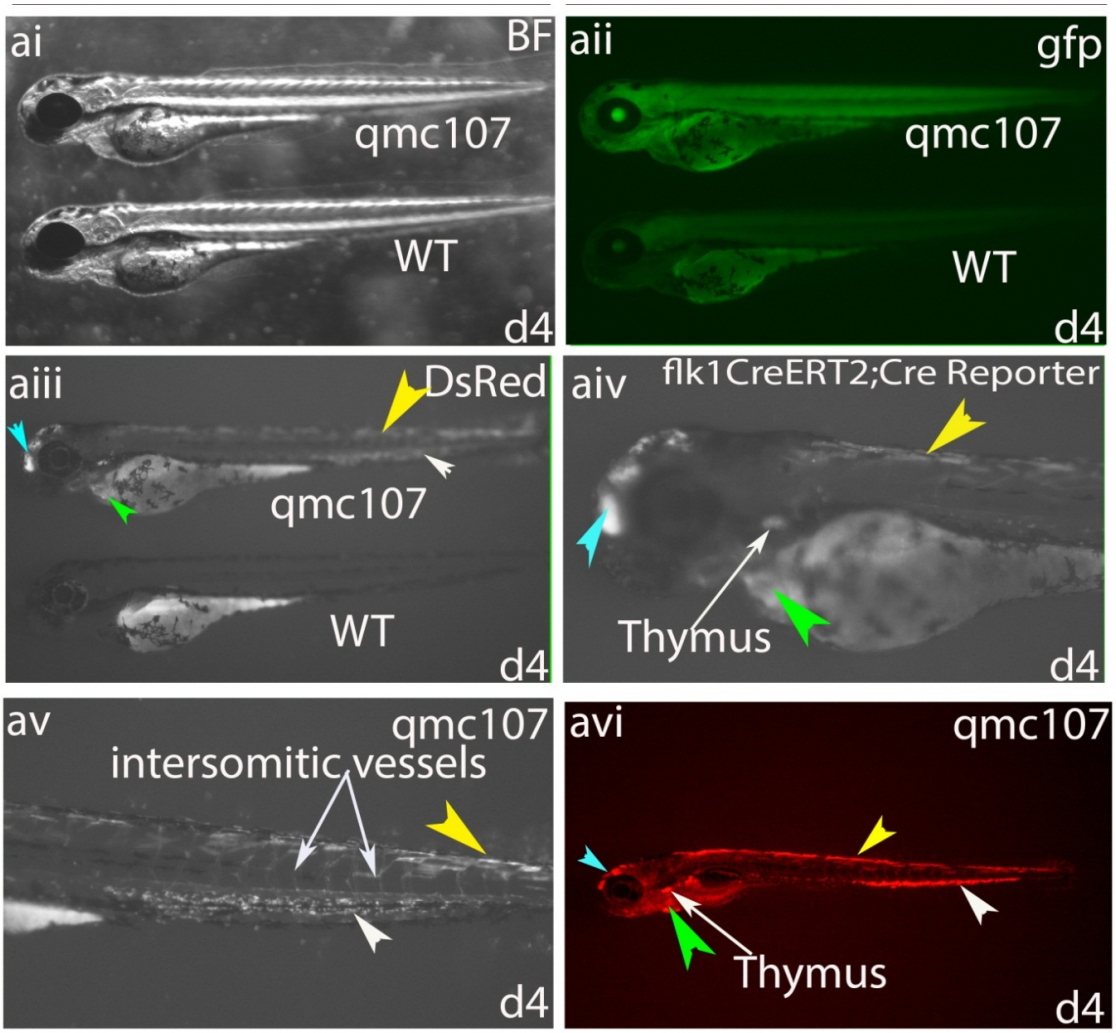
ai) Bright field (BF) image of a qmc107 embryo with a wt embryo

aii) the image was taken in the green channel with a black and white camera and was later pseudocoloured green. The GFP positive embryo is qmc107.

aiii-av) were taken in the red channel and were photographed using a black and white camera. The red pseudocoloured image was not of a very good and convincing quality hence were left in black and white which depicts dsRed positive cells much better. aiii) shows a wt embryo as a negative control and a qmc107 embryo that had ectopic dsRed expression in the neural Crest and in the somatic muscle cells and as part of the normal expression pattern shows dsRed expression in the heart, thymus and Caudal haematopoietic tissue (CHT). aiv) shows a magnified version of the head region of qmc107 expressing dsRed positive cells ectopically in the neural Crest and in the somatic muscle cells and as part of the normal expression pattern shows dsRed expression in the heart and thymus. av) shows a magnified image of the trunk and part of the tail region where dsRed positive cells where expressed ectopically in the somatic muscle cells and normal dsRed expression was observed in cells in the CHT and in intersomitic vessels.

avi) shows a qmc107 embryo taken in the red channel. A different camera was used to take this coloured image. dsRed positive cells were expressed ectopically in the neural Crest

and somatic muscle cells and normal dsRed expression was observed in cells in the CHT, the heart and the thymus. Neural Crest (cyan arrowhead) somatic muscle cells (yellow arrowhead) heart (green arrowhead), and Caudal haematopoietic tissue (CHT) (white arrowhead).



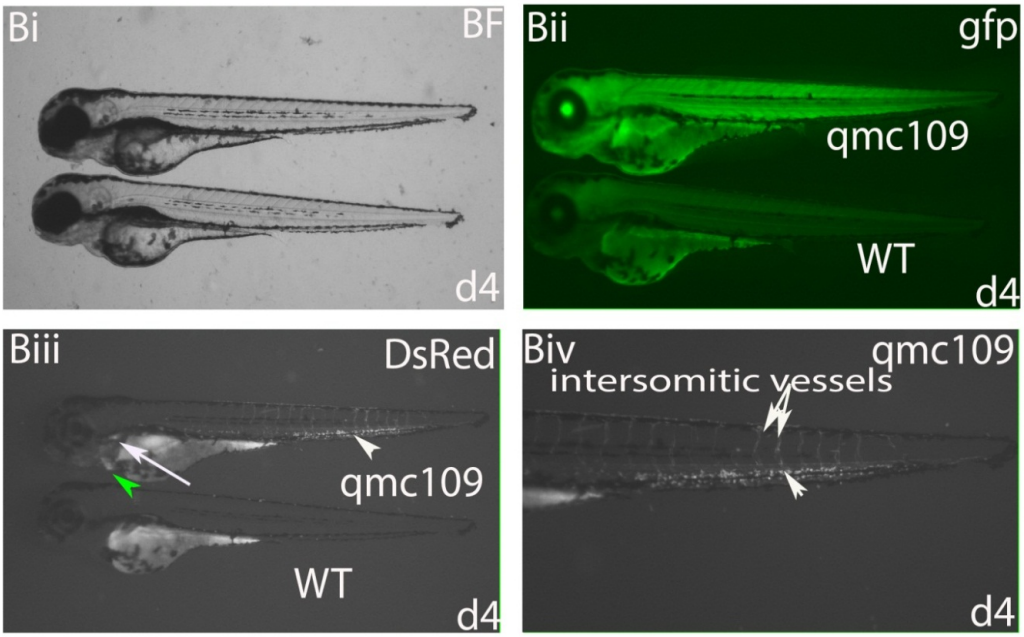


Fig. 15: Progeny of SN9♂6, qmc109. Pictures were taken with a wild type (wt) embryo from the same batch that were treated with 4-OHT.All pictures were taken at 4 dpf

Bi) Bright field image of a qmc109 embryo with a wt embryo,

Bii) the image was taken in the green channel with a black and white camera and was later pseudocoloured green. The GFP positive embryo is qmc109. Images

Biii and Biv) were taken in the red channel and were photographed using a black and white camera. The red pseudocoloured image was not of a very good and convincing quality hence were left in black and white which depicts the dsRed positive cells more convincingly. Biii) shows a qmc109 embryo along with a wt embryo as a negative control. In the qmc109 embryo the heart (green arrowhead) expressed dsRed, the thymus (white arrow) showed dsRed expression and the CHT expressed dsRed positive cells (white arrowhead), the CHT is magnified in Biv. Biv) shows the CHT containing dsRed positive cells (white arrowhead) and also

shows intersomitic vessels that expressed the red fluorescent protein.

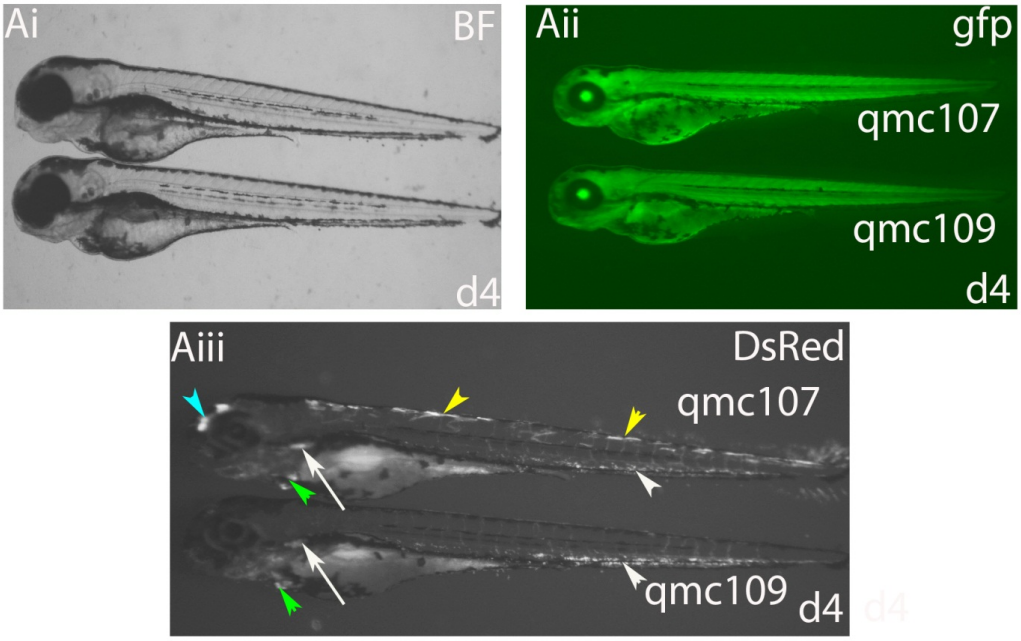


Fig. 16: Depicts the comparison between the 2 lines established by SN9♂6, namely qmc107 and qmc109

Ai) A Bright field (BF) image of qmc107 and qmc109 embryos,

Aii) the image was taken in the green channel with a black and white camera and was later pseudocoloured green. Both lines, qmc107 and qmc109 expressed GFP ubiquitously.

Aiii) was taken in the red channel and was photographed using a black and white camera. The red pseudocoloured image was not of good quality hence was left in black and white which depicts the dsRed positive cells more convincingly. (Aiii) shows the expression pattern differences between qmc107 and qmc 109 embryos. Both embryos expressed dsRed in the heart (green arrowhead), the thymus (white arrow) and showed dsRed positive cells (white arrowhead) in the CHT. Qmc 107 also showed ectopic expression in the somatic muscle cells (yellow arrowheads) and in the neural Crest (cyan arrowhead).

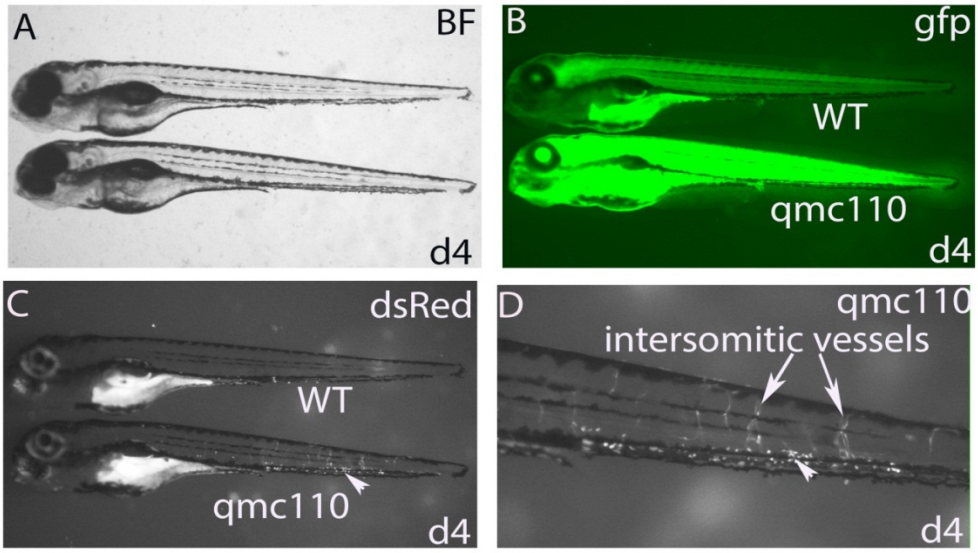
All of the 221 embryos were treated with 4-OHT when they were at around 11-12 hpf (3-6 somite stage). 138 embryos were only GFP positive and 83 embryos were GFP negative (Table 2). Only 1 embryo out of 139 GFP positive embryos showed dsRed expression in the endothelium on 4 dpf (Fig. 17C). Hence 0.7% of the germline of SN9♂5 carried the flk1CreERT2 transgene. The single dsRed positive embryo was given a new line number; qmc110 and SN9♂5 was the founder of qmc110. Unfortunately this embryo did not survive and hence the line qmc110 was discontinued. dsRed expression was not only observed in 1/139 embryos but the dsRed expression observed was relatively weaker (Fig. 17C and D) with only a few cells expressing dsRed in the CHT and a few intersomitic vessels when compared with embryos that expressed dsRed, obtained from SN9♂6 and ♂8, namely qmc105, qmc107 and qmc109. Qmc110 did not express dsRed very convincingly in the heart nor in the thymus (Fig. 17C). This could either be due to the lesser copy number of the flk1CreERT2 transgene present as compared to that present in the other 2 fish SN9♂6 and ♂8. Another reason for the weak expression seen in the qmc110 could be that the flk1CreERT2 transgene could have jumped in a region of a regulatory element where expression of the reporter is supressed.

Fig. 17: Progeny of SN9♂5, qmc110. Pictures were taken with a wild type (wt) embryo from the same batch that were treated with 4-OHT.All pictures were taken at day 4 dpf

A) Bright field (BF) image of a qmc110 embryo with a wt embryo,

B) The image was taken in the green channel with a black and white camera and was later pseudocoloured green. The GFP positive embryo is qmc110. This image was taken at a high exposure time and hence looks over exposed. The qmc110 embryo was photographed along with a wt embryo as a negative control.

C and D) were taken in the red channel and were photographed using a black and white camera. The red pseudocoloured images were not of good quality hence were left in black and white which depicts the dsRed positive cells more convincingly. In the qmc110 embryo the heart did not express dsRed very convincingly and nor did the thymus. The CHT in this line expressed very few dsRed positive cells (white arrowhead) as compared to the other lines, the CHT is magnified in image D. D) shows the CHT containing very few dsRed positive cells (white arrowhead) a few intersomitic vessels also expressed the red fluorescent protein.


A
BF
d4
B
gfp
WT
qmc110
d4
C
dsRed
WT
qmc110
d4
D
qmc110
intersomitic vessels
d4

Together in this experiment we identified 3 SN9 (F0) fish that had the flk1CreERT2 transgene and from these we established 4 lines qmc105, qmc107, qmc109 and ectopic qmc110. However qmc107 gave ectopic expression in the neural Crest and in somatic muscle cells and hence embryos of this line were not grown up. Qmc110 was a single embryo and unfortunately did not survive. Hence only 2 lines are currently growing up namely qmc105 and qmc109.

The reason for crossing the SN9 fish was to identify F0 founders and use them to generate and maintain flk1CreERT2 lines in the future. We had the SN51 qmc104 line which was the F1 generation of the SN9♀2L to work with for our lineage tracing experiments (our next step). However just in case the SN51 fish did not give us a single non-leaky line we would still have the SN9 fish, which were now identified, to go back to.

4.2. Identification of SN51 F1 filial fish of the flk1CreERT2 qmc104 that give rise to progeny in which Cre activity is strictly controlled by the presence of 4-OHT- the derivation of line qmc106

In order to carry out lineage tracing experiments it was important to first essential characterise and identify at least a single fish that carried out recombination only in the presence of the ligand 4-OHT and not otherwise.

As discussed in Chapter 3, the SN51 or qmc104 fish was a line established by crossing the SN9 fish to the Cre reporter line (SN41). The founder of this line was the SN9♀2L. The CreERT2/loxP system is a valuable and efficient tool used to carry out site specific recombination. It has been widely used in the mouse but Hans et al (2010) have shown that this system is in fact extremely efficient even in the zebrafish. We decided to confirm this using our SN51 fish.

We know from previous work done in the lab that 10% of the SN51 GFP positive embryos were also dsRed positive and had carried out recombination in the endothelium. Hence further characterisation of the SN51 fish was carried out. A pedigree of the SN51 fish that were characterised is illustrated in Fig. 18 Since the SN51 fish carry the flk1CreERT2 transgene that is inducible, embryos from this line should carry out recombination only in the presence of the drug tamoxifen or its active metabolite 4-OHT. The latter was used to induce recombination in experiments that were carried out.

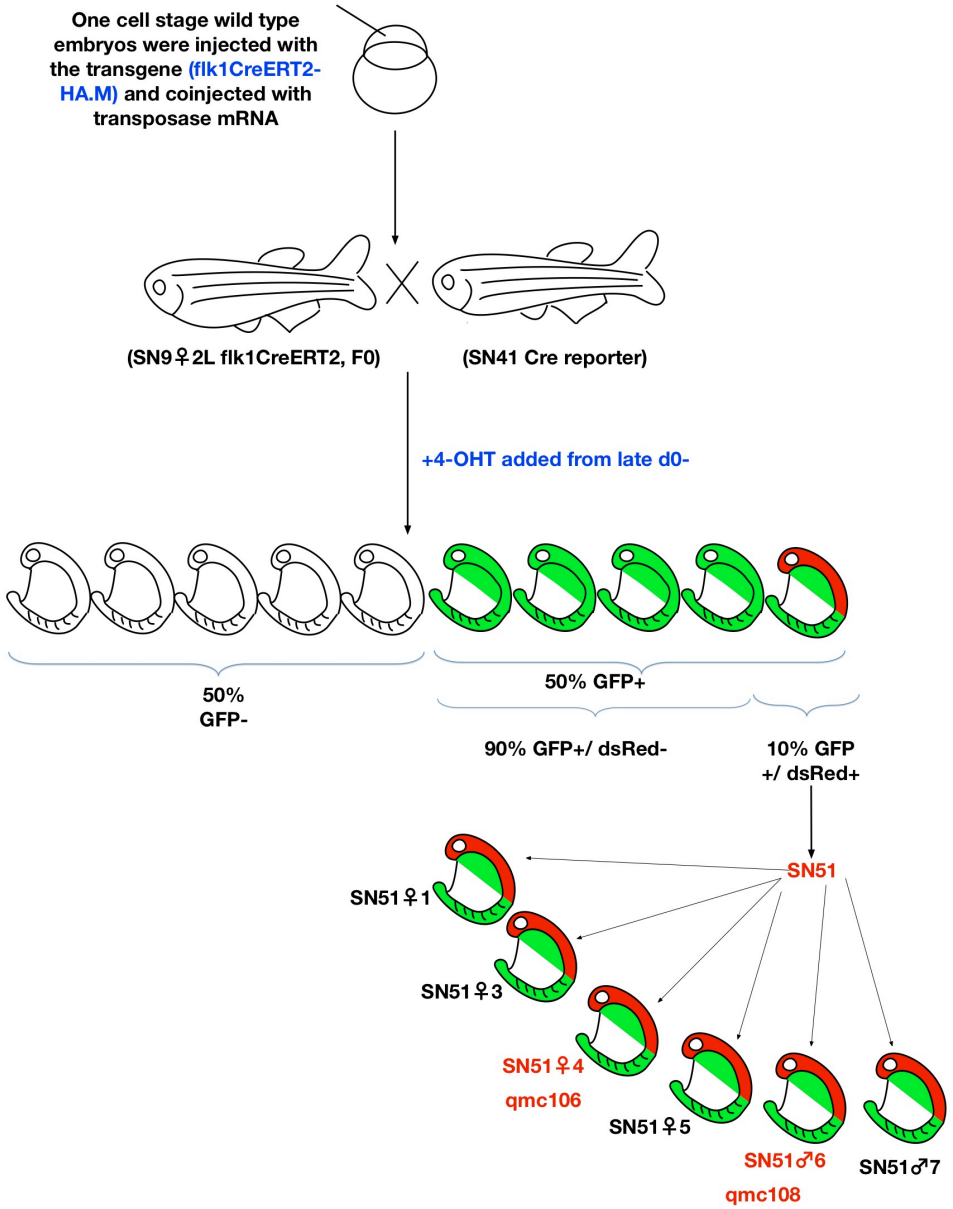


Fig. 18: Pedigree of fish characterised from the SN51 fish

In an inducible CreERT2 system recombination should not occur in the absence of the ligand 4-OHT (Fig. 19). However, if recombination did occur in the absence of the ligand, it could be due to too much of CreERT2 being present in the cytoplasm that the hsp90 protein cannot hold back, which could overwhelm the cellular mechanism thus causing the CreERT2 to escape into the nucleus. In a normal inducible system recombination occurs specifically in the tissue of interest, in our case since the flk1 promoter drives the CreERT2, recombination should take place only in the endothelium and in cells that express flk1. Since the line has an inducible Cre recombination should occur only in the presence of 4-OHT.

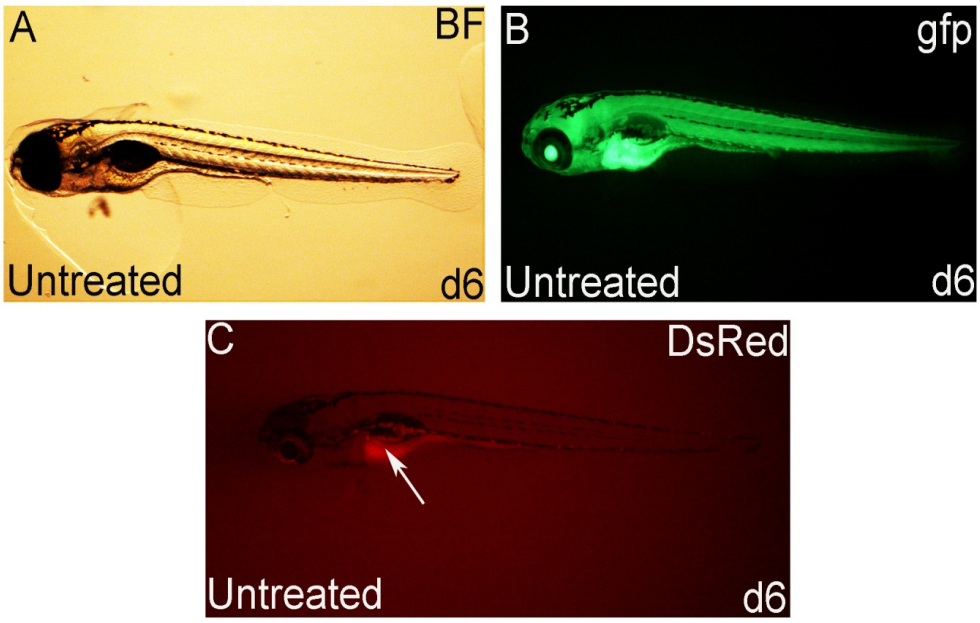


Fig. 19: Absence of dsRed expression in embryos from SN51♀4 that were not treated with 4-OHT

A-C) Images were taken on 6 dpf.

A) Bright field image of an embryo not treated with 4-OHT.

B) Image of the same embryo as seen in the green channel, shows ubiquitous GFP expression.

C) No dsRed expression observed in this embryo. Auto-fluorescence (white arrow) seen in the yolk sac.

Our aim was to identify a fish among the SN51 qmc104 fish that carried out recombination in endothelial and endocardial cells, only in the presence of the ligand 4-OHT.

Since the SN51 fish already carried both the flk1CreERT2 as well as the Cre reporter transgene, we crossed these to wild type (wt) fish, SN26. We set up 6 crosses. In order to characterise and identify a line that carried out recombination only in the presence of 4-OHT and not in its absence, it was important to have a control batch of embryos that were not treated with 4-OHT (Table 3), along with 4-OHT treated embryos (Table 3). This was done for each cross. The embryos that were treated with 4-OHT were treated when they were between the 10-16 hpf stage until 5 dpf.

On 5 dpf we analysed the number of embryos from each cross to check for GFP and dsRed expression among embryos that were treated with 4-OHT and among those that were untreated (negative controls) (Table 3).

Table 3: Shows the number of embryos analysed per cross and indicates the number of GFP^-, GFP^+ only and GFP^++ $dsRed^+$ embryos after treatment with 4-OHT. It also depicts the number of GFP^- and GFP^+ embryos in the control batches that were not treated with 4-OHT and the number of embryos that did the recombination (green to red) in the absence of the ligand 4-OHT.

SN51 fish that were crossed to SN26 wt	Total number of embryos analysed from each cross	+4-OHT (Treated)			-4-OHT (Untreated)		
		GFP^-	GFP^+ only	GFP^+ and $dsRed^+$	GFP^-	GFP^+ only	GFP^+ and $dsRed^+$
♀1	420	55	21	24	151	163	6
♀3	403	62	33	22	143	139	4
♀4	334	64	32	26	100	112	0
♀5	557	97	76	24	178	181	1
♂6	490	135	44	75	115	121	0
♂7	289	70	23	42	78	44	32

Given a scenario where a fish contains one copy of the Cre Reporter transgene and one copy of the flk1CreERT2 transgene we would expect the progeny to express GFP in about 50% of the embryos while 50% should be GFP negative. Half of the GFP positive embryos should express dsRed in the presence of the ligand 4-OHT. Keeping this in mind we analysed our embryos and reasoned out a possible explanation for leaky expression. This helped us make a decision on which fish to carry out future experiments with.

Our numbers (Table 3) show that all crosses gave around a 50% split of GFP positive and GFP negative embryos, which confirms that each fish had a single copy of the Cre reporter transgene. As for the flk1CreERT2 transgene, as discussed above, 50% of the total GFP positive embryos should be dsRed after treatment with 4-OHT. However, the progeny of SN51♀1 expressed dsRed in a slightly higher number of embryos, than the expected 50% (Table 3). This would suggest that SN51♀1 could have more than one copy of the transgene. The same was true for SN51♂6 and ♂7. The progeny of these fish too expressed dsRed in the presence of 4-OHT in more than 50% of the total number of GFP positive embryos (Table 3). This would suggest that SN51♂6 and ♂7 contain a multiple copy number of the flk1CreERT2 transgene in their genome.

It was also observed that in some embryos from the SN51♀1, ♀3, ♀5 and ♂7 fish there was dsRed expression even in the absence of 4-OHT (Table 3). Although embryos from SN51♀1, ♀3, ♀5 that were not treated with 4-OHT gave some leaky dsRed expression, it was only in a few cells that recombination occurred (Fig. 20). SN51♂7 not only expressed dsRed in a high number of embryos in the absence of 4-OHT but it also expressed dsRed in more number of cells. This kind of leaky dsRed expression could suggest that there are more than one copy of the flk1CreERT2 transgene, especially high in SN51♂7 and a positional effect of the transgene in these fish. We therefore decided to exclude these fish for further experiments.

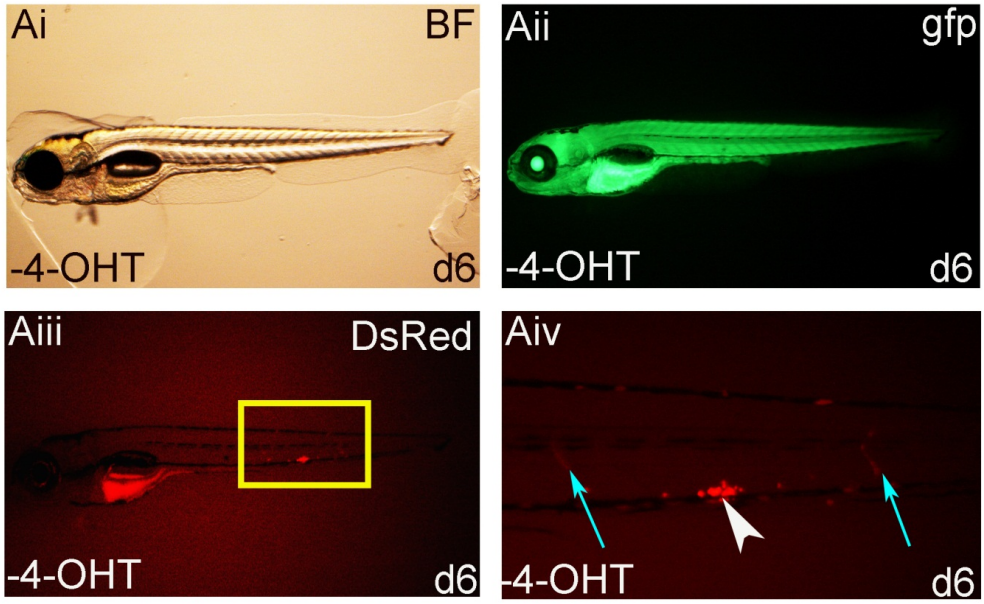


Fig. 20: dsRed expression seen in the progeny of SN51♀5 in the absence of the ligand 4-OHT (-4-OHT)

The images were taken on 6 dpf Ai) bright field (BF) image of the embryo

Aii) This image was taken in the green channel and shows a GFP positive embryo

Aiii and Aiv) were taken in the red channel to view dsRed positive cells.

Aiii) Shows that even in the absence of the ligand 4-OHT a few cells have escaped into the nucleus where they have carried out recombination to express dsRed (yellow boxed area).

Aiv) The yellow boxed area in image Aiii has been magnified. A few dsRed positive cells (white arrowhead) were seen in the CHT and a few intersomitic vessels (cyan arrows) showed dsRed expression even in the absence of 4-OHT.

From the data obtained (Table 3) we decided that SN51♀4 was the best candidate to carry out time point and lineage tracing experiments with. SN51♀4 expressed dsRed in the endothelium, which includes cells in the CHT, intersomitic vessels and the thymus and in the endocardium (Fig. 21) only in the presence of the ligand 4-OHT and never in its absence (Table 3). This indicates that the SN51♀4 is a tight, non-leaky system, suitable to carry out lineage tracing experiments with. The numbers from this cross also suggested that this fish carried one copy of each of the transgenes, the Cre reporter and the flk1CreERT2. SN51♀4 is the founder of a new line qmc106.

SN51♂6 was another prospective candidate that could be used for further lineage tracing experiments and was the founder of qmc108. The progeny of this fish did not express dsRed in the absence of 4-OHT (Table 3). In the presence of the 4-OHT, embryos from this fish expressed dsRed in the endothelium and in endocardial cells (Fig. 22). A few dsRed positive cells are seen in circulation (Fig. 22 d-e yellow arrowheads), these cells seem to be on the migratory route from the CHT to eventually seed the thymus and the kidney. However, as the numbers suggest (Table 3) there could be more than a single copy of the flk1CreERT2 transgene in this fish.

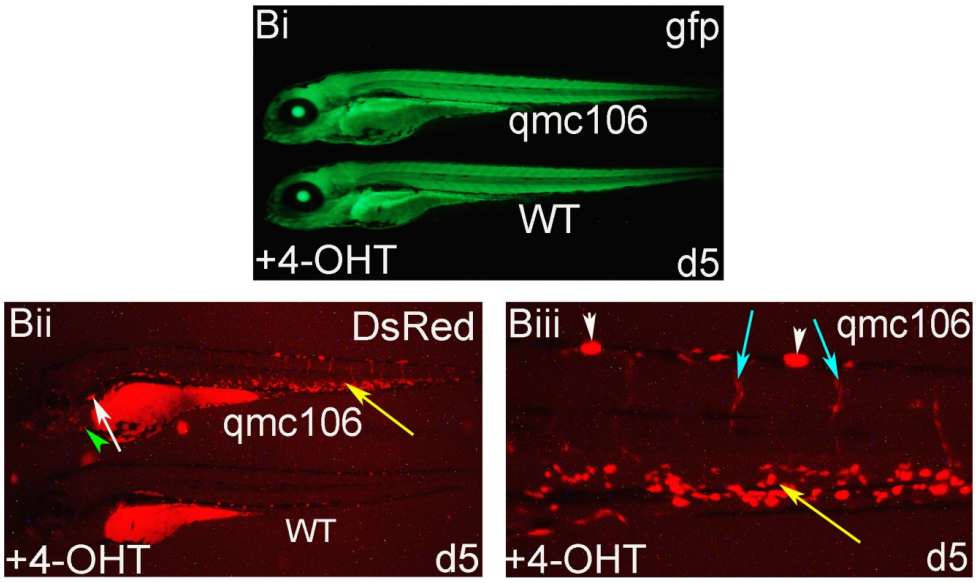


Fig. 21: qmc106 has a tight inducible system when embryos were treated with 4-OHT from late day 0 (10hpf) to late day 5.

Bi) Shows the qmc106 embryo taken along with a control embryo that was GFP positive as well

Bii and Biii) Image was taken in the red channel to view dsRed expression. Bi) shows a qmc106 embryo with a negative control. Qmc106 shows dsRed expression in cells of the CHT (yellow arrows), the thymus (white arrow), while the wt embryo shows no dsRed expression in these areas. Bii) shows a close up image of the trunk region of the qmc106 embryo, cells in the CHT (yellow arrow) have recombined to give dsRed expression; intersomatic vessels (cyan arrows) also express dsRed and some pigment cells (white arrowheads) auto-fluoresce in the red channel.

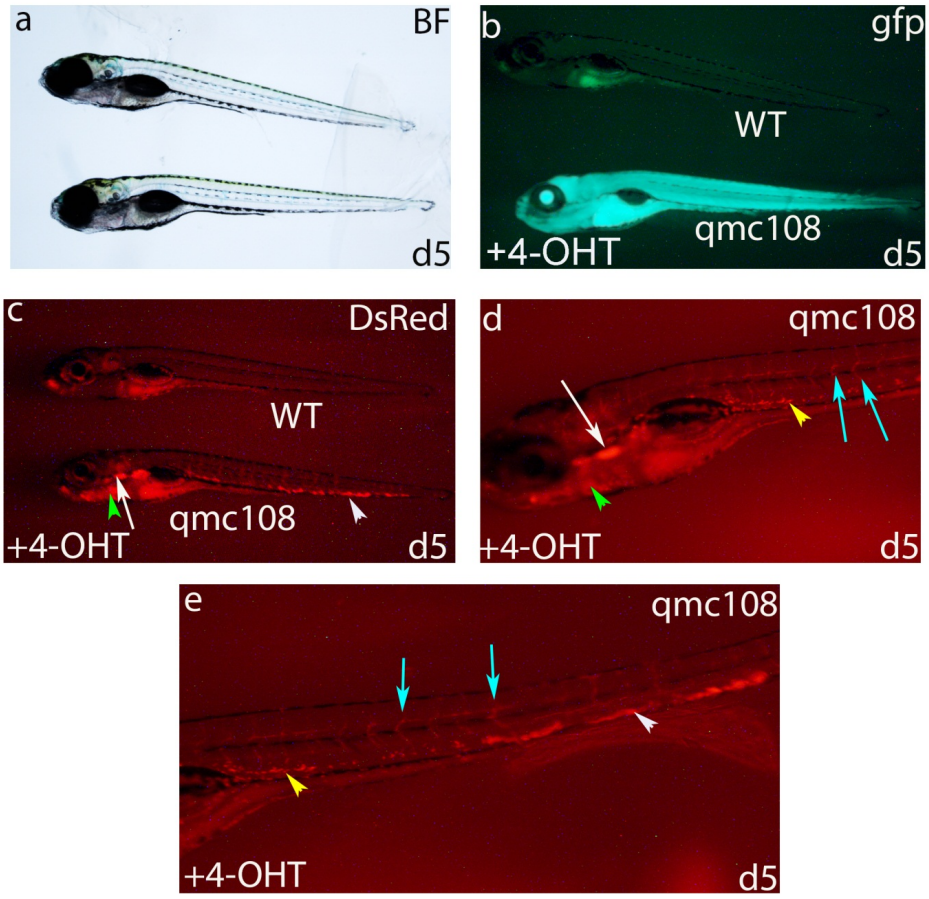
Fig. 22: SN51♂6 founder of qmc108 and a potential candidate for carrying out lineage tracing experiments

Embryos were treated with 4-OHT and were observed and imaged on 5 dpf.

a) Bright field (BF) image of qmc108 taken with a negative control embryo

b) qmc108 with GFP expression as seen on 5 dpf, the wt embryo is GFP negative.

c-e) images were taken in the red channel to observe for dsRed expression. c) shows qmc108 along with the negative control, qmc 108 expresses dsRed in the (c and d) thymus (white arrow), in the endocardium (green arrow head), in (d and e) in intersomitic vessels (cyan arrows) and a few dsRed positive cells are seen in circulation (yellow arrowheads) and dsRed expression seen in cells in the CHT (white arrowheads) in c and e.


a
BF
d5
b
gfp
WT
qmc108
+4-OHT
d5
c
DsRed
WT
qmc108
+4-OHT
d5
d
qmc108
+4-OHT
d5
e
qmc108
+4-OHT
d5

Hence, in summary, this section shows the identification and characterisation of 2 SN51 fish, each on being a founder of a new qmc line. These are SN51 ♀4z founder of qmc106 and SN51♂6 founder of qmc108. The SN51 ♀4z and SN51♂6 both displayed non-leakiness and never carried out recombination in the absence of 4-OHT, hence displaying a tight inducible system.

In order to carry out further experiments on lineage tracing and to check for the efficiency of our inducible CreERT2 system we needed just a single line to work with. We thus chose the qmc106 SN51 ♀4z for future experiments.

4.3. Treating qmc106 embryos with 4-OHT at different time points during development

We had now identified the qmc106 SN51♀4z and decided that it was the most suitable fish with which we could carry out our further experiments.

Now, using the (2010) that carries the flk1CreERT2 transgene, we would like to lineage trace HSCs that arise from the flk1 positive hemogenic endothelium of the ventral wall of the dorsal aorta, to seed the adult kidney. Using our CreERT2 system we could induce recombination in these cells at different time points during embryonic development. Following induction with 4-OHT at particular time points of development would allow us to claim more confidently that the cells later seen in the adult kidney were those that did the recombination at the time recombination was induced.

Hence our next step was to treat embryos obtained from a cross between SN51♀4z and an SN26 wt male fish with 4-OHT at different time points. We treated the embryos with 4-OHT at 2 different time points during embryonic development. One batch of the embryos were treated with 4-OHT from late day 0 (10hpf) to late 1 dpf (d0-d1), at this time point we expect endothelial cells to express flk1 (Liao et al., 1997). Another batch was treated with 4-OHT only from late 4 dpf to late 5 dpf (d4-d5) of embryonic development. 30-60hpf is the expected time of ETH as described by Kissa and Herbomel (2010), hence treating embryos from d4-d5 falls beyond this expected threshold (Kissa and Herbomel, 2010). We also kept a control batch of embryos that were treated with 100% ethanol (Table 4), the diluent used to make a 0.5μM solution of 4-OHT.

Table 4: Depicts the total number of embryos analysed from the cross between SN51♀4 to SN26 wt and treating the embryos at different time-points with 4-OHT, which were then analysed for GFP and dsRed expression on 5 dpf.

Embryos treated with +4-OHT from d0-d1 n=45			Embryos treated with +4-OHT from d4-d5 n=47			Embryos treated with 0.02% ethanol (negative control) from d0-d5 n=46		
GFP-	GFP+ only	GFP+ and dsRed+	GFP-	GFP+ only	GFP+ and dsRed+	GFP-	GFP+ only	GFP+ and dsRed+
24	11	10	21	26	0	20	25	1

On 5 dpf the embryos were analysed for dsRed expression and it was observed that the batch of embryos treated with 4-OHT from day0 to day1 showed dsRed expression in the endothelium and in the endocardium (Fig. 21 Aiii-v). However the number of dsRed positive cells in the CHT, intersomitic vessels and in the heart seems lesser in number when compared to the progeny of the same fish SN51♀4 (Fig. 21 Bii and Biii). This could suggest that recombination does not occur in all cells at the same time.

Referring to Table 4 the 10 GFP^+ and $dsRed^+$ embryos were taken up to the nursery and are being grown under the stock number SN84. The kidneys of these fish will be analysed for dsRed positive cells once these fish are adults.

Embryos that were treated with 4-OHT from late 4 dpf to late 5 dpf could only be analysed for GFP expression and were sorted by dividing GFP positive and GFP negative embryos. There were no dsRed expressing embryos (Table 4) seen as on 5 dpf when the embryos were analysed. 26 embryos only expressing GFP (Table 4) were taken up to the aquarium nursery and are growing up under the stock number SN88. Since we could not distinguish at this stage which of the GFP positive embryos may have done the recombination, we took all 26 embryos to the aquarium nursery. As mentioned earlier dsRed expression is only seen on 4 dpf to in embryos treated with 4-OHT from 10 hpf (tailbud stage). Hence, in embryos treated with 4-OHT from late 4 dpf to late 5 dpf we could expect to see dsRed positive cells on day9 to 10 dpf (if any cells express flk1 at this time point to carry out recombination).

The negative control batch of embryos was treated with 5μl of 100% ethanol. Out of 46 embryos that were treated with 100% ethanol 25 were GFP positive only (Table 4). Unfortunately in this negative control batch of embryos there was a single embryo that expressed dsRed in a one cell in the CHT (Table 4; data not shown). This just goes to show that no system is fool proof and perfect. However, we do not know whether this could have been due to a manual error while transferring embryos from one plate to another. In which case an embryo that might have been exposed to 4-OHT for a little while and might have remained in the pipette while transferring embryos into a fresh dish.

Fig. 23: qmc106 embryos treated with 4-OHT from late day0 (10hpf) to late day1 (d0-d1)

All images were taken on 5 dpf.

Ai-Aiii) shows the qmc106 taken along with a wt embryo from the same batch of treated embryos, as a negative control.

Ai) Bright field image of a qmc106 embryo taken with a wild type (wt) embryo

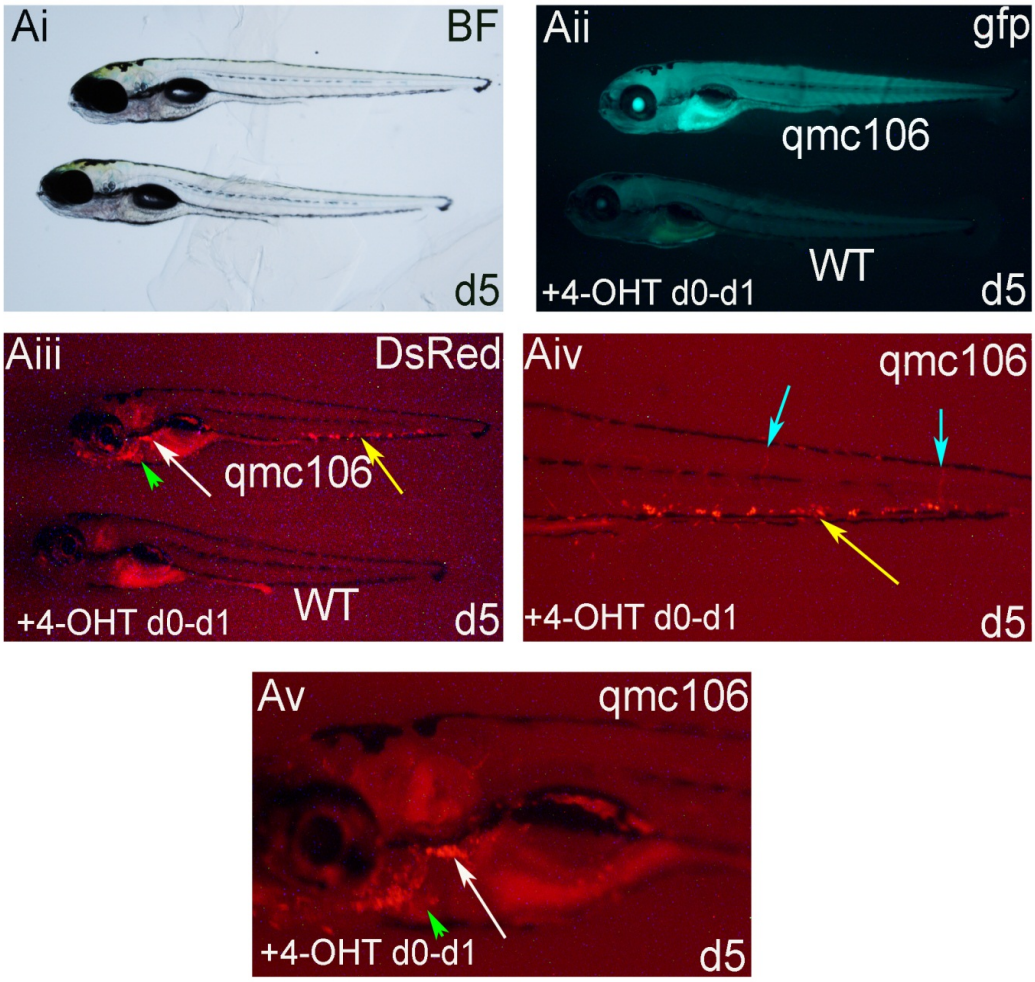
Aii) the same image as seen in the green channel to view GFP expression, as seen qmc106 is the GFP positive while the other embryo is a GFP negative wt embryo.

Aiii-Av) the embryos as viewed in the red channel for dsRed expression.

Aiii) The qmc106 shows dsRed expression in the thymus (white arrow), in the heart (green arrowhead) and dsRed positive cells in the CHT (yellow arrow), whereas the wt embryo displays no dsRed expression.

Aiv) Gives a closer view of the trunk area of the qmc106 embryo, which displays weak dsRed expression in intersomitic vessels (cyan arrows) and in the CHT (yellow arrow)

Av) A magnified view of the head region of the qmc106 embryo that showing dsRed expression in the thymus (white arrow) and weak expression in the endocardium (green arrowhead).


Ai
BF
d5
Aii
gfp
qmc106
WT
+4-OHT d0-d1
d5
Aiii
DsRed
qmc106
+4-OHT d0-d1
WT
d5
Aiv
qmc106
+4-OHT d0-d1
d5
Av
qmc106
+4-OHT d0-d1
d5

Our observations so far are only in the form of number of embryos that expressed dsRed, that too only in the batch that was treated with 4-OHT from d0-d1. Since our fish are still growing up in the aquarium nursery, we have not yet analysed their kidneys to trace our labelled cells. We could however logically analyse what we could expect to see in the kidneys of these fish once they are adults.

Fig. 24: Diagram that shows the expected results of lineage tracing experiments in which a green-to-red Cre reporter line is crossed to two lines that express a constitutively active and a conditional Cre in endothelial cells under the flk1 promoter.

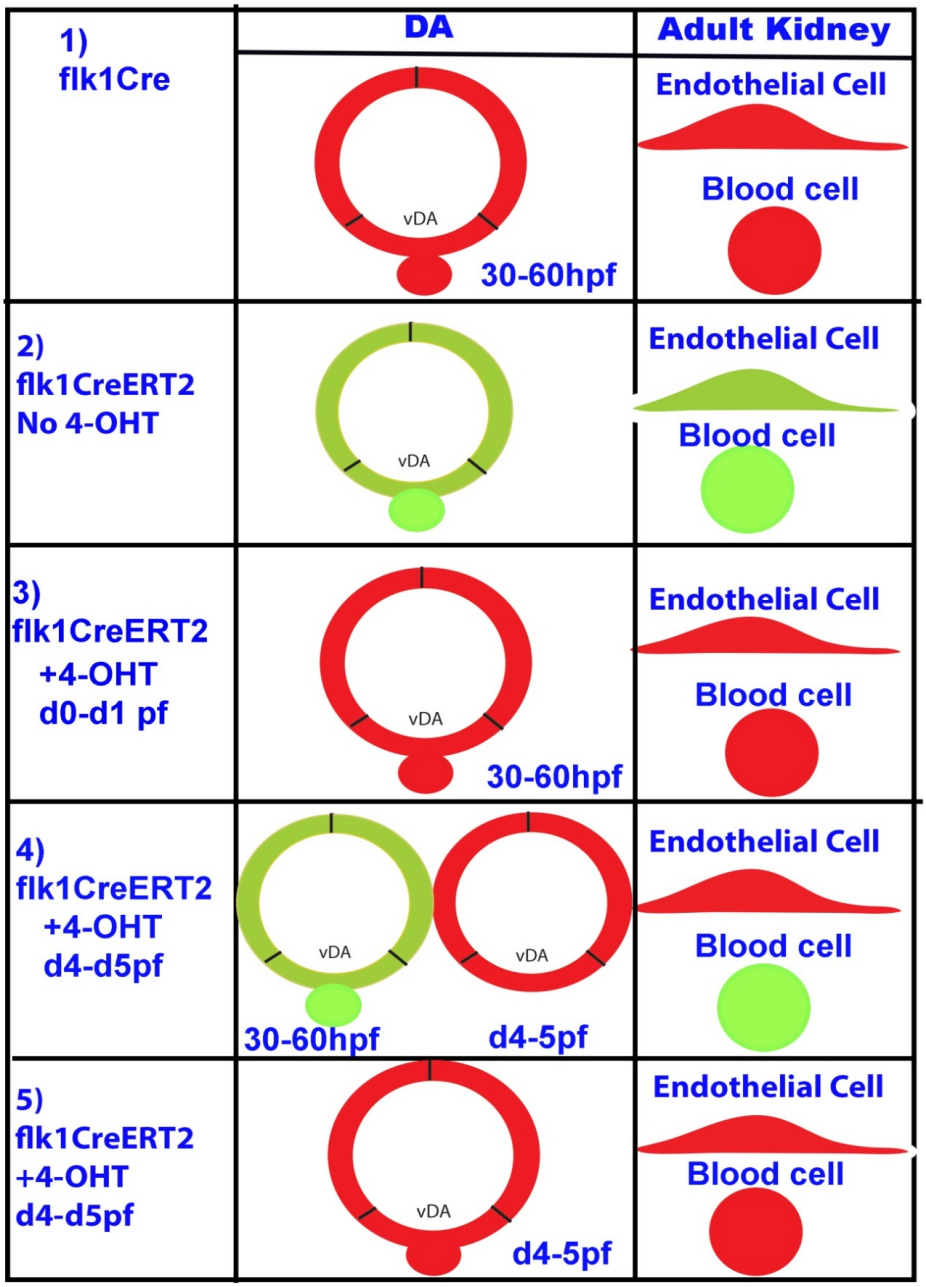
1) Shows recombination when the constitutively active flk1Cre is used. All cells that are Flk1 positive at any time point recombine and hence both endothelial as well as HSCs in the adult kidney would be dsRed positive.

2) Shows no recombination should take place in the absence of 4-OHT and hence both endothelial and HSCs should be only GFP positive

3) When 4-OHT is added from d0-d1 all blood and endothelial cells that express flk1 at this time point would be dsRed positive in the adult kidney, if the vDA gives rise to HSCs at d1-d3.

4) In the case of delayed treatment with 4-OHT from d4-d5 since the suggested time of ETH has already passed the vDA would be only GFP and not dsRed positive and nor would the blood cells that emerged from it, hence the blood cells in the adult kidney would be GFP positive. However the endothelial cells would be dsRed positive because they still express flk1. This would mean most blood cells arise de novo before 5 dpf.

5) If however blood cells are dsRed positive in the adult kidney of embryos treated that were treated with 4-OHT from d4-d5, this would mean that blood cells continue to emerge from the vDA even after the time that has so far been proposed.


1)
flk1Cre
DA
Adult Kidney
vDA
30-60hpf
Endothelial Cell
Blood cell
2)
flk1CreERT2
No 4-OHT
vDA
Endothelial Cell
Blood cell
3)
flk1CreERT2
+4-OHT
d0-d1 pf
vDA
30-60hpf
Endothelial Cell
Blood cell
4)
flk1CreERT2
+4-OHT
d4-d5pf
vDA
vDA
30-60hpf
d4-5pf
Endothelial Cell
Blood cell
5)
flk1CreERT2
+4-OHT
d4-d5pf
vDA
d4-5pf
Endothelial Cell
Blood cell

4.4: Identification of dsRed- and GFP positive cells in kidney sections of transgenic adult zebrafish

One of the seeding sites for haematopoietic stem cells during development of the zebrafish embryo is the kidney. The kidney is also the site for haematopoiesis in the adult fish (Ellett and Lieschke, 2010).

After carrying out time point experiments on embryos from SN51♀4z we now have these fish growing up in the aquarium nursery. Only once these are adults can we analyse their kidneys for the presence of dsRed positive cells that we have labelled at different time-points using the flk1CreERT2 recombination system.

In the meantime, in order to trace the dsRed positive cells from the CHT of the early embryo that seed the kidney in the adult fish there were a number of steps that needed to be established. The following schematic diagram (Fig. 25) gives an over view on the steps that needed to be addressed in order to be able to view dsRed positive cells in the adult zebrafish kidney.

Step 1 involved the dissection and isolation (Fig. 25) of an adult zebrafish kidney that was done according to a paper by Gerlach et al. (2011). The paper describes a step wise method of how a zebrafish can be dissected in order to isolate the kidney (Gerlach et al., 2011). Once dissected and all other internal organs are removed the kidney can be visualised under the dissecting microscope (Fig. 26A). The adult zebrafish kidney is a pinkish, translucent organ with a lot of blackish pigment cells and the dorsal aorta is associated to it (Gupta and Mullins, 2010). The kidney is made up of three

distinct anatomical regions, head, trunk/saddle and tail (Gerlach et al., 2011) (Fig. 26B).

Step 2: The trunk of the fish containing only the kidney after dissection is placed in a container containing cold 4% PFA (fixation solution), and is left overnight (Gerlach et al., 2011). Fixing a tissue is done in order to preserve it and is hence a crucial part of tissue preparation. The next day the kidney is detached from connective tissue in the body cavity in order to isolate it. Technically, kidney isolation is followed by fixation.

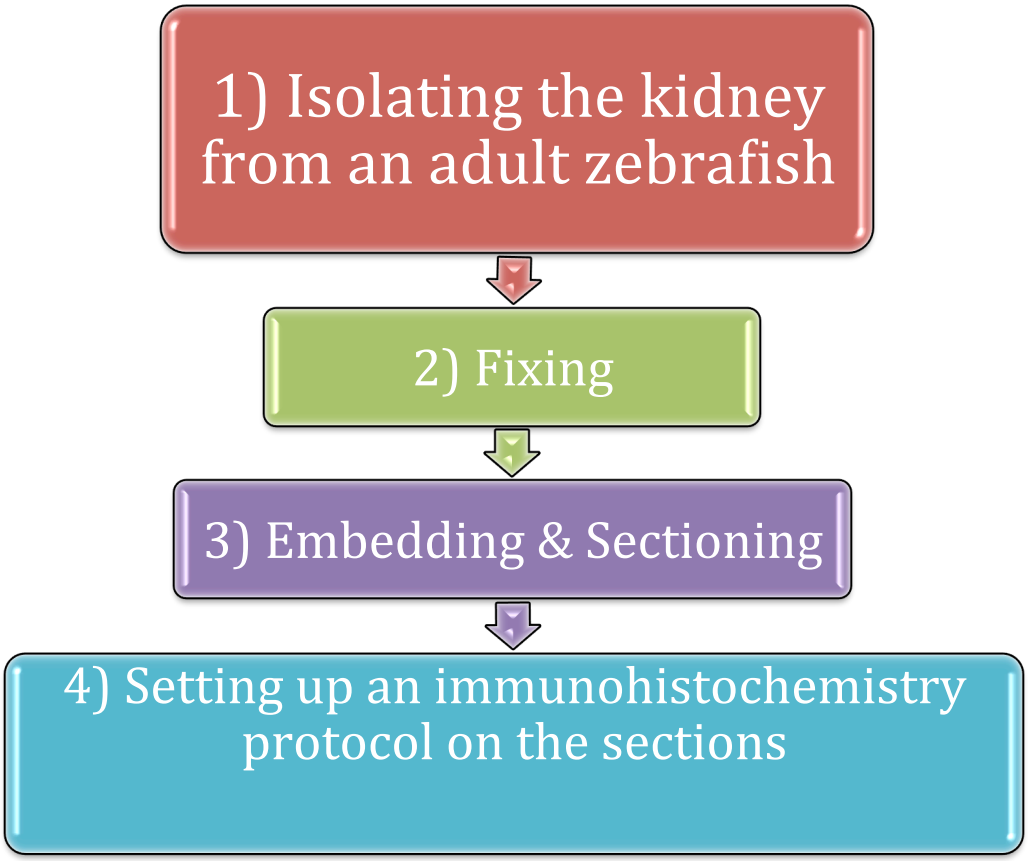


Fig. 25: Schematic representation of the steps involved in setting up procedures to view and analyse an adult zebrafish kidney

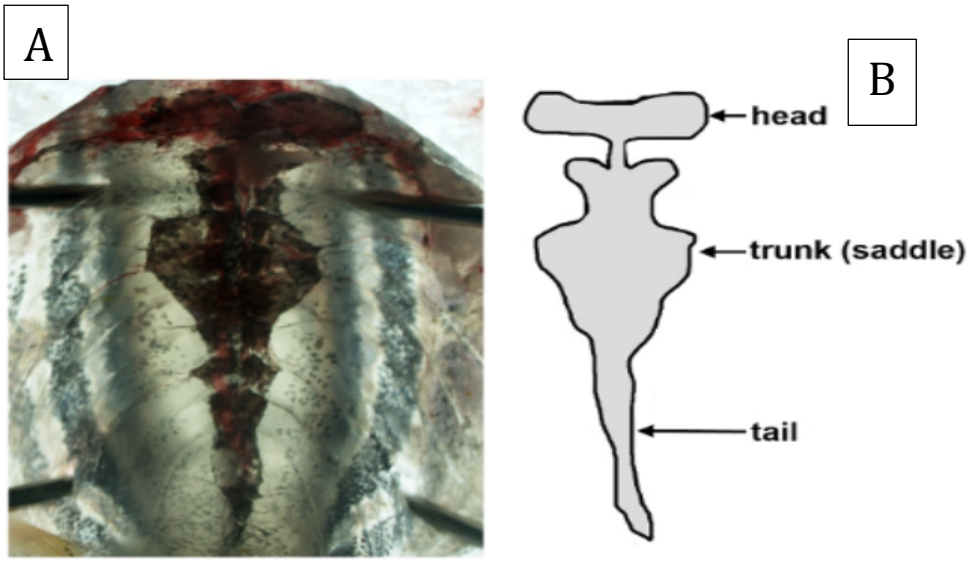


Fig. 26: Adult zebrafish kidney isolation Gerlach et al. (2011)

A) An adult zebrafish kidney as viewed under a dissecting scope before detachment from the dorsal body cavity.

B) Schematic representation of the anatomical shape of the adult zebrafish kidney.

Step 3: After fixation and isolation of the kidney, the kidney was ready to be embedded and sectioned. Initially we thought that paraffin embedding and sectioning would work best, as it has been a method of choice for embryo sectioning. However when we did paraffin embedding and later sectioned, the sections did not seem to remain intact. Considering the kidney is a tissue that unlike an embryo is not tightly bound by structural proteins, could be a possible explanation for why the tissue seemed broken within the paraffin.

We next turned to cryostat sectioning which included embedding the kidney into an optimal cutting temperature (OCT) compound. Embedding involved immersing the kidney into OCT, contained in a plastic mould. The plastic mould containing the OCT and kidney tissue is then placed over dry ice for instant freezing of the tissue. Once frozen the mould was left over night at -80°C for further freezing. The next day the embedded tissue was ready to be sectioned on a cryostat. The embedding procedure for cryostat sectioning is quick and very simple and does not put the tissue through too many procedural steps that may damage the tissue.

The kidneys were sectioned at 10micron sections and were applied on superfrost ++ slides. In order to stain the sections that were embedded in OCT, the OCT had to be first rinsed off the sections before carrying out further staining on them. We stained a section with hemotoxyllin and counter stained with eosin in order to get an over view of the morphology of the kidney (Fig.27). The histology of the adult zebrafish kidney has been described by Menke et al. (2011). Haematopoietic tissue lies within the interstitium of the kidney (Menke et al., 2011).

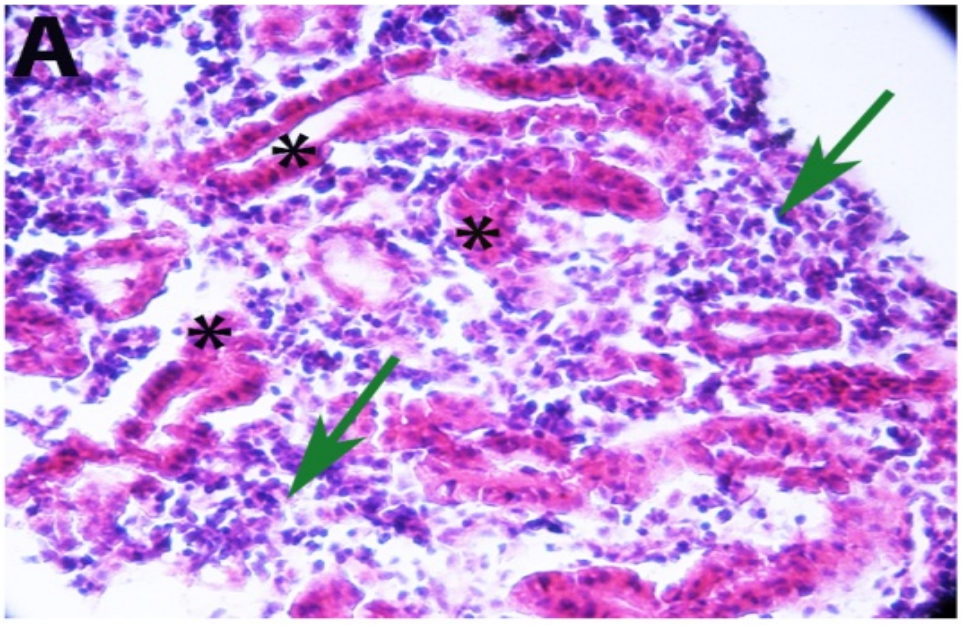


Fig. 27: Morphology of a section of the adult zebrafish kidney. A cryostat section of a wt adult zebrafish kidney that was stained with hemotoxyillin and counter stained with eosin. Black asterisks mark the tubules of the kidney and the green arrows point to the area of haematopoietic tissue in the kidney.

Step 4: In order to be able to detect the recombined cells in the adult kidney from our lineage tracing experiments, it was important for us to standardise immunohistochemistry for GFP as well as for dsRed antibodies.

Since this sort of immunohistochemistry experiments have not really been done on adult zebrafish kidneys there were a lot of trial and errors involved in establishing an effective method of detecting GFP and dsRed positive cells in this organ.

Kidneys of known transgenic zebrafish lines were first analysed:

Our aim is to find cells in the kidney and show that they are stem cells. In order to do that we first tried sectioning and immunohistochemistry on transgenic zebrafish lines that are known to express in the adult kidney.

qmc551:gata1dsRed: To begin with we chose a double transgenic zebrafish line that had both a GFP as well as a dsRed reporter. This line was the qmc551:gata1 dsRed line. In this line the qmc551 has the gfi1:GFP and was used to see if GFP could be detected. The gata1 in the same line was used to establish a method of detecting a red fluorescent protein, namely dsRed.

CD41:GFP was a second transgenic line we used in order to be able to detect GFP positive cells in the kidney. Ma et al (2011) described two populations of CD41-GFP^+ cells in the kidney of CD41:GFP transgenic fish, a GFP (hi) and a GFP (lo). They identified the GFP (lo) cells as cells with undifferentiated morphology, which suggested they are early progenitor cells, while the GFP (hi) cells were described as being mature thrombocytes (Ma et al., 2011).

Flk1:GFP: Labels endothelial cells in the kidney and would give us an idea of what this would look like.

Cre Reporter (GFP): sectioning and staining of the Cre reporter transgenic line would give us an idea of which cells would seed the kidney in the absence of recombination. This would also give us an idea of the activity of the ef1α promoter. Those cells that express GFP in the kidney have the ef1α promoter on in the adult kidney. These should be an entire population of GFP^+ cells.

Qmc101:Cre reporter (SN907): These fish have the qmc101 (flk1CreXERT2) transgene and the Cre reporter. These fish

have the constitutively active Cre protein. The dsRed positive cells in the kidney of these fish should be a subset of the cells seen in the Cre reporter line. The common factor between the SN907 and the Cre reporter is the ef1α promoter. This line would also help us in setting up the immunohistochemistry for a red fluorescent protein, dsRed.

SN919 Wild type (wt): It helped us set up cryostat sectioning. wt zebrafish adult kidney sections were used as negative controls, to distinguish between specific and non-specific staining.

4.5.1. Autofluorescence hampers unequivocal detection of GFP fluorescence in the adult kidney

The qmc551 line is a gene trap line that labels with the GFP reporter the vDA when cells are hemogenic. From inverse PCR results it was seen that the reporter sits in the growth factor independence (gfi) 1.1 gene which is a homologue of the mouse Gfi1 (Thambyrajah, PhD 2012; Ucanok, personnel communication). Gfi1 positive cells would express GFP in myeloid progenitors in the adult kidney. This was done to see if GFP expression could be picked up in the adult kidney. Gata1 is an eryhthroid-specific transcriptional factor (Detrich et al., 1995) and would label erythroid progenitors which could be viewed as dsRed positive cells.

Hence we made use of the double transgenic line to carry out immunohistochemistry on the kidney sections and to establish a method that would allow us to detect GFP and dsRed positive cells and to know what to expect in the kidneys of the fish we have labelled for lineage tracing using flk1CreERT2.

We did this using fluorescent antibody staining, to detect GFP and dsRed (Fig. 28). A single green cell is observed; however there seems to be staining in the kidney tubules as well (Fig. 28A). Since this was the first time we were doing this sort of experiment we were not really sure if what we were looking at was real staining or auto-fluorescence. Although it was more likely that the single green cell was real GFP staining as it lies in the haematopoietic tissue area of the kidney, hence chances of finding progenitors or HSCs is more likely in this region of the section. However the green colouration in the tubules was something that was more confusing. In order to differentiate

between auto-fluorescence and real GFP staining we stained a wt kidney section with the GFP and dsRed antibody (Fig. 29).

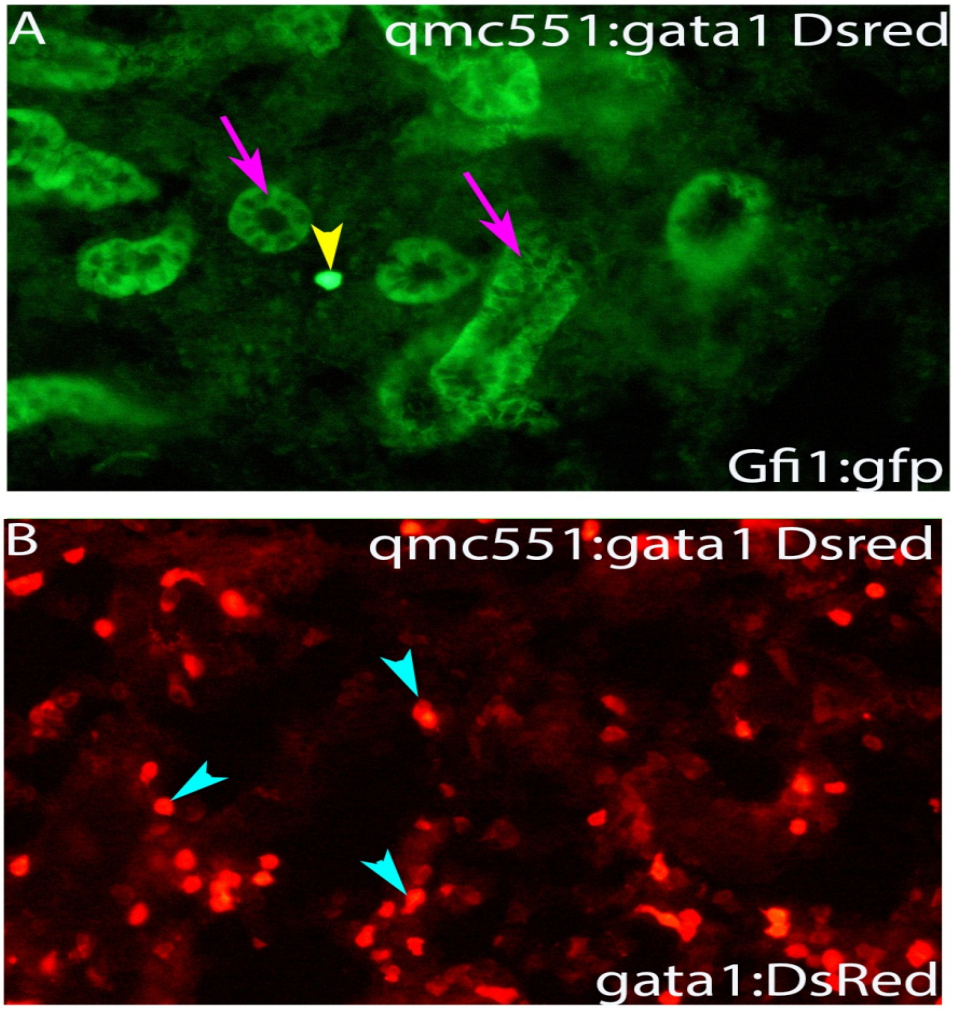


Fig. 28: Fluorescent immunohistochemistry staining done for detecting GFP$^+$ and dsRed$^+$ cells in the kidney section of a qmc551:gata1dsRed double transgenic adult zebrafish kidney.

A) kidney section stained to detect GFP expression for gfi1 cells. A single cell GFP positive cell (yellow arrow) is seen. Tubules (pink arrows) show green colouration.

B) Section stained with dsRed antibody to detect gata1:dsRed positive cells. Many dsRed positive cells (cyan arrows) were observed. Tubules are not visible at this exposure time and were hence not annotated.

In the wild type kidney sections that were stained for GFP and dsRed there did not seem to be individual green or red cells but however the tubules did seem to be stained in both green and red (Fig. 29A and B). This would suggest that what we do see in the tubules of the transgenic lines was auto-fluorescence. However the green fluorescence in the wild type kidney tubules seemed very bright and could thus suggest that there might be some kind of non-specific binding of the GFP antibody to some other protein in the tubules which was later looked into.

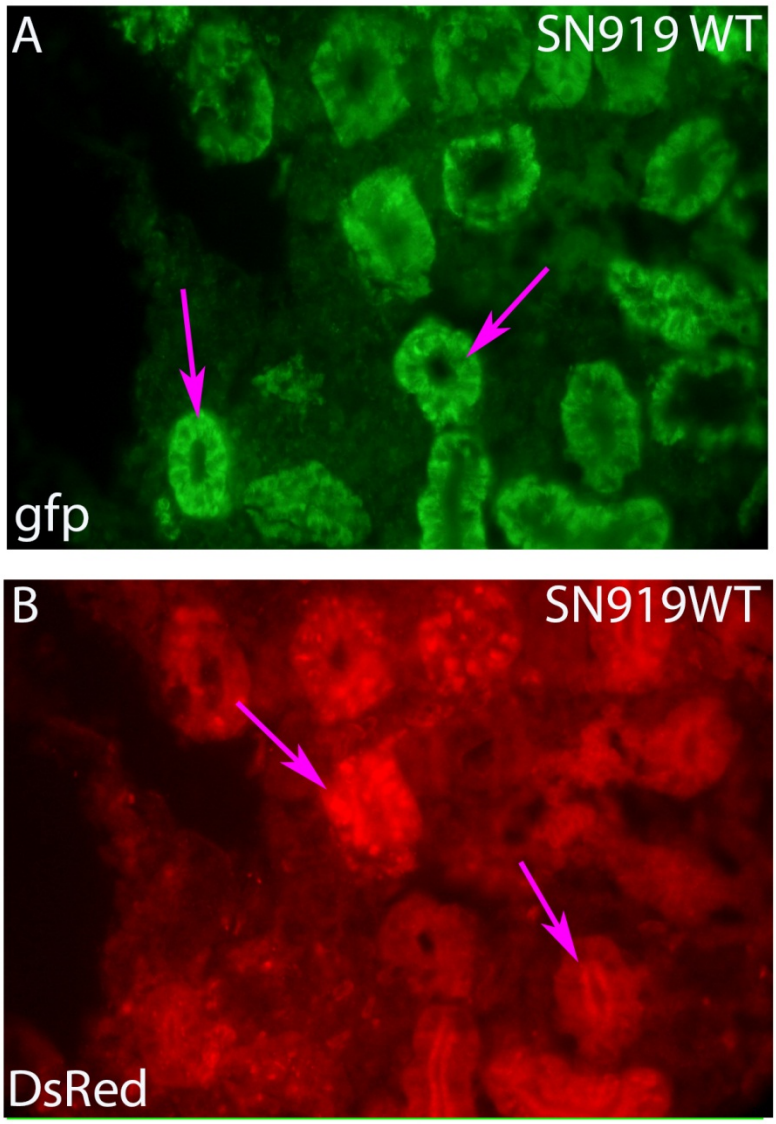


Fig. 29: SN919 wt kidney section stained with GFP and dsRed antibody.

A) Shows the section as seen in the green channel where tubules (pink arrows) are stained green.

B) Shows the section in the red channel where tubules are also stained red.

To differentiate between auto-fluorescence and non-specific binding of the GFP antibody to the kidney tubules, wt, CD41:GFP and flk1:GFP kidney sections were rinsed with buffer and were not stained with the antibody. In the wt section, although the green colour intensity seemed reduced there was still green staining (Fig. 30). This would suggest that what we see in these sections is auto-fluorescence.

Kidney sections of the flk1:GFP and CD41:GFP were also looked at in the absence of staining with the GFP antibody (Fig. 31), there were other cells that were seen along with the green colouration in the tubules. Although the exposure time on the camera setting had to be reduced to image both of these sections due to the high intensity of the green colouration seen in the individual cells seen in the CD41:GFP sections (Fig. 31B) and elongated cells in flk1:GFP sections (Fig. 31A yellow arrowheads), tubules could still be seen in these sections. The morphology of the cells seen in the flk1:GFP kidney section fits with that of endothelial cells and the individual cells seen in the CD41:GFP kidney section could suggest they are actually CD41 expressing cells. However we could not rule out auto-fluorescence that was seen in wt kidney sections (negative controls) and had to be absolutely sure of actual or real GFP expressing cells.

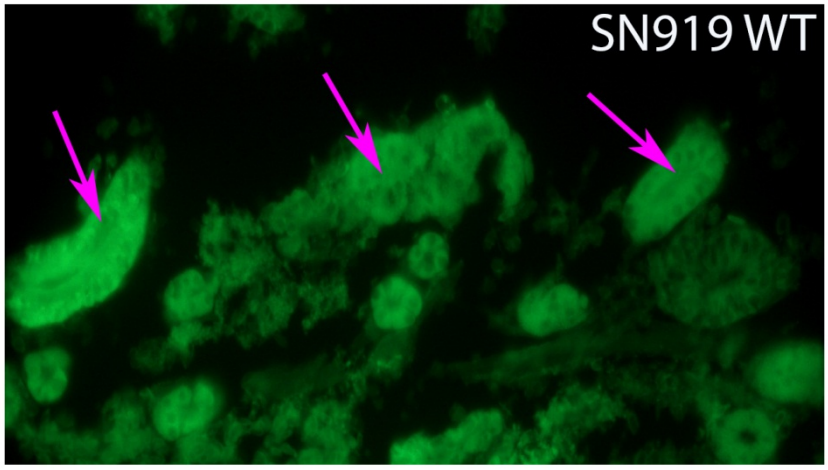


Fig. 30: SN919 wt kidney section rinsed with buffer and not stained.

Auto-fluorescence is seen in tubules (pink arrows) in a wild type (wt) kidney section in the absence of the GFP antibody.

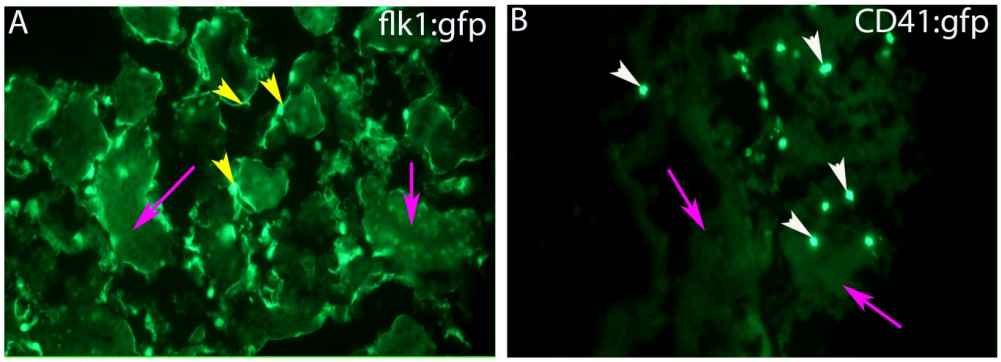


Fig. 31: flk1:GFP and CD41:GFP kidney sections rinsed with buffer and not stained, revealed other cells as well.

A) A flk1:GFP kidney section Bright elongated cells lining tubules (yellow arrowheads) and tubules (pink arrows)

B) Bright single cells (white arrows) and tubules (pink arrows).

4.5.2. Using a streptavidin Alexa fluor 488 conjugate in combination with a primary biotinylated antibody reveal GFP positive cells in the kidney, but also gives non-specific staining of extracellular matrix components

Streptavidin has its drawbacks and is notorious for non-specfic binding. Leaving all other conditions of the immunohistochemistry protocol constant, and by either adding only primary or only secondary GFP antibody to CD41:GFP sections (Fig. 32) we confirmed that non-specific binding was in fact a problem.

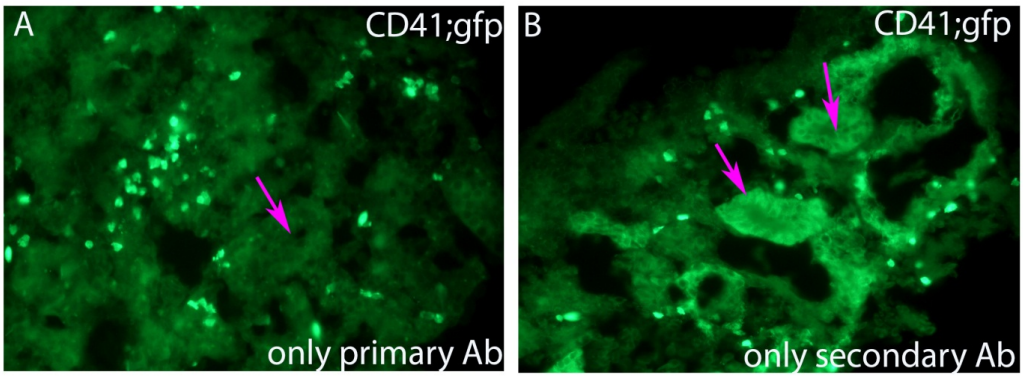


Fig. 32: Non-specific binding of secondary antibody streptavidin tested on CD41:GFP kidney sections

A) Addition of only primary antibody and not secondary antibody give weaker green colouration in the tubules (pink arrow)

B) Addition of only secondary antibody; no primary antibody added gives brighter green colouration in tubules (pink arrows)

Both images were taken using identical camera settings.

Hence with the problem of auto-fluorescence and non-specific binding of the streptavidin we decided to use DAB staining to

get a better overview on what kind of cells were labelled in our transgenic lines.

4.5.3. A combination of a biotinylated primary antibody with the VectastainABC detection system allows unambiguous detection of GFP-positive cells in the adult kidney

After a lot of trials, errors and modifications of available DAB methods we finally settled with a satisfying modified DAB staining protocol to detect GFP cells in adult zebrafish kidney sections. The only problem with using DAB was interference of endogenous peroxidase activity that gave a lot of brown colouration especially in tubules (Data not shown). This however was abrogated using a 3% hydrogen peroxide (H_2O_2) in methanol. All GFP expressing transgenic kidney sections were stained using DAB staining and at the same time a wt kidney section was also stained as a negative control (Fig. 33).

The SN919 wt kidney section showed negligible brown colouration (Fig. 33Ai), which is what we expected. We had managed to eliminate almost all of the endogenous peroxidase activity.

The flk1:GFP kidney section showed brown staining in elongated cells that fit the description of endothelial cells, which are flk1 positive (Fig. 33Aii) and thus these cells are GFP positive cells. The number of cells with this morphology was extremely high. These cells were seen lining the tubules in the kidney. Endothelial cells line blood vessels and blood vessels are associated with all organs. Although we tried to get rid of endogenous peroxidase activity the flk1:GFP sections invariably had a slight brown colouration in some tubules (Fig. 33Aii). Because of the high number of flk1 positive cells, the

time required for staining with DAB was lesser when compared to the other transgenic lines. However the time for staining might need to be reduced further.

CD41:GFP cells are seen as single round cells spread all over the tissue section (Fig. 33Aiii). However as described by Ma et al we could not, atleast from our sections differentiate between the GFP (hi) and GFP (lo) low expressing cells. We could only see the whole population of CD41 cells.

In the Cre reporter kidney sections there seemed to be more than one type of cell expressing GFP (Fig. 33Aiv) some round cells and some elongated cells were also observed in this transgenic kidney section. There also seemed to be a lot of brown patchy staining all over the tissue section. Since the wt kidney section did not show any such patchy staining pattern it could not be ignored completely.

Finally the qmc551 section was stained to detect the gfi1.1 expressing cells in the kidney, which seem to be few in number (Fig. 33Bii). These cells could be myeloid progenitors in the kidney. This kidney section was stained on a different day from the other above described lines. A different wt kidney section was also stained along with it to have a negative control (Fig. 33Bi).

Fig. 33: GFP staining done using DAB on transgenic zebrafish lines

Sections (Ai-iv) were stained together on the same day

Ai) SN919 wt kidney section shows tubules (t) brown staining in this kidney section is negligible. The adult zebrafish kidney is associated with black pigment cells (yellow arrow). Some blood cells (black arrow) can also be seen in this section.

Aii) flk1:GFP kidney section displays a large number of GFP positive elongated cells (blue arrowheads) lining tubules (t). Some blood cells (black arrow) probably erythrocytes are also seen.

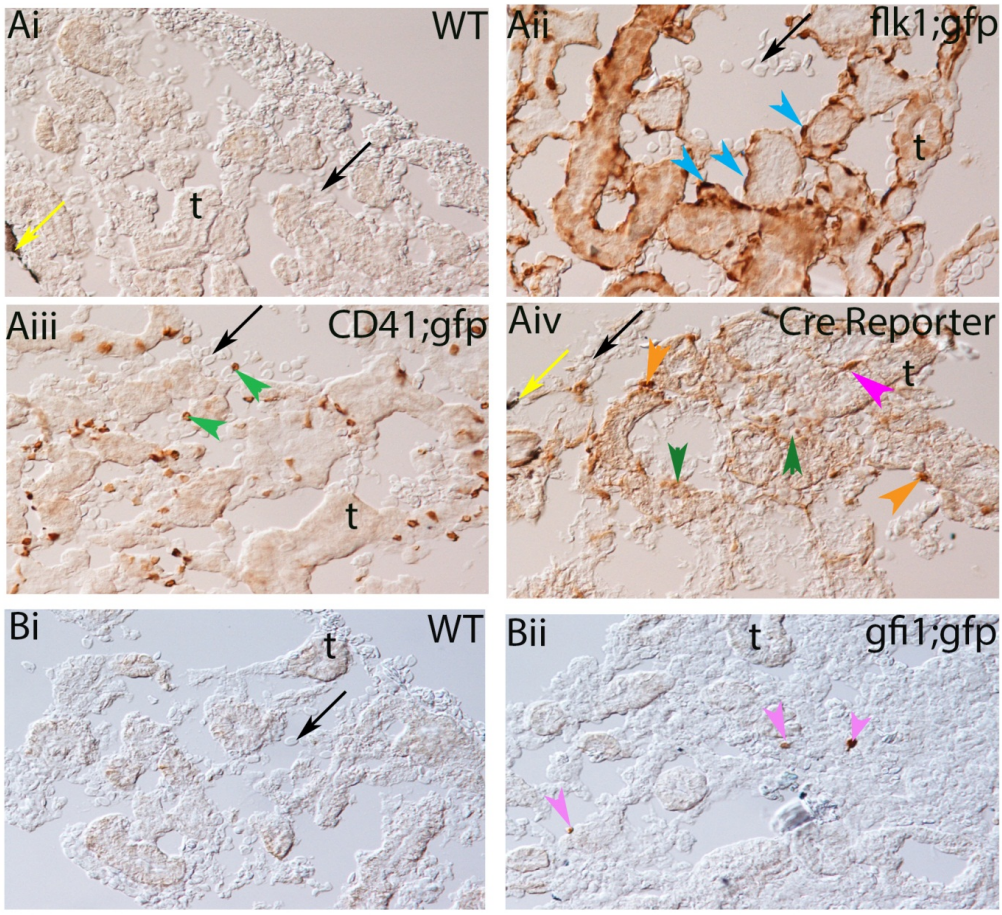
Aii) In the CD41:GFP kidney section a large population of GFP positive cells are scattered as single cells (green arrowheads) all over the tissue section. The tubules (t) seem to have negligible brown colouration just as in the wt kidney section. There seem to be a cluster of unstained blood cells (black arrow) present in the section, which could be erythrocytes.

Aiv) The Cre reporter kidney section shows 3 type of stained cells, round single cells (orange arrowheads), elongated cells (pink arrow head) and patchy staining (green arrowheads). Pigment cells (yellow arrow) are also seen in this particular section and some erythrocytes (black arrow).

Sections (Bi and Bii) were stained on the same day.

Bi) wt kidney section shows negligible colouration in the tubules (t). A blood cell (erythrocyte) (black arrow) is also seen.

Bii) in the qmc551 a very few number of cells are GFP positive (pink arrowheads). The tubules (t) show no brown colouration.



4.6. Analysis of the progeny of the double transgenic of the qmc101;Cre reporter fish reveals that the flk1 promoter is active in the germline

Once we had detected GFP positive cells in transgenic lines that had the green fluorescent reporter, we now had to standardise a method of detecting dsRed positive cells in the adult zebrafish kidney. Finally it is dsRed positive cells that need to be detected in the kidneys of the fish from our lineage tracing experiment. We thus needed to look at transgenic zebrafish lines that would express dsRed in the kidney. We already had the qmc551:gata1 dsRed double transgenic line. The gata1: dsRed would be used to standardise a DAB immunohistochemistry staining to detect dsRed.

We needed one more dsRed transgenic line in order to standardise our immunohistochemistry. We chose the SN907 that carried the qmc101:Cre reporter. These fish carried the constitutively active flk1CreXERT2 (qmc101) along with the Cre reporter. However since we were not very sure of which fish among these were transgenic, we decided to cross them to wt fish to identify a transgenic and only once characterised, use it to isolate the kidney.

On crossing the SN907 to a wt fish we were surprised to see that progeny of the cross gave no GFP expression in any of the embryos, but only expressed dsRed ubiquitously (Fig. 34). A male and a female SN907 fish were crossed to wt fish of the opposite sex.

The progeny of the female expressed robust ubiquitous dsRed expression even at around the 2 somite stage (10.7 hpf) (earliest embryos were checked) which could indicate maternal mRNA contribution. Besides showing robust expression (Fig.

34C) of dsRed all (100%) of the embryos were red and no GFP expression (Fig.32B) was observed. This suggests that the SN907 female parent fish carried more than a single copy of the transgene.

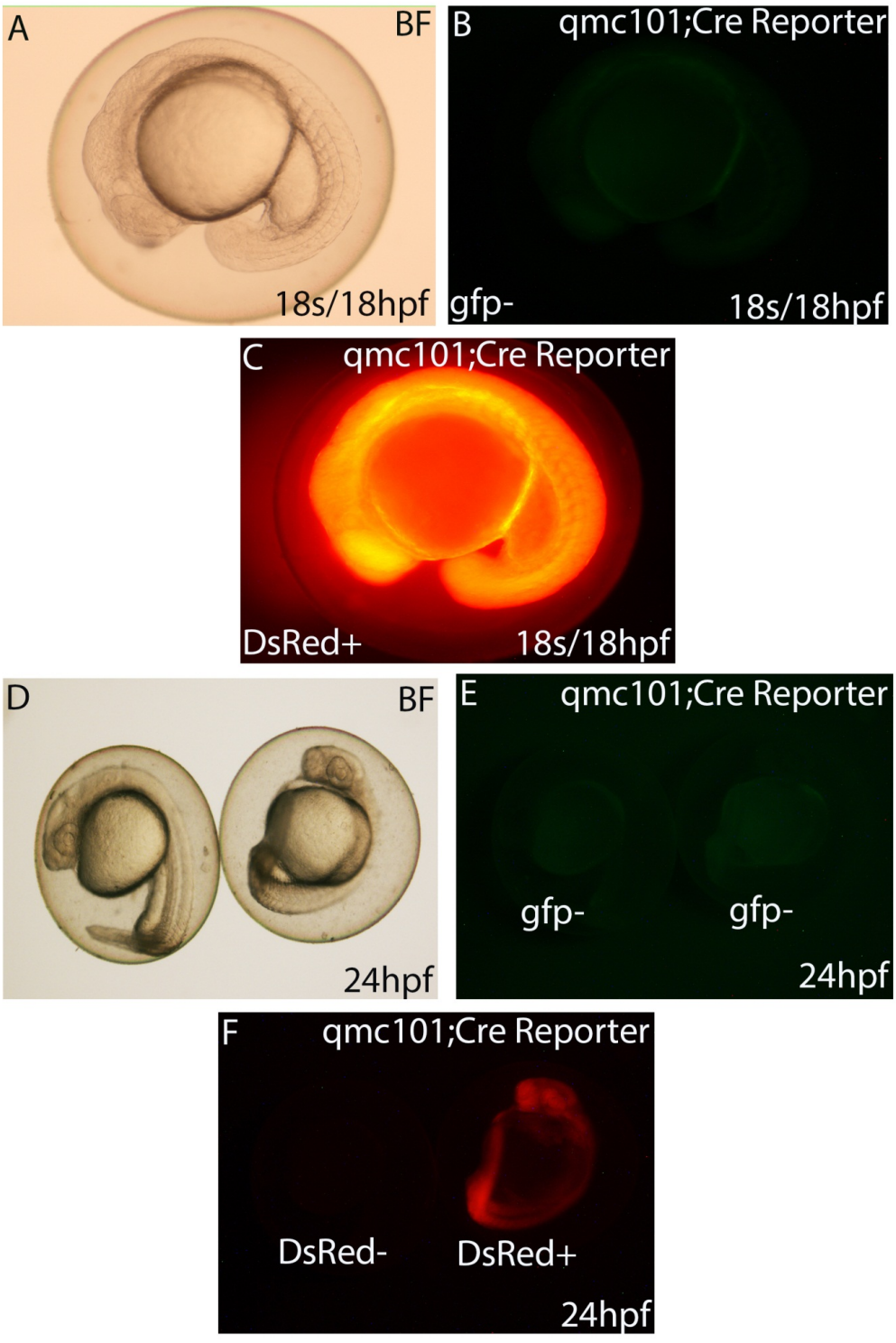
The progeny from the male SN907 fish also expressed only dsRed ubiquitously (Fig. 34F) and no GFP expression (Fig. 34E) was observed. 50% of the embryos expressed dsRed suggesting that the male SN907 carried only a single copy of the flk1CreERT2:Cre reporter transgene.

Overall this experiment unexpectedly showed for the first time, that the flk1 promoter is active even in the germline. Recombination of the loxP sites has taken place at some point in the germline of the parent fish, hence giving ubiquitous expression of the red reporter (dsRed) in the progeny.

Fig. 34: Flk1 is active in the germline of flk1Cre (qmc101):Cre reporter (SN907) fish

A-C) Progeny of the SN907 female fish. (A) Embryo as seen in bright field (BF), (B) as seen in the green channel, the embryo is GFP negative, slight green colour seen is due to bleed through of robust dsRed expression. (C) same embryo as seen under the TRITC filter for dsRed expression, displays robust dsRed expression throughout the embryo.

D-F) Progeny of the SN907 male fish (D) Embryos as seen in bright field (BF) (B) as seen in the green channel, both embryos are GFP negative, sl. (C) same embryos as seen under the TRITC filter for dsRed expression, ubiquitous dsRed expression seen in one; the other embryo was a sibling that was dsRed negative.


A
BF
18s/18hpf
B
qmc101;Cre Reporter
gfp-
18s/18hpf
C
qmc101;Cre Reporter
DsRed+
18s/18hpf
D
BF
24hpf
E
qmc101;Cre Reporter
gfp-
gfp-
24hpf
F
qmc101;Cre Reporter
DsRed-
DsRed+
24hpf

4.7. Immunohistochemistry allows detection of dsRed positive cells in the kidney of adult flk1Cre;Cre-reporter double transgenic zebrafish

In order to be able to detect kidney cells in the flk1Cre;Cre-reporter fish that had undergone loxP recombination earlier during development a method to detect dsRed-positive cells had to be established. For this purpose, a combination of a rabbit primary dsRed antibody with a horseradish peroxidase-conjugated anti rabbit secondary antibody was tested on gata1-dsRed transgenic fish. In this line, dsRed is expressed in primitive and definitive red blood cells (Ellett and Lieschke, 2010). Using DAB as a substrate, weakly dsRed-positive cells were detected scattered throughout the kidney section of this line (Fig. 35B), but were not found in kidney sections of wt controls (Fig. 35A

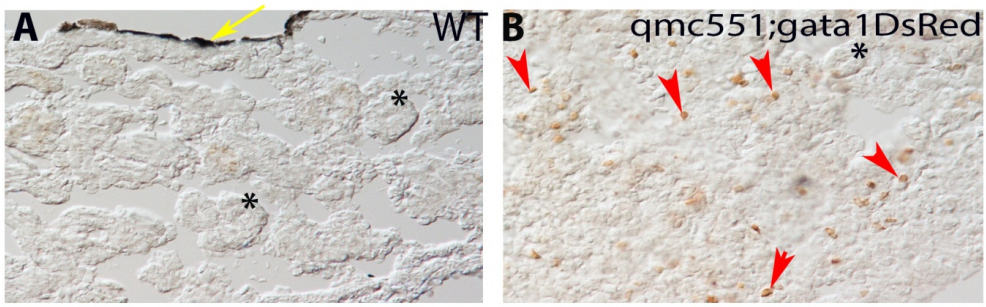


Fig. 35: dsRed-positive cells were detected in the kidney of gata1:dsRed transgenic fish by immuno-histochemistry

A) A wildtype kidney section reveals the presence of tubules (black asterisks) and melanocytes (yellow arrow), but contains no DAB stained cells.

B) dsRed-positive, DAB-stained cells were detected in between the tubules (asterisk) of the kidney of qmc551:gata1-dsRed double transgenic fish.

Having established the dsRed detection method, dsRed expression was next examined in the flk1CreXERT2^{qmc101};Cre-reporter fish. First, dsRed expression was looked at in the transparent 6 day-old embryo. At 6 dpf, double transgenic embryos showed dsRed expression in the endothelium and endocardium, as well as in the thymus and in the kidney (Fig. 36). This is consistent with the idea that cells from the CHT seed the thymus and the kidney by this stage.

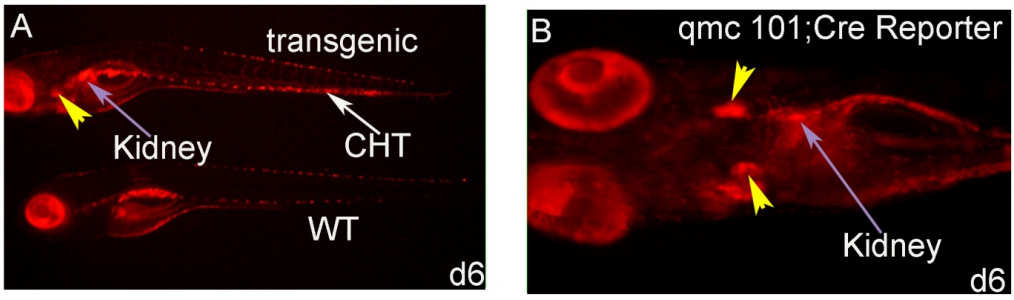


Fig. 36: dsRed expression pattern in 6 dpf flk1Cre(qmc101);Cre-reporter double transgenic embryos.

A) A qmc101;Cre-reporter double transgenic embryo expresses dsRed in cells in the CHT, in the thymus (yellow arrowhead) and in the kidney. Taken with a wt negative control that showed no dsRed expression. The red fluorescence observed in the eyes of the transgenic and wt embryos is due to autofluorescence.

B) A close up view of the head region the transgenic embryo (in image A), where the dsRed expression is clearly seen in the kidney and in the thymus (yellow arrowheads).

Next, assuming that the ef1α promoter was also active in adult blood cells, dsRed expression was analysed in the kidney of adult double transgenic fish.

For this experiment, two negative controls were used: (a) a section of a wt kidney and (b) a kidney section of a Cre-reporter single transgenic fish (Fig. 37A and B). In these, no dsRed-positive cells were expected. Our results confirmed our expectations.

Two kidney sections isolated from an SN907 double transgenic fish were taken from two different anatomical regions of the kidney, namely the head kidney (Fig. 37Ci) and the saddle (Fig. 37Cii). In both sections, individual dsRed-positive cells in between the tubules were detected (Fig. 37Ci and Cii). This shows that at least some blood cells are derived from cells that have expressed Cre under the control of the flk1 promoter and have undergone recombination of the loxP sites.

Where the strong background staining visible in the section of the kidney saddle of the double transgenic animal came from is not clear as it was neither observed in the head kidney section of the double transgenic fish nor in the sections of the control kidneys.

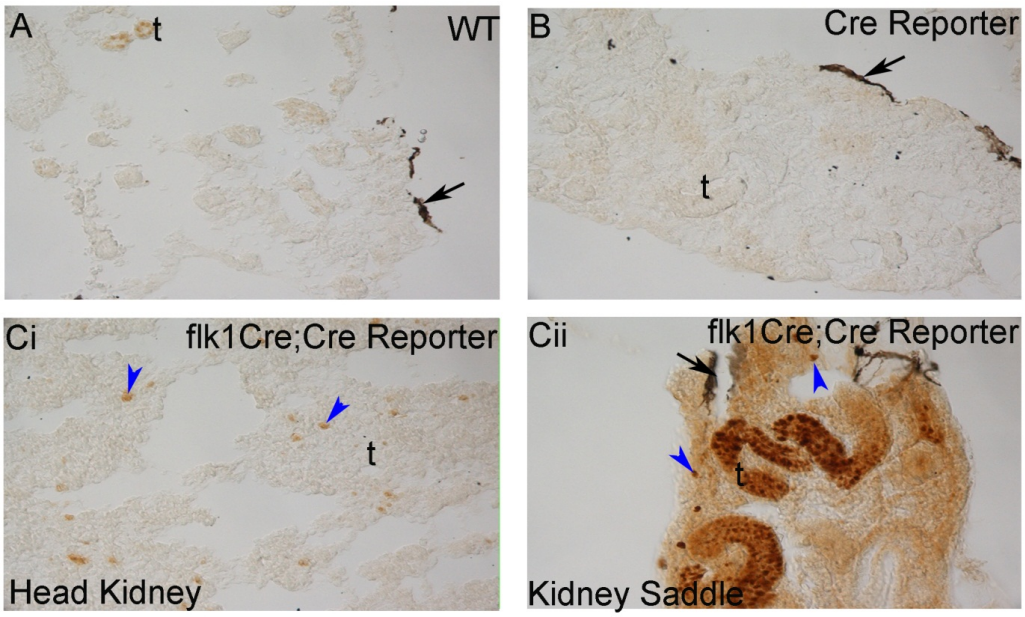


Fig. 37: dsRed expression in the kidney of flk1Cre:Cre reporter (SN907) double transgenic adult zebrafish.

Sections of kidneys of wild type (A) and Cre reporter single transgenic (B) fish showed no staining with the dsRed antibody.

Sections through the head (Ci) and saddle (Cii) portion of the kidney of the SN907 (flk1CreXERT2;Cre reporter) double transgenic fish revealed the presence individual dsRed positive cells (blue arrowheads) between the tubules (t). Black pigmentation reveals the presence of melanocytes (black arrows) associated with the kidney. Strong background was observed in the section of the kidney saddle (Cii).

Chapter 5: Discussion

5.1. dsRed expression level and pattern differences seen in the progeny of SN9 fish

dsRed expression level differences were observed in the progeny of each of the 3 fish that were identified (SN9 ♂5, ♂6 and ♂8). There could be a number of possible explanations for this difference. This could be due to the differences in copy number of the flk1CreERT2 transgene present in the chromosome. Higher the copy number, greater is the dsRed expression. Positional effects could also play a role in expression pattern differences, like in the qmc107 line that showed normal as well as ectopic dsRed expression. This would mean that the gene could have jumped into the vicinity of a regulatory element during integration into the genome.

5.2. The SN51♀4z (flk1CreERT2) is a tight, non-leaky system

We have seen that the flk1Cre in our qmc101 line labels more cells than our inducible flk1CreERT2, however looking at the progeny of the SN907 (flk1Cre;Cre reporter) (Fig. 34) we can be more confident of the inducible system in the SN51♀4z fish which is completely dependent on 4-OHT to carry out recombination. Even if the flk1 promoter is active in the germline of the SN51 fish never showed dsRed positive embryos as was seen in the progeny of the SN907 (Fig. 34). This indicates that no recombination takes place in the SN51♀4z fish in the absence of 4-OHT even in the germline. Hence our system is suitable to carry out lineage tracing experiment.

5.3. Immunohistochemistry is the method of choice to detect GFP positive cells in the adult zebrafish kidney

Fluorescent immunohistochemistry on kidney sections has the risk of auto fluorescence and hence making it difficult to distinguish between GFP positive cells and false positives. Although the staining with the dsRed antibody was still better, sections looked messy due to too the auto-fluoresce.

Dab staining is a better method of choice because most of the background cause by endogenous peroxidase activity was abrogated with H_2O_2 treatment. Hence making the sections look much neater making it easier to visualise dsRed or GFP positive cells.

5.4. Is the ef1α promoter active even in the adult fish kidney?

The ef1α has been seen to be most active in proliferating cells (Thummel et al., 2006) and hence its activity in the adult zebrafish kidney may be affected. From the SN907 (flk1Cre:Cre-reporter) kidney sections (Fig. 37) that were stained to be able to visualise dsRed positive cells a very small number of cells seemed to be labelled. If there are HSCs that are quiescent and non-proliferative present in the kidney, then we might not be able to stain and hence visualise them if the ef1α promoter is not active in these cells. However these are sections of the kidney and not the entire kidney, so what we see are a very small percentage of the total cells actually present in the kidney.

5.5. Future experiments

As mentioned above visualising stained cells in kidney sections of the flk1CreERT2;Cre reporter or the flk1Cre;Cre-reporter

(SN907) is not enough to give us an idea of the kind of cells we are labelling, although they are assumed to be HSCs and endothelial cells. In order to do this we would like to flush cells out of the adult kidney and carry out a fluorescence-activated cell sorting (FACS) on the sorted dsRed-positive cells. Once we have these cells, we would like to spin them down on a cytospin and visualise the cells. This would help us identify, morphologically, under a microscope the type of cell. This would be done first on the SN907 adult kidney before looking at our fish from the lineage tracing experiments.

If after further investigation we find that the ef1α promoter is causing us a problem we could try making new constructs using may be a tissue specific promoter, like the β-actin promoter used by Bertrand et al. (2010).

Chapter 6. Introduction to the the phosphotidylinositol 3-kinase/protein kinase B signal transduction pathway

6.1. The phosphotidylinositol 3-kinase/protein kinase B signal transduction pathway

Here we are interested in studying the effects of the activating or blocking the phosphoinositide-3-kinase (PI3K) pathway specifically in the endothelium.

Phosphotidylinositol/phosphoinositide-3-kinase (PI3K)/ protein kinase B (PKB/AKT) signalling pathway is known to be an important mediator of cytokine signalling implicated in regulation of haematopoiesis. PI3K are lipid kinases that phosphorylate the 3’ hydroxyl group of phosphatidylinositol, for example: e.g: the membrane-anchored and most important substrate for class I PI3Ks is phosphatidyl inositol-4,5-bisphosphate which gets phosphorylated to phosphatidyl inositol-3,4,5-triphosphate. The reaction catalysed by PI3K can be reversed by PTEN and SHIP2.

3 distinct subclasses of phosphotidylinositol have been identified, however class I isoforms may be playing a role in haematopoiesis. ClassIA are heterodimers of a regulatory subunit p50α, p55α, p55γ, p85α and p85β and a catalytic subunit as p110α or p110β. Most PI3K are inactive in a cell and only small fractions get activated upon growth factor stimulation.

PI3K is activated by a number of different membrane proteins that are receptors for various ligands, either directly like in platelet-derived growth factor receptor (PDGFR) or indirectly via ras as in case of epidermal growth-factor receptor (EGFR) and fibroblast growth-factor receptor (FGFR) (Jiang and Liu, 2009).

AKT1 also known as protein kinase B (PKB) are a 57kDa serine/threonine kinase and has a number of different targets. In the presence of PIP3 AKT is moved to the membrane. At the membrane PIP3 binds to the pleckstrin homology (PH) domain of AKT. At the membrane AKT gets phosphorylated and thus activated at the T308 position on the threonine residue and at the S473 position on the serine residue by mTOR. AKT1 has a number of different targets and is hence involved in a number of cellular and metabolic functions. It plays a role in cell survival and inhibition of apoptosis (Jiang and Liu, 2009).

6.2. Role of the phosphotidylinositol 3-kinase/protein kinase B signal transduction pathway in blood vessel formation.

Although functionally distinct, arteries and veins arise from a common progenitor, the angioblast. The angioblast gives rise to both a functionally competent complex network of arteries and veins, that together comprise the vasculature.

Hey2 is a transcriptional repressor in zebrafish (Hong et al., 2006). The gridlock mutation is a mutation in Hey2, which encodes a hairy/enhancer-of-split related basic helix-loop-helix protein. Hey2 has been seen to play a vital role in the specification of arteries; and the gridlock mutation results in an insufficient number of arterial cells which in turn either causes a defect or loss of the aorta (Zhong et al., 2001).

In an attempt to identify factors that are involved in arterial and venous fate decision Hong et al. (2006) treated gridlock mutants with a battery of chemical compounds like GS4012 which was found to supress vascular defects in gridlock/hey2 mutants by increasing vegf signalling. They saw that blood

circulation to the tail was restored via normal restored connection to the bilateral DA and the single medial DA. They also saw that GS4012 increased vegfA (vascular endothelial growth factor A) expression in gridlock embryos.

Hey2 has been shown to be expressed downstream of vegf (Rowlinson and Gering, 2010). Hong et al then treated gridlock mutants with another compound GS4898 and this was also able to rescue gridlock mutants. This compound is a flavone compound like the PI3K inhibitor LY294002, suggesting that GS4898 must be targeting a kinase. The LY294002 compound was also seen to rescue gridlock phenotype. Hong et al's study suggested that AKT acts downstream of the PI3K pathway.

In 2009 Herbert et al used flk1:GFP transgenics to track GFP positive angioblast cells during vascular development in the zebrafish embryo. They found that some of the angioblasts were seen to form sprouts from the dorsal aorta and connected to neighbouring cells to form the caudal vein. This suggested that arteries and veins share a common progenitor. They then used the PI3K inhibitor on the flk1:GFP transgenics and found that even though there was a vein, it did not express flk1:GFP, they observed that there were many cells retained in the DA.

Thus the data from both the studies of Hong et al (2006) and Herbert et al (2009) suggests that PI3K/AKT plays a role in the specification of arteries and veins.

6.3. Phosphotidylinositol 3-kinase pathway plays a role in vein formation and haematopoietic stem cells specification.

The rescue experiments done by Hong et al. (2006) led us to carry out LY294002 treatment on zebrafish embryos to study its effects on arterial/venous and blood cell formation. A 30μM solution of LY294002 (in DMSO) was used to treat embryos. This study showed that venous markers msr showed reduction while arterial markers seemed unaffected. Flk1 was not expressed however fli1 was relatively unaffected. Moreover there was a massive reduction in runx1, which would suggest that HSC formation is affected by blocking PI3K.

Studying the effect of the PI3K inhibitor on the whole embryo gave a reduction in venous markers and in runx1, it would now be interesting to study the role of blocking or activating PI3K/AKT signal transduction pathway in the endothelium using a transgenic approach.

Previously in the lab 2 constructs were cloned into a bluescript vector. The construct were; ef1α-loxP-Cerulean-terminator-loxP-HA-AKT1-K179M which is a dominant negative AKT or kinase dead AKT (KDAKT). The second construct was the constitutively active AKT myristoylated (myrAKT) ef1α-loxP-Cerulean-terminator-loxP-myrAKT. These constructs were injected into one-cell stage wt embryos (Fig. 38C). Both the transgenes contained a blue fluorescent reporter protein, cerulean which is floxed (Fig. 38A and B) and only in the event of recombination should there be gain or loss of function of PI3K/AKT signalling.

After injection the embryos were checked for transient cerulean expression. Embryos that expressed cerulean were taken up to the aquarium nursery to be grown.

Embryos that were injected with the transgene (ef1α-loxP-Cerulean-terminator-loxP-HA-AKT1-K179M) transgene were

given the stock number SN44 and embryos that were injected with the transgene (ef1α-loxP-cerulean-terminator-loxP-myrAKT) were given the stock number SN47. These fish were the F0 fish, which further needed to be characterised.

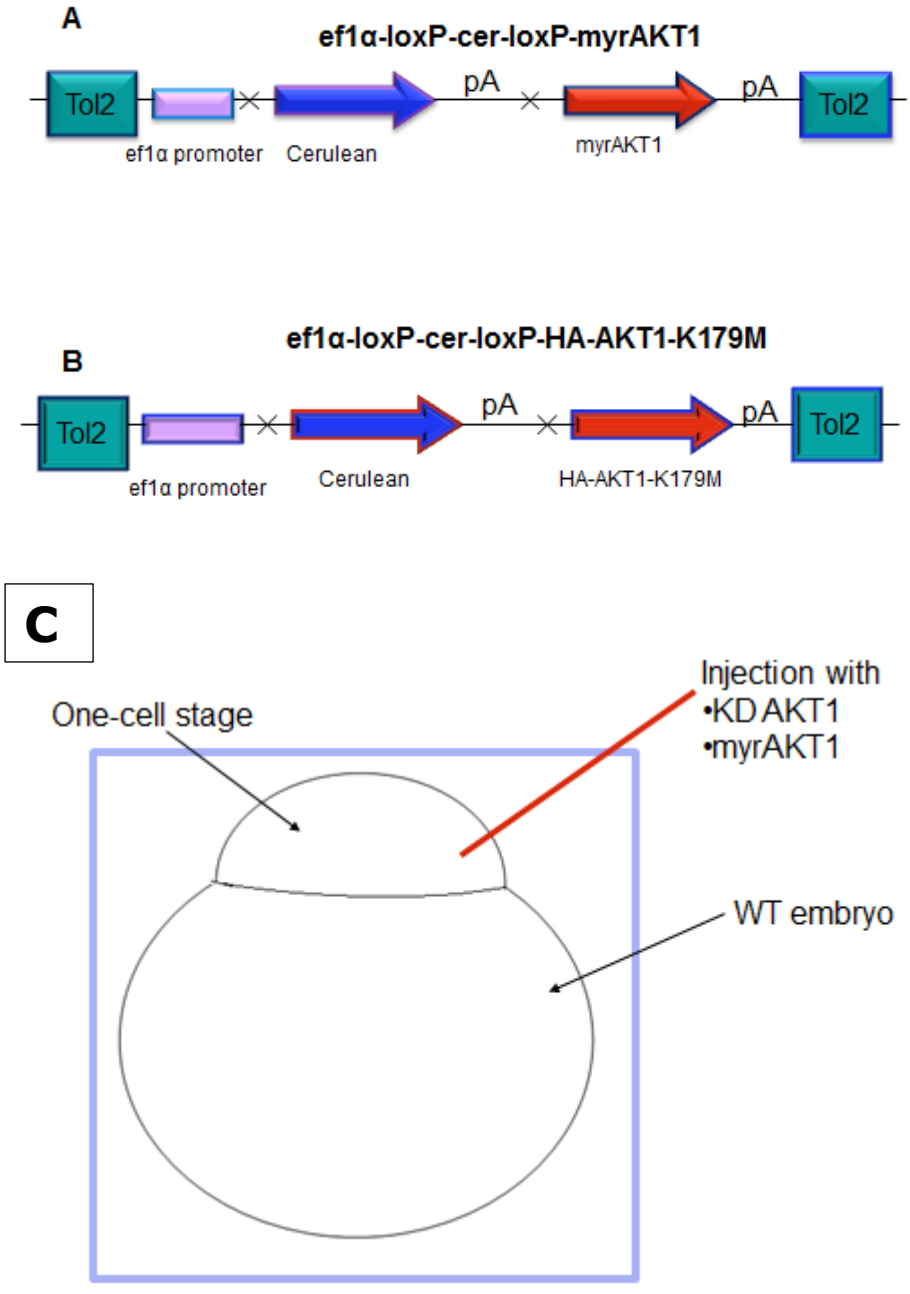


Fig. 38: Generation of 2 new transgenic founders

A-B) The two constructs prepared previously in the lab.

A) is the myrAKT construct

B) is the KDAKT construct that were injected into wt embryos

(C) to generate two new transgenic lines

Chapter 7. Results

Previous work carried out by Imran Khan and Christina Rhodes (Khan, MRes 2010, Rhodes, MRes 2010) in the lab included the treatment of wt embryos with LY294002 compound that is a PI3K inhibitor.

This treatment was done on the whole embryo and not specifically on the endothelium. Further to their studies we were interested in studying the effect of activating or blocking the PI3K pathway specifically in the endothelium using a transgenic approach.

7.1. Identification of KDAKT and myrAKT carriers amongst the SN44 and SN47 F0 fish

In order to identify transgenic founders amongst the SN44 (KDAKT) fish and the SN47 (myrAKT) fish, the SN44 and SN47 were crossed with SN26 wt fish. Their progeny was examined for cerulean expression at 1 dpf. Because the integration of the transgene occurs after the first embryonic cleavages, the tissues of the founders are mosaic with respect to the transgene. One would therefore assume that as little as a few per cent of the gametes could be transgenic. Thus, we were required to get at least 100 embryos from each cross to find a transgenic embryo.

Out of 16 crosses that were set up of the SN47 myrAKT fish only 2 fish gave embryos that displayed cerulean expression (see Table 5). These were ♂10z and ♀13L.

It was observed that the single embryo that expressed cerulean among the progeny of the ♂10z SN47 myrAKT fish was an abnormal embryo (Fig. 39A). This had a heartbeat, however had no blood circulation and hence did not survive.

Cerulean expression however seemed robust (Fig. 39A). Abnormalities in the embryo and a high cerulean expression level could suggest that a high number of copies of the myrAKT transgene may have integrated into the genome of this embryo. This could have caused over expression of AKT, which caused the abnormalities in the embryo. Another explanation for this could be that there might have been some read through which causes the over expression of the AKT. Cerulean expression seems to be higher in the head, neck and tail regions of the embryo (Fig. 39A). However, because this was only a single embryo from a single cross no more could be said regarding this. Sibling embryos analysed were all cerulean-negative and showed no abnormalities or defects as were seen in the single cerulean-positve embryo. SN47 was the founder of a new qmc line, qmc161.

Another fish was identified as a potential founder of the myrAKT transgene and this was the SN47♀13L. 9 out of 39 (table 5) embryos from this cross were cerulean positive (Fig. 39B). 8 of these were normal and were taken up to the nursery to be grown under a new line number qmc162. The fact that this line did not show any significant abnormalities like in the qmc161 embryo could suggest that the terminator in the construct is sufficient to block most if not all expression of AKT before recombination.

Table 5: Depicts the number of embryos analysed and the percentage of the germline that is transgenic in each line when the SN47myrAKT fish were crossed to wt fish.

SN47 fish DOB (22.6.11)	Total no. of embryos analysed	No. of cerulean+ embryos	% of the germline that is transgenic	qmc line established
♂10z	265	1	0.38%	qmc161
♀13L	39	9	23.08%	qmc162

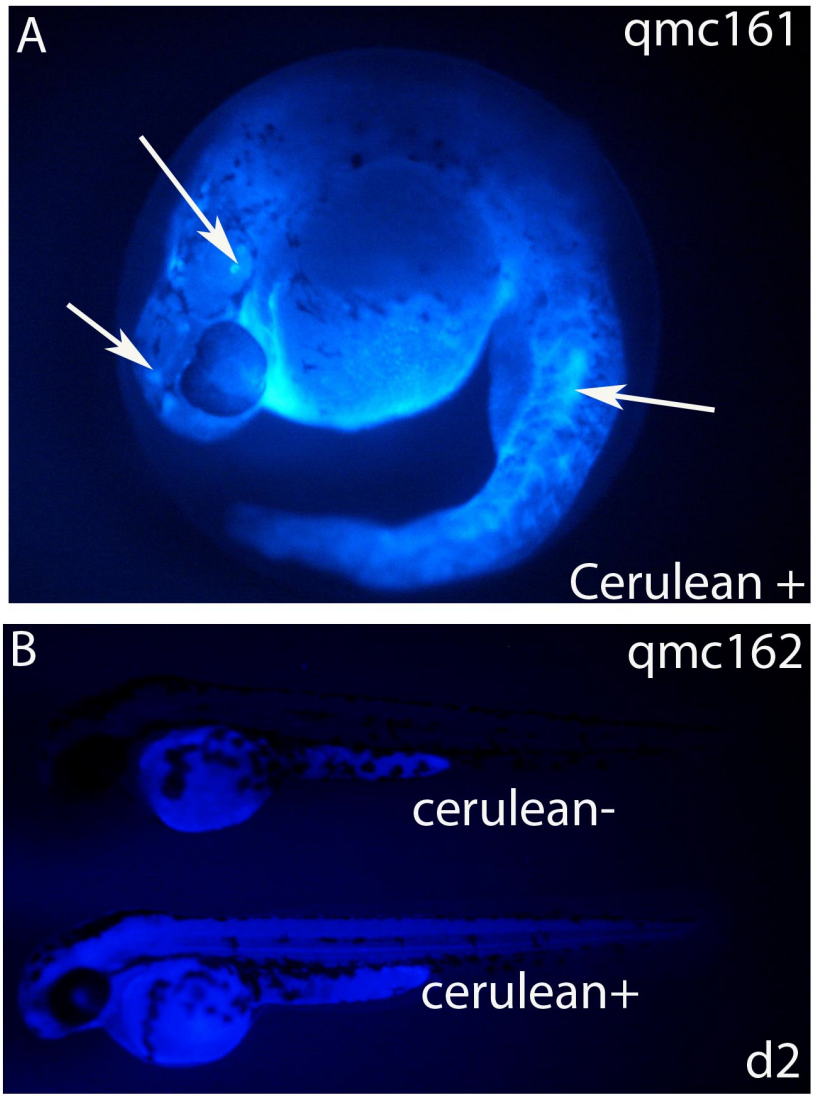


Fig. 39: qmc161 and qmc162 were identified by cerulean expression

A) The qmc161 was the single cerulean positive, abnormal embryo that expressed cerulean more robustly in parts of the head neck and tail regions (white arrows) as observed at around 30-32hpf.

B) The qmc162 were identified by ubiquitous cerulean expression on 2 dpf. Cerulean expression in qmc162 was confirmed by comparing cerulean positive embryos to a sibling cerulean negative embryo.

7.2. Identification of founders from SN44 KDAKT fish

A total of 16 crosses were set up out of which 4 fish were identified as being potential carriers of the KDAKT transgene. The founders were identified by the expression of cerulean among the progeny. Cerulean expression was confirmed by comparing the cerulean positive embryos to a cerulean negative control taken from the same batch (Fig. 40).

The lines generated were qmc171, qmc172, qmc173 and qmc174. The number of embryos and the percentage of the germline that was transgenic are displayed in Table 6.

Cerulean expression in the embryos from all the lines at around 25-28hpf was very convincing (data not shown) and hence the cerulean positive embryos were separated from their cerulean negative siblings. However, on 4 dpf cerulean expression seemed to have diminished in qmc171 and qmc172 (Fig. 40A and B), as compared to qmc 173 and qmc 174 (Fig. 40C and D), which made it extremely difficult to distinguish between a cerulean positive embryo and the negative control (Fig. 40A and B). Hence once the embryos from qmc171 and qmc172 are adults further investigation would be required in order to confirm whether they are transgenic or not. All cerulean positive embryos were grown up.

Expression level differences could be due to the number of copies of the transgene in the germline of the parent fish. As mentioned earlier injection of the transgene at the one cell stage results in mosaicism due to which the percentage of the germline which is transgenic is relatively low in these fish. Furthermore positional effects could also be a possible explanation for expression level differences.

Table 6: Shows the results obtained from crossing the SN47 KDAKT to wt fish in terms of numbers and the percentage of the germline that was transgenic

SN44 fish KDAKT	Total no. of embryos analysed	No. of cerulean+ embryos	Level of cerulean expression observed (strong (+)/weak(w)	% of the germline that is transgenic	qmc line established
♀1z	71	5	w	7.04%	qmc171
♂2z	32	2	w	0.63%	qmc172
♂4z	234	25	+	10.68%	qmc173
♂16L	202	13	+	6.3%	qmc174

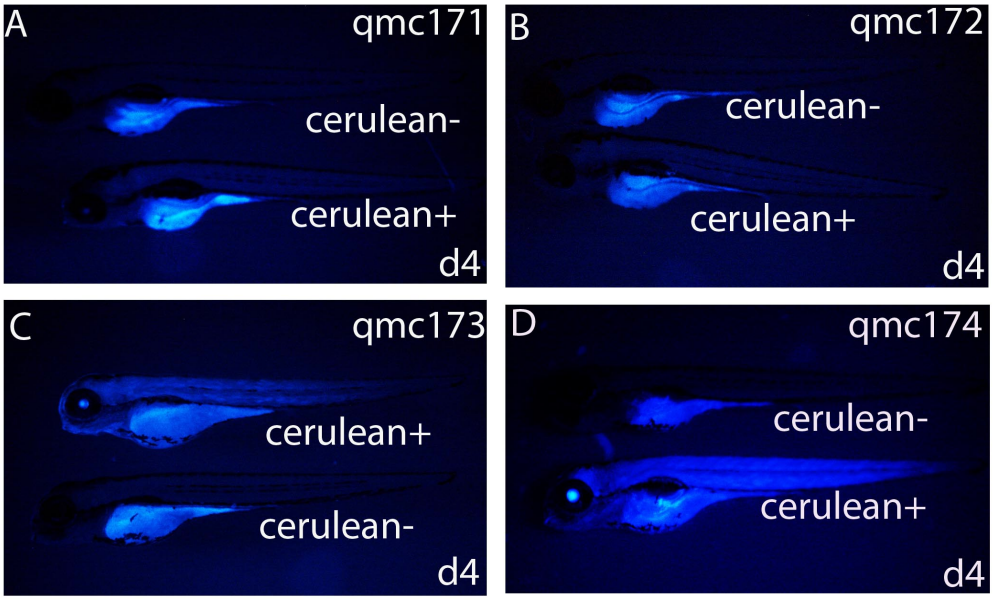


Fig. 40: 4 qmc lines generated by crossing the SN44 KDAKT fish to wild type fish

Cerulean expression as seen on 4 dpf

A-B) qmc171 and 172 show very weak cerulean expression and can barely be distinguished from the negative control (cerulean- embryo)

C-D) qmc 173 and 174 show convincing cerulean expression and can be easily distinguished from the cerulean negative control embryo

In summary we have identified and established new qmc lines, qmc161 and qmc162 for the myrAKT fish. However, qmc161 was an abnormal embryo that had not survived, and the parent was recrossed later but did not yield embryos in the next cross. Qmc162 were 8 embryos that expressed cerulean and had no defects, hence we discontinued the qmc161 line and decided to work with the qmc 162 line.

qmc171, 172, 173 and 174 were established by the KDAKT fish. Although cerulean expression levels were not convincing in qmc171 and 172 we grew them to analyse them in the next generation.

For further reference and convenience the stock numbers of the qmc162 fish is SN69 and that of the qmc174 fish is SN81.

However in order to carry out our further studies a single fish from each line would be sufficient. We could only decide which fish we wanted to work with and which lines we could discontinue by looking at the F2 fish.

7.3. Analysis of the F1 generation of myrAKT and KDAKT fish

Once we had established the myrAKT qmc 162 and the KDAKT1 qmc 171, 172, 173 and 174 lines and these fish that were the F1 generation were now adults and ready to breed, we decided to cross them to wild type fish and analyse the progeny (F2 generation).

7.4. Analysis of F2 embryos from F1 qmc162 myrAKT fish

To analyse the F1 fish we crossed them to wild types (wt).

Out of a total of 5 crosses that were set up 3 were successful, however, the progeny of only one fish expressed cerulean. This was the qmc162 ♀1L. The progeny of this female showed expression level differences, where 130 embryos were cerulean positive (hi) and 57 embryos were cerulean positive (lo) (Table 7) (Fig. 41B). Cerulean expression was not very convincing on 2 dpf as seen in (Fig. 41B). However cerulean expression was stronger in younger embryos in the same batch (Fig. 41C). Younger embryos may be present in the same batch if the fish would have laid at 2 or 3 different occasions while in the breeding tanks. Could the EF1α promoter be responsible for the decrease in cerulean levels as time progressed?

The ratio of cerulean negative to the total number of cerulean positive [cerulean+ (hi) + cerulean+ (lo)] embryos, which is not a 50-50% split (Table 7), would indicate that the parent fish has more than one copy of the transgene.

Currently, there are 25 high expressing and 25 low expressing cerulean positive embryos were taken up stairs to the nursery to be grown the stock number given to them was the same SN101, although kept in separate tanks. This is the F2 generation of myrAKT fish.

Table 7: shows the number of embryos analysed for cerulean expression when myrAKT qmc162 ♀1L was crossed to wt fish

Total no. of embryos analysed	Cerulean negative	Cerulean positive	
		Cerulean+ (hi)	Cerulean+ (lo)
290	103	130	57

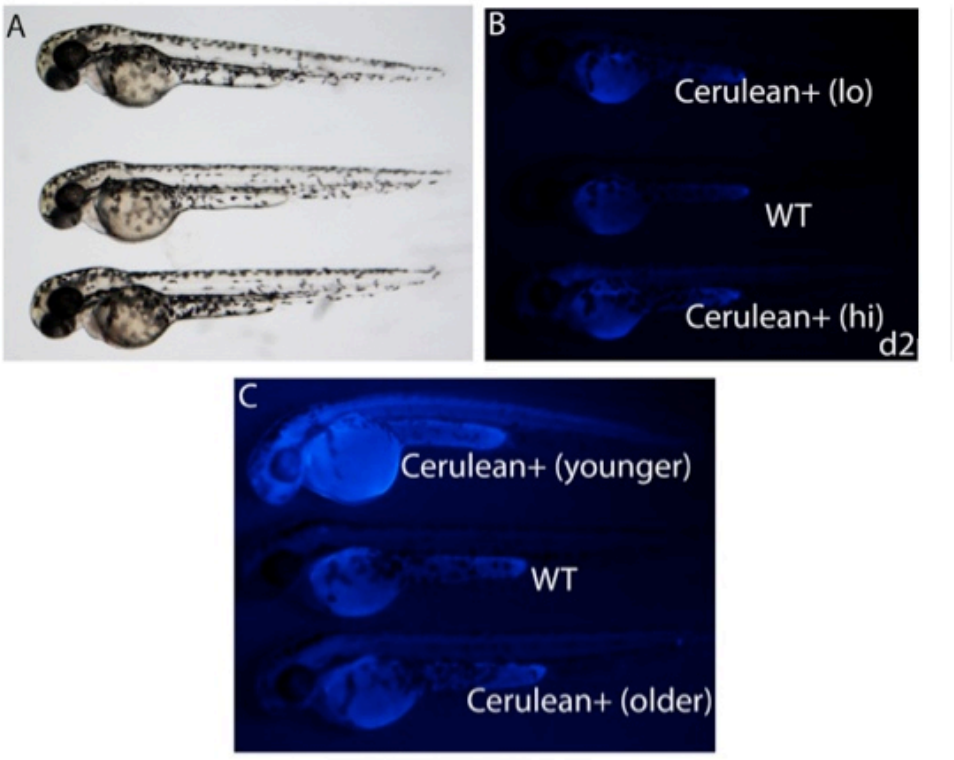


Fig. 41: qmc162 F2 generation

A) A bright filed image of three sibling embryos

B) Shows cerulean+ (hi) and (lo) expressing embryos. Cerulean expression was not very convincing on 2 dpf.

C) a younger embryo from the same batch expresses cerulean at a higher level than an older sibling.

Cerulean expression was confirmed by comparing to cerulean negative siblings.

There seems to be leakiness of myrAKT observed in the male germline. Although this was only an observation and needs further investigation, the progeny of myrAKT males rarely expressed cerulean, even though the parent had expressed cerulean as an embryo. On rare occasions, when cerulean expression was observed, it would be in1 or 2 embryos that expressed cerulean at high levels but were abnormal embryos (data not shown), similar to the qmc161 embryo (Fig. 39A). This shows that the fish is still a carrier. Also, considering the fact that the parent had expressed cerulean, indicates that the parent must have the transgene. This makes us wonder whether the transgene has an adverse effect in the male germline when passed on? This needs further investigation.

7.5. Analysis of F2 embryos from F1 KDAKT fish

Although 4 qmc lines were established with the KDAKT fish, namely qmc171, 172, 173 and 174, we decided to maintain and work with only qmc174 line.

Fish from qmc171, 172, 173 and 174 were crossed to check for cerulean expression; however no expression was seen in the progenies of qmc171 and qmc172 fish. Which was expected as the embryos did not express cerulean very convincingly before they were grown (Fig. 40A and B). Surprisingly although qmc173 gave decent expression of cerulean (Fig. 40C) its progeny did not express cerulean very convincingly (data not shown). As seen above (Fig. 40) the most convincing cerulean expression was seen in the qmc174 (Fig. 40D) embryos and will hence be used to carry out further experiments.

The other three qmc lines qmc171, 172 and 173 were discontinued.

7.6. Analysis of F2 embryos from F1 qmc174 KDAKT fish

A total of four crosses were set up with wt fish. Three of these were successful. The progeny of all of the fish were then analysed for cerulean expression on 1 dpf. Cerulean expression was observed in progeny from all of the 3 fish crossed, which confirmed the parents were all transgenic. For convenience, in Table 8 embryos are broadly divided as cerulean positive and cerulean negative. The numbers (Table 8) suggest that ♀2z has more than one copy of the transgene, hence there isn't a 50-50% split between cerulean positive and cerulean negative embryos. The cerulean positive embryos were more in number. The female is more than 90% transgenic. ♂4z is 52.3% transgenic. ♂3z seems to have only one copy of the transgene where the ratio of cerulean positive embryos to cerulean negative embryos suggests it is almost 50% transgenic.

A variation in expression levels was observed among the progenies of all three fish (data shown only of ♂3z) Fig. 42B). This could be due to positional effects of the transgene.

Table 8: shows the number of embryos analysed from the 3 crosses set up SN81KDAKT qmc174 X wt

SN81 KDAKT qmc174 fish	Total number of embryos analysed	Cerulean negative	Cerulean positive
♀2z	182	17	165
♂3z	289	148	141
♂4z	287	137	150

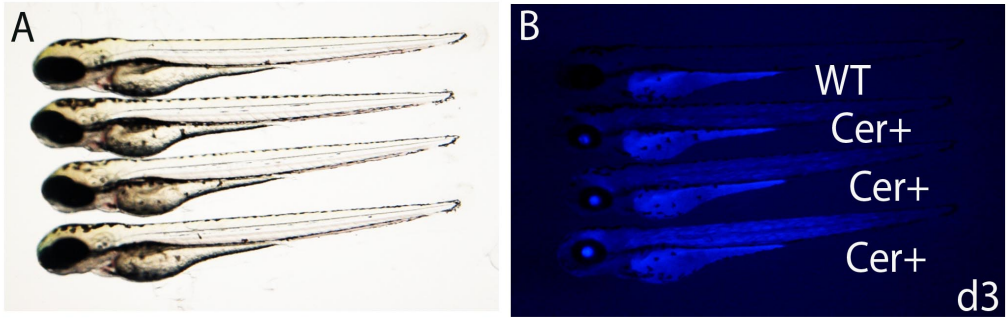


Fig. 42: The progeny of SN81qmc174♂3z shows variations in cerulean expression among its progeny

A) Bright field image of 4 embryos taken together.

B) Three different levels of cerulean expression were observed among siblings. The expression level differences were compared to a wt embryo from the same batch.

Since the embryos from SN81♂3z gave 50% cerulean positive and 50% cerulean negative embryos 50 of the cerulean positive embryos were taken to the aquarium nursery and were given the stock number SN112. These were the F2 generation of the KDAKT fish.

In summary we now had F2 generation fish from both the transgenic lines, namely the myrAKT and the KDAKT lines. These fish would be used to carry out further experiments.

7.7. Recombination of the loxP sites in both the myrAKT and the KDAKT did not give a phenotype

As we already know the 2 constructs for the KDAKT and the constitutively active myrAKT have loxP sites in them (Fig. 38A and B). Recombination of the loxP sites, using a constitutively active Cre protein (flk1Cre) should either block (in case of the KDAKT transgene) or activate (myrAKT) the PI3K pathway in the endothelium.

Hence to see if this actually works the KDAKT was crossed to a qmc101 (flk1Cre). However we did not get any significant phenotype in the embryos obtained from this cross.

Since there were no obvious phenotypes observed in the endothelium of the embryos for the cross between the KDAKT fish and the qmc101 (flk1Cre) line this could either indicate that there might be some subtle defects with respect to blood vessel formation or HSC specification, which cannot be visualised without a reporter. We thus decided to cross the myrAKT and the KDAKT fish to fli1:GFP in order to be able to see defects in the endothelium. Fli1 like flk1 is an endothelial marker. These fish are now growing up.

In summary recombination using the constitutively active Cre protein did not show any significant phenotypic defect. This needs further investigation.

Chapter 8. Discussion

We have identified founders of the myrAKT and KDAKT fish and have upto F2 generations fish growing. We would in the future use these to study the effect of blocking or activating the PI3K/AKT signal transduction pathway on the endothelium and HSC specification.

8.1. Cerulean expression absent in progeny of male myrAKT fish

As seen in the result section the progeny of male myrAKT fish most often failed to express cerulean. In case cerulean was expressed it was expressed at high levels in a mere 1 or 2 embryos that were abnormal. If these fish were cerulean positive as embryos then what would be the reason of them not giving cerulean positive embryos? The fact that there were occasional cerulean expressing abnormal embryos would indicate that the transgene might be present. This would need to be investigated by carrying out a PCR on the cerulean negative progeny of the male fish to check for the presence of the transgene. Furthermore, abnormal embryos seen with high cerulean expression could indicate that there might be some read through of the myrAKT which causes these defects. Hence this could be addressed either by increasing the polyA tails or putting a stop cassette.

8.2. The ef1α promoter could be replaced

Previously in the lab constructs were made for the NICD and dominant negative rbpj to study the Notch pathway. The myrAKT and the KDAKT were made using the same constructs where NICD or the dominanant negative rbpj were replaced by the myrAKT or the KDAKT. Hence Mohammad who is working on the notch lines faced similar problems with cerulean

expression levels which in our constructs are driven by the ef1α promoter. Mohammad is working towards replacing the ef1α promoter with the ubiquitin promoter, in order to get more robust cerulean expression. The fact that the cerulean expression diminishes in embryos as time progresses could be abrogated by using another promoter.

8.3. Recombination using flk1Cre qmc101 line gave no phenotypic defect in KDAKT fish

In order to get a loss or gain of function in the KDAKT or the myrAKT embryos we need to recombine the loxP sites in the transgene. As mentioned earlier this was done on the KDAKT fish that were crossed to the flk1Cre qmc101 line in order to get a loss of function. We however did not see any obvious phenotype. This made us wonder if it could be due to a mutation in base pairs of the KDAKT fragment. However we got both the myrAKT and KDAKT fragments sequenced and sequencing data revealed that both sequences are fine and have no mutations. Hence we could rule out that the myrAKT or KDAKT were not functioning properly.

Mohammad also tried injecting Cre mRNA into the embryos to get recombination and still got no significant phenotype.

Are there subtle defects that cannot be visualised due to the absence of a second reporter? How much of the KDAKT or myrAKT is required to be translated in order to give a significant phenotype of loss or gain of function?

Subtle defects if any could be viewed by crossing the myrAKT or KDAKT fish to a fli1:GFP fish. This double transgenic fish can then be crossed to the flk1Cre in order to get recombination. Now that there is a fli1:GFP transgene in the background that labels the endothelium, we would be able to

view any loss or gain of function in blood vessel formation. In a similar way by crossing the myrAKT or KDAKT fish to the enhancer trap line qmc551 (gfi1:GFP) we could visualise the effect of blocking or activating PI3K/AKT on HSC specification.

8.4. PI3K/AKT Cell autonomous or non-cell autonomous?

Past studies done in the lab with the LY294002 compound that is a known PI3K/AKT inhibitor have shown significant reduction in venous and HSC transcriptional factor runx1. Is PI3K/AKT signalling a requirement of the cell itself or is it a requirement of the cell next door? This is a question that needs to be addressed in future experiments with the help of blocking or activating the PI3K/AKT signalling pathway using the lines established qmc174 and qmc162.

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