THE EVOLUTIONARY ORIGINS OF VERTEBRATE HAEMATOPOIESIS: INSIGHTS FROM NON-VERTEBRATE CHORDATES

A thesis submitted to the University of Manchester for the degree of MPhil Evolutionary Biology in the Faculty of Life Sciences.

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LIST OF ABBREVIATIONS

AGM – Aorta-Gonad-Mesonephros

BP – Base Pairs

CHT - Caudal Haematopoietic Tissue

DNA – DeoxyriboNucleic acid

DPF – Days Post-Fertilization

EST – Expressed Sequence Tag

GRN – Gene Regulatory Network

HPF – Hours Post-Fertilization

HSC – Haematopoietic Stem Cell

ICM – Intermediate Cell Mass

MM – Millimoles

MYA – Million Years Ago

NL – Nanolitres

PBI – Posterior Blood Island

PG – Picograms

PLM – Posterior Lateral Mesoderm

PSC – Posterior Signalling Centre

RNA – RiboNucleic Acid

TCR – T-Cell Receptor

TPM = Transcripts Per Million

VLR – Variable Lymphocyte Receptor

WGD – Whole Genome Duplication

ABSTRACT

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The evolutionary origins of vertebrate haematopoiesis: insights from non-vertebrate chordates

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Both vertebrates and non-vertebrates have haematopoietic systems; however the vertebrate system appears to produce a greater diversity of blood cell types. Erythrocyte and lymphocytes may be examples of vertebrate-specific novelties. To understand how they evolved, the haematopoietic gene regulatory network of the vertebrate ancestor must be inferred from studying extant organisms. In this thesis gene expression analysis was used to identify which non-vertebrate chordate species is the best candidate to represent the vertebrate ancestor.

Homologues of *ikaros,* a transcription factor involved in vertebrate haematopoiesis, were expressed in the developmental precursors of haemocytes in ascidian embryos and what appear to be primordial germ cells in amphioxus embryos. Reanalysis of publicly available transcriptome data suggest that homologues of a number of genes expressed in vertebrate haematopoietic cells or blood cells were expressed in ascidian haemocytes. These data indicate that ascidians represent the better candidate organism.

In addition, the evolution of the transcription factors gata1, gata2 and gata3 was investigated. gata1 plays critical roles in the development of erythrocytes, a lineage which appears to be absent from non-vertebrate chordates. Knockdown and rescue experiments revealed that amphioxus gata1/2/3 and zebrafish gata1 are functionally equivalent in erythrocyte development. This highlights the importance of cis-regulatory changes in the evolution of gata1 and erythrocytes. A 934bp regulatory region of ascidian gata1/2/3 did not produce expression in zebrafish haematopoietic cells. However, now that the method has been successfully established, it could be used to investigate a number of questions related to the evolution of vertebrate haematopoiesis.

DECLARATION

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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INTRODUCTION

Explaining the origins of complex features is one of the oldest and biggest challenges in evolutionary biology. Charles Darwin wrote of intricate organs, such as the eye, evolving through "numerous, successive, slight modifications" (Darwin 1859). It is only very recently – thanks to advances in genomics and developmental biology – that we are able to shed light on many of these modifications. Alterations to developmental programs and their gene regulatory networks (GRNs) may explain much of the diversity found within multicellular organisms. This perspective forms the basis of the field of 'evo devo' (evolutionary developmental biology).

Many organs and systems are composed of several cell types. The elaboration of these features can often be traced back to the functional segregation of cell types (Arendt 2008). The transition from one generalist cell type to many specialist cell types may have adaptive benefits similar to those of a 'division of labour'. The challenge is to explain how GRNs that produce cellular uniformity are rewired to produce cellular diversity.

A popular dichotomy of the animal kingdom is that of vertebrates and invertebrates. This seems unnatural, as invertebrates (hereafter referred to as 'non-vertebrates') do not form a monophyletic group. But perhaps the basis of this is the commonly held view that vertebrates are considerably more complex than their closest cousins. Features specific to vertebrates include neural crest cells, sensory placodes, an endoskeleton and an adaptive immune system (Donoghue & Purnell 2006; Heimberg *et al* 2008). Associated with this is a considerable increase in the number of transcription factors (caused by two rounds of whole genome duplication (WGD)) (Putnam *et al* 2008), and microRNA families (invented *de novo*) (Heimberg *et al* 2008). It remains to be seen what effects these genomic changes may have had on vertebrate evolution.

It is likely that functional segregation of cell types played a major role in vertebrate-specific innovations. The skeleton appears to be an example of this. The oral cirri GRN in amphioxus (an non-vertebrate chordate) contains elements of the osteogenesis and chondrogenesis GRNs in jawed vertebrates. This suggests that mineralised bone and cartilage evolved from a common ancestral skeletal cell type (Kaneto & Wada 2011).

Another complex feature that may have evolved through this process is the haematopoietic (blood cell development) system. Vertebrates have several blood cell types with a variety of functions. Erythrocytes (also known as red blood cells) transport oxygen, thrombocytes (known as platelets in mammals) are involved in blood coagulation, myeloid cells (including granulocytes and macrophages) contribute to the innate immune response, and lymphocytes (including B-cells and T-cells) contribute to the adaptive immune response. Some non-vertebrates, such as bloodworms (Glomski *et al* 1990) and sea cucumbers (Hoffmann *et al* 2012), have independently evolved erythrocytes. However, for the most part the blood cells of non-vertebrates are limited to innate immune cells (Holkenstein 2006). If the vertebrate blood cells evolved and diversified from an ancestral innate immune cell, then this would represent a striking example of the importance of cellular diversification in vertebrate evolution, one which would be ripe for investigation. Moreover, the vertebrate-specific blood cell types can be considered evolutionary novelties, and understanding the origin of novelties is central theme of evo devo. In addition, there is considerable interest in the origins of the adaptive immune system, as highlighted by some recent high-profile papers on the subject (Alder, *et al* 2005, Guo *et al* 2009, Bajoghli *et al* 2011). The evolution of lymphocytes would have been a critical part of this.

The evolution of oxygen transport would have probably facilitated the growth of larger brains and bodies, and an adaptive immune system would have been advantageous when entering new environments. Both may have been essential for the successful expansion and diversification of

the vertebrates. The historical significance of the invention of novel blood cell types cannot be easily overstated. This demands a comprehensive comparison of the blood cells and their development across the animal kingdom.

Fruit flies were chosen for inclusion in this introduction because they are the non-vertebrate that has the best characterised haematopoietic system. The study of sea urchin haematopoiesis has only begun quite recently; however, as deuterostomes, they are more closely related to the vertebrates than the fruit flies. The closest relatives of vertebrates are tunicates and cephalochordates, the non-vertebrate chordates. Both have been suggested to be the best representative of the ancestor of vertebrates. The advantages and disadvantages of both are described below. Cyclostomes (jawless vertebrates) have some very interesting similarities and differences to the gnathostomes (jawed vertebrates), also outlined below. The description of gnathostome haematopoiesis focuses on zebrafish because it is now considered the most useful and tractable organism for studying haematopoiesis in detail (Carroll & North 2014).

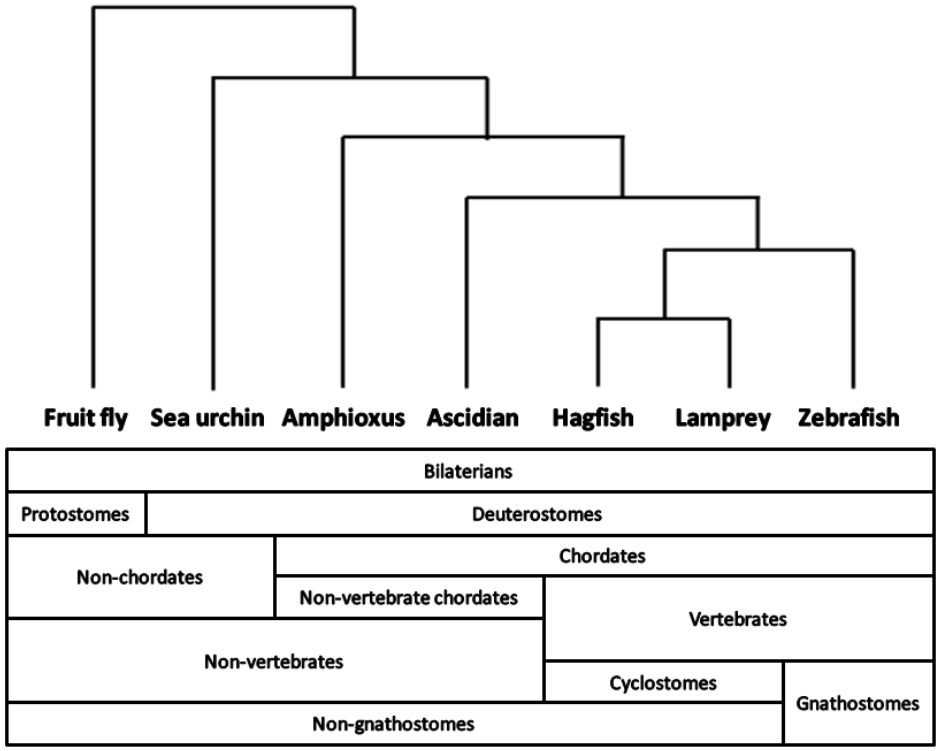

Figure 1 – Phylogenetic relationships between the organisms studied in this thesis.

Haematopoiesis in gnathostomes

All gnathostomes (vertebrates with jaws) experience two waves of haematopoiesis in their development: the early-embryonic 'primitive' wave, producing mainly erythrocytes and some myeloid cells, and the later 'definitive' wave, producing all of the blood cells. The lineages are roughly the same too: erythrocytes (for oxygen transport), thrombocytes (for wound repair), myeloid cells (for innate immunity) and lymphoid cells (for adaptive immunity). The molecular regulation is also well-conserved across the gnathostomes. The timing and positioning of different developmental events do, however, vary between species (Carradice & Lieschke 2008; Paik & Zon 2010). In this introduction, only zebrafish (*Danio rerio*) haematopoiesis has been described in this respect.

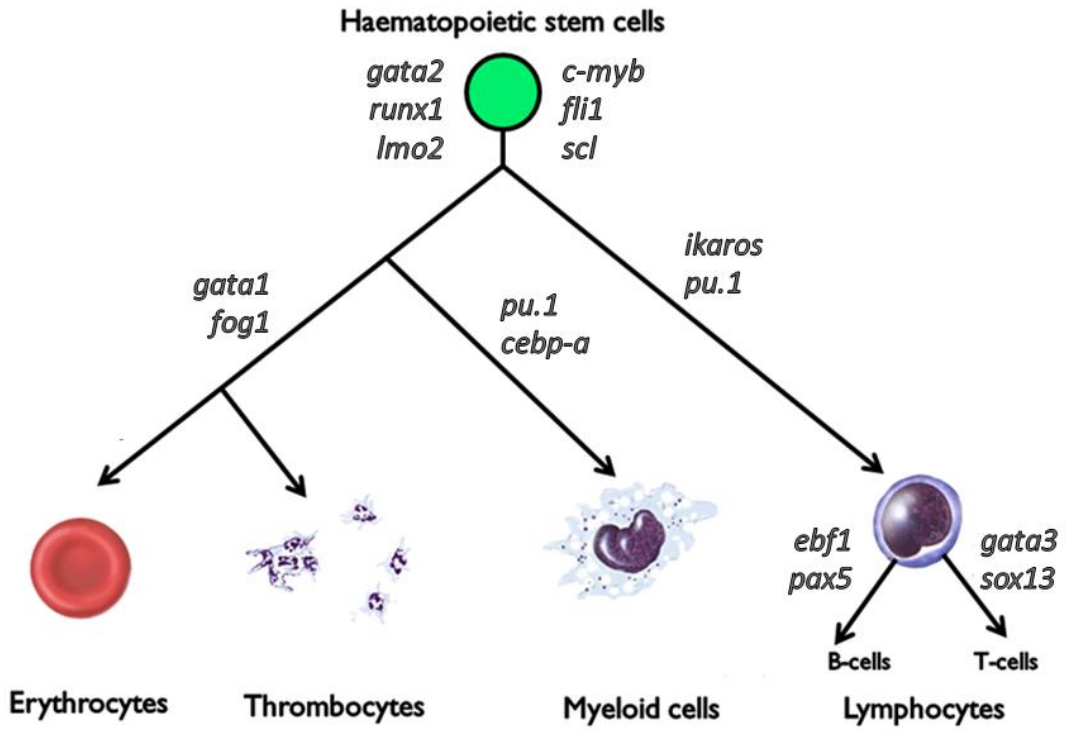

Figure 2 – A simplified overview of definitive haematopoiesis in gnathostomes.

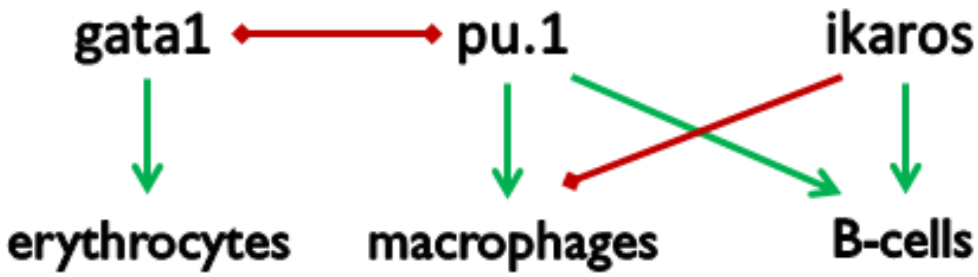

Figure 3 – A schematic for gene regulation in major haematopoietic cell fate decisions. gata1 promotes the development of erythrocytes and at high levels pu.1 promotes the development of macrophages (a type of myeloid cell). gata1 and pu.1 repress each other's expression. ikaros represses the development of macrophages and promotes the development of B-cells. At low expression levels, pu.1 also promotes the development of B-cells. (Adapted from Solek 2012).

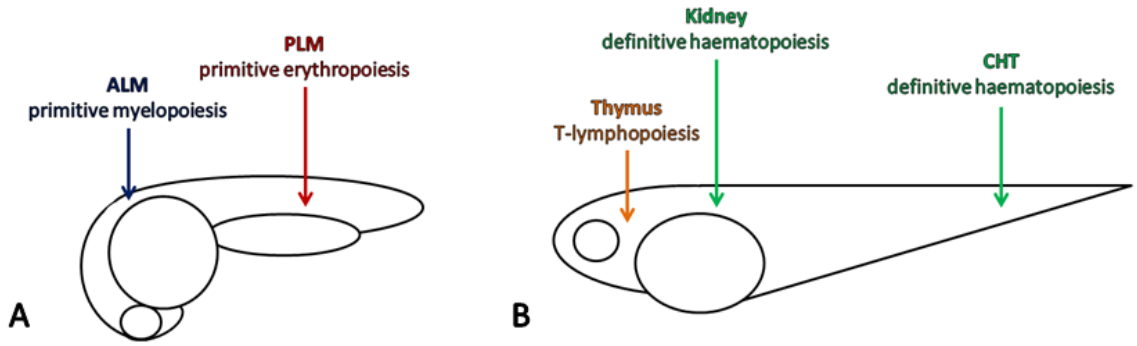

Figure 4 – Sites of haematopoiesis in zebrafish embryos at A) 24hpf and B) 72hpf. (Adapted from Chen & Zon 2009).

Haemangioblasts

Haemangioblasts are progenitors common to angioblasts (which give rise to blood vessels) and blood cells from the primitive wave of haematopoiesis. Bmp signalling, vegf signalling and *cloche* are important in the specification of haemangioblasts from the ventral mesoderm (Thompson *et al* 1998; Liang *et al* 2001; Liu *et al* 2008). This occurs at the 2-somite stage – around 10 hours post fertilisation (hpf) (Paik & Zon 2010). These induce expression of the transcription factor *fli1*, which in turn induces expression of the tyrosine receptor kinase *flk1* and the transcription factors *gata2, etsrp, runx1*, *scl* and *lmo2*. Severe reductions of haemangioblasts are seen in mice with *fli1* -/- mutations and *Xenopus* (clawed frogs) and zebrafish injected with morpholinos that knock down *fli1* gene expression. gata2 may also drive *scl* expression in conjunction with fli1 (Liu *et al* 2008). *gata2* -/- mutant mice die early from severe anaemia (Tsai *et al* 1994). scl and lmo2 then form a complex to direct haemangioblasts towards the fate of blood cells (Patterson *et al* 2007). The first blood cells emerge at the 4-somite stage (11 hpf) in the posterior lateral mesoderm (PLM) and the anterior lateral mesoderm (ALM) (Paik & Zon 2010). *scl* -/- mutant mice, *scl* morphant zebrafish, *lmo2* -/- mutant mice and *lmo2* morphant zebrafish all fail to develop primitive blood cells (Patterson *et al* 2007). In zebrafish, *gata4, gata5* and *gata6* are all essential for haemangioblast specification (Peterkin *et al* 2009).

Primitive erythropoiesis

At the 4 somite stage, a subset of the *scl*+, *lmo2*+, *gata2*+ cells in the PLM in zebrafish start expressing the transcription factor *gata1*, the ubiquitin ligase *tif1γ* and the Kruppel-like factor *biklf* (Patterson *et al* 2007). tif1γ up-regulates *gata1* expression, and gata1 autoregulates itself, as well as

upregulating *tif1γ* and repressing expression of the transcription factor *pu.1* (Monteiro *et al* 2011). Loss of either *gata1* or the transcription factor *fog1* leads to increased expression of myeloid-specific genes in the ICM (Amigo *et al* 2009). At the 12-somite stage (16 hpf), the proerythroblasts express erythroid-specific genes such as globin genes, and migrate medially forming the intermediate cell mass (ICM). By 24 hpf the erythroblasts enter circulation and express genes such as the heme synthetic enzyme *alas2* and *carbonic anhydrase* as they develop into erythrocytes (Galloway *et al* 2005). *meis1*, a transcription factor important in primitive erythrocyte maturation, has been shown to be independent of gata1 but under the control of scl (Cvejic *et al* 2011). The ratio of erythropoiesis to myelopoiesis is maintained by sumoylation of cebpα (Yuan *et al* 2011).

Some thrombocytes have also been detected in the ICM, but how they are specified and regulated has not yet been studied in detail (Warga *et al* 2009).

Primitive myelopoiesis

The transcription factor *pu.1* is expressed in a subset of the *scl*+ *lmo2*+ *gata2*+ cells in the ALM from the 6-somite stage (12 hpf). *pu.1* morphants express the erythroid-specific *gata1* and globin genes in ALM, suggesting that pu.1 represses *gata1* expression, and thus maintains the myeloid fate (Rhodes *et al* 2005; Monteiro *et al* 2011). *pu.1*+ cells migrate towards the midline and over the yolk (Lieschike *et al* 2002). They then differentiate into granulocytes expressing *mpo* (Bennett *et al* 2001), or macrophages expressing *l-plastin* (Herbomel *et al* 1999). *pu.1* morphants show a loss of both macrophages and granulocytes in primitive myelopoiesis (Su *et al* 2007). Irf8, which is downstream of *pu.1*, has been shown to promote the macrophage fate (Li *et al* 2011). Knockdown of microRNA *mir146a*, a target of pu.1, inhibited formation of macrophages (Ghani *et al* 2011).

A small number of *pu1*+ myeloid progenitors form in the ICM, and migrate to the midline at the 14-somite stage. These also differentiate into *mpo*+ neutrophils or *l-plastin*+ macrophages (Bennett *et al* 2001).

The transient wave

Between around 24 to 36 hpf, there is a 'transient' wave of haematopoiesis in the posterior blood island (PBI), which later becomes the caudal haematopoietic tissue (CHT; see below). *gata1* and *pu1* expression is detected here. The progenitors at the stage can only produce two blood lineages, and so cannot be described as haematopoietic stem cells (HSCs) (Bertrand *et al* 2007). Therefore, definitive haematopoiesis has not yet begun.

Haematopoietic stem cells

Definitive HSCs have the ability to develop into any of the blood lineages. They first arise in a region of the ventral wall of the dorsal aorta known as the aorta-gonad-mesonephros (AGM) at around 30 hpf. The immature HSCs express the transcription factors *c-myb* and *runx1* (Kalev-Zylinska *et al* 2002) and the surface marker CD41 (Kissa *et al* 2008). Some of these cells then migrate to a posterior region of the tail known as the CHT at 2 days post-fertilisation (dpf). There is further migration to the thymus at 3 dpf (these are *ikaros*+ (Willet *et al* 2001)) and to the pronephros in the kidney at 4 dpf (Murayama *et al* 2006; Jin *et al* 2007). *runx1* morphants in zebrafish show lower levels of *c-myb* in the dorsal aorta (Burns *et al* 2005). *runx1* -/- mutants in mice lack fetal liver haematopoiesis and die after around 12.5 days (Okuda *et al* 1996). *c-myb* mutant zebrafish fail to begin definitive haematopoiesis (Soza-Ried *et al* 2010). Notch signalling is important in specifying HSCs. The Notch signalling mutant in zebrafish, *mind bomb,* has normal primitive haematopoiesis but fails to develop definitive HSCs. *Mind bomb*-specific

morphants lose *c-myb* and *runx1* in the dorsal aorta (Burns *et al* 2005). This is also found in the morphants of *scl* (Patterson *et al* 2005), which is also expressed in definitive HSCs (Chen & Zon 2009). In mice, both *runx1* and *runx3* have been shown to be direct targets of scl (Landry *et al* 2008).

Definitive myelopoiesis

Definitive myelopoiesis begins in the CHT at 3 dpf and then switches to the kidney at 4 dpf. In the CHT, pu.1 autoregulates itself and represses the expression of erythroid-specific genes (Monteiro *et al* 2011). In mice, pu.1 induces expression of *egr-2* and *nab-2*, which repress *gfi1*expression. gfi1 induces expression of *cebpα* and represses *pu.1*. The relative levels of pu.1 and cebpα determine whether the common myeloid progenitors become macrophages/monocytes (higher pu.1) or granulocytes (higher cebpα) (Dahl *et al* 2003; Spooner *et al* 2009). Again the macrophages/monocytes express *l-plastin* and the granulocytes express *mpo* (Bennett *et al* 2001). In mice, c/ebpα and c/ebpξ interact to promote granulopoiesis (Friedman 2007).

Definitive erythropoiesis

Expression of *gata1* and haemoglobin genes begins at 3.5 dpf in the CHT, marking the beginning of definitive erythropoiesis (Jin *et al* 2009). As with definitive myelopoiesis, erythropoiesis moves to the kidney. Haemoglobin gene expression is first detected here around 5 dpf (Jin *et al* 2009). In the CHT, tif1y upregulates expression of *gata1* but downregulates expression of *pu.1*. gata1 downregulates both *tif1y* and *pu.1,* and autoregulates itself. This promotes the erythroid fate (Monteiro *et al* 2011). gata1 regulates erythropoiesis by its interactions with other transcription factors such as scl, lmo2 and fog1 (Kerenyi & Orkin 2010). Direct targets of gata1 include globin

genes, heme biosynthesis enzymes, *epo* (the primary growth factor for erythroid cells) and its cell-surface receptor *epoR*, *gata2* (Ferreira *et al* 2005), and the locus of microRNAs *mir144* and *mir451* (Dore *et al* 2008). mir451 regulates a number of genes (Rasmussen *et al* 2010), including *gata2* (Pase *et al* 2009). *mir451* morphants have impaired maturation of erythrocytes (Dore *et al* 2008). sox6 regulates the survival of definitive erythrocytes and is also involved in globin gene switching (Hagiwara 2011).

Definitive lymphopoiesis

T-cell progenitors are first detected in the thymus around 3 dpf. T-cell-specific markers such as *ikaros*, *gata3*, *lck*, *rag1, rag2* and *tcrα* are expressed from around 4 dpf (Danilova *et al* 2000; Trede *et al* 2001). In *ikaros* mutants, T-lymphopoiesis is absent up to 14 dpf, when *rag1*+ cells finally appear in the thymus (Schorpp *et al* 2006). Studies in mice have shown that the tcr signal induces *gata3*, *runx3* and *thpok* expression. gata3 upregulates *thpok,* and thpok and runx3 antagonistically inhibit each other. gata3 activates CD4 lineage genes as to promote the T-helper cell lineage. runx3, on the other hand, activates CD8 lineage genes to promote the cytotoxic T cell lineage (Muroi *et al* 2008; Wang *et al* 2008; Egawa *et al* 2008). fog1 represses gata3-mediated T-helper cell development (Kurata *et al* 2002). sox13 promotes T-γδ cell development at the expense of T-αβ cell development (Melichar *et al* 2007).

The recombination activating gene *rag1* is expressed in the pancreas at 4 dpf, and *cu*, which encodes a subunit of the IgM immunoglobin, is expressed in the pancreas at 10 dpf. This suggests that the pancreas is the first site of B-lymphopoiesis. *rag1* expression is not detected in the kidney until 3 weeks post fertilisation (Danilova & Steiner 2002). In mice, the transcription

factors ebf1, e2a and pax5 have been shown to be important in the development of the B cell lineage (Nutt & Kee 2007).

Definitive thrombopoiesis

Thrombopoiesis begins in the CHT at 2 dpf, with the detection of strong expression of CD41, a platelet-specific marker. The thrombocytes first enter circulation around 3 dpf. Thrombopoiesis shifts from CHT to the kidney at around 5 dpf (Lin *et al* 2005). MicroRNAs mir126 and mir150 are required to regulate the cell fate decisions between erythrocyte and thrombocyte lineages. Both reduce *c-myb* levels, promoting thrombopoiesis at the expense of erythropoiesis (Lu *et al* 2008; Grabher *et al* 2011). Thrombopoietin is a hormone important in the production of megakaryocytes (the precursors of platelets) in mammals (Kaushansky 1995). Although zebrafish do not have megakaryocytes (Carradice & Lieschke 2008), knockdown of *c-mpl*, the thrombopoietin receptor, inhibits the production of thrombocytes. This is also found in *scl* knockdown morphants (Lin *et al* 2005) and *runx1* truncation mutants (Sood *et al* 2010), showing these genes are necessary for thrombopoiesis. *gata1* is expressed in immature thrombocytes; however this reduces as they mature, and is replaced by *fli1* expression (Jagadeeswaran *et al* 2010). fog1 has an essential role in thrombopoiesis, which requires interaction with either gata1 or gata2 (Amigo *et al* 2009).

Haematopoiesis in cyclostomes

Lampreys and hagfish are members of the superclass *Cyclostomata*. These fishes have most characteristic vertebrate features, but lack jaws (Shimeld & Donoghue 2012). Hagfish even lack true vertebrae (Ota *et al* 2011). They separated from gnathostomes around 500 Mya (Hedges *et al* 2009). Whether or not the cyclostomes experienced both of the WGDs is unclear, as different

conclusions can be made from different gene families. A recent analysis of the lamprey genome indicated that both WGDs probably did occur before the cyclostome/gnathostome split (Smith *et al* 2013).

Cyclostomes have lymphocytes. However, these blood cells contribute to the adaptive immune response via variable lymphocyte receptors (VLRs) rather than the B-cell receptors (BCRs) and T-cell receptors (TCRs) of gnathostome lymphocytes. The cyclostome lymphocytes come in three types: VLRA+, VLRB+ and VLRC+. The expression profiles of these cell types are similar to those of gnathostome T-αβ cells, B-cells and T-γδ cells, respectively. For example, the VLRA+ cells express a *gata1/2/3* homologue, the VLRB+ cells express a *pax5* homologue, and the VLRC+ cells express a *sox13* homologue (Guo *et al* 2009).

Cyclostomes also have thrombocytes, macrophages, granulocytes and erythrocytes (Page & Rowley 1983). Their erythrocytes use haemoglobins which are homologous to the cytoglobins of gnathostomes, and are optimised for oxygen transport in a structurally different way (Hoffmann *et al* 2012). This makes it seem as if the cyclostome erythrocytes and lymphocytes are not homologous to their vertebrate counterparts. However, it is possible that the different cell types evolved *before* they were optimised (at the molecular level) for their specialised functions. If they are homologous at this level, we would expect them to have very similar GRNs for development. Expressed sequence tags (ESTs) corresponding to *ikaros* and *spi* have been obtained from the lymphocytes of lampreys (Mayer *et al* 2002); though it is not yet known whether these genes have similar functions to their gnathostome orthologues.

Very little recent work has been done on haematopoietic development in lampreys. Based on observational work, haematopoiesis appears to start in the blood islands of the posterior lateral plate mesoderm of the embryo, then moves on to the typhlosole (an internal fold of the

intestine), supraneural body (a fat column along the central nervous system), and kidney (Amemiya *et al* 2007). Lampreys have thymus-like structures in their gill baskets known as thymoids. Only in these thymoids is a homologue of a gnathostome thymopoietic gene (*foxn1*) coexpressed with VLRA genes. This suggests the thymoids function in a similar way to the T-lymphocyte-producing thymus of gnathostomes (Bajoghli *et al* 2011). Expression of e*ts1b* and *myb* has been reported in haematopoietic precursors in the blood islands (Sauka-Spengler *et al* 2007; Onimaru *et al* 2011). But this is the extent of the molecular work done on lamprey haematopoiesis.

Haematopoiesis in ascidians

Tunicates (previously known as urochordates) are non-vertebrate chordates. They are a diverse group, but their common feature is the cellulose-containing tunic which covers their bodies (Lemaire 2011). Despite being the most closely related non-vertebrates to the vertebrates (Delsuc *et al* 2006), they are not commonly thought of as good representatives of the chordate or vertebrate ancestor (Holland & Gibson-Brown 2003, Lemaire 2011). While the swimming larvae have a typical chordate body plan, the adult forms of different species are diverse and often very uncharacteristic of chordates (Lemaire 2011). They are also genomically quite derived, with many gene losses and signs of rapid evolution (Holland & Gibson-Brown 2003). However, rudimentary neural crest cells have been identified in a tunicate but not in any other non-vertebrate (Abitua *et al* 2012). It may be that tunicates are poor representatives of the vertebrate ancestor in terms of morphology but good in terms of internal systems. Like the vertebrates, tunicates have a heart and circulating blood cells (Wright 1981).

One major group of the tunicates are the ascidians (sea squirts), which are vase-like filter feeders that spend their adult life attached to rocks or shells (Lemaire 2011). The solitary ascidian *Ciona*

intestinalis has been widely used for developmental studies. Its blood cells develop from mesenchyme during metamorphosis. The specific precursor lineages are labelled A7.6, B7.7 and B8.5 (Tokuoka *et al* 2004). In adults, new blood cells form from nodules in the pharyngeal wall (Di Bella & De Leo 2010). Blood cells have been categorised as either phagocytic (phagocytose pathogens, like the macrophages of vertebrates) or cytotoxic (produce toxins, like some of the lymphocytes of vertebrates) (Arizza & Parrinello 2009). This has also been described in the colonial ascidian *Botryllus schlosseri* (Ballarin & Cima 2005). No specialised oxygen transport cells (i.e. erythrocytes) have been observed and tunicates lack an adaptive immune system (Nonaka & Satake 2010).

Homologues of *l-plastin,* a myeloid cell marker in zebrafish, and *fog1*, which plays important roles in erythropoiesis, have been shown to be expressed in the blood cells of young *C. intestinalis* adults (Wakoh *et al* 2004; Ogasawara *et al* 2006). In the colonial ascidian *B. schlosseri,* an antibody against CD57, which marks T-cells and Natural Killer cells in vertebrates, recognised only the cytotoxic blood cells (Ballarin & Cima 2005). In *C. intestinalis,* an antibody against CD94, which marks Natural Killer cells in vertebrates, did the same (Arizza & Parrinello 2009). However, this is as far as the blood cells have been characterised at the molecular level in ascidians. No functional experiments have been done related to haematopoiesis. Embryonic expression patterns of varying quality are available for many genes. Two that are notable are *gata1/2/3* and *fli/erg-a*, which are both expressed in the mesenchyme (i.e. the developmental precursors of haemocytes) (Imai *et al* 2004).

Haematopoiesis in amphioxi

Amphioxi (also known as lancelets) are the only living cephalochordates, and, like the tunicates, are non-vertebrate chordates. They are filter-feeders, but unlike the ascidians, they are free-living even in adulthood. They burrow into the sand or gravel of tropical and temperate waters (Bertrand & Escriva 2011). They are widely considered the best living representatives of the vertebrate ancestor. They have had fewer independent gene losses than the tunicates (Louis *et al* 2012), and are more morphologically similar to fossils of the putative basal chordate *Pikaia* (Schubert *et al* 2006). They also have classic chordate structures, including a notochord, a neural tube, a pharynx with gill slits, segmented axial muscles, a post-anal tail, and a pronephric kidney. Where vertebrates have 2 or more paralogues of a transcription factor, amphioxus usually has one (Putnam *et al* 2008). This has made amphioxus the most commonly used model organism for studying the evolution of vertebrates (Bertrand & Escriva 2011).

Amphioxi do not have a true heart, nor do they have circulating blood cells. However, phagocytic cells have been observed in the gut of adults (Rhodes *et al* 1982, Rowley *et al* 1984, Han *et al* 2010), which appear to develop from the lining of the coelom (Rhodes & Ratcliffe 1983). In addition, some cells which resemble lymphocytes have been observed in the gills (Rowley *et al* 1984, Huang *et al* 2007, Han *et al* 2010), but these cells have not yet been shown to have immune activity. Other than this observational work, haematopoiesis has not been studied in amphioxus.

The embryonic expression patterns of a few genes that are potentially related to haematopoiesis have already been published, but in papers mainly focusing on other aspects of development. *gata1/2/3* was expressed in *B. belcheri* (the Chinese species of amphioxi) in the cerebral vesicle, anterior gut and weakly in a posterior region, possibly the hindgut (Zhang & Mao 2009). In the

European species of amphioxi, *B. lanceolatum, gata1/2/3* was expressed in right and left coelomic diverticula, the club-shaped gland, the endostyle and the pharynx. There was some overlap of expression with *scl* and *vegfr* in the pharyngeal mesoderm. vegfr was then expressed in migrating cells, which could be haematopoietic precursor cells. However they could also be endothelial-like cells. Amphioxi do have vessels and hemal fluid, although they lack would could be described as true endothelium and a true heart (Pascual-Anaya *et al* 2013). Macrophage-like cells appear in the extracellular matrix (ECM)-filled tubular space between the basal surfaces of the endoderm and mesoderm in 14-day old larvae. This positioning is similar to the angioblasts in vascular lumen formation in the mouse. The macrophage-like cells then clear the ECM by proteolysis and phagocytosis to generate vascular lumen as part of the development of cardiovascular tubes (Kucera *et al* 2009). Migrating cells present in the alimentary canal of 72 hours old larvae have been suggested as the progenitors of the macrophage-like cells (Lin *et al* 2011).

The rest of the genes were analysed in *B. floridae* (the American species of amphioxi). *myb* does not seem to have a specific embryonic expression pattern (Onimaru *et al* 2011). *runx* was expressed in the hindgut, notochord and neural tube (Hecht *et al* 2008). p*ax2/5/8* was expressed in optic support cells, nephridium, thyroid-like structures and pharyngeal gill slits (Kozmik *et al* 1999). *ebf* was expressed in the central nervous system, epidermal sensory neurons and pharyngeal gill slits (Mazet *et al* 2004).

In the adults of *B. belcheri*, *ikaros* is expressed in ovary and gills, and *ebf* is expressed in the ovary, gills and intestine (Huang *et al* 2007). Injection of bacteria into *B. belcheri* adults leads to an upregulation in expression of *ikaros*, *ebf*, *spi/pu.1*, *fli/erg* and *cebp* (Huang *et al* 2007; Huang *et al* 2011).

Haematopoiesis in sea urchins

Far from being specific to chordates, it actually seems that phagocytic cells and an innate immune response are present in virtually all animals (Hartenstein 2006).

As well as the chordates, the deuterostomes include the echinoderms and the hemichordates. Sea urchins, which are echinoderms, have two main types of blood cell: blastocoelar cells, which are phagocytic and come in several sub-types, and pigment cells, which migrate to sites of infection and injury (Solek 2012, Solek *et al* 2013). Both derive from secondary mesenchyme cells (Calestani *et al* 2003). *gata1/2/3, runx, spi/pu.1, ikaros*, *scl, lmo2, fl/erg* are all expressed in the blood cells or their progenitors (Pancer *et al* 1999; Hilbino *et al* 2006; Rizzo *et al* 2006; Solek 2012, Solek *et al* 2013). Knockdown of *gata1/2/3* caused problems with blastocoelar development, whereas knockdown of *scl* disrupted the segregation of pigment cell and blastocoelar cell precursors (Solek 2012, Solek *et al* 2013). This suggests that there is some conservation of the haematopoietic GRN across the deuterostomes.

Haematopoiesis in fruit flies

Similar to vertebrates, fruitfly (*Drosophila*) haematopoiesis occurs in two waves: first from the head mesoderm in the early embryo and then from the lymph gland in the larva. The haematopoietic progenitors in *Drosophila* are known as prohaemocytes. These then differentiate into the three types of haemocyte: plasmatocytes, crystal cells and lamellocytes. Plasmatocytes are the macrophages. Crystal cells are involved in wound healing and encapsulation of invaders. Lamellocytes are also involved in encapsulation (Waltzer *et al* 2010).

serpent (a *gata4/5/6* homolog) is the first transcription factor expressed in embryonic prohaemocytes (Rehorn *et al* 1996). *gcm* and *gcm2* are also initially expressed in all prohaemocytes.

However, *gcm* and *gmc2* are downregulated in the most anterior prohaemocytes and instead express *lozenge* (a *runx* homologue) (Bataille *et al* 2005). lozenge and serpent form a complex to induce prohaemocytes to become crystal cells. In the rest of the prohaemocytes, continued expression of *gcm* and *gcm2* induces these cells to become the plasmatocytes. Crystal cell development is unaffected by mutations to *gcm* and *gcm2* (Alfonso & Jones 2002), but is absent in *lozenge* loss-of-function mutants (Lebestky *et al* 2000). Plasmatocytes, on the other hand, are severely reduced by *gcm* and *gcm2* double-mutants, and there is an increase in the number of crystal cells (Alfonso & Jones 2002). Gcm and gcm2 regulate the size of the crystal cell population by inhibiting lozenge expression (Bataille *et al* 2005). *u-shaped* (a *fog* homologue) is highly expressed in prohaemocytes and during plasmatocyte development, but is downregulated during crystal cell development. U-shaped represses crystal cell production, and *u-shaped* loss-of-function mutants overproduce crystal cells (Fossett *et al* 2001).

In larval haematopoiesis, the haemocytes originate from the lymph gland. serpent, u-shaped (Sorrentino *et al* 2007), receptor tyrosine kinase pvr (a *pdgf/vegf* homologue) (Bruckner *et al* 2004; Mondal *et al* 2011), Ras/raf signalling (Asha *et al* 2003; Zettervall *et al* 2004), Hedgehog signalling (Mandal *et al* 2007), Wnt signalling (Sinenko *et al* 2009), myb (Davidson *et al* 2005), hand (Han *et al* 2006), and microRNA mir-7 (Tokusumi *et al* 2011) are all involved in prohaemocyte proliferation and/or maintenance. Pvr is also involved in haemocyte migration (Cho *et al* 2002). One of the earliest transcription factors expressed in the lymph gland is *homothorax* (a homologue of *meis1*). In the lymph gland, there is a cluster of signalling cells known as the posterior signalling centre (PSC). The Hox factor antennapedia is required for initial expression of *collier* (a homologue of *ebf1*) in the PSC (Mandal *et al* 2007). collier maintains JAK/STAT signalling to prevent premature differentiation of prohaemocytes, and is itself maintained by serrate-mediated Notch signalling (Krzemien *et al* 2007). Crystal cell differentiation in the lymph gland is dependent on lozenge, which is also induced by serrate/Notch signalling (Lebestky *et al* 2002). In

the lymph gland, serpent and u-shaped interact to suppress lamellocyte differentiation (Sorrentino *et al* 2007). JAK/STAT signalling (Sorrentino *et al* 2004), collier (Crozatier *et al* 2004), Toll signalling, rac1 and anterior open (Zettervall *et al* 2004) have all been suggested as been involved in lamellocyte formation. Neither *gcm* nor *gmc2* is expressed in the lymph gland (Bataille *et al* 2005). Plasmatocyte differentiation is instead controlled by JAK/STAT signalling and pannier (a *gata4/5/6* homolog) (Minakhina *et al* 2011).

THE EVOLUTION OF HAEMATOPOIESIS

The evolution of haematopoiesis and innate immune cells

All bilaterians have some kind of innate immune system (Hartenstein 2006). Several genes expressed in gnathostome haemangioblasts, HSCs or myeloid cells have homologues in non-vertebrate species which are either expressed in innate immune cells, are involved in innate immune cell development in some way, or change expression in response to bacterial infection. These gene families are *gata1/2/3, gata4/5/6, spi/pu.1, lmo2, scl, runx, cebp, myb* and *fli/erg*. This suggests that many significant elements of the basic haematopoietic GRN were established well before the evolution of vertebrates, possibly even in the ancestor of bilaterians.

The evolution of lymphocytes

The adaptive immune systems of cyclostomes and gnathostomes vertebrates are different at the molecular level (VLR-based and Ig/TCR-based, respectively), but they both have three lymphocyte cell types with similar gene expression profiles. The transcription factors *ikaros, pu.1, gata3*, *pax5* and *sox13* are known to be expressed in the lymphocytes in both gnathostomes and

cyclostomes. The most likely scenario therefore seems to be that these three cell types evolved in the ancestral vertebrate before the cyclostome-gnathostome vertebrate split (Hsu 2011; Hirano *et al* 2013). This means that lymphoid cells probably evolved before adaptive immune systems. If an adaptive immune system is not a prerequisite for a lymphoid haematopoietic lineage, then rudimentary lymphoid-like cells may be present in non-vertebrates.

Amphioxus possesses many of the genes necessary for innate and adaptive immune systems, including homologues of receptor gene families such as TLRs and NLRs, complement components, transcription factors, and even elements of immunoglobins and recombination activating genes (Holland *et al* 2008; Huang *et al* 2008). Amphioxi also have cells which morphologically resemble lymphocytes. The amphioxus homologue of *ikaros*, a transcription factor required for lymphocyte development in gnathostomes, is expressed in the gills, the area where these lymphocytes reside.

The evolution of erythrocytes

All vertebrates, including cyclostomes, have erythrocytes. Synteny analysis has shown that the globin genes in vertebrates (including the haemoglobins) duplicated in the WGDs. And, in fact, this is an example of functional divergence after genome duplication, as the different duplicates took on different functions (Hoffmann *et al* 2011). Surprisingly, haemoglobins evolved from different precursor globins in gnathostomes and cyclostomes. The haemoglobins of lamprey are homologous to the cytoglobin of vertebrates, which does not have an oxygen-transport function (Hoffman *et al* 2010).

It is conceivable that erythrocytes evolved independently in gnathostomes and cyclostomes, but a more likely scenario is that erythrocytes evolved prior to the WGDs and different orthologues

became the globin genes that remained in erythrocytes. This is similar to the scenario given for the evolution of lymphocytes: evolution of the cell type(s) before molecular optimisation.
We should be cautious in inferring evolutionary events from developmental events. The relationship between ontogeny and phylogeny has been a topic of much debate (e.g. Gould 1977). However, what is currently known about haemocytes and their development in the animal kingdom, does suggest that the evolution of erythroid and myeloid cells may have evolved in a similar way to how they develop. Erythroid and myeloid cells develop from a common precursor. Perhaps erythroid cells evolved from a lineage of immune cells that lost immune functions and became specialised for oxygen transport. Non-vertebrate erythrocyte-like cells, which have evolved independently, often have other functions besides oxygen transport (Glomski & Tamburlin 1990). The erythroid lineage may have evolved before or after the evolution of globin expression within haemocytes.

Although fruit flies, sea urchins, amphioxi and ascidians do not have identifiable erythrocyte-like cells, globin gene expression has not been studied in detail in these species, and fruit fly and ascidian homologues of *fog1* and the sea urchin homologue of *gata1* are all expressed in haemocytes. Therefore, the existence of rudimentary erythroid cells in living non-vertebrates remains very possible.

AIMS

The basic aim

The transition from the non-vertebrate haematopoietic system to the vertebrate haematopoietic system is very relevant to the field of evolutionary biology. It could be argued that it is related to the evolution of novelties (Wagner & Lynch 2010), complex features (Lenski *et al* 2003), immune

systems (Cooper & Alder 2006; Rodriguez *et al* 2012), vertebrates (Donoghue & Keating 2014), developmental gene regulatory networks (Erwin & Davidson 2009), and gene and genome duplications (Crow & Wagner 2006).

There appears to a great deal of similarity between the GRNs involved in vertebrate and non-vertebrate haematopoiesis. This may allow researchers to identify the key changes responsible for the evolution of novel blood cell types such as erythrocytes and lymphocytes. It may even be possible to characterise the changes at the molecular level – for example, finding new domains in transcription factor proteins or new binding sites in enhancers.

However, there is not enough published data available to be able to infer what haematopoietic GRN the ancestor of vertebrates had. The only non-vertebrate which has had its haemocytes studied in characterised in detail is the fruit fly, so we can only speculate as to when erythroid-like and lymphoid-like lineages may have originated.

This thesis includes some data about non-vertebrate gene expression patterns and the evolution of the gata1/2/3 proteins. One motivation of the research was to 'fill in the gaps' (see Figure 5), but the primary aim was to demonstrate the success of various approaches that could be used to study this topic. It is not immediately obvious from the published literature whether amphioxi or ascidians are better representatives of the ancestor of vertebrates (in the context of haematopoiesis). This thesis aimed to resolve this.

Gnathostome genes	Published evidence for expression in haematopoietic or immune cells: Fruit flies	Sea urchins	Amphioxi	Ascidians	Cyclostomes	Inferred origin of expression
cebp-a, cebp-b						Deuterostome
c-myb						Bilaterian
ebf1						Bilaterian
fli1						Deuterostome
fog1						Bilaterian
gata1, gata2, gata3						Deuterostome
gata4, gata5, gata6						Bilaterian
haemoglobin						Vertebrate
ikaros, helios, aiolos, eos						Deuterostome
lmo2						Deuterostome
l-plastin						Olfactore
pax5						Vertebrate
pu.1, spi-b, spi-c						Deuterostome
runx1, runx3						Bilaterian
scl						Deuterostome
sox6, sox13						Vertebrate

Bilaterians
Deuterostomes
Chordates
Olfactores
Vertebrates

Figure 5 – Evidence for expression in haematopoietic or immune cells of each of the organisms and each of the genes studied (see 'Choice of genes' below) from the published literature. Green denotes that there is evidence of some kind; grey denotes that there is no evidence. The origin of this haematopoietic/immune expression is inferred from the most basal organism for which there is evidence.

Choice of genes

The genes that were chosen were either transcription factors which played key roles in haematopoietic development/differentiation, or were markers of erythrocytes (globin genes) or leukocytes (white blood cells) (*l-plastin*). For every gene family chosen there was some published evidence of expression in haematopoietic or immune cells in at least one non-gnathostome species. See Figure 5 for the list of genes studied.

Identify non-gnathostome homologues of genes involved in gnathostome haematopoiesis

It is essential to know which genes are present in which species in order to identify promising organisms and gene families to study. Although there have been papers published which have searched for homologues of gnathostome haematopoietic genes in the genomes of sea urchins (Hilbino *et al* 2006) and amphioxi (Huang *et al* 2008), there are some genes which have become relevant in the light of new evidence (e.g. *sox13* (Hirano *et al* 2013)). And this has not been done to the same degree for fruit flies, ascidians and cyclostomes. Blast searches were performed using published genomes and other resources.

Analyse EST collections

One rich source of expression data is Expressed Sequence Tag (EST) collections. These are partial cDNAs typically produced by next generation sequencing (Morozova & Marra 2008). Tissue-specific EST collections can be particular informative as they can provide preliminary expression data for genes. If a gene is only represented by ESTs in the liver-specific EST collections, then this suggests the gene is expressed exclusively in the liver. RT-PCR or in situ hybridisation assays can then be used to confirm or refute this.

Haemocyte-specific EST collections are available for fruit flies (Nakanishi *et al* 2004), sea urchins (Cameron *et al* 2000), ascidians (Satou *et al* 2002), lampreys (Pancer *et al* 2004) and hagfishes (Suzuki *et al* 2004). None of these collections have been analysed with regards to the evolution of haematopoiesis or even to look for homologues of genes involved in gnathostome haematopoiesis. Although the primary focus of this thesis is on non-vertebrate chordates, it is important to look a broad range of species in order to understand the evolution of this system.

For example, if a gene is expressed in sea urchin and lamprey haemocytes, then this suggests an expression domain common to all deuterostomes, so it may be expressed in amphioxus and ascidian haemocytes.

There are sufficient additional tissue-specific collections for fruit flies and ascidians to compare the number of ESTs to the total number of tissue-specific ESTs. It should be noted, however, the numbers of ESTs for a particular gene should not be a taken as a direct measure of that gene's expression level.

Nevertheless, if there were ESTs representing one of these genes present in a haemocyte-specific collection, then this should be taken as preliminary evidence that the gene is expressed in haemocytes. This is a quicker and easier method of identifying promising species and genes than, say, cloning and doing *in situ* hybridisations for every gene in every species.

Analyse amphioxus and ascidian gene expression using *in situ* hybridisations

RNA *in situ* hybridisations are an effective technique for determining expression patterns, so they should be done for genes which look promising based on a combination of published data and the results of the EST analysis.

To establish that the resources and technique are working appropriately in the laboratory, *in situ* hybridisations were performed on amphioxus and ascidian embryos. The genes chosen to be analysed were homologues of *ikaros* and *l-plastin*. To ensure that the expression patterns were reliable, positive controls were chosen for each species for which there are unambiguous, published patterns. *brachyury2* was used for amphioxi (Bertrand & Escriva 2011) and *fli/erg-a* was used for ascidians (Imai *et al* 2004).

ikaros and *l-plastin* were chosen because they are used as markers of immune cells in zebrafish (Willett *et al* 2001). Also, *ikaros* is expressed in the adult amphioxus gill (where cells resembling lymphocytes reside) (Huang *et al* 2007) and *plastin* is expressed in the haemocytes of adult ascidians (Ogasawara *et al* 2006). Of the genes studied in this thesis, these are two that seem most likely to be expressed in the embryonic haematopoietic cells of non-vertebrate chordates. If the expression of either overlapped with the published expression patterns for *gata1/2/3* then may indicate that *gata1/2/3* is involved in haematopoiesis in non-vertebrate chordates.

The ascidian haematopoietic system has potential for developmental as well evolutionary research. Ascidian larvae have simple body plans with relatively few cells (2600) (Tolkin & Christiaen 2012), and most post-metamorphosis tissues have been mapped onto their precursor larval cells. This simplicity can provide advantages over more complicated model species such as zebrafish and mice. Targeted expression of fluorescent reporters can be introduced into ascidians easily and quickly by simultaneously electroporating hundreds of fertilised ascidian eggs (Wang & Christiaen 2012). This contrasts to the slow and expensive process of producing and rearing stable transgenic lines of zebrafish. The relatively compact genomes of *Ciona* species (Lemaire 2011) means that the identification and cloning of regions of regulatory DNA (i.e. promoters and enhancers) should be simpler than in vertebrates.

Ascidian may be of additional interest to regeneration and wound healing research. Ascidians have significant regenerative capacities: for example, *C. intestinalis* is able to regenerate its oral siphon (Auger *et al* 2010), and colonial ascidians (e.g. *Bortrylloides violaceus)* can undergo whole body regeneration (i.e. they have the ability to form a new individual from vascular tissue) (Brown *et al* 2009). Myeloid cells are known to be important in regeneration and wound healing in vertebrates, so if ascidian immune cells were known to be homologous to myeloid cells , then

ascidians could become a useful new experimental system for studying these processes. The whole body regeneration of colonial ascidians is particular interesting because the process seems to originate from totipotent blood cells (Rinkevich *et al* 1995; Brown *et al* 2009). Moreover, the regenerative capacities of *C. intestinalis* decline with age (Jeffery 2012), which may or may not be the result of similar processes which cause wound-healing to decline with age in humans (Gosain & DiPietro 2004).

Attempt rescue of vertebrate gata1 knockdown with non-vertebrate gata1/2/3

gata1/2/3 genes play key roles in the haematopoiesis of sea urchins and gnathostomes, and there is some evidence this may also be the case in ascidians and cyclostomes. This suggests that gata1/2/3 may be involved in haematopoiesis in all deuterostomes. Although sea urchins have cells that could be described as HSC-like and myeloid-like, no erythrocyte-like or lymphocyte-like cells have yet been identified. gata1 and gata3 are key regulators of erythrocyte and T-lymphocyte development, respectively. It is therefore not unreasonable to describe the gata2 function (in progenitor cells) as 'ancestral', and the gata1 and gata3 functions as 'novel'.

One of the motivations of this research is to better understand the molecular changes behind the evolution of novel cell types. gata1 and gata3 are the products of the WGDs, which coincide with the possible origin of erythrocytes and lymphocytes. It has been previously suggested that the WGDs may have facilitated the evolution of the vertebrate novelties (Feiner *et al* 2013), and this may be an example. However, it could be a coincidence, and it is not possible to directly test how important the WGDs were.

There has been some debate in the evo devo field as to the relative contributions of protein-coding and cis-regulatory changes (Hoesktra & Coyne 2007, Carroll 2008). gata1 is arguably the

most important transcription factor in erythrocyte development. How did it gain such an important function that is very distinct from its paralogues (gata2 and gata3)? If the erythropoietic GRN is simplified down to gata1 regulating haemoglobin genes, then how did gata1 gain this regulatory function? Were there changes to the binding domains of gata1 or is it unique just because of its unique expression pattern (i.e. due to *cis*-regulatory changes). There is a paradigm in evo devo that many morphological changes are due to 'toolkit' genes being expressed in new regions (Carroll 2005). However, it is not yet known whether *cis*-regulatory changes are sufficient for the evolution of new cell types. spi-b can functionally replace pu.1 in myeloid but not lymphoid development (Dahl *et al* 2002). If we take myeloid development to be representative of an ancestral function and lymphoid to be novel then it appears as if protein-coding changes were important in the evolution of the novel function. This might also be the case for gata1's role in erythrocyte development.

If protein-coding changes were important in the evolution of gata1, then the non-vertebrate gata1/2/3 should not be able to functionally replace gata1 in erythrocyte development. To test this, gata1 expression was knocked down in zebrafish using morpholinos. The zebrafish (*Danio rerio*) is an ideal system for this experiment. Zebrafish embryos are transparent, and their haematopoietic system is well characterised (Jing & Zon 2011). The first erythrocytes form in the intermediate cell mass (ICM) adjacent to the dorsal aorta; and the first myeloid cells form in the anterior lateral mesoderm (ALM) (Davidson & Zon 2004). It is therefore very easy to detect if there has been a reduction in primitive erythropoiesis. The amphioxus *gata1/2/3* gene was used to attempt the rescue because tunicates are evolving much more rapidly at the molecular level than amphioxi and vertebrates (Ferrier 2011; Berna & Alvarez-Valin 2014). This means that the ascidian gata1/2/3 may be very different to the gata1/2/3 of the ancestor of vertebrates. The amphioxus *gata1/2/3* was put under the control of the zebrafish *gata1* regulatory region to ensure that expression is directed to the correct regions. *gata1* knockdown reduces the numbers

of primitive erythrocytes (Rhodes *et al* 2005; Galloway *et al* 2005); if *gata1/2/3* can restore this number then this suggests that protein-coding changes were not important in the evolution of gata1 in erythropoiesis.

A full research project would repeat this rescue using gata1/2/3 homologues from other species, and would investigate the additional functions of gata1, including its repression of pu.1 and myeloid development. It would also repeat the experiment for gata2 and gata3, pu.1 and its orthologues, ikaros and its orthologues, and other genes. The rescue experiment presented in this thesis aimed to demonstrate the success of the methodology, which could then be easily modified by future researchers to build up a full picture of the molecular changes behind the evolution of the vertebrate haematopoietic GRN.

This type of experiment has been performed in zebrafish once before. Van Otterloo *et al* (2012) knocked down *tfap2* and rescued it with non-vertebrate tfap genes. However, this was related to the evolution of the neural crest. The method has not been previously shown to be successful in the haematopoietic system.

Use non-vertebrate gata1/2/3 to drive reporter expression in vertebrates

If gata1 is functionally equivalent to gata1/2/3 at the level of protein structure, then this would highlight the importance of *cis*-regulatory changes. There could have been regulatory neofunctionalisation – after duplication, gata1 gained new erythroid enhancers – or subfunctionalisation – after duplication gata2 and gata3 lost erythroid enhancers. Subfunctionalisation would imply that there must have been some kind of erythroid-like lineage and an erythroid-like GRN before the WGDs occurred.

Several amphioxus sequences have been used to drive tissue-specific reporter expression in mice; and some have provided evidence for regulatory subfunctionalisation (Holland *et al* 2008). *C. intestinalis* non-coding sequences have driven tissue-specific expression in zebrafish, including one which was expressed in myeloid cells. The sequence that drove this expression was adjacent to the ascidian homologue of *h-hex* (haematopoietically-expressed homeobox protein) (Doglio *et al* 2013). These results indicate that many *cis*-regulatory sequences are conserved between non-vertebrates and vertebrates, possibly including some that drive expression in haematopoietic cells.

To test which scenario is more likely – neofunctionalisation or subfunctionalisation (or elements of both) – would require the entire regulatory region of amphioxus or ascidian gata1/2/3 to be used to drive reporter expression in zebrafish. If there was, for example, expression in erythroid cells, HSCs and lymphoid cells, then this would indicate subfunctionalisation. The more erythroid-like enhancers would have been lost from gata2 and gata3 but maintained by gata1, and so on. Whether or not regulatory subfunctionalisation could be taken as indicative of cellular subfunctionalisation would depend on where this regulatory region drove expression in the non-vertebrate. Expression in a single lineage would suggest that the separate vertebrate lineages had divided from a single non-vertebrate lineage.

Distinguishing between neofunctionalisation and subfunctionalisation in the evolution of gata1/2/3 would require a large scale set of experiments in multiple species. This thesis aims to demonstrate the effectiveness of a methodology that could be easily modified to test different sequences in different species. pTransgenesis is a recently developed cloning and transgenesis system which can be used to create appropriate reporter constructs. pTransgenesis can be expressed in fruit flies, *Xenopus* tadpoles and zebrafish (and theoretically into ascidians and

amphioxi), and regulatory sequences can be inserted and removed quickly and simply (Love *et al* 2011).

Khoueiry *et al* (2010) identified a 934bp non-coding region 5' to the start codon of *Ci-gata1/2/3* in a computational search of the *C. intestinalis* genome for clusters of ETS and GATA binding sites. This sequence drove *LacZ* reporter expression in mesenchyme and a-epidermis cells in developing *C. intestinalis* embryos. This is probably not the entire regulatory region for ascidian gata1/2/3, but it does drive expression in the mesenchyme, which are the pre-metamorphosis precursors of haemocytes. Therefore, it is possible that it contains haematopoietic enhancers. It was inserted into pTransgenesis and injected into zebrafish embryos to see if could produce tissue-specific expression in zebrafish.

METHODS

Homologue identification

Homo sapiens protein sequences were taken from the NCBI Genbank collection. They were then blasted against non-redundant protein sequences using the NCBI blastp algorithm (expect threshold: 10, word size: 3, matrix: BLOSUM62, gap costs: existence 11 extension 1, conditional compositional score matrix adjustment) limited to the appropriate species (*D. melanogaster*, *S. purpuratus, B. floridae, C. intestinalis, P. marinus*). They were also blasted against the genomes using the NCBI tblastn algorithm (same conditions as NCBI blastp) or the Ensembl tblastn (search sensitivity: distant homologies, culling limit: 5, maximum e-value: 1e-1, word size: 3, matrix: BLOSUM62, gap costs: existence 11 extension 1, conditional compositional score matrix adjustment). Hits were then blasted against the *H. sapiens* non-redundant protein sequences using NCBI blastp or blastx (same conditions as NCBI blastp). If the top hits from this reverse-blasting were the original *H. sapiens* genes then the non-gnathostome genes were designated orthologues of the vertebrate genes.

Analysis of EST data

Protein or DNA sequences corresponding to each of the genes were blasted against expressed sequence tags using NCBI tblastn or blastn (same conditions as NCBI blastp) for the following haemocyte-specific EST libraries: dbEST_15895 (*D. melanogaster),* dbEST_19874 (*S. purpuratus*), dbEST_11975 (*C. intestinalis*), LIBEST_016049 (*P. marinus*), LIBEST_016544 (*E. burgeri*). Hits were then blasted against all non-redundant protein sequences using NCBI blastp or blastx (same conditions as NCBI blastp). If the top hits from this reverse-blasting were the original genes or homologues of them then the EST was assigned to that gene. Haemocyte transcripts

per million (TPM) were calculated using the total number of ESTs in each collection. Total site-specific TPM were taken from Unigene pages corresponding to the genes, where available.

Collection of amphioxus embryos

Sexually mature *B. lanceolatum* adults were collected from Banylus, France in June 2012 and induced to spawn by electric stimulation (Holland & Holland 1989). Embryos were raised in the laboratory at 19°C until they reached the desired stages (late neurula and larva with one opened gill slit). For *in situ* hybridisation, embryos were fixed in 4% PFA in MOPS buffer for 24 hours at 4˚C, dehydrated into 70% ethanol, and then stored at -20˚C. For RNA extraction, embryos and adults were frozen in RNase Later at -80˚C.

Collection of ascidian embryos

Sexually mature *C. intestinalis* adults were collected from Arbroath, Scotland in July 2013. They were dissected to expose the gametes, and the sperm from one adult was mixed in a dish of filtered seawater with the eggs of another and allowed to fertilise for 10 minutes. The fertilised eggs were rinsed through egg baskets, and then embryos were raised in the laboratory at 15°C until they reached the mid-tailbud stage. For *in situ* hybridisation, embryos were fixed in 4% PFA in MOPS buffer for 24 hours at 4˚C, dehydrated into 70% ethanol, and then stored at -20˚C.

Collection of zebrafish embryos

Zebrafish (*Danio rerio*) adults were kept in tanks at 28.5˚C. Fertilised embryos were allowed to develop in dishes at 28.5˚C. For *in situ* hybridisation, embryos were fixed in 4% PFA in PBS at 4˚C for 24 hours, dehydrated into 70% methanol and stored at -20˚C.

Cloning genes

RNA was extracted from *B. lanceolatum* embryos using Invitrogen TRI reagent solution, and cDNA was synthesised using Invitrogen Superscript III Reverse transcriptase, according to manufacturers' instructions. Primers were designed to amplify fragments of protein-coding sequences based on *B. lanceolatum* ESTs or *B. floridae* predicted protein-coding regions (*plastin-a:* AGGTAAAGCCGGGAGTTGTT, GGCAGGGTCAACAAGAAGTT, TCTTTCTGGCCATGGAGATG, TGACTTCCACCAGGTCTTCC; *ikaros:* CGAGTGCATTAATCCCAAGA, GACTGACACATTCGCCCTTT, TCACACAGTCCAACAGGTACG). The PCR program used was 35 cycles of 1 minute 94˚C, 1 minute 60˚C and 1 minute 72˚C. Fragments were separated by gel electrophoresis, cut, extracted using the QIAGEN Gel Extraction Kit, and ligated into pGEMTeasy vectors, transformed onto LB+ampicillin plates, white colonies picked and cultured in LB+ampicillin medium, miniprepped and then sequenced. All other plasmids used for *in situ* hybridisations were obtained from other laboratories.

Whole mount *in situ* hybridisations

Plasmids were linearised using appropriate restriction digest enzymes and cleaned up using the GeneClean Kit. Antisense probes were then synthesised using appropriate RNA polymerases, treated with DNase, purified using QIAGEN spin columns, and stored at -80˚C.
In situ hybridisation of amphioxus, ascidian and zebrafish was performed essentially as described in Thisse & Thisse (2008). Essential steps included rehydration into PBS, treatment with 10mg ml-1 Proteinase K for 10 minutes, fixation in 4% PFA, washes in PBS, washes in hybridisation buffer, leaving overnight in hybridisation buffer with probe at 65˚C (60 ˚C for amphioxus),

washes in PBS, washes in 2% blocking solution, leaving overnight in 2% blocking solution with anti-DIG antibody, washes in PBS, washes in AP solution, and then leaving in BM Purple to stain. After staining was complete, embryos were fixed in 4% PFA in PBS then stored in glycerol at 4˚C.

gata1 knockdown and rescue

The predicted protein-coding sequence of *B. floridae gata1/2/3* was removed from an EST clone and the *D. rerio gata1* protein-coding sequence was removed from a Dr-gata1:Dr-gata1 construct (a transposon-based transgene (Takeuchi *et al* 2010) under the control of the *D. rerio gata1* regulatory elements (Kobayashi *et al* 2001)) using restriction digestions. DNA fragments were separated by gel electrophoresis, cut from the gel, and extracted using the QIAGEN Gel Extraction Kit. *Bf-gata1/2/3* was inserted into the vector by ligation, then transformed. Colonies were picked, miniprepped and sequenced to ensure successful insertion. *Tol2* mRNA was synthesised using the Ambion® mMESSAGE mMACHINE® RNA Transcription Kit. 1-cell stage zebrafish embryos were injected with 1nl of 0.4mM splice-blocking morpholino targeted against the first intron/exon boundary of *D. rerio gata1a* (5'-GTTTGGACTCACCTGGACTGTGTCT-3'), then injected with 0.5nl of a mix of 25pg *Tol2* mRNA and 12.5pg of either the original *Dr-gata1:Dr-gata1* construct or the *Dr-gata1:Dr-gata1/2/3* construct.

gata1/2/3:mCherry reporter

Construction of the γ-crystallin:mCherry/Ci-gata1/2/3:mCherry reporter was done using Invitrogen's Gateway Multisite Cloning Kit. The γ-crystallin promoter, 934bp Ci-gata1/2/3 regulatory region (from (Khoueiry *et al* 2010), mCherry coding regions, and pTransgenesis vector

were combined using the LR recombination reaction (Love *et al* 2011). 1nl of a 20 pg/nl solution of the reporter + 25 pg of Tol2 mRNA was injected into 1-cell stage zebrafish embryos. Embyos were allowed to develop at 28.5˚C. After 72hpf, embryos were fixed in fixed in 4% PFA in PBS at 4˚C for 24 hours then washed into PBS. Fluorescence was detected using the Leica DFC310 FX microscope set to RFP (red), GFP (green) or CFP (blue) filters.

RESULTS

Non-gnathostome homologues of genes involved in gnathostome haematopoiesis

The genomes of five non-gnathostomes (fruitflies [*D. melanogaster*], sea urchins [*S. purpuratus*], amphoxi [*B. floridae*], ascidians [*C. intestinalis*], and lampreys [*P. marinus*]) were analysed to determine the number of homologues of key genes involved in gnathostome haematopoiesis that were present. EST collections were analysed to see if any of these homologues had ESTs in haemocyte-specific collections, which could be indicative of a role in haematopoiesis.

Gnathostome genes	*D. melanogaster*	*S. purpuratus*	*B. floridae*	*C. intestinalis*	*P. marinus*
cebp-a, cebp-b	*slow border cells*	*cebp*	*cebp*	*cebp1, cebp2*	*cebp*
c-myb	*myb*	*myb*	*myb*	*myb*	*myb*
ebf1	*knot*	*ebf*	*ebf*	*ebf*	*ebf1, ebf2/3a, ebf2/3b*
fli1	*ets at 65a*	*fli/erg*	*fli/erg-a, fli/erg-b*	*fli/erg-a, fli/erg-b, fli/erg-c*	*fli/erg*
fog1	*u-shaped*	*fog*	*fog*	*fog*	*fog1*
gata1, gata2, gata3	*grain*	*gata1/2/3*	*gata1/2/3*	*gata1/2/3*	*gata1/2/3a, gata1/2/3b*
gata4, gata5, gata6	*pannier, serpent, gata-d, gata-e*	*gata4/5/6*	*gata4/5/6a, gata4/5/6b*	*gata4/5/6*	*gata4/5/6*
haemoglobins	3 globin genes	*globin*	15 globin genes	4 globin genes	23 globin genes*
ikaros, helios, aiolos, eos	-	*ikaros*	*ikaros*	*ikaros*	*ikaros/aiolos, helios, eos*
lmo2	*beadex*	*lmo*	*lmo*	-	*lmo*
l-plastin	*fimbrin*	*plastin*	*plastin-a, plastin-b*	*plastin*	*plastin*
pax5	*shaven*	*pax2/5/8*	*pax2/5/8*	*pax2/5/8a, pax2/5/8b*	*pax2/5/8*
pu.1, spi-b, spi-c	-	*spi/pu.1*	*spi/pu.1-a, spi/pu.1-b*	*spi/pu.1*	*spi/pu.1***
runx1, runx3	*runx-a, runx-b, lozenge*	*runx*	*runx*	*runx*	*runx-a, runx-b*
scl	*hlh3b*	*scl*	*scl*	-	*scl*
sox6, sox13	*sox102f*	*sox5/6/13*	*sox5/6/13*	*sox5/6/13*	*sox5/6/13*

Figure 6 – The presence of homologues of gnathostome genes in the genomes of non-gnathostomes. A dash (-) denotes that there is no direct homologue of that gene family present in that species' published genome. There are a large number of globin genes in vertebrates, so for brevity they have not been listed in full. * Number of cyclostome globin genes taken from Schwarze et al (2014). ** Not present in the genome published online, but identified in Shintani *et al* (2000) and Mayer *et al* (2002).

The *D. melanogaster* genome contained homologues of all the studied vertebrate gene families with the exception of *ikaros* and *pu.1* (Figure 6). Of those genes, 9/24 (37.5%) had tissue-specific transcripts, 4/24 (16.67%) had haemocyte-specific transcripts (*fog1* homologue *u-shaped, gata4/5/6* homologue *pannier, globin1*, and *l-plastin* homologue *fimbrin*), and 0/24 had only haemocyte-specific transcripts (Figure 7). Three gene families had duplicated independently of

the vertebrate duplications: there were four orthologues of *gata4/5/6*, three orthologues of *runx*, and three globin genes.

Gnathostome genes	*D. melanogaster* gene	Haemocyte TPM	Total site-specific TPM	Haemocyte %
cebp-a, cebp-b	*slow border cells*	0	0	-
c-myb	*myb*	0	732	0
ebf1	*knot*	0	0	-
fli1	*ets at 65a*	0	223	0
fog1	*u-shaped*	301	706	43
gata1, gata2, gata3	*grain*	0	0	-
gata4, gata5, gata6	*pannier*	903	1461	62
	serpent	0	380	0
	gata-d	0	312	0
	gata-e	0	0	-
haemoglobins	*globin1*	150	1223	12
	globin2	0	125	0
	globin3	0	0	-
lmo2	*beadex*	0	0	-
l-plastin	*fimbrin*	1807	2468	73
pax5	*shaven*	0	0	-
runx1, runx3	*runx-a*	0	0	-
	runx-b	0	0	-
	lozenge	0	0	-
scl	*hlh3b*	0	0	-
sox6, sox13	*sox102f*	0	0	-

Figure 7– Tissue-specific ESTs for each of the studied genes in fruit flies. TPM = transcripts per million.

The *S. purpuratus* genome contained homologues of all the studied vertebrate gene families, with no independent duplications (Figure 6). There were insufficient data and resources to analyse the total number of tissue-specific transcripts for these genes; however only 1/16 genes (6.25%) had haemocyte-specific transcripts (*globin*) (Figure 8).

Gnathostome genes	*S. purpuratus* gene	Haemocyte TPM
cebp-a, cebp-b	*cebp*	0
c-myb	myb	0
ebf1	*coe*	0
fli1	*fli/erg*	0
fog1	*fog*	0
gata1, gata2, gata3	*gata1/2/3*	0
gata4, gata5, gata6	*gata4/5/6*	0
haemoglobin	*globin*	1986
ikaros, helios, aiolos, eos	*ikaros*	0
lmo2	*lmo2*	0
l-plastin	*plastin*	0
pax5	*pax2/5/8*	0
pu.1, spi-b, spi-c	*spi/pu.1*	0
runx1, runx3	*runx*	0
scl	*scl*	0
sox6, sox13	*soxd*	0

Figure 8 – Tissue-specific ESTs for each of the studied genes in sea urchins. TPM = transcripts per million.

The *B. floridae* genome contained homologues of all the studied vertebrate gene families (Figure 6). Five had independently duplicated: there were two orthologues of *fli/erg,* two orthologues of *gata4/5/6*, two orthologues of *l-plastin*, two orthologues of *spi/pu.1*, and 15 globin genes. There were insufficient data and resources to analyse tissue-specific transcripts.

The *C. intestinalis* genome contained homologues of all the studied vertebrate gene families with the exception of *lmo2* and *scl* (Figure 6). Of those genes, 17/23 (73.91%) had tissue-specific transcripts, 12/23 (52.17%) had haemocyte-specific transcripts (*cebp1, cebp2, fli/erg-a, globin1, globin2, ikaros, plastin, pax2/5/8a, pax2/5/8b, spi/pu.1,* and *sox5/6/13*), and 3/23 (13.04%) had only haemocyte-specific transcripts (*fli/erg-a, pax2/5/8a,* and *sox5/6/13*) (Figure 9). Four gene

families had duplicated independently of the vertebrate duplications: there were two orthologues of *cebp*, three orthologues of *fli/erg*, two orthologues of *pax2/5/8,* and three globin genes.

Gnathostome genes	*C. intestinalis* homologue	Haemocyte TPM	Heart TPM	Total site-specific TPM	Haemocyte %	Heart %
cebp-a, cebp-b	*cebp1*	740	500	2179	34	23
	cebp2	172	0	875	20	0
c-myb	*myb*	0	0	0	-	-
ebf1	*ebf/coe*	0	0	105	0	0
fli1	*fli/erg-a*	137	0	137	100	0
	fli/erg-b	0	41	41	0	100
	fli/erg-c	0	0	0	-	-
fog1	*fog*	0	0	0	-	-
gata1, gata2, gata3	*gata1/2/3*	0	41	41	0	100
gata4, gata5, gata6	*gata4/5/6*	0	0	31	0	0
haemoglobin	*globin1*	68	125	193	35	65
	globin2	620	333	1068	58	31
	globin3	0	0	0	-	-
	globin4	0	0	0	-	-
ikaros, helios, aiolos, eos	*ikaros*	137	0	168	82	0
l-plastin	*plastin*	1998	208	2601	77	8
pax5	*pax2/5/8a*	34	0	34	100	0
	pax2/5/8b	51	0	304	17	0
pu.1, spi-b, spi-c	*spi/pu.1*	86	0	201	43	0
runx1, runx3	*runx*	0	250	1363	0	18
sox6, sox13	*sox5/6/13*	551	0	551	100	0

Figure 9 – Tissue-specific ESTs for each of the studied genes in ascidians. TPM = transcripts per million.

The *P. marinus* genome contained homologues of all the studied vertebrate gene families (Figure 6). *spi/pu.1* could not be detected in the published genome or in the EST databases available online; however its existence has been confirmed in previous publications (*Shintani et al 2000; Mayer et al 2002*). The number of globin genes was based on the most recent publication on the subject to date (Schwarze *et al* 2014). There were insufficient data or resources to analyse the total number of tissue-specific transcripts for these genes. Additionally, it was not possible in most cases to distinguish between different cyclostome orthologues, so the EST analysis was limited to one orthologue per vertebrate gene family. 4/17 (23.53%) had lamprey "lymphocyte"-specific transcripts (*globin1, lmo, l-plastin* and *runx-a*), 6/17 (35.29%) had hagfish "leukocyte"-specific transcripts (*fli/erg, gata1/2/3a, gata4/5/6, globin1, l-plastin* and *scl*), and 2/17 (11.77%) had haemocyte-specific transcripts in both species (*globin1* and *l-plastin*) (Figure 10).

Gnathostome genes	Cyclostome homologue	Lamprey haemocyte TPM	Hagfish haemocyte TPM
cebp-a, cebp-b	*cebp*	0	0
c-myb	*myb*	0	0
ebf1	*ebf1*	0	0
fli1	*fli/erg*	0	126
fog1	*fog1*	0	0
gata1, gata2, gata3	*gata1/2/3a*	0	754
gata4, gata5, gata6	*gata4/5/6*	0	670
haemoglobin	*globin1*	14519	1214
ikaros, helios, aiolos, eos	*ikaros/aiolos*	0	0
lmo2	*lmo*	115	0
l-plastin	*plastin*	115	42
pax5	*pax2/5/8*	0	0
pu.1, spi-b, spi-c	*spi/pu.1*	0	0
runx1, runx3	*runx-a*	115	0
scl	*scl*	0	84
sox6, sox13	*sox5/6/13*	0	0

Figure 10 – Tissue-specific ESTs for each of the studied genes in cyclostomes (lampreys and hagfish). TPM = transcripts per million.

Gene expression in ascidian and amphioxus embryos

Analysing the embryonic expression pattern of a gene can provide information about its possible functions. To demonstrate the success of this technique in non-vertebrate chordates, the expression patterns of homologues of *ikaros* and *l-plastin* (and controls) in ascidians and amphioxi were analysed. As described in the introduction, these genes were chosen because they seem the most likely candidates for markers of haematopoietic cells in these organisms.

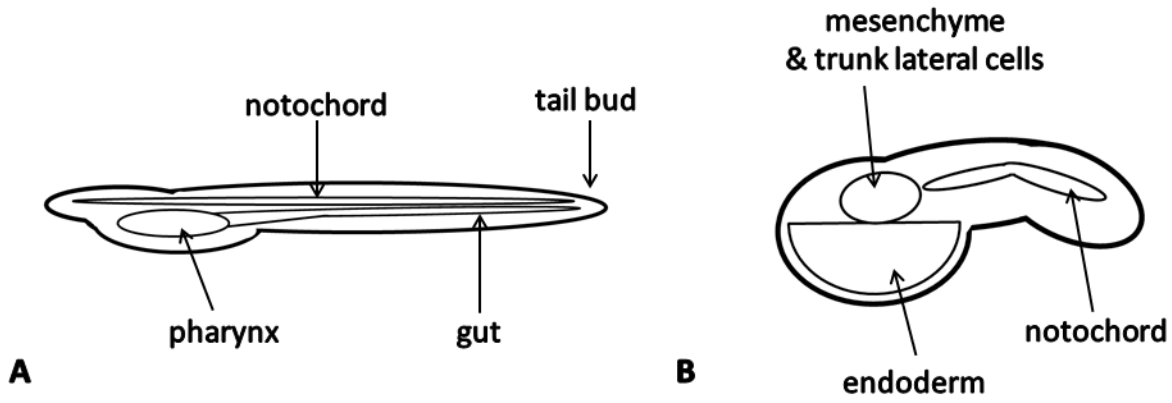

Figure 11 – Diagrams of A) an amphioxus embryo (at the larval stage) and B) an ascidian embryo (at the tailbud stage), with salient tissues labelled. (Adapted from Bertrand & Escriva 2011 and Shi *et al* 2005.)

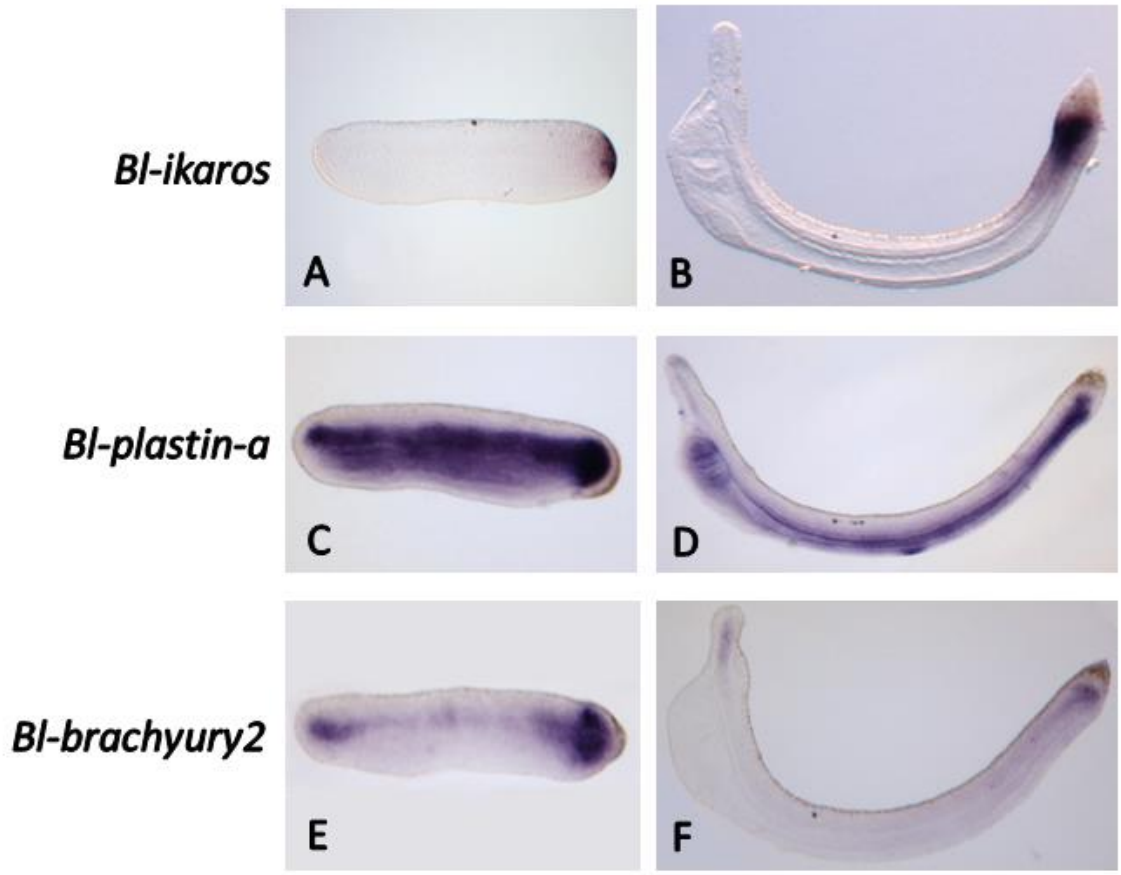

Figure 12 – Gene expression in amphioxus embryos. In situ hybridisation staining for *ikaros* (A, B), *plastin-a* (C, D) and *brachyury2* (E, F) in *B. lanceolatum* embryos at late neurula (A, C, E) and larva with one opened gill slit (B, D, F) stages.

Bl-ikaros was expressed in the most posterior cells of the embryo at the late neurula stage, and then in a region around the end of the hindgut at the larval stage. *Bl-plastin* was expressed in the notochord and posteriorly at the late neurula stage, and then throughout the walls of the gut and in the pharynx at the larval stage, with some weak expression in the anterior end of the notochord. *Bl-brachyury2* was expressed in the notochord and posteriorly at the late neurula, and then at the anterior and posterior ends of the notochord at the larval stage (Figure 12, also see Figure 11).

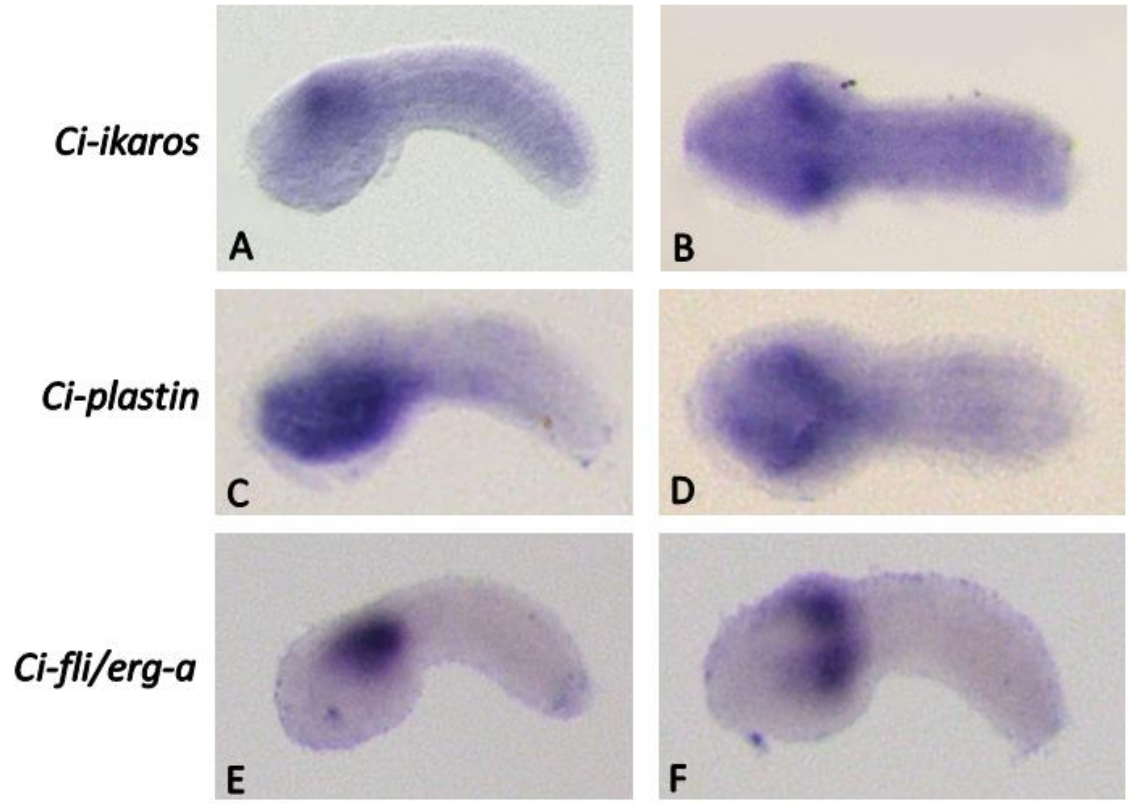

Figure 13 – Gene expression in ascidian embryos. *In situ* hybridisation staining for *ikaros* (A, B), *plastin* (C, D) and *fli/erg-a* (E, F) in *C. intestinalis* embryos at the mid-tailbud stage viewed lateral ly (A, C, E) and dorsoventrally (B, D, F).

All ascidian expression patterns were at the mid-tailbud stage. *Ci-ikaros* was expressed in the mesenchyme and trunk lateral cells, *Ci-plastin* was expressed in the endoderm, and *Ci-fli/erg-a* was expressed in the mesenchyme and trunk lateral cells (Figure 13, also see Figure 11).

Knockdown of gata1 and rescue with gata1/2/3

In order to investigate the importance of protein-coding changes to the evolution of *gata1*, a key erythropoietic transcription factor, *gata1* expression was knocked down in zebrafish using morpholinos, and was replaced by an amphioxus *gata1/2/3* transgene, to see if erythrocyte development could be restored. A haemologlobin gene was used as a marker of erythrocytes.

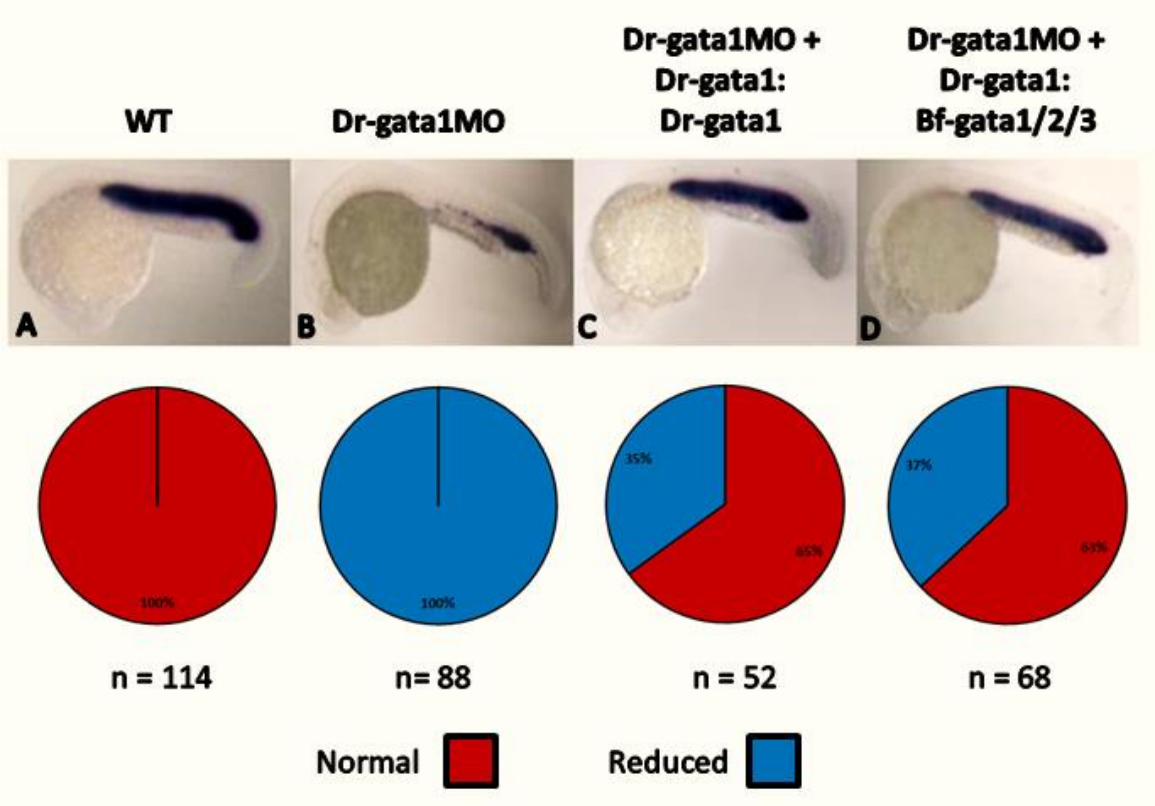

Figure 14 – Amphioxus gata1/2/3 rescues primitive erythrocyte development in a gata1 knockdown. Expression of *βe1-Globin* in 24hpf zebrafish embryos either A) uninjected (i.e. WT), injected with just the gata1 morpholino (B), injected with the gata1 morpholino and the gata1:gata1 transgene (C), or injected with the gata1 morpholino and the gata1:gata1/2/3 transgene (D). Pie charts represent proportion of phenotypes with normal expression and reduced expression.

Morpholino injections were used to knockdown *gata1* expression, and injection of gata1:gata1 and gata1:gata1/2/3 transgenes were used to attempt to rescue the *gata1* expression (Figure 14). Knockdown of *gata1* caused consistent reductions in β*e1-globin* (marker of erythrocytes) in the ICM at 24hpf. The gata1:gata1 transgene rescued β*e1-globin* expression expression to wild-type levels 65% of the time. The gata1:gata1/2/3 transgene produced rescues of 63%. These success rates are not significantly different from each other (chi-square test: $p = 0.8494$). However, both genes produced wild-type expression levels significantly more than in gata1MO only embryos (chi-square tests: gata1:gata1: $p < 0.0001$; gata1:gata1/2/3: $p < 0.0001$.).

gata1/2/3:mCherry reporter expression

In order to investigate the importance of cis-regulatory changes to the evolution of *gata1*, an ascidian *gata1/2/3* cis-regulatory region was used to drive fluorescent reporter gene expression in zebrafish, to see if it would be expressed in similar manner to any of the vertebrate gata1/2/3 genes.

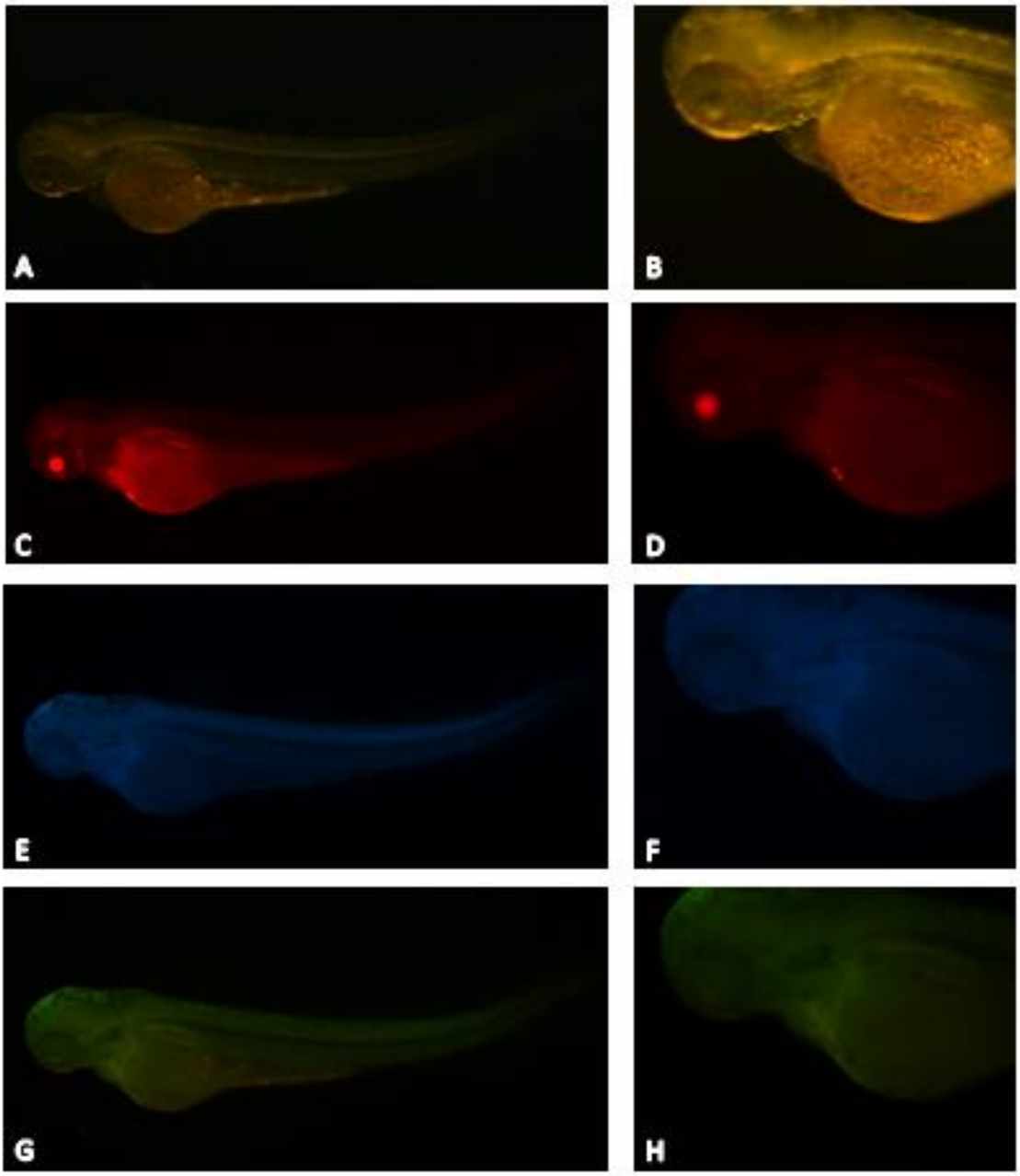

Figure 15 – A *ci-gata1/2/3* regulatory region drives reporter expression in cells near the zebrafish heart. Zebrafish injected with the Dr-γ-crystallin:mCherry/Ci-gata1/2/3:mCherry transgene show red fluorescence (C, D) at 72hpf in the eye lens and cells near the beating heart. This fluorescence is not detectable in normal light (A, B) or under blue (CFP wavelengths) (E, F) or green (GFP wavelengths) filters (G, H).

Injection of crystallin:mCherry/Ci-gata1/2/3-1kb:mCherry into zebrafish embryos produced red fluorescence in the eye lens in 19/36 embryos (52.78%). 6/36 (16.67%) had expression in both in the eye lens and in cells in the area of the heart (Figure 15).

DISCUSSION

Gene expression in non-chordates

Four genes were represented in the *Drosophila* haemocyte EST collection (Figure 6). *u-shaped,* a homologue of vertebrate *fog1,* and *pannier* and *serpent,* homologues of vertebrate *gata4, gata5* and *gata6,* were previously known to be expressed in haemocytes (Minakhina *et al* 2011). The presence of *fimbrin*, a homologue of vertebrate *l-plastin,* suggests that fimbrin/plastin genes are expressed in haemocytes throughout the bilaterians. Both fruit flies (Figure 7) and sea urchins (Figure 8) had a single globin gene and in both cases they were presented by a relatively large number of haemocyte ESTs. Erythrocyte-like cells have not been identified in either species (Hartenstein 2006). There was a general lack of ESTs representing the genes studied in both sea urchins and fruit flies, limiting the conclusions that can be made from these species.

Gene expression in amphioxi

Amphioxi have at least one orthologue of all of gene families studied (Figure 6). None of these genes have been confirmed to have haematopoietic functions or expressions, although adult *cebp, ikaros, fli/erg-a, fli/erg-b, ikaros, spi/pu.1-a* and *spi/pu.1-b* expression levels change after bacterial injection (Huang *et al* 2011).

The expression pattern of *brachyury2* matched the published expression pattern (Bertrand & Escriva 2011) indicating that the collection, fixation and *in situ* hybridisation of amphioxus embryos were working successfully (Figure 12). The posterior expression of *ikaros* and *plastin* resembles the hindgut expression of *gata1/2/3* (Zhang *et al* 2009; Pascual-Anaya *et al* 2013). This suggests that this region may be involved in the haematopoietic development and/or the

immune system in amphioxus embryos. In order to determine if the similar posterior expressions overlap then double fluorescent *in situ* hybridisations and sectioning would need to be performed. Even then, co-expression of e.g. *gata1/2/3* and *ikaros* would not prove that the tissue is involved in haematopoiesis. Vertebrate ikaros does have non-haematopoietic functions: for example, ikaros plays a role in regulating early-born neuronal fates in the mammalian cerebral cortex (Alsio *et al* 2013). In gnathostomes, *gata2* and *gata3* are expressed in the developing nervous system (Nardelli et al 1999) as well as in haematopoietic lineages, but expression around the hindgut seems unlikely to be related to this. Expression in the gut of gnathostomes is characteristic of gata4/5/6 genes rather than gata1/2/3 (Bossard & Zaret 1998). In the annelid Platynereis dumerilli, gata1/2/3 expression is restricted to ectodermal tissues (Gillis et al 2007), although in another annelid, Capitella teleta, it is expressed in the foregut (Boyle & Seaver 2008).

One possibility is that gata1/2/3 and ikaros are involved in gonad development. Genes characteristic of primordial germ cells (PGCs) in other species, including *vasa* and *piwi*, were expressed in this posterior region of the embryo (Zhang *et al* 2013). gata1/2/3 genes are expressed in the testes of mice (Siggers *et al* 2002; Ketola *et al* 2002); and *ikaros* is expressed in the ovaries of adult amphioxi (Huang *et al* 2007) and lampreys (Mayer *et al* 2002), and induces cell migration in human ovarian cancer cells (He *et al* 2012). If the *ikaros*-expressing cells in the amphioxus embryo were PGCs rather than haematopoietic cells then this would explain why these cells do not express *scl* (Pascual-Anaya *et al* 2013), which has important haematopoietic functions in sea urchins and vertebrates (Solek 2012; Solek *et al* 2013) (although has been lost from the *C. intestinalis* genome).

Identifying amphioxus haematopoietic cells by gene expression alone is a very difficult task as most transcription factors have multiple functions in multiple tissues, and it does not necessarily follow that tissues that are functionally and anatomically similar in different species will have

similar GRNs. Finding diagnostic genes for haematopoiesis across the chordates may prove impossible. Ultimately, amphioxus immune cells and their developmental precursors must be thoroughly and conclusively characterised through a combination of immunological, anatomical and developmental work before the evolutionary importance of the amphioxus haematopoietic system can be analysed. Even so, gene expression patterns in isolation are still a useful tool for searching for candidate haematopoietic cells. One example is the *scl*+ *vegfr*+ cells discovered in a previous study (Pascual-Amaya *et al* 2013). These cells are just as likely to be endothelial-like cells rather than haematopoietic cells; however they would probably not have been identified at all if it were not for gene expression analysis.

The conclusions that can be drawn from amphioxus *plastin-a* expression are limited because it is only one of two *plastin* orthologues in amphioxus. *plastin-a* was expressed near the developing gills in amphioxus embryos (Figure 10). This may be significant as there cells residing in the gills of adult amphioxus which resemble lymphocytes. Some relevant genes have been found to be expressed in the gills, including *pax2/5/8* (Chen et al 2010), *ikaros*, *ebf* (Huang et al 2007) and *runx* (Hecht et al 2008). However, as of yet, no research has conclusively shown that these cells in the gills are immune cells, let alone homologues of lymphocytes.

Gene expression in ascidians

Ascidian *fli/erg-a* was expressed in an identical fashion to the patterns previously produced (Imai *et al* 2004) (Figure 13). Although the *ikaros* expression pattern was not as clear as the one produced for *fli/erg-a,* they appear to both be expressed in the mesenchyme. As previously mentioned, the mesenchyme cells are the pre-metamorphosis precursors of the adult haemocytes. *plastin* was not expressed in these cells, despite published results which show expression in adult haemocytes (Ogasawara *et al* 2006) and the presence of *plastin* ESTs in the

haemocyte-specific collection. This lack of mesenchymal/TLC expression does not preclude *plastin* from being expressed in these lineages at later developmental stages or during metamorphosis.

The embryonic *in situ* patterns for *ikaros* and *fli/erg-a* are consistent with the findings that *ikaros* and *fli/erg* are expressed in sea urchin haemocytes and the EST data presented here which indicates *ikaros* and *fli/erg-a* are probably expressed in ascidian haemocytes. The overall conclusion should be that *ikaros* and *fli/erg* genes are probably expressed in haematopoietic lineages throughout the deuterostomes.

Ascidians have orthologues of all of the genes except *lmo2* and *scl*, which appear to have been lost from the genome (Figure 6). Of the genes that are present, the majority have tissue-specific ESTs (Figure 9), indicating that EST data is a valuable resource for understanding adult ascidian gene expression. Of those genes, the majority have haemocyte-specific transcripts, including homologues of genes associated with vertebrate HSCs (*fli/erg-a*), erythroid cells (*globin1, globin2*), myeloid cells (*cebp1, cebp2, spi/pu.1*, *plastin*), and lymphoid cells (*ikaros, pax2/5/8a, pax2/5/8b, sox5/6/13*). Of the five that do not, three have heart-specific transcripts. The adult heart is full of haemocytes (own observations – data not shown), so while these ESTs may represent expression in heart cells, there is a possibility they actually represent haemocyte expression. The fact that several of the genes with haemocyte-specific transcripts also have heart-specific transcripts suggests this may be a genuine possibility. That said, vertebrate homologues of the three genes that have ESTs from the heart but none from the haemocytes (*fli/erg-b, gata1/2/3, runx*) do have some cardiovascular functions/expressions (Levanon & Groner 2004; Val & Black 2009). Two of the globin genes have haemocyte ESTs, despite no erythrocyte-like cells being described in any tunicates. The haemocyte ESTs representing *plastin* are consistent with previously published *in situ* hybridisations showing expression in haemocytes (Ogasawara *et al*

2006). This makes it probable that *in situ* hybridisations for *cebp1, cebp2, fli/erg-a, ikaros, pax2/5/8-a, pax2/5/8-b, spi/pu.1* and *sox5/6/13* would show haemocyte expression.

If a gene has an EST present in a collection from a specific tissue that does not necessarily mean that it is expressed at a significant level there. Nor does a gene being absent from a collection mean that the gene is not expressed there. To demonstrate proper expression would require RT-PCRs or *in situ* hybridisations. The limitations of using EST data such as these are that not every mRNA transcript expressed in a tissue will necessarily be represented, there is a possibility of contamination between tissues, and transcripts per million comparisons cannot be used as a true measure of expression levels. Nevertheless, they do represent good preliminary data. If there are haemocyte-specific ESTs for a gene then there is a good chance that the gene is expressed in haemocytes. Expression in haemocytes is not necessarily indicative of a haematopoietic function, but it does strongly suggest it. Vertebrate *ikaros* is expressed in both developing and mature lymphocytes (Gomez-del Arco *et al* 2005).

The ascidian haematopoietic system has potential for developmental as well evolutionary research. Ascidian larvae have simple body plans with relatively few cells (2600) (Tolkin & Christiaen 2012), and most post-metamorphosis tissues have been mapped onto their precursor larval cells. This simplicity can provide advantages over more complicated model species such as zebrafish and mice. Indeed, identifying and tracking the precursor cells of zebrafish is the subject of current research.

The major implication of this research for developmental biology is the finding that ascidians have haemocytes that seem to express similar genes to vertebrate blood cells. This means that the haematopoietic systems of non-vertebrate and vertebrate chordates may have similar GRNs. It is likely that there are many regulatory genes important in vertebrate haematopoiesis that have

not yet been identified. It is conceivable that one of these genes might be identified because a homologue was involved in ascidian haematopoiesis.

In the introduction, the possibility was raised that if ascidian haemocytes were shown to be homologous to vertebrate myeloid cells then ascidians may of use to wound healing and regeneration research. Although it cannot be said that the data presented in this thesis has shown such a homology, it does provide preliminary evidence for it. Primitive myeloid cells expressing *spi-b* migrate to wounds in *Xenopus* (clawed frog) embryos (Costa *et al* 2008). Ascidian haemocytes appear to express *spi/pu.1*.

The lymphocytes of lampreys have been studied through functional characterisation (Mayer *et al* 2002), establishment of molecular markers (Pancer *et al* 2005), gene expression profiling by ESTs (Mayer *et al* 2002; Pancer *et al* 2004), and gene expression profiling by separation and then RT-PCRs (Guo *et al* 2009; Hirano *et al* 2013). The best way to study ascidian haemocytes is to emulate this order of research. The first three steps have been done, so the next step would be to separate haemocytes using the CD markers and then check expression of the genes investigated in this thesis. It would also be useful to make EST collections from separate subsets of ascidian haemocytes, as this may provide more information on their immunological activities.

Gene expression in cyclostomes

The lamprey *spi/pu.1* gene, as cloned by Shintani *et al* (2000), is not present in the published *P. marinus* genome, despite *in situ* hybridisation confirming that it is expressed. Sequencing of the lamprey genome was done fairly recently, and there were challenges presented by the number of repetitive elements and GC bases, and the programmed genome rearrangements that the lamprey undergoes during embryogenesis (Smith *et al* 2013). This means that if this analysis was repeated

after the publication of better genomic assemblies then it is possible new orthologues would be identified. There was at least one lamprey orthologue for all of the genes studied (Figure 6). *myb, gata1/2/3a,* haemoglobins, *ikaros/helios, pax2/5/8, spi/pu.1* and *sox5/6/13* have already been shown to be expressed in haemocytes or haematopoietic cells of some description (Mayer *et al* 2002, Onimaru *et al* 2011; Hirano *et al* 2013; Schwarze et al 2014). Of these, haemoglobins and *gata1/2/3a* have ESTs present in the lamprey or hagfish haemocyte collections (Figure 10). The analyses presented in this thesis show that *fli/erg, gata4/5/6, lmo, plastin, runx-a* and *scl* also had ESTs in these collections. The presence of haemoglobin transcripts in the lamprey lymphocyte collection more likely indicates that the samples were contaminated with erythrocytes rather than lamprey lymphocytes expressing globin genes. The cells were sorted by light-scattering characteristics (Mayer *et al* 2002; Pancer *et al* 2004), which does not have the specificity of sorting by expression of a specific antigen or GFP reporter. Interestingly, although *ikaros* and *spi/pu.1* transcripts were present in the Mayer *et al* (2002) EST collection (and their expression was confirmed by RT-PCR), neither were present in the Pancer *et al* (2004) lamprey lymphocyte or hagfish leukocyte collections.

The data in this thesis and previous studies of lamprey gene expression combine to suggest that the majority of the key transcription factors involved in haematopoiesis are conserved between jawed and jawless vertebrates.

gata1/2/3 rescue experiment

Haemoglobin expression, indicating the presence of primitive erythrocytes in the zebrafish embryo, was successfully reduced by the gata1 morpholino and then successfully restored with the gata1:gata1/2/3 transgene (Figure 14). The expression patterns matched those produced by

gata1 morpholino injections and in situ hybridisations previously (Rhodes *et al* 2005; Galloway *et al* 2005).

A positive control for the experiment was achieved by co-injecting the gata1 morpholino and a gata1:gata1 transgene. If the experiment was designed correctly gata1 should have rescued knockdown of gata1 because that is essentially replacing gata1 with itself. And indeed, that is what occurred. The majority of the embryos injected showed wild-type haemoglobin expression. 100% of embryos injected with just the morpholino showed a significant reduction of haemoglobin expression in the PLM. This means that embryos that appear 'rescued' are unlikely to actually be WT embryos in which the morpholino has failed.

The finding that amphioxus gata1/2/3 can rescue zebrafish the primary function of gata1 fits in with the paradigm that the changes to development usually evolve through changes to cis-regulatory elements (Carroll 2008). Amphioxus tbx (Minguillon et al 2009) and tfap2 (Van Otterloo et al 2012) have also been shown to be able to functionally substitute for their paralogues in vertebrates. The results here show that the unique function of gata1 in promoting erythropoiesis seems to be primarily due to a unique expression pattern.

Ideally, a gata1 mutant would have been used, such as Vlad Tepes (Lyons *et al* 2002), but, unfortunately, one could not be acquired. Nevertheless, the morpholino and the subsequent rescue produce a consistent enough results to be convincing. When a gata1 construct was used to rescue erythrocyte development in the Vlad Tepes mutant, the rescue rate was around 26% (Takeuchi et al 2010). The rescue success rates in this experiment were >60%. This could be due to differences in the strength of the knockdown between the morpholino and the mutant, differences in the way the rescues were assayed, or differences in injection technique.

gata1/2/3 reporter experiment

pTransgeneis gateway cloning and injection produced successful fluorescent reporter expression in zebrafish embryos (Figure 15). This methodology could be modified to drive expression in other species (Love *et al* 2011) or to analyse other non-coding regulatory elements.

No mCherry fluorescence was detected in haematopoietic cells at any stage observed. In some embryos there was fluorescence in cells near or part of the heart. These cells did not migrate or move with the beating of the heart when observed in live embryos. The identity and function of these cells are currently unknown. To determine whether these cells are involved in the cardiovascular system, this reporter would have to be injected into zebrafish embryos of a stable transgenic line which expressed a reporter in cardiovascular tissues, such as *fli1:gfp,* to see if the cells expressed both reporters.

Given that many embryos displayed mCherry fluorescence in the eye lens – indicating successful injection – but not in these cells, does raise the possibility that the additional expression pattern is an artefact of the experiment. It could be some kind of misexpression due to injection technique. To rule this out, a stable transgenic line would have to be produced to see if homozygous offspring also displayed expression in these cells.

There are some reasons to believe that the expression may be biologically significant. There were *CI-gata1/2/3* transcripts present in the ascidian heart EST collection, and none from any other tissue, including haemocytes. In vertebrates, gata1 (Fan *et al* 2009), gata2 (Connelly *et al* 2006) and gata3 (Raid *et al* 2009) are expressed in endothelial cells.

Evolution of erythrocytes

Globin genes appear to be expressed in the haemocytes of fruit flies, sea urchins, ascidians, cyclostomes and gnathostomes. This suggests that haemoglobin might be ubiquitous to the bilaterians. This is significant for comparative zoology, as there has been a consensus that fruit flies, sea urchins and ascidians do not have cellular haemoglobin-based oxygen transport systems (Glomski & Tamburlin 1990; Hartenstein 2006; Gruner 2013). It has previously been assumed that insects do not need a cellular oxygen transport system due to the diffusion of gases through spiracles and tracheae (Wigglesworth 1990). Globin molecules do have functions other than oxygen transport in vertebrates, such as myoglobin, found in muscle tissue, and neuroglobin, found in the nervous system (Wystub *et al* 2004); however it is difficult to imagine what the function of globin might be in haemocytes other than for oxygen transport. It would be interesting to block the expression of these globin genes in fruit flies, sea urchins and ascidians to see what the negative implications were. Would they have immunological deficiencies or anaemia-like symptoms or other defects?

Whatever the function of these haemoglobins, it is easy to imagine scenarios for how erythrocytes may have evolved. For example, the ancestral cell type may have had immune functions as well as haemoglobin expression. Gradually these cells would lose their immune functions and become more optimised for oxygen-transport. It is possible that such a cell type could be found in some extant species. It could be argued that the EST data already indicates the existence of that cell in fruit flies, sea urchins and ascidians. The next step would be to confirm expression through RT-PCR and *in situ* hybridisation analyses. If haemocytes in these species definitely do produce haemoglobin, then it should be determined whether this is true of all haemocytes or just certain lineages. Fruit flies have at least three distinct lineages (Williams 2007) and sea urchins and ascidians have at least two (Ballarin & Cima 2005; Solek *et al* 2013).

In the introduction, the scenario that erythroid and myeloid lineages evolved from a single ancestral lineage was proposed. It is a very difficult hypothesis to test, given that we cannot check the gene expression of transitional forms. Nevertheless, the presence of haemoglobins in species without distinct erythroid cells may make it possible to find some evidence to support the hypothesis. If the development of a single haematopoietic lineage involved elements of both the myeloid GRN and erythroid GRN, and resulted in a haemocyte that had immune functions and produced haemoglobin, then this would suggest the evolutionary scenario is correct.

gata1/2/3 transcription factors have been shown to be expressed in gnathostome erythrocytes, haematopoietic stem cells and T-lymphocytes (Patient & McGhee 2002), lamprey VLRA+ lymphocytes (Hirano *et al* 2013), the cells which are the developmental precursors of ascidian haemocytes (Imai *et al* 2004), sea urchin haemocytes (Solek 2012; Solek *et al* 2013) and sea scallop haemocytes (Yue *et al* 2014). The data presented in this thesis do not provide any evidence that *gata1/2/3* genes are expressed in the haemocytes of ascidians or the haematopoietic cells of amphioxi. The expression of *fli/erg-a* and *ikaros* in the developmental precursors of haemocytes, does suggest that the expression of *gata1/2/3* in these cells may be relevant to haematopoiesis. The work on sea urchin haematopoiesis remains the best current evidence for a haematopoietic role for gata1/2/3 genes across the deuterostomes.

Globin gene transcripts were present in the haemocyte-specific EST collections of every species that had one. It is possible that each of these species independently evolved such an expression. However, the presence of erythrocyte-like cells in sea cucumbers and bloodworms would suggest that haemoglobin is a feature which is ubiquitous in metazoans.

Two scenarios for the evolution of the erythrocytes were given in the introduction: either a rudimentary erythroid lineage evolved before haemoglobin production or after. The data

presented in this thesis strongly suggest the former. *gata1/2/3* and *globin* are expressed in sea urchin haemocytes, which raises the possibility that the gata-globin regulation key to gnathostome erythropoiesis may have even evolved before erythrocytes did.

First it should be established that *gata1* regulates haemoglobin across the vertebrates. Two gata1/2/3 genes were found in the lamprey genome, but neither showed any obvious direct homology with any single gnathosome paralogue (i.e. gata1, gata2 or gata3). There are two possibilities: the two lamprey paralogues are the result of an independent duplication, or that one corresponds to *gata1* and the other to *gata2/3* (*gata2* and *gata3* were the result of the second WGD). One paralogue has already been shown to be expressed in lymphocytes, similar to *gata3*. So if the latter scenario is true then we should expect to see expression of the other in erythrocytes. If this is the case then morpholinos targeting this gene should reduce haemoglobin expression.

If cyclostomes do have a gata1-like gene that regulates erythropoiesis, then it would be interesting to see if gata1/2/3 regulates globin gene expression in sea urchins and ascidians. This would require chromatin immunoprecipitation (ChIP) sequencing, which would determine whether gata1/2/3 was binding to non-coding genomic regions near globin genes (Liu *et al* 2010). Complementary experiment would be to clone the *cis*-regulatory elements of sea urchin and ascidian globin genes and use them to drive reporter expression in zebrafish. If there was expression in the haemocytes then this would show that haemoglobin regulation is very similar in vertebrates and non-vertebrates. Expression in erythrocytes would indicate that non-vertebrate haemoglobin is probably regulated by gata1/2/3.

Whatever the results of those experiments, it would be necessary to characterise which subsets of ascidian haemocytes express globin genes. If it was all of them then this would suggest that a

specialised erythroid lineage evolved after the tunicate-vertebrate split. If it was just in one subset then it probably evolved before. If ascidians or sea urchins have a subset of haemocytes that express haemoglobin then this does not necessarily mean these cells are homologous to vertebrate erythrocytes. Although, it will always be impossible to completely rule out the possibility that erythrocyte-like cells evolved independently in different taxa, a plausible evolutionary scenario could be built up by studying haemoglobin expression in species from a number of different taxa, including non-deuterostomes such as fruit flies and annelids.
Erythroid and myeloid cells develop from a common precursor. gata1 and fog1 promote the development of the erythroid/thromboid lineage at the expense of the myeloid lineage; and pu.1 and cebpa do the vice-versa (Mancini *et al* 2012). gata1 binds to the DNA sequence [AT]GATA[AG], and its targets include most erythrocyte-specific genes (Welch *et al* 2004). gata1 also has a function in repressing myeloid development. It not only represses the transcription of *pu.1* (Chou *et al* 2009), but also prevents pu.1 from binding to its co-factor *C-jun* by directly interacting with it. pu.1 acts similarly on gata1 to repress erythropoiesis (in addition to activating myeloid-specific genes). The domains of interaction are the C-terminal zinc finger of gata1 and the ETS DNA-binding domain of pu.1 (Liew *et al* 2006). Knockdown of *gata1* reduces the number of erythrocytes and increases the number of myeloid cells; pu.1 does the opposite (Rhodes *et al* 2005).

We do not yet know which genes are regulated by gata1/2/3 and spi/pu.1 in non-vertebrates, but both are expressed in sea urchin innate immune cells. Perhaps the two transcription factors regulated different sets of genes in a common immune cell, and then they evolved to repress each other. This could have led to the segregation of two subsets of immune cells, one of which later became erythroid and the other myeloid. It would therefore be interesting to see if non-vertebrate gata1/2/3 was able to replace gata1 in pu.1-repression. This would be a repeat of the

rescue experiment in this thesis but with myeloid gene expression assayed instead of haemoglobin.

Evolution of lymphocytes

To test whether ascidians have rudimentary lymphoid cells would require a number of steps, which are similar to those used to identify rudimentary neural crest cells in ascidians (Abitua *et al* 2012). Firstly, cells must be found that have anatomical and functional characteristics that could be reasonably described as homologous to vertebrate lymphocytes. Ascidians do not have an adaptive immune system. However work in lampreys has suggested that adaptive immune cells may have evolved from cytotoxic innate immune cells (Wu *et al* 2013), and ascidians have cells that perform this function (Ballarin & Cima 2005). Next, the development of these cells must involve a similar basic GRN as vertebrate lymphocytes. The development of melanocytes in both ascidians and vertebrates (in which they are neural crest cell derivatives) involves activation of *tyrp1* by mitf (Abitua *et al* 2012).

ikaros transcription factors have been shown to be expressed in gnathostome lymphocytes (Schorpp *et al* 2006), lamprey lymphocytes (Mayer *et al* 2002) and sea urchin haemocytes (Solek *et al* 2012). The data presented here suggest they are also expressed in ascidian haemocytes and their developmental precursor cells. The embryonic expression of amphioxus ikaros did not indicate any haematopoietic expression; however published data showed that it is expressed in the gills of adult amphioxi (Huang *et al* 2007) and its expression level changes after bacterial infection (Huang *et al* 2007; Huang *et al* 2011). The gills are the site of T-like lymphocyte development in lampreys (Bajoghli *et al* 2011), and the site of T-lymphocyte development in jawed vertebrates is the thymus which is developmentally originates from pharyngeal pouches

(Zou *et al* 2006). These findings coalesce to suggest that ikaros factors are expressed in immune cells across the deuterostomes.

The expression of *ikaros* in ascidian haemocytes is consistent with the hypothesis that rudimentary lymphoid-like cells existed before adaptive immune systems evolved. If true lymphocytes did evolve from cytotoxic immune cells then *ikaros* might be specifically or preferentially expressed in ascidian cytotoxic haemocytes. There is no direct evidence for this; however different types of haemocytes have been shown to express different types of genes (Ogasawara *et al* 2006), and it would be consistent with data from other species and the evolutionary scenario posited in this thesis.

Expression of *ikaros* in cytotoxic cells would not provide enough evidence by itself to conclude that those cells were homologous to vertebrate lymphocytes. However, if there were a number of homologues of lymphoid genes expressed in cytotoxic cells then that would be more convincing. Cyclostome VLRA, VLRC and VLRA cells can be described as homologous to gnathostome T-αβ cells, T-γδ cells, and B-cells, respectively, because of similarities in both function and gene expression (Hirano *et al* 2013). Because non-vertebrates lack adaptive immune systems, the level of functional similarity between vertebrate lymphocytes and non-vertebrate lymphocyte-like cells cannot reach that of gnathostome and cyclostome lymphocytes. This means we must places greater emphasis on similarities in gene expression. Homologues of *sox13, pax5, ikaros, pu.1, gata3* are expressed in both gnathostome and cyclostome lymphocytes, so it is very likely they were expressed in the lymphocyte-like cells of their common ancestor. Therefore, expression of some, or all, of homologues of these genes in the cytotoxic cells of ascidians would provide evidence that these cells may be homologous to lymphocytes. It is significant that all of these genes have haemocyte-specific ESTs in ascidians.

There is no evidence to suggest that extant non-vertebrates have rudimentary T-αβ-like cells, T-γδ-like cells, and B-like cells. But equally there is no evidence to suggest they do not. The extinct ancestor of gnathostomes and cyclostomes must have had these three lineages, and the exact number of haematopoietic lineages in ascidians is disputed (Ballarin & Cima 2005; Cooper 2014). If some of the genes listed in the previous paragraph were expressed in cytotoxic cells, it would be interesting to see whether there are subsets of these cells that express certain combinations of genes. In vertebrates, *sox13* is indicative of T-γδ/VLRC+ cells, *gata3* is indicative of T-αβ/VLRA+ cells, and *pax5* is indicative of B/VLRB cells (Hirano *et al* 2013).

There remains the possibility that multiple types of haemocytes has evolved independently in vertebrates and ascidians, or that the haemocytes of the two groups have been subject to so much independent evolutionary change, that it is not possible to infer anything about the evolutionary history of this system. I believe the data presented in this thesis suggest that this is probably not the case, although greater analysis of gene expression could find combinations which are difficult to place into particular evolutionary scenarios.

If non-vertebrate chordates do have rudimentary lymphoid cells then it would be interesting to know the evolutionary origin of these cells. Fruit flies do not have a direct homologue of *ikaros*, although it derives from the hunchback family. Across the protostomes, hunchback is involved in mesoderm development and neurogenesis (and anterior-posterior patterning in insects) (Kerner *et al* 2006). It has been proposed that the ancestral function of hunchback was in mesoderm development (Large & Mathies 2010). Given that *ikaros* has been found to be expressed in either the gonad or haemocytes (or both) in a range of bilaterian species (Margolis *et al* 1994; Huang *et al* 2007; Large & Mathies 2010; data presented in this thesis), and that both of these tissues are mesodermal in origin, then this scenario seems likely.

Fruit flies also do not have any direct homologues of *pu.1*, *spi-b* or *spi-c*. These genes are members of the ETS-domain transcription factor family, which includes *fli1* (Laudet *et al* 1999). In fruit flies, the ETS transcription factors pointed and anterior open are involved in haemocyte specification (Williams 2007), a homologue of *ese* is expressed sea scallop haemocytes (Ma *et al* 2009), and a homologue of *fli1* is expressed in annelid haemocytes (Bocquet-Muchembled *et al* 2002). Because the ETS family is so large, it is difficult to infer what the ancestral function of *spi/pu.1* may have been. It should be noted, however, that *spi/pu.1* is only present in deuterostomes (Laudet *et al* 1999; Anderson *et al* 2001) and has been linked to the immune system in a range of deuterostome species (Mayer *et al* 2002; Huang *et al* 2011; Solek 2012).

Given that protostomes do not have homologues of two of the key transcription factors involved in lymphocyte development, the best candidate to look for the earliest origins of the lymphoid lineage would be a basal deuterostome. Currently, in the context of haematopoiesis, sea urchins must be considered the best representatives of the ancestors of deuterostomes. Sea urchins have several types of haemocytes, and at least some of them express homologues of *ikaros* and *spi/pu.1* (Solek 2012; Solek *et al* 2013). Future research could reveal which haemocyte subtypes do and do not express these genes, and what immune functions these subtypes have.

The methodology used for the rescue and reporter experiments could also be used to investigate questions about the evolution of lymphocytes. In mice, spi-b can replace pu.1 in myeloid development but not in lymphoid development (Dahl *et al* 2002), which shows that the protein-coding evolution may have been important in the evolution of vertebrate pu.1. The equivalent experiment has not been attempted with ikaros, although similar research has shown that the ikaros/hunchback-like genes of roundworms are not completely functionally equivalent (Large & Mathies 2010). Non-vertebrate gata1/2/3 may be able to functionally replace gata1 in erythrocyte development; however the gata-globin GRN may have been established before the

evolution of vertebrates. ikaros is involved in both B-cell and T-cell receptor gene recombination (Collins *et al* 2013). Non-vertebrates do not have B-cell and T-cell receptors and lack many other important genes involved in lymphocyte function (Cooper & Alder 2006).

It is unclear how important *cis*-regulatory neofunctionalisation was in the evolution of lymphoid transcription factors. Take *pax2/5/8*, for example. *pax5* is expressed in B-cells in gnathostomes (Medvedovic *et al* 2011), whereas neither *pax2* nor *pax8* have haematopoietic expression patterns. The single lamprey *pax2/5/8* is expressed in the B-cell-like VLRB+ cells (Hirano *et al* 2013), and pax2/5/8 genes are probably expressed in ascidian haemocytes. This suggests that *pax2* and *pax8* lost lymphoid enhancers, rather than *pax5* gaining them. The non-haematopoietic expression patterns of amphioxus, ascidian and lamprey *pax2/5/8* genes suggest considerable subfunctionalisation after the whole genome duplications (Goode & Elgar 2009; Hirano *et al* 2013).

Most of the key lymphoid transcription factors show no signs of gain of lymphoid enhancers. All of the gnathostome *ikaros* paralogues are expressed in lymphocytes (Merkenschlager 2010), and a single, non-duplicated *ikaros* is expressed in the lymphocytes of lampreys (Mayer *et al* 2002) and the haemocytes of ascidians, sea urchins (Solek 2012) and possibly amphioxi (Huang et al 2007, 2011). Gonadal expression is found in the single *ikaros* of lampreys (Mayer *et al* 2002b), ascidians (according to ESTs – data not shown) and amphioxi (Huang *et al* 2007). Similarly, all of the gnathostome *spi/pu.1* paralogues are expressed in lymphocytes (Shintani *et al* 2000; Mayer *et al* 2002), and a single, non-duplicated *spi/pu.1* is expressed in the lymphocytes of lampreys (Mayer *et al* 2002) and the haemocytes of ascidians, sea urchins (Solek 2012; Solek *et al* 2013) and possibly amphioxi (Huang *et al* 2011). The expression of *pu.1*, *spi-b* and *spi-c* is largely restricted to the haematopoietic system (Anderson *et al* 2001). There were, however, *spi/pu.1* ESTs from the ascidian 'digestive system' collection (data not shown). This may merit further investigation, as

plastin, *cebp-a* and *cebp-b* also had ESTs from this collection (in addition to haemocytes) (data not shown).

If non-vertebrate *cis*-regulatory sequences for *ikaros, spi/pu.1,* etc. were made to drive reporter expression in zebrafish, as described in this thesis, then there is a possibility that one may drive expression in lymphocytes. This would provide further evidence for rudimentary lymphoid-like cells in non-vertebrates.

Given that the lymphocytes of gnathostomes and cyclostomes use receptors of very different phylogenetic origins (Cooper & Alder 2006), it may not be possible to find a molecular marker of vertebrate lymphocytes that is not a regulatory gene. Erythrocyte evolution can be investigated through studying haemoglobin regulation in a range of species, but it may prove impossible to design similar research for the evolution of lymphocytes.

Conclusion

Ascidians appear to be the most promising candidate organism for studying the evolution of vertebrate haematopoiesis. Sea urchin and fruit fly haematopoiesis have been studied in more detail, but ascidians are phylogenetically much more closely related to the vertebrates. Cyclostomes have all the same blood cell types as gnathostomes, so they cannot be used to identify rudimentary or evolutionarily intermediate lineages. There is too little research done on amphioxus haematopoiesis to make any solid conclusions.

Most of the genes chosen to be this studied either had ESTs from haemocytes or their *in situ* hybridisation expression patterns were observed in cells that become haemocytes later in development. The haemocytic expression of globin genes and homologues of lymphoid genes

(e.g. *ikaros, pu.1, pax5, sox13*) suggests that ascidians may have rudimentary erythroid or lymphoid cells. Future research should focus on characterisation of ascidian haemocyte subtypes (and their development) by gene expression.

A variety of sources of data highlight the importance of gata1/2/3 transcription factors to the evolution of haematopoiesis in general, and erythrocytes and T-lymphocytes in particular. In this thesis, evidence was presented that suggested that gata1's unique function in erythropoiesis originated primarily from regulatory changes rather than protein-coding changes. The techniques used in this thesis could be modified to complete this line of enquiry and then expand it to the other functions of gata1 and other genes altogether.

The data in this thesis should be considered preliminary evidence in what could become a much larger project looking at the evolution of vertebrate haematopoiesis. Nevertheless, taken as they are, they 'fill in the gaps' considerably (see Figure 16).

	Evidence for expression in haematopoietic or immune cells					Inferred origin
Gnathostome genes	**Fruitflies**	**Sea urchins**	**Amphioxi**	**Ascidians**	**Cyclostomes**	**of expression**
cebp-a, cebp-b				*		Deuterostome
c-myb						Bilaterian
ebf1						Bilaterian
fli1				*	*	Deuterostome
fog1						Bilaterian
gata1, gata2, gata3						Deuterostome
gata4, gata5, gata6					*	Bilaterian
haemoglobin	*	*		*		Bilaterian*
ikaros, helios, aiolos, eos				*		Deuterostome
lmo2						Deuterostome
l-plastin	*				*	Bilaterian*
pax5				*		Olfactore
pu.1, spi-b, spi-c				*		Deuterostome
runx1, runx3						Bilaterian
scl					*	Deuterostome
sox6, sox13				*		Olfactore*
	Bilaterians					
		Deuterostomes				
			Chordates			
				Olfactores		
					Vertebrates	

Figure 16 – Evidence for expression in haematopoietic or immune cells of each of the organisms and each of the genes studied (see 'Choice of genes' below) from the published literature and the data in this thesis. Green denotes that there is evidence of some kind; grey denotes that there is no evidence. The origin of this haematopoietic/immune expression is inferred from the most basal organism for which there is evidence. Stars (*) indicate the presence of evidence or an inferred origin that has been changed because of the data presented in this thesis.

Several genes had never been linked to haematopoiesis or haemocytes in several species. In many cases, the inferred origin of their haematopoietic expression has been pushed back considerably. For example, previously the origin of haemoglobin could only be traced back to the vertebrate ancestor; now we can conclude that it may have been in the ancestor of bilaterians.

To conclude: this thesis adds to our understanding of the origins of vertebrate haematopoiesis. The system remains a promising case study for understanding the evolution of novelty and complexity.

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