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Exploring the role of *wnt10b* as leukemia driver in zebrafish model

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EXPLORING THE ROLE OF *WNT10B* AS LEUKEMIA DRIVER IN ZEBRAFISH MODEL

ABSTRACT

Acute myeloid leukemia (AML) is a heterogeneous clonal disorder characterized by a misregulation in the differentiation program of myeloid progenitor cells that proliferate abnormally and arrest in their maturation. In the effort to reveal the mechanism that confers self-renewal potential to Leukemia Stem Cells (LSCs), Wnt pathway came out as a candidate. The Wnt pathway has a central role in the hematopoiesis regulating the hematopoietic stem cells (HSCs) self-renewal. A recent study showed an increase of WNT proteins (i.e. WNT10B) expression and release within the microenvironment in both leukemic blasts and stromal-like cells of AML patients, indicating its possible autocrine/paracrine involvement in the bone marrow microenvironment, and suggesting that the regenerative WNT signaling is a stem cell-associated function altered in AML stem cell fraction. The aim of this work is to elucidate the role of WNT10B in AML generating an animal model in which this signaling is disregulated. The striking similarities of zebrafish and human hematopoiesis make this fish an excellent model for elucidating physiologic and pathological mechanisms. Furthermore, various hematopoietic zebrafish mutants, mimicking the human hematopoietic system diseases, have been generated. *wnt10b* was transiently upregulated in zebrafish embryos and effects of such misregulation on embryonic hematopoiesis were analyzed. *In-situ* hybridization assays, performed using myeloid and HSCs markers (*pu.1* and *scl*, respectively), and *in vivo* observation of transgenic fish lines expressing fluorescent proteins in erythroid progenitors and mature neutrophils - *Tg(gata1:dsRed)* and *Tg(mpx:EGFP)* - demonstrated an abnormal accumulation of HSCs, myeloid and erythroid progenitors in the embryo hematopoietic tissue, at the expense of more differentiated cells. Moreover, *wnt10b* overexpression affects the migration of myeloid cells in the ALM (Anterior Lateral Mesoderm). Taken together, these results support the notion of an involvement of *wnt10b* in vertebrate hematopoiesis. A plasmid construct with zebrafish *wnt10b* under the control of *runx1* (a pivotal gene in definitive hematopoiesis) promoter and flanked by trasposase-responsive elements has been generated; injection of this plasmid in zebrafish embryos together with trasposase mRNA will lead to the generation of a transgenic line in which *wnt10b* expression can be induced only in definitive HSCs. Future plans include the temporal control of the transgene expression through the insertion in the plasmid of a lox-P cassette flanking a STOP signal and the outcross of the resulting transgenic line with a line expressing Cre recombinase under the control of hsp70 (heat-shock protein) promoter. This model will

provide the chance to better understand the Wnt-mediated early mechanisms that lead to leukemia development.

INTRODUCTION

ACUTE MYELOID LEUKEMIA

Acute myeloid leukemia (AML) is a heterogeneous clonal disorder that results from an abnormal expansion of uncontrollably proliferating myeloid progenitors that lose the ability to differentiate (Sykes *et al.*, 2015: Estey and Döhner, 2006). The consequence of this abnormal proliferation is the lack of differentiated blood cells, leading to infection, bleeding or organ infiltration. The incidence is greater in adults 65 years of age and older.

Treatment in younger adults consists of two phases, an induction therapy that aims to produce complete remission, and a post-remission therapy, in order to maintain remission (Estey and Döhner, 2006). Elderly patients are characterized by unfavourable prognostic factors, in addition to their inability to tolerate cytotoxic chemotherapy, making it difficult to have a complete remission (Lowenberg *et al.*, 1999).

Different classifications of AML have been proposed: the FAB (French – American – British) classification (1976) is based on the myeloid lineage involved and the degree of leukemic cell differentiation (Table 1) (Lowenberg *et al.*, 1999), whereas the more recent WHO classification (Corradini and Foà, 2008) looks at the morphologic, clinical, genetic and molecular features of the various disorders. The European LeukemiaNet (ELN) (Mrózek *et al.*, 2012) and the National Comprehensive Cancer Network (NCCN) (NCCN Clinical Practice Guidelines in Oncology, 2014) classifications integrate genetic findings with treatment outcome in AML, and divide cases in prognostic risk groups.

Secondary AML arises from myelodysplastic syndrome (MDS) and myeloproliferative neoplasms (MPNs) or after treatment with cytotoxic chemotherapy, whereas the etiology of *de novo* (or primary) AML remains poorly understood. Several risk factors have been associated with its development, such as benzene and pesticides exposures, cigarette smoking, and genetic disorders (Sykes *et al.*, 2015; Corradini and Foà, 2008; Estey and Döhner, 2006).

GENETICS OF AML

Both *de novo* and secondary AML are characterized by chromosomal abnormalities, such as deletions and translocations (Deschler and Lübbert, 2006).

Many chromosomal translocations have been associated with AML, more than the known leukemic phenotypes. So the fusion proteins originating from these rearrangements likely misregulate similar transcription or transduction pathways involved in the normal

hematopoietic development. Members of this group of transcription factors are Core Binding Factor (CBF), retinoic acid receptor alpha (RARα), and HOX family components (Kelly and Gilliland, 2002).

TABLE 1. THE FRENCH–AMERICAN–BRITISH (FAB) CLASSIFICATION OF AML AND ASSOCIATED GENETIC ABNORMALITIES.

FAB SUBTYPE	COMMON NAME (% OF CASES)	RESULTS OF STAINING			ASSOCIATED TRANSLOCATIONS AND REARRANGEMENTS (% OF CASES)	GENES INVOLVED
		MYELOPEROXIDASE	SUDAN BLACK	NONSPECIFIC ESTERASE		
M0	Acute myeloblastic leukemia with minimal differentiation (3%)	−	−	−*	inv(3q26) and t(3;3) (1%)	*EVI1*
M1	Acute myeloblastic leukemia without maturation (15–20%)	+	+	−		
M2	Acute myeloblastic leukemia with maturation (25–30%)	+	+	−	t(8;21) (40%), t(6;9) (1%)	*AML1-ETO, DEK-CAN*
M3	Acute promyelocytic leukemia (5–10%)	+	+	−	t(15;17) (98%), t(11;17) (1%), t(5;17) (1%)	*PML-RARα, PLZF-RARα, NPM RARα*
M4	Acute myelomonocytic leukemia (20%)	+	+	+	11q23 (20%), inv(3q26) and t(3;3) (3%), t(6;9) (1%)	*MLL, DEK-CAN, EVI1*
M4Eo	Acute myelomonocytic leukemia with abnormal eosinophils (5–10%)	+	+	+	inv(16), t(16;16) (80%)	*CBFβ-MYH11*
M5	Acute monocytic leukemia (2–9%)	−	−	+	11q23 (20%), t(8;16) (2%)	*MLL, MOZ-CBP*
M6	Erythroleukemia (3–5%)	+	+	−		
M7	Acute megakaryocytic leukemia (3–12%)	−	−	+†	t(1;22) (5%)	Unknown

*Cells are positive for myeloid antigen (e.g., CD13 and CD33).

†Cells are positive for α-naphthylacetate and platelet glycoprotein IIb/IIIa or factor VIII–related antigen and negative for naphthylbutyrate.

Table 1. The FAB classification of AML and associated genetic abnormalities

(from Lowenberg *et al.*, 1999)

CBF is composed of a DNA binding component, named AML1 (also known as RUNX1) and a second subunit (CBFβ) that regulates the transcriptional activity of AML1. The translocations involving CBF are t(8;21), inv(16), t(12;21), with resulting fusion proteins AML1/ETO, CBFβ/SMMHC, and TEL/AML1, respectively, in which CBF transcriptional activity is repressed. The most common translocation involving RARα produces the fusion protein PML/RARα. A common feature in the mechanism of transcriptional repression mediated by these proteins is the aberrant recruitment of the nuclear corepressor/histone deacetylase

complex. As a result, the transcription of genes pivotal in hematopoietic development is blocked and therefore blood cell differentiation is impaired (Kelly and Gilliland, 2002).

Translocations involving HOX family members probably lead to the constitutive activation of proteins which activity normally decreases during differentiation; the consequence is the abnormal increase of progenitors at the expense of mature blood cells (Forrester *et al.*, 2011).

Even if these translocation events alter hematopoietic development, they are not sufficient to drive AML. Several studies demonstrated the requirement for a second mutation to induce the disorder (Kelly and Gilliland, 2002).

Gilliland and Griffin (Gilliland and Griffin, 2002) proposed a "2-hit model" of leukemogenesis (Fig. 1) and identified two different classes of mutations: class I is represented by mutations that confer proliferative and survival advantage without affecting differentiation, whereas class II mutations impair hematopoietic differentiation. AML develops when mutations from both classes are present. AML1/ETO, CBFβ/SMMHC, TEL/AML1, PML/RARα and HOX genes mutations are members of class II. In class I we can find *RAS*, *KIT*, and *FLT3* genes, that have a role in normal hematopoiesis, and whose mutations lead to the constitutive activation of tyrosine kinases or their downstream effectors, with subsequent impairment of cell proliferation (Beghini, 2000; Gilliland and Griffin, 2002; Grimwade *et al.*, 2015).

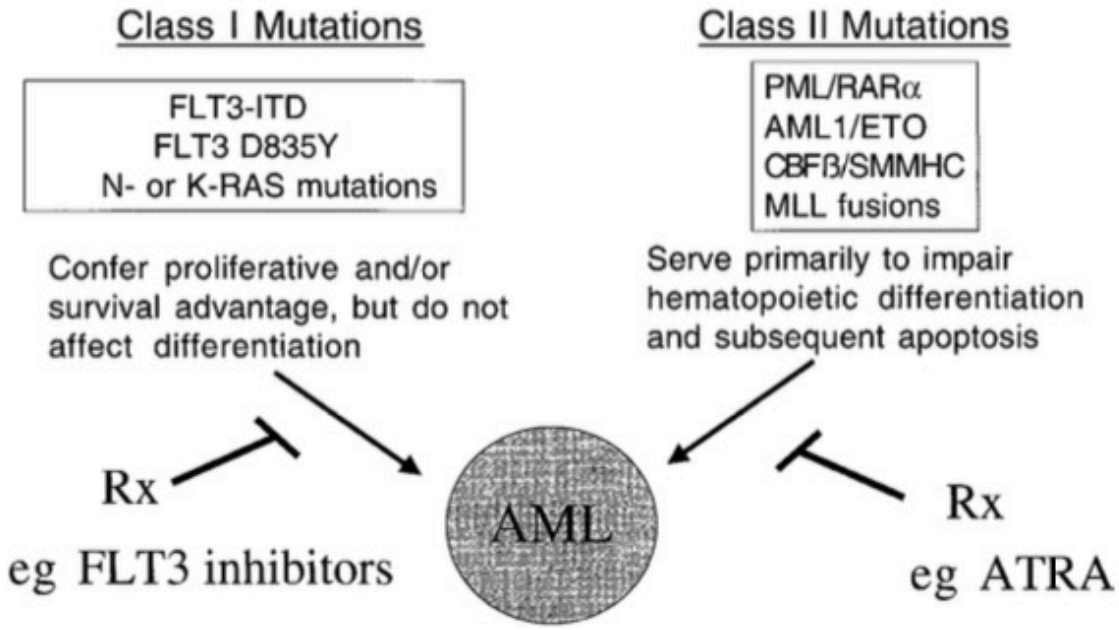

Fig. 1. "Two-hit" model of leukemogenesis.

(from Gilliland and Griffin, 2002)

Jan and collaborators (Jan *et al.*, 2012) proposed that hematopoietic stem cells accumulate serial mutations in "landscaping genes" with roles in DNA methylation, chromatin modification, and cohesin complex, resulting in the formation of a pre-leukemic hematopoietic stem cells population. These cells remain functionally normal until they acquire other mutations in proliferating genes: these additional event leads to AML development (Sykes *et al.*, 2015).

The maintenance of leukemia is guaranteed by the presence of a subset of AML cells characterized by limitless self-renewal (Leukemia stem cells, LSCs), a property shared with normal HSCs (Hematopoietic Stem Cells). Since these features make LSCs potential therapeutic targets, several studies focused on those signaling pathways whose control their development (Wang *et al.*, 2010). One of the strongest candidates is the Wnt pathway.

THE WNT PATHWAY IN DEVELOPMENT, HEMATOPOIESIS AND BLOOD DISEASES

The Wnt pathway is highly evolutionarily conserved and plays roles in several developmental processes. Its genes encode secreted glycoproteins that interact with membrane receptors (the Frizzled family) activating different intracellular pathways. The canonical Wnt pathway leads to beta-catenin activation: instead of being degraded, as in the absence of Wnt ligands, beta-catenin is translocated in the nucleus, where it acts as a transcription activator. This pathway is highly regulated through various molecules that act in the different steps of the cascade, interfering with the receptors, the beta-catenin degradation complex, or the transcriptional complex (Mikesch *et al.*, 2007).

The Wnt pathway is involved in many processes during embryogenesis, acting on cell fate specification, cell differentiation and proliferation, and determination of dorso-ventral and antero-posterior axis (Hikasa *et al.*, 2013). It also plays a pivotal role in the homeostasis of many tissues and organs, regulating the determination and maintenance of stem cell compartments. That said it is not surprisingly that malignancies involving different organs, as heart, intestine, mammary gland, and CNS have been associated with misregulation of the pathway (Ring *et al.*, 2014).

In particular, the Wnt signaling has a function in the control of hematopoietic progenitors and stem cells self-renewal, thereby regulating their proliferation (Mikesch *et al.*, 2007). Congdon and coworkers (Congdon *et al.*, 2008) found that the Wnt signaling is highly activated after injury during hematopoietic regeneration; interestingly, the regeneration process is

supported by bone marrow microenvironment through an increased expression of the Wnt ligand WNT10B (Congdon *et al.*, 2008).

WNT10B is upregulated in various malignant disorders (for a complete review see Wend *et al.*, 2012) and several lines of evidence point to a correlation between the Wnt signaling misregulation and the AML pathogenesis; indeed, many AML cases are characterized by an aberrant expression of Wnt ligands, that upregulate the signaling pathway and stimulate LSCs proliferation (Mikesch *et al.*, 2007).

Beghini and colleagues (Beghini *et al.*, 2012) reported an overall upregulation of the Wnt signaling in AML cells, with the notably increase in the expression of *WNT10B* in AML patients bone marrow microenvironment, suggesting that the regenerative Wnt signaling is altered in AML.

ZEBRAFISH AS A MODEL SYSTEM

Given that a good animal model has to be easy and cheap to maintain, share functional processes with human, and guarantee about rapid and reproducible experiments, zebrafish (*Danio rerio*) has became, in the last years, fundamental in developmental biology, pharmacology and biomedicine for the study of various diseases.

This freshwater teleost originated in the shallow waterways of the Indian subcontinent, and is easy and cheap to maintain thanks to its small size. More than 200 eggs can be obtained from a single mating event, the fertilization is external and the embryo develops quite rapidly: in just 24 hours the main organs are laid out (Fig. 2). The embryos are transparent during their first day of development, and later pigmentation can be chemically inhibited, making it easy to analyze embryogenesis and organogenesis. Pathways and gene functions are conserved between zebrafish and the other Vertebrates, and almost all the genes associated with human diseases have their homologue in zebrafish (Santoriello and Zon, 2012).

Zebrafish embryos adsorb chemicals through their water, so they can be used for *in-vivo* drug screenings characterized by unbiased/high-throughput outcomes of the *in-vitro* systems. Many chemicals, or different concentrations of one compound, can be simply tested using a multi-well plate and observing siblings development (Garcia *et al.*, 2016).

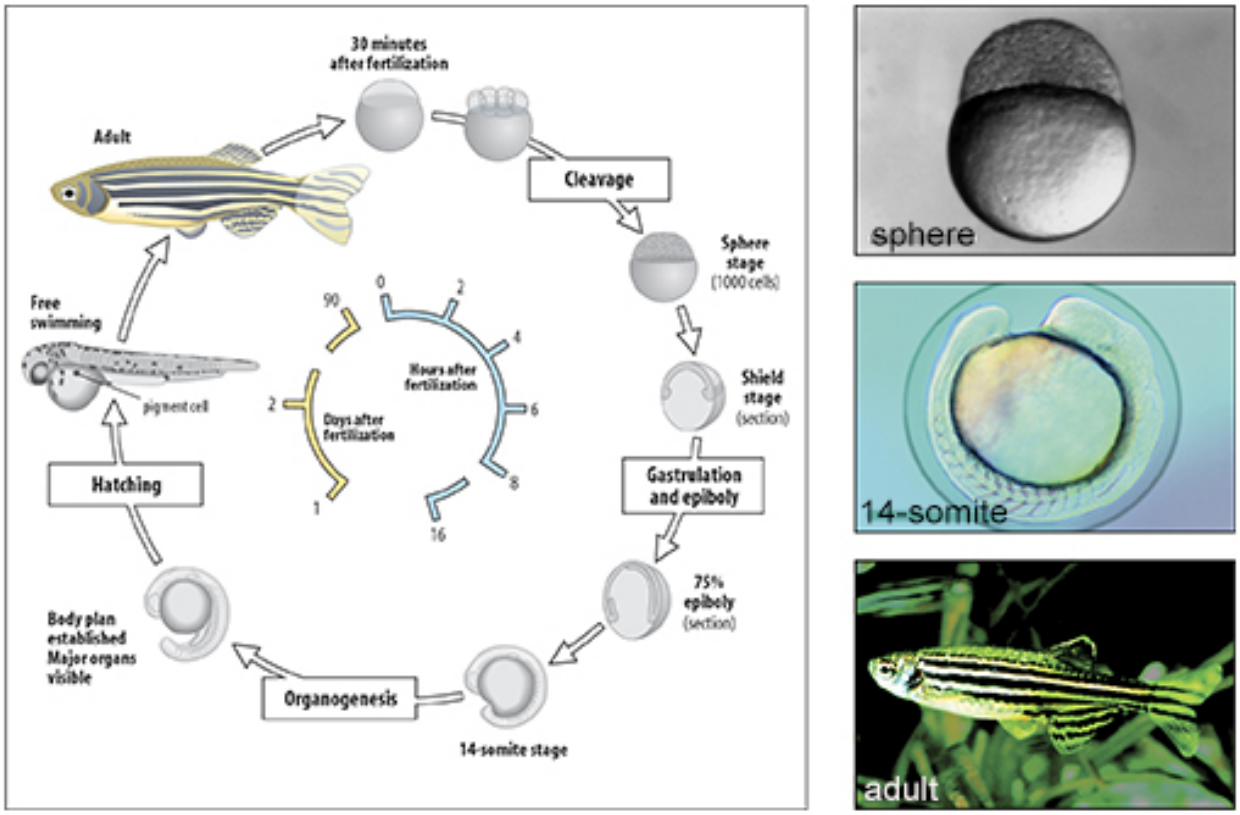

Fig. 2. Zebrafish life cycle. On the left: stages of zebrafish development. On the right: live images of representative stages (modified from Allison D'Costa and Iain T. Shepherd, 2009).

Alteration of gene expression

Zebrafish embryos can be easily injected with nucleic acids in order to alter gene expression. Gene overexpression can be performed by the injection into the one-cell embryo (zygote) of a specific synthetic transcript (Fig. 3), whereas the injection of an antisense oligonucleotide (morpholino, MO) complementary to a target mRNA can block its splicing or its translation into protein (gene expression knock-down). MO knock-down and mRNA overexpression can be performed individually or together; the following analysis on embryo development at phenotypic, cellular, and molecular level provide useful information concerning the involvement of the gene of interest in a specific pathway.

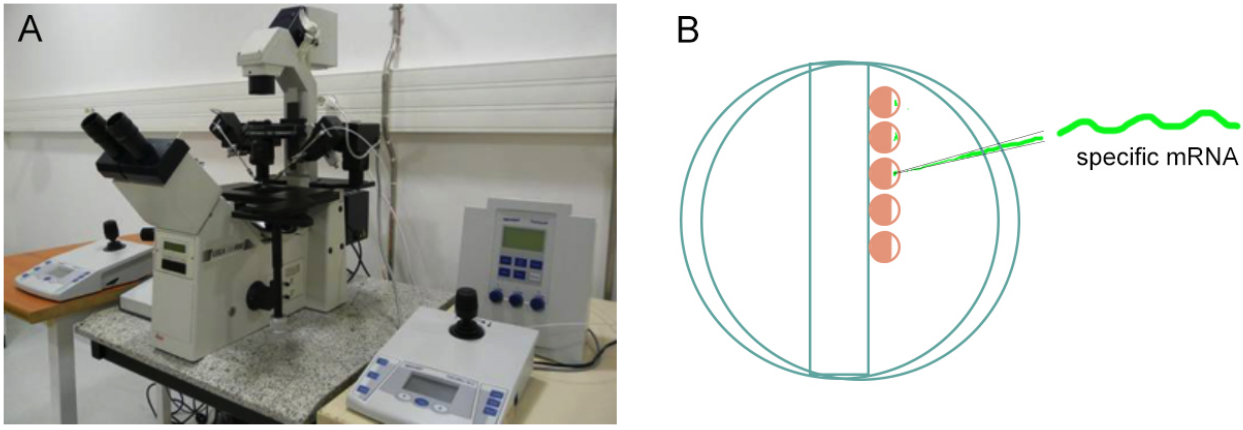

Fig. 3. Zebrafish embryos micromanipulation. A) Microinjection apparatus. **B**) Semplified scheme of microinjection: a synthetic transcript (in green) is injected into zygotes (pink spheres) laid on a microscope glass (in blue).

Recent studies pointed out the possibility to easily generate stable gene knock-out (KO), using the CRISPR-Cas9 system, that returns targeted double strand breaks followed by the error-prone DNA repair mechanism recruitment that causes deletion/insertion mutations with high frequency (Garcia *et al.*, 2016).

In order to analyse *in vivo* cell behaviour and organogenesis, as well as the effects on development caused by gene misregulation, many transgenic lines have been generated (Kawakami *et al.*, 2007). Different methods exist to deliver a specific construct into the embryo. The first developed method was the plasmid-based transgenesis, characterized by the injection into the zygote of a plasmid; due to a mosaic expression of the transgene in F0, only a small percentage of the offspring receive the transgene (Thermes *et al.*, 2002). A subsequent approach, proposed by Thermes and coworkers (Thermes *et al.*, 2002) was based on the coinjection of I-SceI meganuclease, a restriction enzyme, with a plasmid that contains the construct flanked by corresponding restriction sites; with this method mosaic expression of transgene in F0 diminished and germline transmission rates increased, improving transgenesis efficiency.

Kawakami and colleagues presented an efficient transgenesis method, in which the mRNA coding the Tol2 transposase is coinjected in the zygote with a plasmid containing a transposon construct; the transgene is expressed ubiquitously through the body in F0, with very high transgenesis frequency (Kawakami, 2007).

Whatever is the method, the plasmid injected in the zygote can carry a reporter gene (for example the green fluorescent protein, GFP) under the control of a specific tissue promoter, allowing the observation, under a fluorescent microscope, of that tissue development and cells behavior. Furthermore, the zygote can be injected with a plasmid that contains a gene associated with a human disease under the control of a promoter regulating the expression in the organ involved in that disease; the resulting transgenic line would be characterized by stable overexpression of the wild type or the mutated form of the gene of interest, allowing a more detailed analysis of the involvement of such gene in the onset and progression of the disease.

ZEBRAFISH AND HUMAN HEMATOPOIESIS RESEMBLANCES

Hematopoiesis is the process of blood cellular components formation that begins during embryogenesis to create a functional blood system and continues throughout adulthood to replenish it.

This process shares many steps among Vertebrates. First of all, there are two phases in hematopoiesis: the primitive wave and the definitive wave. The aim of the primitive wave is to generate non-pluripotent progenitors that give rise to erythrocytes and macrophages, in order to permit growth and survival; this wave is transient and occurs in blood islands of the extra-embryonic yolk sac of mammals and avians (Fig. 4-step 1) (Jagannathan and Zon, 2013).

Human definitive HSCs (multipotent cells that give rise to all blood lineages) arise from the ventral wall of the dorsal aorta in a region called aorta – gonad – mesonephros (AGM) (Fig. 4-step 2), migrate to fetal liver and finally to thymus and bone marrow (Fig. 4-step 3), where adult hematopoiesis takes place (Jagannathan and Zon, 2013).

On the other hand, zebrafish primitive hematopoiesis (Fig. 5-yellow panel) is intra-embryonic; it occurs during the first day of development in the anterior (ALM) and posterior (PLM) lateral mesoderm. The ALM is the site of myelopoiesis, and gives rise to two different types of myeloid cells: granulocytes, and monocytes and macrophages (Chen and Zon, 2009). As for mammals, the master regulator of primitive myeloid cell development is the transcription factor *pu.1/spi1* (Jagannathan and Zon, 2013).

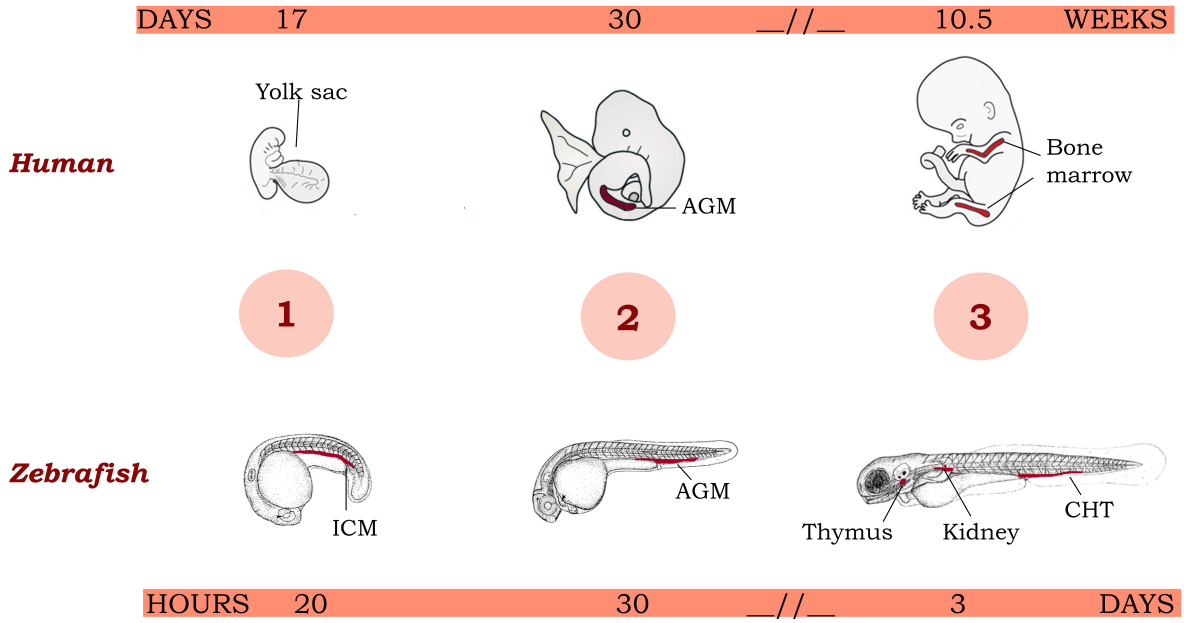

Fig. 4. Comparison of human and zebrafish hematopoiesis. Human hematopoiesis: 1- embryonic hematopoiesis takes place in the yolk sac; 2- during definitive wave HSCs arise from AGM; 3- definitive hematopoiesis takes place in bone marrow and thymus. Zebrafish hematopoiesis: 1- primitive wave occurs in lateral mesoderm and ICM; 2- during definitive wave HSCs arise from AGM; 3- definitive HSCs colonize thymus and kidney (Images from Baron *et al*, 2012; Medvinsky *et al.*, 2011; Kimmel *et al.*, 1995).

The PLM cells, expressing the evolutionary conserved transcription factor *gata1* (Jagannathan and Zon, 2013), migrate medially to form the intermediate cell mass (ICM) (Fig. 4-step 1) and differentiate into proerythroblasts that enter the circulation at 24 hours post fertilization (hpf) (Chen and Zon, 2009).

From 24 to 36 hpf erythromyeloid progenitors, without self-renewal capability, are found in the posterior blood island (PBI) (Fig. 5-pink panel), also known as the caudal hematopoietic tissue (CHT); these cells give rise to erythroid and myeloid cells during the transient definitive wave (Chen and Zon, 2009).

Definitive wave of zebrafish hematopoiesis (Fig. 5-blue panel) originates in the AGM (Fig. 4-step 2), where HSCs emerge from the endothelium of the ventral wall of the aorta and differentiate into progenitors that migrate and seed the CHT, the thymus and the pronephros (Fig. 4-step 3), where they proliferate in order to expand the stem cell niche. CHT

hematopoiesis stops at 6 days post fertilization (dpf), as the primary site of definitive hematopoiesis in the adult is the kidney marrow (Chen and Zon, 2009).

Like in mammals, zebrafish definitive hematopoiesis is dependent on the transcription factor *runx1*, expressed in HSCs during embryogenesis and adulthood (Chen and Zon, 2009); mice lacking *runx1* expression lose all blood cell lineages (Jagannathan and Zon, 2013), and zebrafish embryos injected with *runx1* morpholino display defects in vasculogenesis and primitive and definitive hematopoiesis, with an enlargement of the ICM (due to an accumulation of hematopoietic progenitors), lack of normal circulation, and reduced expression of *myb*, a transcription factor essential for the formation of definitive blood lineages (Kalev-Zylinska *et al.*, 2002).

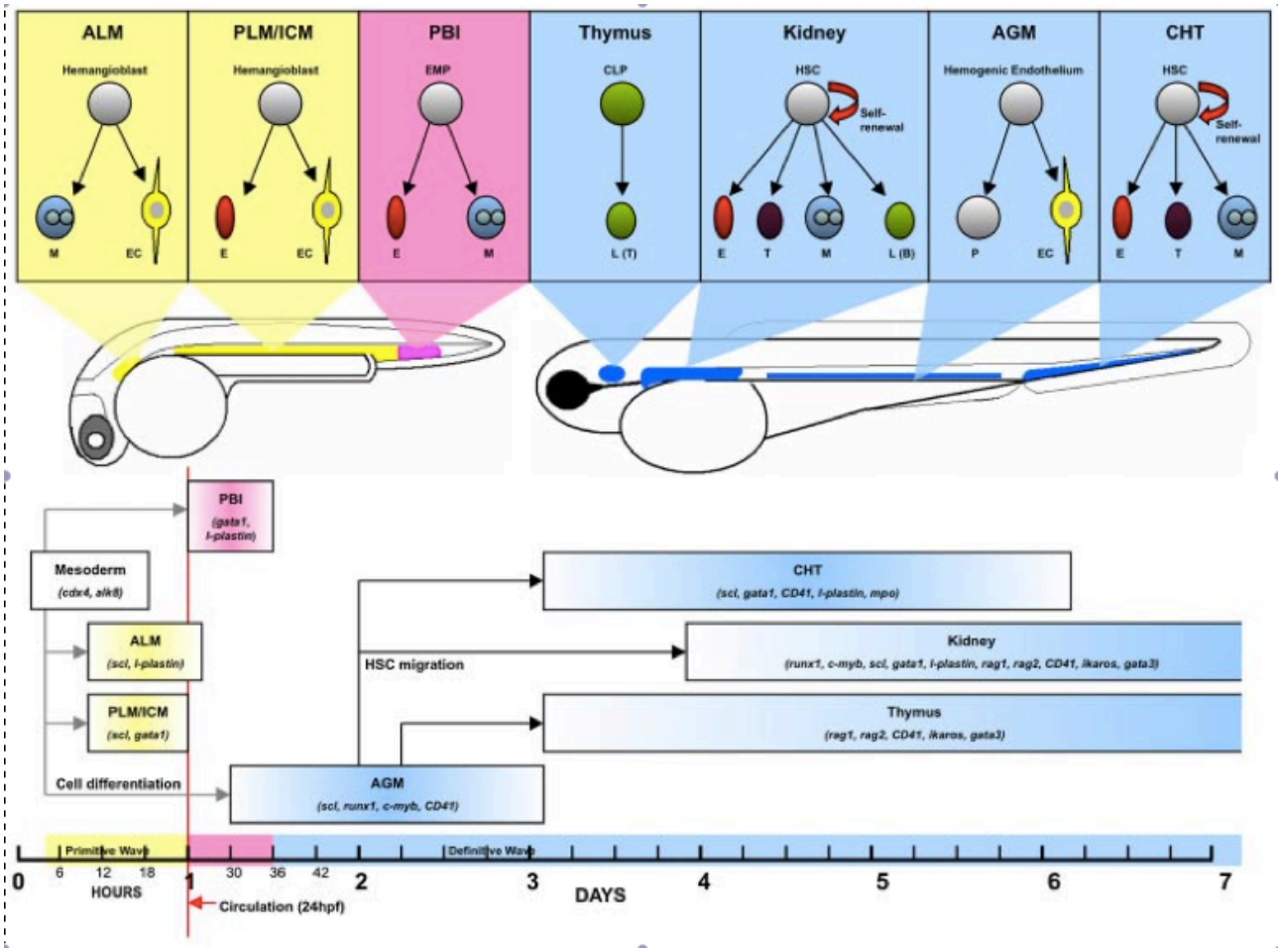

Fig. 5. Spatial and temporal representation of hematopoiesis in the zebrafish. Primitive wave (yellow) consists of hemangioblasts that give rise to myeloid (M) and endothelial cells

(EC) in the anterior lateral mesoderm (ALM), and to erythroid (E) and ECs in the posterior lateral mesoderm (PLM), which later becomes the intermediate cell mass (ICM). In the posterior blood island (PBI), the erythromyeloid progenitors (EMPs) give rise to erythroid and myeloid cells in a transient wave (pink). The definitive wave (blue), that contains the long-term self-renewing hematopoietic stem cells (HSCs), originates in the aorta-gonad-mesonephros (AGM). ECs and hematopoietic progenitor cells (P) that originate in the AGM migrate to and colonize other sites of definitive hematopoiesis. In the caudal hematopoietic tissue (CHT) HSCs proliferate and give rise to erythroid, myeloid and thromboid (T) lineages. Kidney marrow contains all the different blood lineages, including the lymphoid B cell (L(B)) lineage. The common lymphoid progenitor (CLP) mature into lymphoid cells (L(T)) in the thymus. The locations of different sites of hematopoiesis are depicted in a 24 hpf and a 72 hpf embryo (from Chen and Zon, 2009).

ZEBRAFISH AS A MODEL FOR BLOOD DISORDERS

The evolutionary conservation of transcription factors and their role in hematopoiesis between zebrafish and mammals makes of zebrafish an excellent model to study the pathways involved in blood system development and their misregulation in blood diseases. Several transgenic lines, characterized by the expression of fluorescent proteins in different blood cell types, have been generated, such as *spi1*:EGFP for myeloid progenitors (Ward *et al.*, 2003), *mpx*:GFP for neutrophils (Renshaw *et al.*, 2006), and *gata1*:DsRed for erythroblasts (Traver *et al.*, 2003).

In addition, zebrafish has been used to mimic human leukemia by transient or stable overexpression and knock-down of genes involved in its pathogenesis. The first transgenic zebrafish generated to model leukemia expressed the mouse *Myc* gene (involved in definitive hematopoiesis) under the control of the lymphoid marker *Rag2*; this transgenic line displayed the typical features of T-cell acute lymphoblastic leukemia (T-ALL) (Jing and Zon, 2011).

AML has also been modeled in zebrafish (Jing and Zon, 2011). A transgenic line expressing the fusion gene AML/ETO under the control of *hsp70* promoter has been generated. AML/ETO overexpression led to the misregulation of the myeloid-erythroid balance at the expense of the erythroid cell lineage (Payne and Look, 2009). Another model involved the human oncogene MOZ/TIF2, expressed in this AML-zebrafish under the control of *spi1*, which led to the accumulation of myeloid cells in the kidney (Rasighaemi *et al.*, 2015).

Forrester and colleagues (Forrester *et al.*, 2011) reported the generation of a transgenic zebrafish line expressing the human fusion gene NUP98-HOXA9, normally associated with poor prognosis in AML, under the control of the *spi1* promoter in a Cre/lox inducible fashion; since Cre recombinase is expressed only following the activation of Hsp70, overexpression of the fusion gene is temporally and spatially regulated. Transgenic fish displayed aberrant hematopoiesis and developed a myeloproliferative neoplasm in adulthood (Forrester *et al.*, 2011).

Given the similarities between human and zebrafish hematopoiesis, together with the above mentioned unique features of zebrafish, the aim of this study was to analyze the effects of *wnt10b* overexpression on hematopoiesis and AML pathogenesis employing zebrafish as a model.

WNT PATHWAY AND *wnt10b* IN ZEBRAFISH

The Wnt pathway in zebrafish regulates different processes depending on the moment of its activation. Maternal Wnts, present during the early steps of development, are essential for the formation of dorsal structures, and their inactivation leads to the dramatic ventralization of the embryos, that display a reduced or absent head (Lu *et al.*, 2011; Beghini *et al.*, 2012). Conversely, activation of the Wnt pathway after the mid-blastula transition stage (MBT), when the zygotic genome starts to be expressed, has a role in determining the posteriormost structures of the embryo, and its aberrant activation causes posteriorized phenotypes, that miss the anterior regions of the head (van de Water *et al.*, 2001; Beghini *et al.,* 2012).

Zebrafish *wnt10b* has been described for the first time by Lekven and colleagues (Lekven *et al*., 2003), and comparison of the protein sequence among Vertebrates showed a high degree of conservation. Gene expression analysis performed through *in-situ* hybridization during embryonic development showed that *wnt10b* expression is present in the midbrain – hindbrain boundary (MHB), in the epiphysis, cerebellum and rhombomeres, overlapping the expression of another member of the family, *wnt1*. Indeed, functional experiments showed that the two genes are partially redundant in regulating the expression of those genes involved in the maintenance of the MHB (Lekven *et al*., 2003).

AIM OF THE STUDY

WNT10B upregulation in various cancer disorders (Wend *et al.*, 2012), particularly in bone marrow microenviroment of AML patients (Beghini *et al.*, 2012), and its role in hematopoietic regeneration (Congdon *et al.,*2008), led us to deepen our knowledge about its role in normal and malignant hematopoiesis. Thanks to its unique features, we decided to use zebrafish as a model. Given the high degree of similarity between the human and zebrafish proteins (73% aminoacid identity) (Lekven *et al.*, 2003), zebrafish *wnt10b* was used.

At first we asked if zebrafish represented a feasible model for the study; to answer this question, the functional analysis was performed by means of transient overexpression; the effects of the forced *wnt10b* upregulation was investigated using *in-situ* hybridization or live imaging of transgenic lines for genes that are markers of blood cell development (*scl, pu.1, mpx, gata1*).

Our results show that transient *wnt10b* overexpression affects zebrafish hematopoiesis, thus we planned to generate a stable zebrafish transgenic line expressing, in a Cre – lox inducible fashion, *wnt10b* under the control of the *runx1* promoter, leading to a temporally and spatially restricted overexpression of the gene of interest in the embryos and adult fish for following analyses.

MATERIALS AND METHODS

ZEBRAFISH MAINTEINANCE

Different zebrafish strains were used for experiments:

- AB wt strain
- Tg(*mpx*:EGFP) (Renshaw *et al.*, 2006)
- Tg(*gata1:*DsRed) (Traver *et al.*, 2003)

Adult fishes were maintained at 28°C and on a 14 h light/10 h dark cycle.
Embryos were obtained through natural matings, raised at 28°C in Fish Water with PTU (1-phenyl-2-thiourea) to avoid pigmentation, and staged as described (Kimmel *et al.*, 1995).

The Animal Welfare Act (AWA) covers fish that are able to feed on their own and no longer are reliant on the yolk sac. Agreeing to "The Zebrafish Book", the embryos use up their yolk sacs in about 4 days, after which they must feed, until reaching nutritional independence at 8 days post fertilization (dpf). According to the general guidelines of the "University of Maine Institutional Animal Care and Use Committee Guidelines for Use of Zebrafish in Research, Teaching, and Testing", a IACUC protocol for studies using zebrafish embryos younger than 4 dpf is not required. Also important, the same Guidelines state that early stages (4–7 dpf) do not feel pain or distress.
The higher percentage of the animals employed in this Research will be embryos same age or younger than 4 dpf.
Our animal facility strictly adheres to the relevant Italian laws, rules and regulations (D.to L.vo 116/92), as also confirmed by the Autorizzazione Comunale of the city of Milan (D.Lvo 27.1.1992 n°116, art.10).

RT-PCR

Given the similarity between human and zebrafish *wnt10b* sequence and, probably, function, we decided to make use of the zebrafish gene for all the experiments.
Total RNA from 1-cell stage to 4dpf embryos was extracted with ReliaPrep™ RNA Tissue Miniprep System (Promega) and reverse transcribed with the ImProm-II™ Reverse Transcriptase Kit (Promega), according to manufacturers' instructions.
Subsequent PCR was performed using the following primers:

zf_*wnt10b*_START GGGCCCGGGCCGCCATGGAGTTACCCCACAGACAG

zf_*wnt10b*_STOP GGTCCCGGGCCTACTTGCACACATTAACCCACTC

The zf_*wnt10b*_START/ zf_*wnt10b*_STOP amplicon (1282 bp) has been gel extracted with Wizard® SV Gel and PCR Clean-Up System (Promega) and cloned in pCMV-SC vector (Strataclone).

SITE DIRECTED MUTAGENESIS

Mutagenesis was performed according to Zheng *et al.* (2004) on pCMV-SC_*wnt10b* to remove the SacI restriction site from *wnt10b* sequence. The following primers were used:

FF_*wnt10b*Mut: AGGAGAGAACACACCTGAGCTTAGCTCGCTCCACGGCAGCCTT

RR_*wnt10b*Mut: AAGGCTGCCGTGGAGCGAGCTAAGCTCAGGTGTGTTCTCTCCT

Following plasmid denaturation, annealing with mutagenic primers and extension, the product was digested with *Dpn*I, that cleaving at methylated sites destroyed the template plasmid. The PCR product was transformed in competent cells.

EXPRESSION ANALYSIS

RT-PCR was performed on embryos at 28hpf and 3dpf or on their tails, using all the above mentioned primers. The 579 bp products were run on 1% agarose gel.

mRNA MICROINJECTION

Capped *wnt10b* mRNA was synthesized from zf_*wnt10b*_START/ zf_*wnt10b*_STOP amplicon with mMESSAGE mMACHINE® T3 Transcription Kit (TermoFischer). Embryos microinjection at one-cell stage was performed with FemtoJet precision pressurized injector (Eppendorf). *wnt10b* mRNA (1000 pg/embryo) or control EGFP/DsRed mRNA(1000 pg/embryo) were injected together with the dye tracer rhodamine dextran (Invitrogen).

In situ HYBRIDIZATION

Whole mount *in situ* hybridization was carried out as previously described (Thisse *et a.l,* 1993) on embryos fixed overnight in 4% paraformaldehyde/PBS, then rinsed with PBS-Tween, dehydrated in 100% methanol and stored at -20°C until processed for WISH (Jowett and Lettice, 1994). Antisense riboprobes for *wnt10b, pu.1* (Lieschke *et al*., 2002) and *scl* (Liao *et al.,* 1998) were used.

o-DIANISIDINE staining

Zebrafish embryos at 3dpf were stained for 15 min in the dark in *o*-dianisidine staining solution, as previously described (Detrich *et al.*, 1995). Stained embryos were cleared with benzyl benzoate/benzyl alcohol (2:1, vol/vol).

IMAGING

In situ hybridization and *o*-dianisidine-stained embryos were imaged by Digital Camera DFC310 FX (Leica) and software LAS (Leica Application Suite) on MZ 10F microscope (Leica). Live imaging of transgenic embryos was carried out using Axiovert 200M (Zeiss). To determine the number of neutrophils in the *mpx*:GFP transgenics each embryo was photographed on 4-5 different focal planes, then the pictures were assembled in a single image on which the cell count was performed using the ImageJ 1.47 software.

STATISTICAL ANALYSIS

χ^2 test was used to test the significance of the differences in *in-situ* hybridization signals between control and *wnt10b*-injected embryos.

RESULTS

wnt10b EXPRESSION ANALYSIS

In order to deepen our knowledge concerning *wnt10b* expression during zebrafish development, we performed RT-PCR on 24 and 72 hpf (hours post fertilization) embryos total RNA. The result shows the presence of the transcript during these stages of development (Fig. 6).

Whole mount *in-situ* hybridization results by Lekven and co-workers (Lekven *et al.,* 2003) revealed the expression of the gene at the level of the midbrain-hindbrain boundary (MHB) at the 5-somite stage; at 24 hpf the transcript is present in the epiphysis, dorsal midline of the optic tectum, the anterior half of the MHB constriction, and in all rhombomeres.

We asked whether *wnt10b* was expressed only in these regions or if its transcript could be present in more posterior hematopoietic regions, like the Caudal Hematopoietic Tissue (CHT). For this reason we isolated the tails from embryos at 24 and 72 hpf and searched for the presence of the transcript through RT-PCR; the mRNA, not detectable by *in-situ* hybridization, is present in the tail region of 24 an 72 hpf embryos (Fig. 6).

wnt10b OVEREXPRESSION EFFECTS ON EARLY DEVELOPMENT

wnt10b in-vitro synthesized transcript was injected in zebrafish eggs at the 1-cell stage, in order to distribute the mRNA in all cells of the developing embryo. The optimal concentration of mRNA was evaluated performing various injections with different concentrations and observing the resulting phenotype and counting the number of dead embryos. One-thousand pg/embryo of mRNA was chosen as the optimal concentration as its injection causes the death of about 5% of the embryos compared with control embryos injected with Green Fluorescent Protein (GFP) mRNA (that has no effects on development), and phenotypic alterations in 30% of the embryos; the remaining siblings, which showed normal development at a first glance, were employed to analyse cell migration, tissues formation, and organ development, in comparison to controls.

Embryos injected with *wnt10b* mRNA showed alterations in the development of the dorso/ventral axis and hematopoietic system. At 24 hpf, compared with control embryos, they displayed the expansion of the caudalmost tissues, and lack or reduced development of the head and the tail.

At 72 hpf, axis development alteration is evident and reflects the timing of the upregulation of the Wnt pathway. In control embryos we saw a straight and extended body axis, with head and tail at the extremities (Fig. 7A-D). Twenty % of the embryos injected with the *wnt10b* transcript were characterized by a larger head, absence of one or both eyes, and absent or reduced tail (Fig. 7C), typical features of dorsalization, that occur when maternal Wnts are overexpressed. Ten % of the embryos showed smaller heads, and enlarged tails (Fig.7B-E); these alterations mirror the upregulation of the zygotic Wnt signaling.

wnt10b OVEREXPRESSION EFFECTS ON BLOOD CIRCULATION

Embryonic blood circulation was *in-vivo* analysed under a stereomicroscope.
The caudal hematopoietic tissues of injected embryos, analysed at 120 hpf, showed accumulation of cells, that often produced alteration or block of blood circulation.
Thirty % of *wnt10b*-injected embryos was characterized by the typical severe axes aberrations reported above, making it impossible to analyse blood circulation and compare it to control embryos; 23% of the injected embryos displayed the decrease in blood flow velocity compared to control embryos, and in 5% the blood cells were motionless.

wnt10b OVEREXPRESSION ALTERS THE EXPRESSION PATTERN OF *scl*

In order to analyse the effects of *wnt10b* overexpression on different blood cell populations, *in-situ* hybridization were performed using various hematopoietic markers probes. All the analyses were performed exclusively on those *wnt10b* mRNA-injected embryos displaying the general body architecture comparable to controls, in order to avoid any bias due to malformations.
scl is expressed in the Anterior (ALM) and the Posterior (PLM) Lateral Mesoderm during somitogenesis, then marks posterior progenitors that migrate towards the midline to form the ICM (Gering *et al.*, 1998), and is present until 30 hpf in the PBI (Fig. 8A).
Analysis of progenitor populations in the PBI at 28 hpf (Fig. 8B-C) showed that *wnt10b* overexpression caused an expansion of the number of these cells in 70% of the embryos injected with the transcript (n=62), compared to controls (n= 34). χ^2 test confirmed that the result was extremely significant ($p<0.05$).

wnt10b OVEREXPRESSION ALTERS THE EXPRESSION PATTERN OF *pu.1*

Myeloid progenitors were examined by means of *pu.1* expression at different stages of development, during somitogenesis and at 28 hpf.

Control embryos showed the normal expression pattern of *pu.1* during somitogenesis: from 14 hpf (12-somite stage), myeloid progenitors are localized mostly in two lateral clusters in the ALM (Fig. 9B); at 15-somite stage these cells migrate toward the centre (Fig. 9E).

Embryos injected with *wnt10b* transcript are characterized by the anomalous dispersion and the premature migration of myeloid progenitors in the ALM (Fig. 9H-I).

From 16 hpf myeloid progenitors migrate laterally and spread into the yolk sac (Davidson and Zon, 2004). In control embryos at 28 hpf, the expression of *pu.1* was still present in the yolk sac (Fig. 10A), and myeloid progenitors were also found in the ICM and the PBI until 30 hpf (Fig. 10A).

Injection of *wnt10b* mRNA led to the expansion of the myeloid cell population, in the ALM and in the PBI, as shown in Fig. 10B-C. This expansion occured in 54% of embryos injected with the *wnt10b* transcript (n=114) compared to controls (n=66). χ^2 test confirmed that the result was extremely significant ($p<0.05$).

wnt10b OVEREXPRESSION AFFECTS ERYTHROID PROGENITOR NUMBER

The transgenic line Tg(*gata1*:DsRed) was used to better analyse the onset of circulation, since embryos are characterized by the expression of the red fluorescent protein (DsRed) in erythroid progenitors (Traver *et al.*, 2003). Thirty % of *wnt10b*-injected embryos (n=58) at 28 hpf showed an increase in the number of *gata1* positive cells localized in the PBI (Fig. 11C), compared to controls (n=41) (Fig. 11B). χ^2 test confirmed that the result was significant ($p<0.05$).

wnt10b OVEREXPRESSION ALTERS THE NUMBER OF NEUTROPHILS

Analysis of differentiated myeloid population was performed at 48 hpf through *in-vivo* observation of transgenic *mpx:*GFP embryos, characterized by expression of the GFP in neutrophils (Renshaw *et al.*, 2006).

Fifty-two % of embryos injected with *wnt10b* transcript (n=18), compared to controls (n=10) (Fig. 12A), shows a reduction in the number of neutrophils (Fig. 12B).
Image data were further analysed through CellProfiler 2.1.1 software, and the reduction in the number of neutrophils in *wnt10b*-injected embryos was also statistically confirmed (Fig. 12C).

wnt10b OVEREXPRESSION DOES NOT DISPLAY MACROSCOPIC EFFECTS ON MATURE ERYTHROCYTES NUMBER

Mature red blood cells were analysed through *o*-dianisidine staining at 72 and 120 hpf.
Wnt10b overexpression did not change the number or distribution of mature erythrocytes (Fig. 13).

GENERATION OF THE PLASMID CONSTRUCT FOR ZEBRAFISH ZYGOTE TRANSGENESIS

To analyse the effects of *wnt10b* overexpression in zebrafish myeloid cells in a stable fashion, we decided to generate a transgenic line in which *wnt10b* is under the control of the *runx1* promoter. The pBluescript II SK plasmid, containing the *runx1P2* zebrafish promoter driving the expression of the EGFP (pBluescript II SK_*runx1P2*_EGFP), was kindly donated by professor Crosier (Lam *et al.*, 2009). The EGFP coding sequence was removed from the pBluescript II SK_*runx1P2*_EGFP by digestion with *Bam*HI and *Xho*I restriction enzymes (pBluescript II SK_*runx1P2,* Fig. 14A). *Xho*I and *Sac*I restriction sites of pCMV-SC_*wnt10b* were removed by PCR-based Site Directed Mutagenesis, and the *wnt10b* coding sequence with the downstream polyA region was amplified by PCR using forward and reverse primers with *Bgl*II and *Xho*I restriction sites at their 5' ends, respectively (Fig. 14B). The amplicon was then digested with *Bgl*II and *Xho*I restriction enzymes (Fig. 14C) and cloned into the pBluescipt II SK_*runx1P2* plasmid digested with *Bam*HI (*Bgl*II compatible) and *Xho*I restriction sites (FIG. 14D).
pGEM-Teasy was linearized with *Sal*I (Fig. 15B) and ligated to pT2AL200R150G linearized with *Xho*I (Fig. 15A). A PCR with T3 and SP6 primers was performed directly on the ligation reaction (Fig. 15C), resulting in the L200 fragment with *Sac*I restriction sites at both ends. L200 sequence was digested with *Sac*I and ligated with the pBluescript II SK_*runx1P2_wnt10b*+polyA linearized with *Sac*I (Fig. 15C-D). The R150 sequence was excised

from pT2AL200R150G by *Xho*I and *Kpn*I digestion and ligated into pBluescript II SK_*runx1P2_wnt10b*+polyA_L200 digested with same restriction enzymes (Fig. 15E-F).

The final plasmid pBluescript II SK_*runx1P2_wnt10b*+polyA_L200_R150 is ready to be coinjected in the zygote with the transposase synthetic mRNA in order to increase the efficiency of the transgenesis process (Kawakami, 2007).

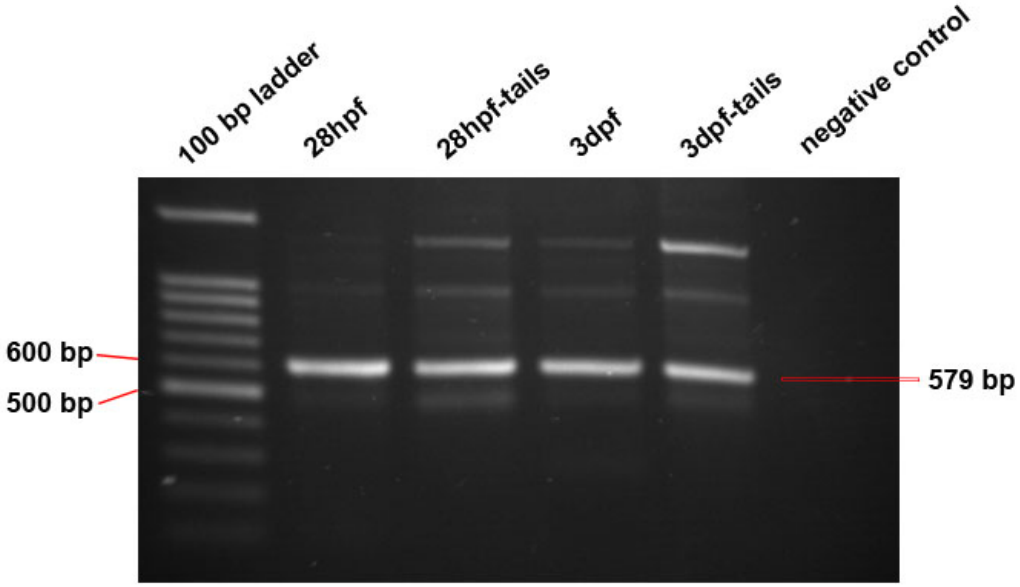

Fig. 6. Expression profile of zebrafish *wnt10b*. RT-PCR was performed at different developmental stages (28hpf and 3dpf) and in tails isolated from 28 hpf and 3dpf embryos. All samples analysed are positive for *wnt10b* expression, as confirmed by the 579 bp specific amplicon.

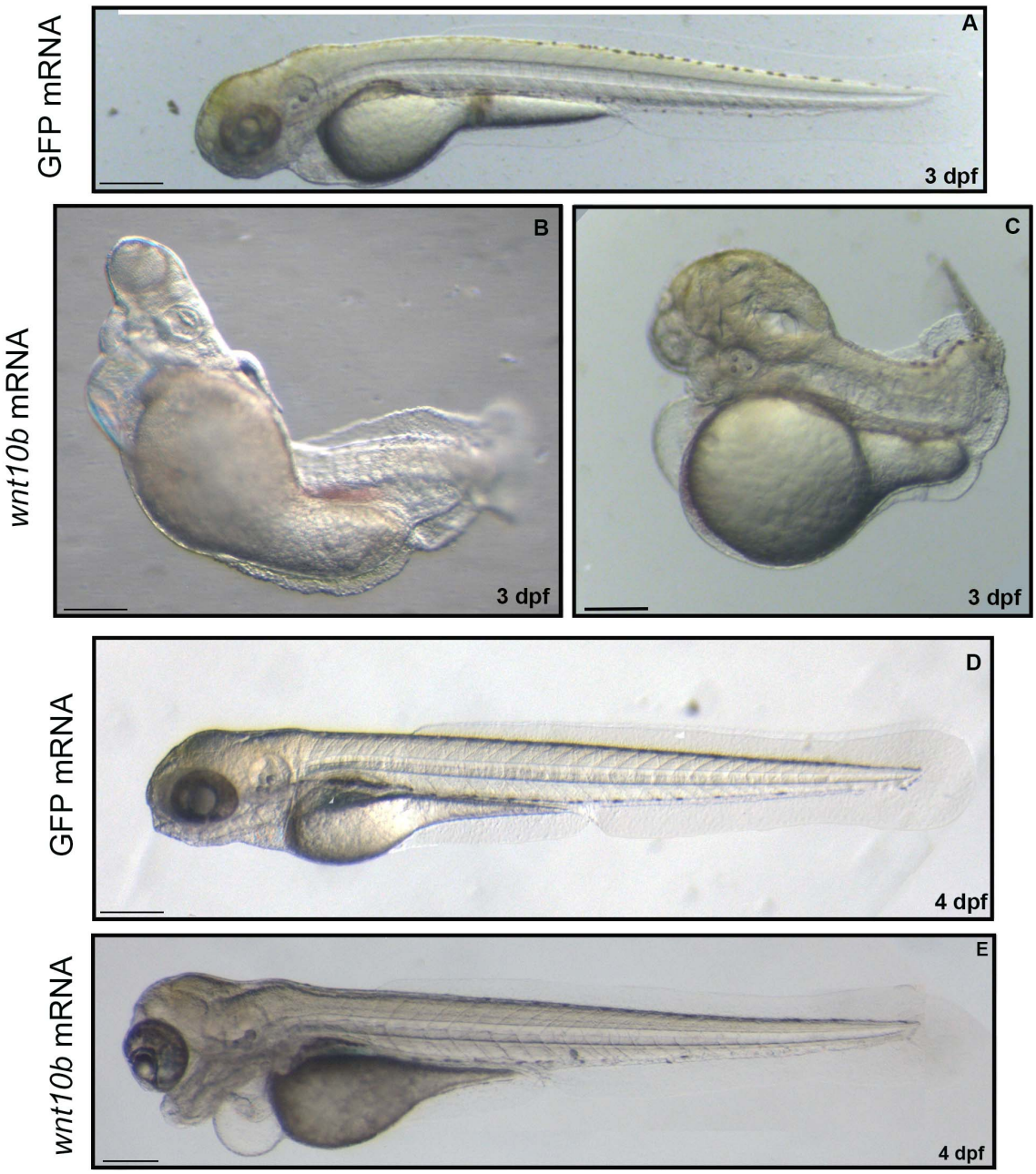

Fig. 7. *wnt10b* overexpression effects on body axis development. A and D) Control embryos at 3 and 4 dpf. **B, C, E)** *wnt10b*-injected embryos show ventral tissues expansion and absence of anteriormost part of the head (B), dorsalization (C) and hypodevelopment of the cephalic region also in presence of a straight body axis (E). All lateral views, anterior to the left. Scale bar corresponds to 500 μm in A and E, 600 μm in D, 250 μm in B and C.

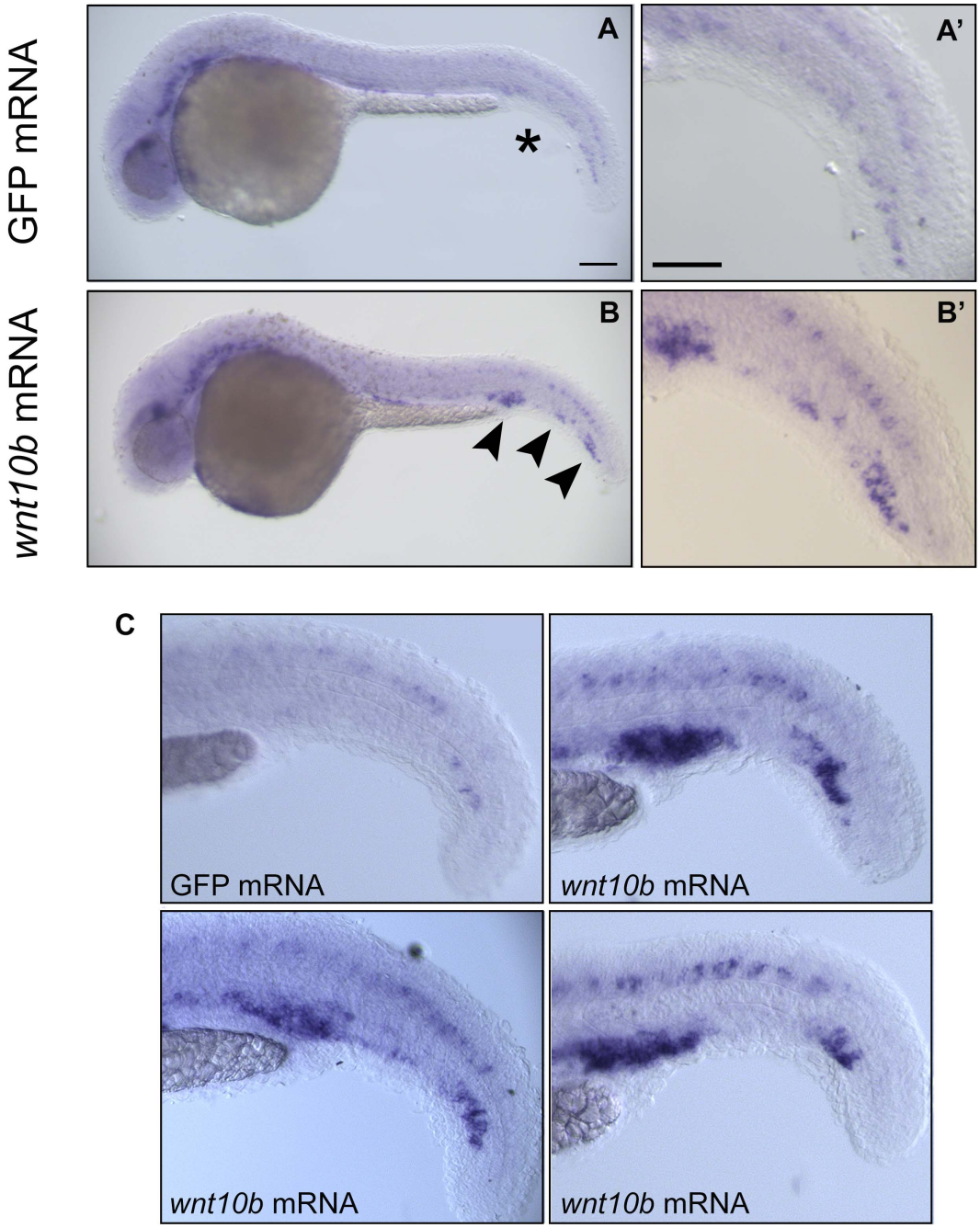

Fig. 8. *wnt10b* overexpression alters the expression pattern of *scl*. A and B) *In-situ* hybridization for *scl* on 28 hpf embryos injected with control (A) or *wnt10b* (B) transcript. Arrowheads in B show the increase of hematopoietic stem cells compared to A (asterisk). Scale bar 100 μm. A' and B' are magnifications; scale bar 50 μm. **C)** PBI magnifications of control and *wnt10b*-injected embryos. Lateral views, anterior to the left.

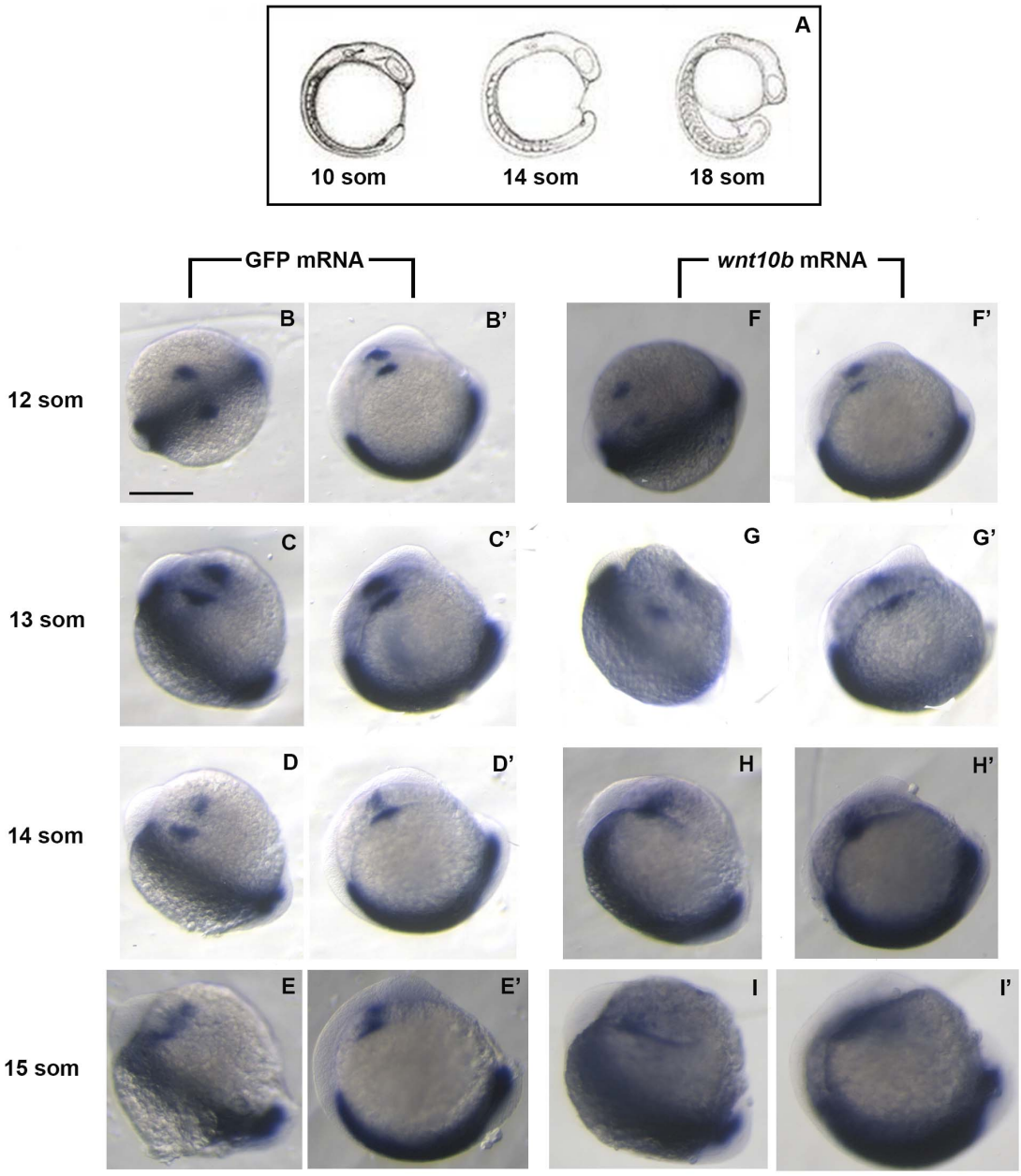

Fig. 9. *wnt10b* overexpression alters the expression pattern of *pu.1* during somitogenesis. A) Scheme representing selected somitogenesis stages. **B-I')** *In-situ* hybridization for *pu.1* in embryos injected with control (B-E') or *wnt10b* (F-I') mRNA. Frontal (B-E and F-I) and lateral (B'-E' and F'-I') views, anterior to the top. Scale bar 300 μm.

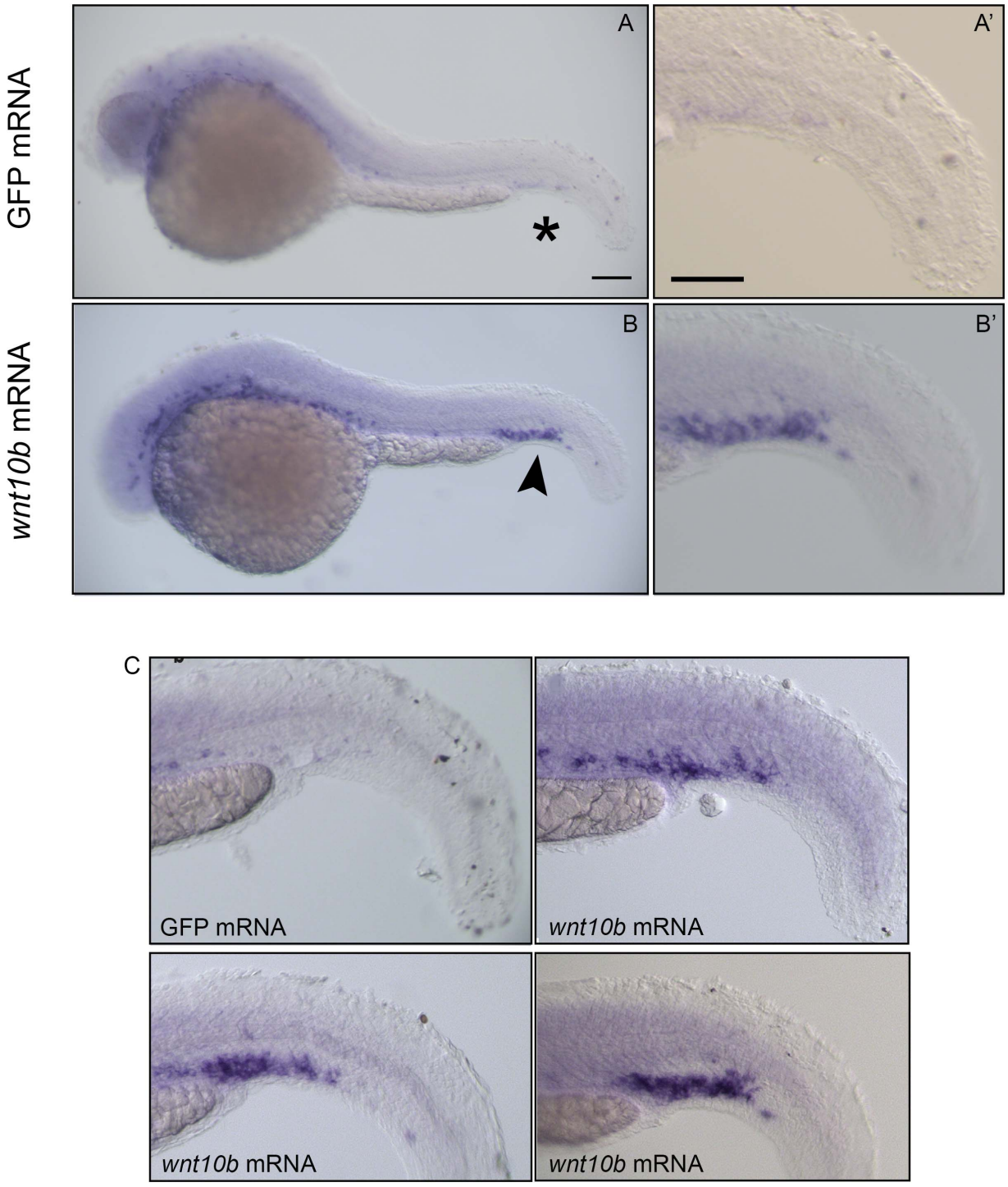

Fig. 10. *wnt10b* overexpression alters the expression pattern of *pu.1*. A and B) *In-situ* hybridization for *pu.1* on 28 hpf embryos injected with control (A) or *wnt10b* (B) transcript. Arrowhead in B shows the increase of myeloid progenitors compared to A (asterisk). Scale bar 100 μm. A' and B' are magnifications; scale bar 50 μm. **C)** PBI magnifications of control and *wnt10b*-injected embryos. Lateral views, anterior to the le

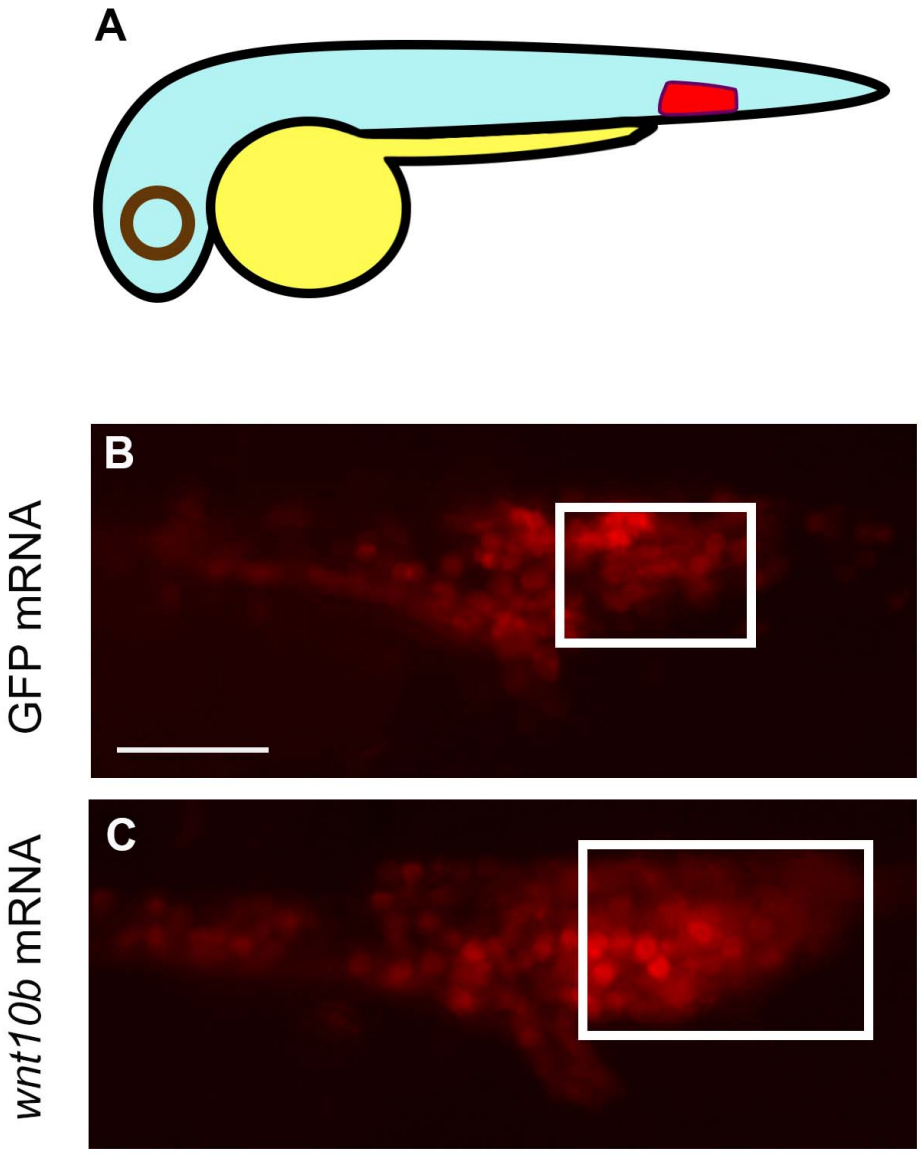

Fig. 11. *wnt10b* overexpression increase the number of erythromyeloid progenitors.
A) Schematic representation of a 28hpf embryo with PBI evidenced in red. **B and C)** Live imaging of 28 hpf transgenic *gata1*:DsRed embryos PBI. *wnt10b*-injected embryos show an increase of erythroid progenitors compared to controls. Scale bar 125 μm. Lateral views, anterior to the left.

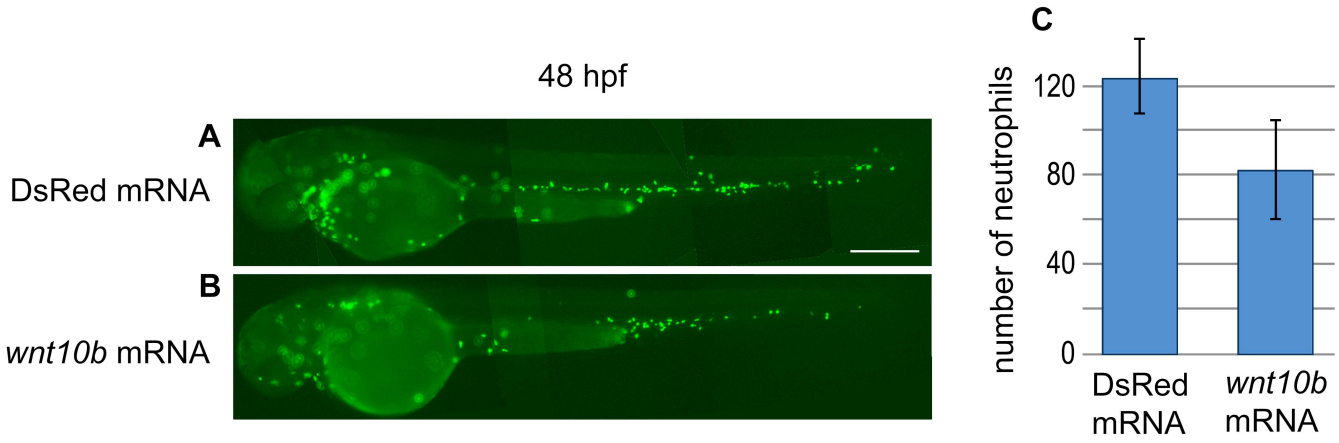

Fig. 12. *wnt10b* overexpression alters the number of neutrophils.

A and B) In vivo imaging of *mpx*:GFP embryos at 48hpf. Green fluorescent neutrophils decrease in *wnt10b*-injected embryo (B) compared to control (A). Scale bare 300 μm. Lateral views, anterior to the left.

C) Statistical analysis performed on 18 *wnt10b*-injected and 11 control embryos. Results confirm the reduction in the number of neutrophils of *wnt10b*-injected embryos compared to controls. **<0.01 with Student's t-test.

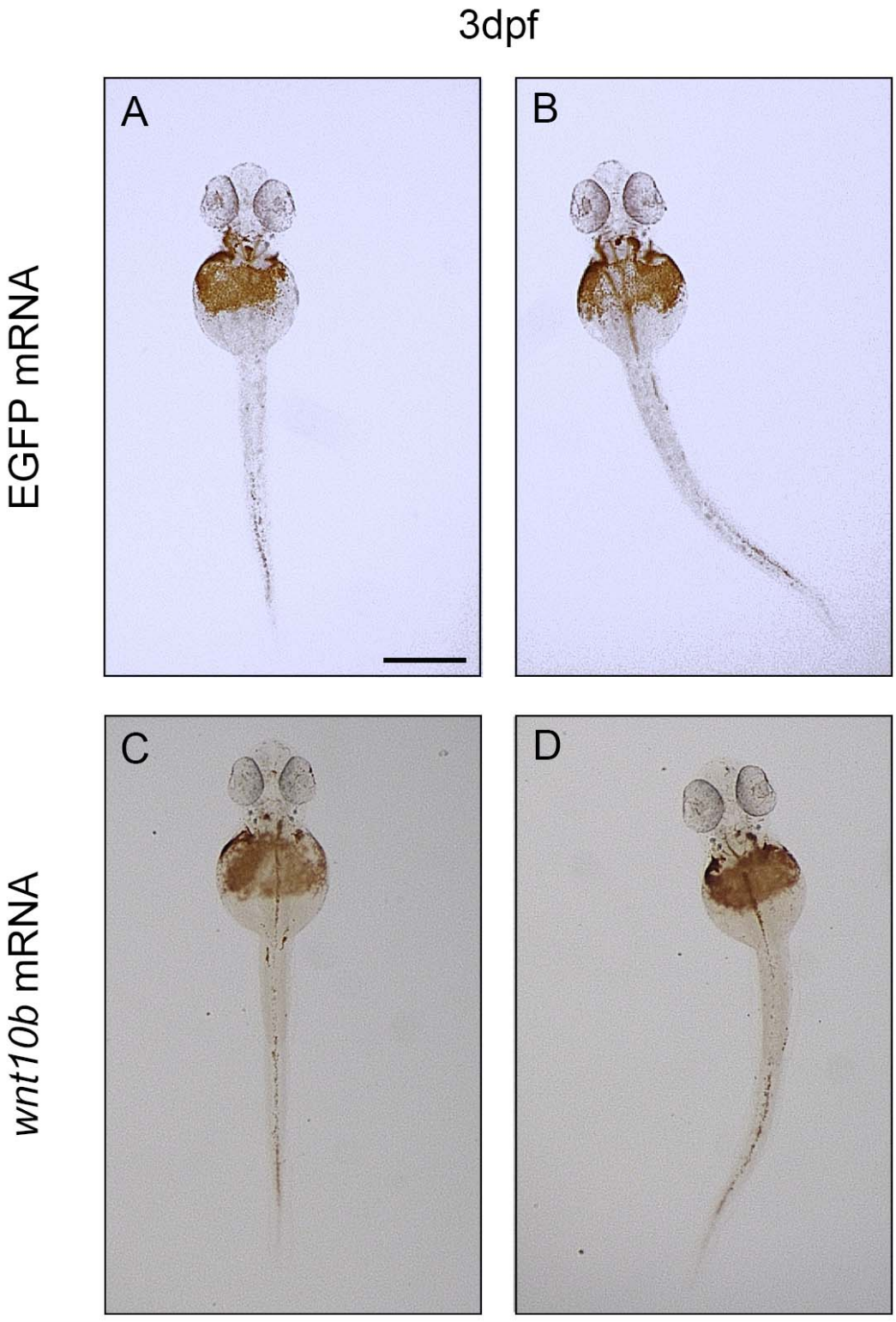

Fig. 13. *o*-dianisidine staining of 3dpf embryos. Embryos injected with *wnt10b* mRNA (**C,D**) do not show differences in the number of erythrocytes compared to control embryos (**A,B**). Scale bar 150 μm. Ventral views, anterior to the top.

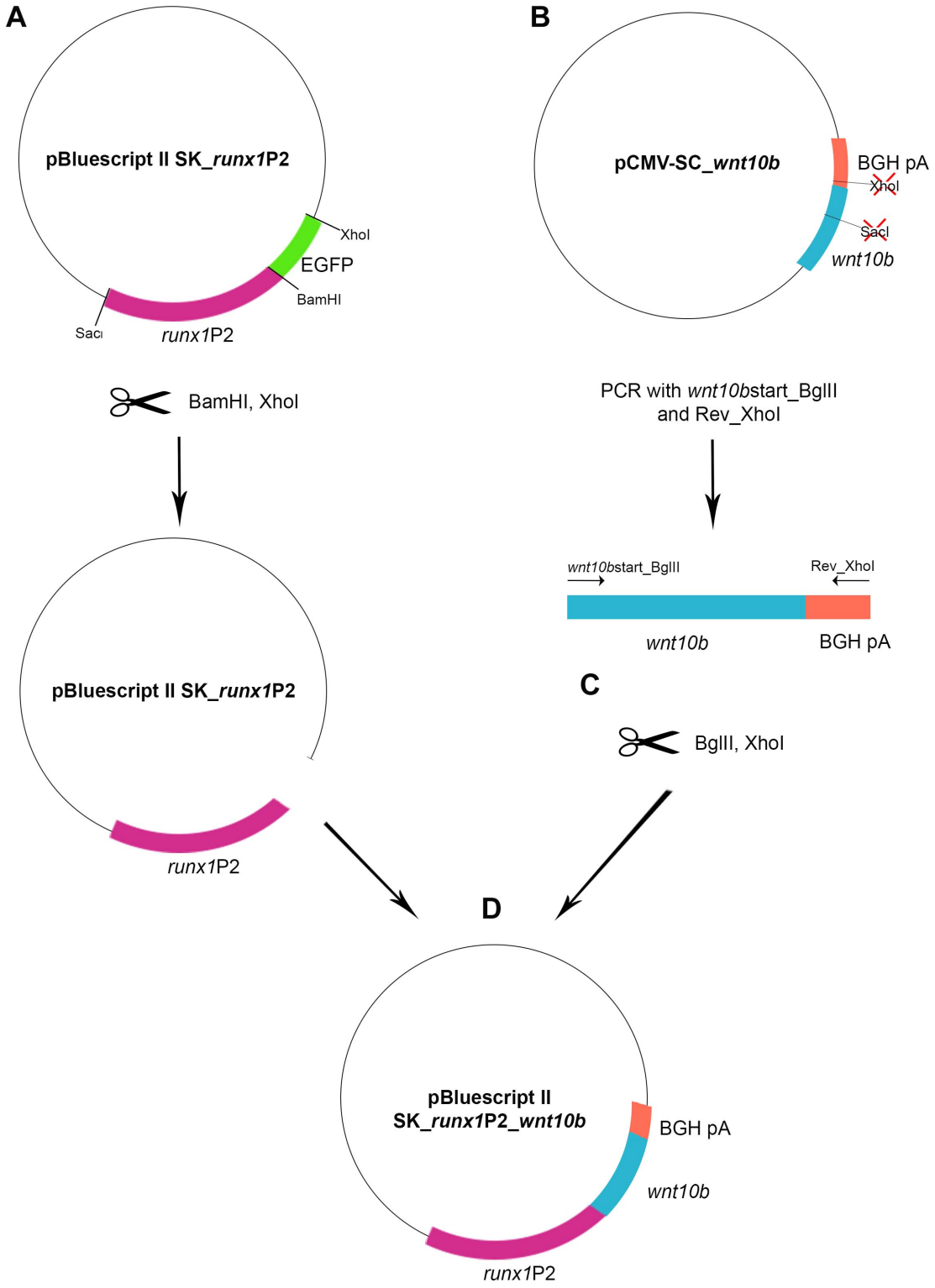

Fig. 14. Schematic representation of the generation of pBluescript II SK_*runx1*P2_*wnt10b*. A) *BamH*I and *Xho*I digestion of pBluescript II SK_*runx1*P2. **B)** PCR starting from pCMV_SC_*wnt10b* with primers with *Bgl*II and *Xho*I consensus sequences at the extremities. **C)** PCR digestion with *Bgl*II and *Xho*I. **D)** Ligation and formation of final plasmid.

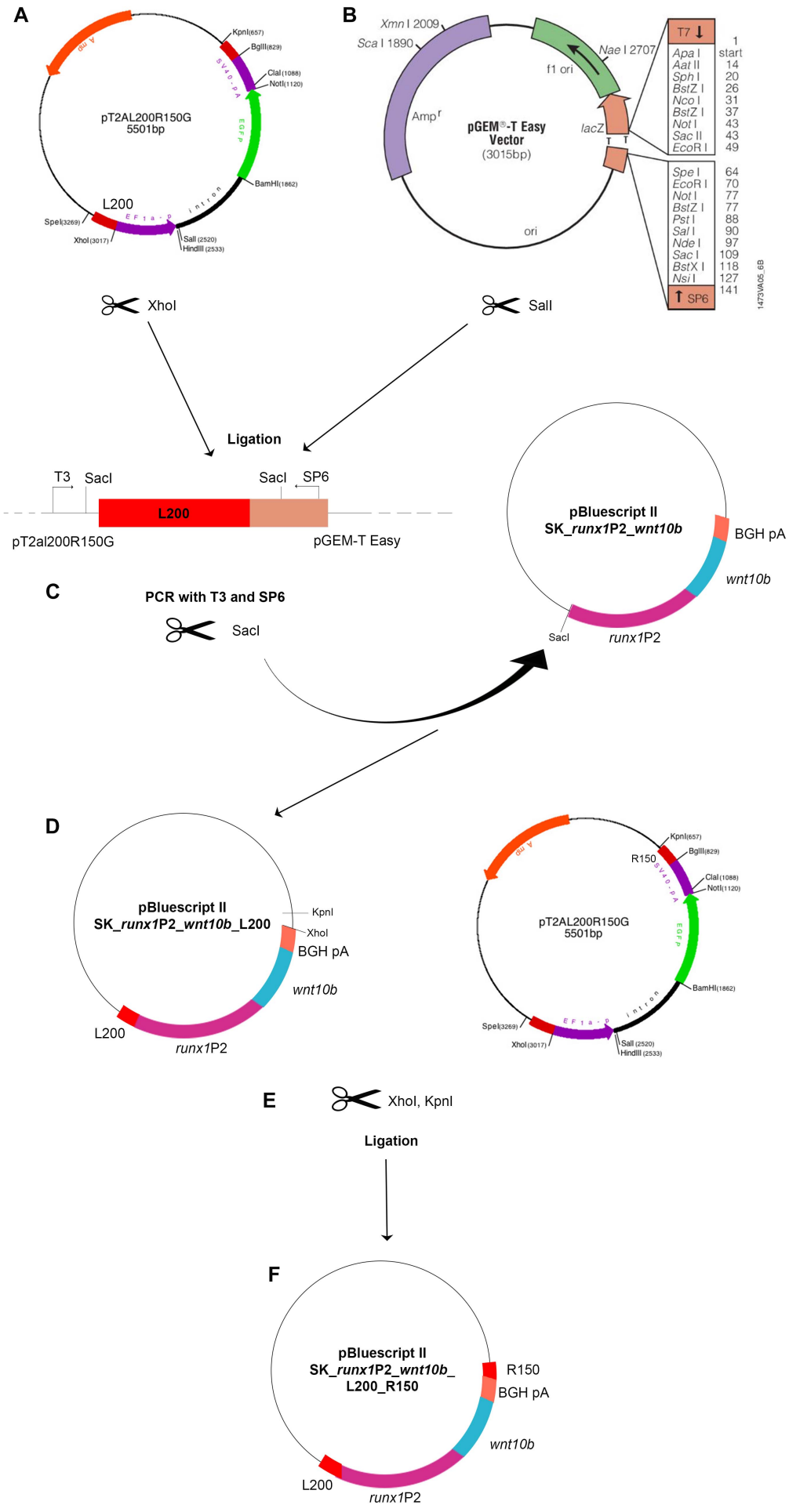
A
pT2AL200R150G
5501bp
L200
B
pGEM®-T Easy
Vector
(3015bp)
T7
SP6
XhoI
SalI
Ligation
T3
SacI
SacI
SP6
L200
pT2al200R150G
pGEM-T Easy
pBluescript II
SK_runx1P2_wnt10b
BGH pA
wnt10b
runx1P2
C
PCR with T3 and SP6
SacI
D
pBluescript II
SK_runx1P2_wnt10b_L200
KpnI
XhoI
BGH pA
wnt10b
L200
runx1P2
pT2AL200R150G
5501bp
R150
E
XhoI, KpnI
Ligation
F
pBluescript II
SK_runx1P2_wnt10b_
L200_R150
R150
BGH pA
wnt10b
L200
runx1P2

Fig. 15. Generation of pBluescript II SK_*runx1*P2_*wnt10b*_L200_R150. A) *Xho*I linearization of pT2AL200R150G. **B)** *Sal*I linearization of pGEM-T easy. **C)** T3/SP6 PCR on resulting ligation. **D)** *Sac*I digestion of PCR and pBluescript II SK_*runx1*P2_*wnt10b*, ligation and formation of pBluescript II SK_*runx1*P2_*wnt10b*_L200. **E)** *Xho*I and *Kpn*I digestion of pBluescript II SK_*runx1*P2_*wnt10b*_L200 and pT2AL200R150G. **F)** Final plasmid resulting from ligation.

DISCUSSION

The aim of this study was to elucidate the role of *WNT10B* as a leukemia driver in a zebrafish model, exploring its role in normal and misregulated zebrafish hematopoiesis.

Human and zebrafish WNT10B aminoacidic sequences are highly similar; furthermore, *WNT10B* maps in close proximity to *WNT1*, and this synthenic organization is maintained among Vertebrates (Lekven *et al.*, 2003). These indications are suggestive of an evolutionary conserved function between zebrafish and human. *WNT10B* is up-regulated in various malignant disorders (for a complete review see Wend *et al.*, 2012), supporting a role for this gene in carcinogenesis. Interestingly, the *WNT1-WNT10B* cluster expression is modulated in human breast cancer (Qurrat-ul-Ain *et al.*, 2011).

According to the *in-situ* hybridization analyses previously performed (Lekven *et al.*, 2003; Lu *et al.*, 2011), zebrafish *wnt10b* is expressed starting from the 1-cell stage, when the zygotic genome is still silent, proving the maternal origin of the transcript; at 24hpf, when the zygotic DNA is already activated, its expression is restricted to discrete brain regions (Lekven *et al.*, 2003). Zebrafish *wnt10b* is essential for the maintenance of gene expression in a portion of the midbrain-hindbrain boundary (MHB), and function redundantly with *wnt1*; only down-regulation of both genes leads to cell death in the tectum and absence of MHB markers expression (Lekven *et al.*, 2003). Given the central role of *WNT10B* in hematopoietic regeneration after injury that has been pointed out by Congdon and co-workers (Congdon *et al.*, 2008) and the up-regulation of *WNT10B* in AML patients bone marrow microenvironment reported by Beghini and colleagues (Beghini *et al.*, 2012), we asked whether zebrafish *wnt10b* expression was actually present also in the hematopoietic tissues but resulted undetectable because of the low sensitivity of the *in-situ* hybridization technique previously employed (Lekven *et al.*, 2003; Lu *et al.*, 2011). Our RT-PCR assays on the RNA selectively extracted from isolated embryo caudal tissues, comprising hematopoietic territories (PBI, ICM, and CHT), revealed *wnt10b* expression also in these regions, supporting the hypothesis of a physiologic *wnt10b* role also in zebrafish hematopoiesis.

Our forced overexpression of *wnt10b* during the embryonic development led, as expected, to specific alterations of the embryo body axis, confirming that our approach truly acted misregulating the Wnt pathway. Indeed, the Wnt signaling in zebrafish is essential for the formation of the dorso-ventral and the antero-posterior axes, depending on the timing of its activation. Maternal Wnts, expressed in the very early phases of development, are essential for the formation of dorsal structures (Lu *et al.*, 2011), while expression of the zygotic *Wnt* genes plays a crucial role in determining the posteriormost structures of the embryo; indeed, their aberrant activation cause posteriorized phenotypes lacking the anteriormost regions of

the head (van de Water *et al.*, 2001; Beghini *et al.,* 2012). Therefore, the phenotypes we obtained following *wnt10b* mRNA injection, as enlarged head or, on the other hand, absence of cephalic structures, mirror misregulation, respectively, of maternal and zygotic Wnt signaling. (the effects of the forced expression of Wnt factors during zebrafish embryonic development are nicely covered in Beghini *et al.*, 2012).

Wnt10b-injected embryos also show an accumulation of cells in ICM and the severe blockade of blood circulation. Interestingly, this result resembles what previously reported by Yeh and colleagues (Yeh *et al.,* 2008), which generated the leukemic hsp:AML1-ETO zebrafish transgenic line expressing the human AML-associated translocation (Kelly and Gilliland, 2002) in a temporal controlled fashion. Indeed, in the 24 hpf embryos of the hsp:AML1-ETO line a great number of hematopoietic cells accumulated in the ICM region, blocking blood circulation. Thus, our results show that *wnt10b* over-expression affect zebrafish embryonic hematopoiesis.

To explore more deeply the role of *wnt10b* in zebrafish hematopoiesis and the effects of its misregulation, we performed *in-situ* hybridization assays and took advantage of some transgenic reporter line to perform live imaging of blood cell markers.

Analysis of *scl* (a hematopoietic stem cells marker) expression pattern after *wnt10b* over-expression showed an increase in the number of *scl*-expressing cells in the PBI. This result corresponds with the findings by Shen and collaborators (Shen *et al.*, 2013), who generated a zebrafish transgenic line expressing the murine *MYCN* oncogene (Kawagoe *et al.*, 2007) under the control of a heat shock promoter. The transgenic embryos, mimicking human AML, showed the increase in proliferation of myeloid cells and accumulation of immature hematopoietic stem cells, with an up-regulation of *scl* and *pu.1* (a marker of myeloid progenitors) expression.

Our analysis then focused on myeloid progenitors, through investigation of *pu.1* expression in two different stages of development: somitogenesis and 28 hpf. During somitogenesis, myeloid progenitors are present in ALM and PLM; in ALM, they are initially localized in two lateral clusters, which later migrate toward the centre and disperse over the yolk mass. Rhodes and co-workers (Rhodes *et al.*, 2005), analysing *pu.1* expression and its regulation through *pu.1:*GFP transgenic embryos, found out that myeloid progenitors cells, when begin dispersing over the yolk, decrease *pu.1* expression and activate more mature myeloid markers, indicating that migration/cell movement are hallmarks of differentiation. The overexpression of *wnt10b* affected the myeloid progenitor migration pattern during somitogenesis, leading to a more precocious movement of *pu.1* cells towards the yolk.

Interestingly, while such earlier migration was not accompanied by the decrease of *pu.1* expression as expected, we observed the increase in the number of myeloid progenitors; our hypothesis is that *wnt10b* upregulation expands the myeloid progenitors population - which maintain high level of *pu.1* expression - leading to a defective migration process.

During zebrafish normal embryonic development *pu.1* physiological expression decreases in the course of the first day of development, until it disappears at 28 hpf (Lieschke *et al.*, 2002). *Pu.1* activity is also directly dependent on *scl* expression: indeed, embryos injected with *scl*-specific morpholino (*scl*-deficient embryos) displayed a decrease in *pu.1* and *gata1* (a erythroid progenitors marker) expression at 1 dpf (Patterson *et al.*, 2005). The analysis of myeloid and erythroid progenitors at 1 dpf in *wnt10b*-injected embryos showed an increase in the number of these cell populations. On the basis of the higher number of myeloid and erythroid progenitors and the increase in the *scl*-positive cells we determined by means of *wnt10b* forced expression, we propose that *wnt10b* might function upstream of *scl* in the genetic hierarchy required for proper zebrafish hematopoiesis. Indeed, a similar hierarchy was also proposed by Tarafdar and colleagues, that using a HSCs *in-vitro* model in which beta-catenin was upregulated, pointed out that *scl* was positively regulated by the Wnt pathway (Tarafdar *et al.*, 2013).

Since human AML is characterized by an increase of myeloid cells arrested in their maturation, resulting in granulocytopenia or anemia (Lowenberg *et al.*, 1999), we decided to analyse neutrophils and erythrocytes number and distribution following *wnt10b* injection. Through live imaging of transgenic *mpx*:GFP embryos (characterized by fluorescent neutrophils) we pointed out the decrease in the number of differentiated neutrophils in *wnt10b*-injected embryos; this result is in contrast with those presented so far (Yeh *et al.*, 2008, Patterson *et al.,* 2005, Shen *et al.,* 2013), which pointed out the direct dependence of *mpx* expression upon *pu.1*: mutants with deregulated *pu.1*, as well as zebrafish models of leukemias developed by now, show the simultaneous increase in both *pu.1* and *mpx* expression. Besides the fact that our approach is based on transient misregulation of the gene of interest, while the zebrafish leukemia models reported above are generated through stable transgenesis or mutation, these partially discordant results could also be explained by the fact that *WNT10B* is heavily involved in hematopoiesis, hematopoietic regeneration, and disease states (Mikesch *et al.*, 2007, Congdon *et al.*, 2008, Beghini *et al.*, 2012), so it might have a more widespread role, affecting different pathways, with multifaceted effects on zebrafish blood development. The hypothesis is that, also in zebrafish, *wnt10b* may regulate the proliferation/differentiation balance of HSCs, acting upstream of *scl. pu.1, gata1* and other

hematopoietic markers. Moreover, the accumulation of myeloid progenitors in hematopoietic tissues and the decrease of more differentiated myeloid cells in *wnt10b*-injected embryos resemble granulocytopenia, one feature of leukemic disorders, caused by the over-proliferation of myeloid progenitors (Lowenberg *et al.*, 1999).

Our subsequent analysis regarded the number and distribution of erythrocytes population; the results obtained through *o*-dianisidine staining did not show a significant decrease in the differentiated population after *wnt10b* overexpression. Additionally, more sensitive analyses, as RT-PCR for globin genes, need to be performed.

Taken together, our results support the involvement of *wnt10b* in Vertebrate hematopoiesis, which might regulate the delicate proliferation/differentiation balance of blood cell progenitors.

On the basis of the encouraging results presented in this thesis, our next step will be the generation of a zebrafish transgenic line expressing *wnt10b* in a temporal inducible fashion, specifically in the hematopoietic stem cells. To restrict the expression of the transgene in the hematopoietic territories, the zebrafish *runx1* promoter has been selected. *Runx1* is essential in definitive hematopoiesis, and gene structure and function are conserved among Vertebrates. *Runx1* is expressed in all definitive hematopoiesis sites in mammals and zebrafish (North *et al.*, 2002; Kalev-Zylinska *et al*., 2002), as also demonstrated through the construction of a transgenic *runx1*:EGFP zebrafish line, in which EGFP expression marks all sites of *runx1* expression during development and adulthood (Lam *et al.*, 2009).

We have generated a construct, named pBluescript II SK_runx1P2_wnt10b+polyA_L200_R150, in which wnt10b coding sequence is under the control of the runx1 promoter that will allow to drive wnt10b expression in definitive hematopoietic tissues, as the embryonic AGM and the adult kidney (Lam *et al.*, 2009). The transgene is flanked by the transposable elements in order to dramatically increase the rate of transgenesis (Kawakami et al., 2007).

The generation of this transgenic line will give the opportunity to deepen our knowledge about the involvement of *WNT10B* in Vertebrate hematopoiesis and, possibly, to elucidate the mechanism by which this gene is involved in the onset and progression of AML.

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