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# Molecular profiling of the embryonic hematopoietic stem cell niche

Olivera Miladinović

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**Sorbonne Univeristé**

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Team: Migration and differentiation of hematopoietic stem cells

# **Molecular profiling of the embryonic hematopoietic stem cell niche**

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Speciality: Cell Biology and Development

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„Znanje je svetlost koje osvetljava nas put kroz život.”

*Mihajlo Pupin*

“Knowledge is the light that illuminates our path through life”

*Mihajlo Pupin*

*To my parents, who always believed...  
Dok je nama nas!*

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**Abbreviations:**

AGM – Aorta Gonad Mesonephros

BM – Bone Marrow

BMP – Bone Morphogenic Protein

CFU-S – Colony Forming Unit in the Spleen

CMP – Common Myeloid Progenitor

CLP – Common Lymphoid Progenitor

CFCs – Colony Forming Cells

CHT – Caudal Hematopoietic Tissue

DA – Dorsal Aorta

DT – Dorsal Tissues

Dcn – Decorin

E – Embryonic Day

ECs – Endothelial Cells

ESCs – Embryonic Stem Cells

ECM – Extracellular Matrix

EHT – Endothelial to Hematopoietic Transition

FL – Fetal Liver

Gdf6 – Growth differentiation factor 6

HSCs – Hematopoietic Stem Cells

HSPCs – Hematopoietic Stem and Progenitor Cells

HE – Hemogenic Endothelium

HSCT – Hematopoietic Stem Cell Transplantation

Hpf – Hours post fertilization

IAHCs – Intra Aortic Hematopoietic Clusters



hiPSCs – human induced Pluripotent Stem Cells

iPSCs – Induced Pluripotent Stem Cells

ID – Immune deficient

LT – HSCs – Long Term Hematopoietic Stem Cells

LTC – IC – Long Term Culture-Initiating Cells

LMPs – Lympho-Myeloid Progenitors

MPP – Multipotent progenitor

MSCs – Mesenchymal stem/stromal cells

NO – Nitric oxide

PNS – Parasympathetic Nervous System

PSM – Presomitic Mesoderm

PGE2 – Prostaglandin 2

ROS – Reactive oxygen species

ST – HSCs – Short Term Hematopoietic Stem Cells

SNS – Sympathetic Nervous System

Scf – Stem Cell Factor

SDECs – Somite Derived Endothelial Cells

SLAM – Signaling Lymphocyte Activation Molecule

Sp – Splanchnopleure

Tnc – Tenascin C

VT – Ventral Tissues

VEGFR – Vascular Endothelial Growth Factor Receptor

VECDN - Vascular Endothelial Cadherin

WISH – Whole Mount In Situ Hybridization

YS – Yolk Sac

# **INTRODUCTION**

## **1. History of the hematopoietic stem cell research**

First concept of hematopoietic stem cells (HSCs) was established more than 100 years ago. In 1908, a Russian histologist Alexander Maximov put forward a Unitarian theory claiming that all blood cells derive from a unique precursor that he named “Stammzelle” (stem cell). It was only few years later in 1917 when Franz Ernst Christian conducted the first study of the hematopoietic system. In his work he described the bone marrow (BM) as the organ of blood cell formation.

By the end of World War II, in 1945 the United States detonated two nuclear bombs over Hiroshima and Nagasaki that lead to death of a large number of people. These people suffered from hematopoietic failure due to a lethal dose of irradiation. That was the starting point for primeval discoveries in the field of hematopoietic cell transplantation. During the early 1950's two scientist Jacobson and Lorenz demonstrated that adult mouse spleen and bone marrow cells were able to fully reconstitute the hematopoietic system of a lethally irradiated animal, proving that there is an existence of cells responsible for replenishing hematopoiesis (Jacobson et al., 1951; Lorenz et al., 1951).

In 1960, Till and McCulloch revealed that the injection of BM cells into lethally irradiated mice led to formation of hematopoietic nodules within the spleen. These nodules were designated as Colony Forming Unit in the Spleen (CFU-S) (Till & McCulloch, 1961). These nodules were essentially formed of myeloid, erythroid and a few megakaryocytic cells but also less differentiated hematopoietic cells. Further experiments showed that CFU-S were not able to maintain the long-term hematopoiesis that led to conclusion that they did not contain HSCs, but rather hematopoietic progenitors with an important multiplication and differentiation potentials (Hodgson & Bradley, 1979).

In the adult amniotes and amphibians, HSCs reside within the BM in contrast to fish where HSCs are found in the kidney. Both organs provide microenvironments for HSC survival and maintenance.

In both embryo and adult, HSCs are rare and their characterization is challenging. The paradigm of HSCs is that it is still impossible to isolate them at purity since no specific markers have been identified so far. Using a combination of antibodies recognizing specific membrane surface molecules, cell fractions enriched in HSCs can be isolated, and their respective hematopoietic potential evaluated retrospectively using appropriate *in vivo* and/or *ex vivo* assays.

### **1.1. Hematopoiesis and the adult hematopoietic hierarchy**

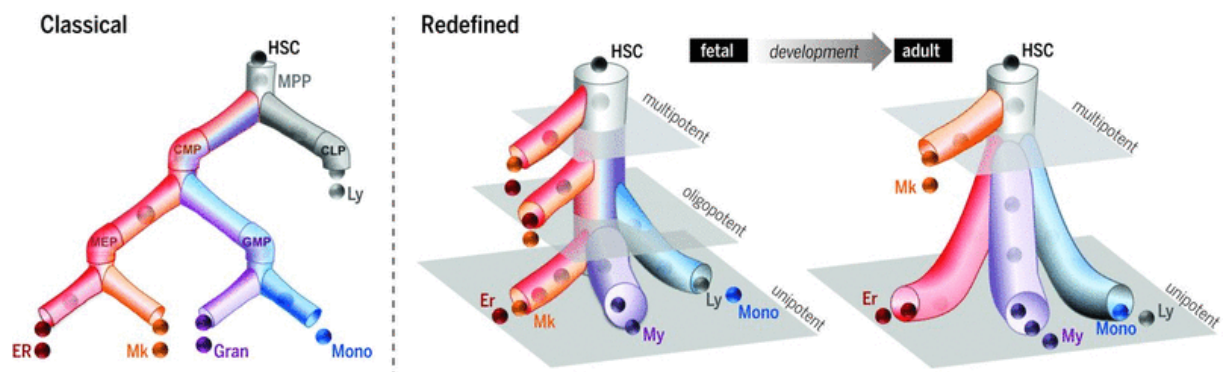
Hematopoiesis is the process of forming blood cell components. This process occurs very early during embryonic development and also through adult life to produce and replenish the blood system. Blood is a circulating fluid in the body of eukaryotic animals. The liquid part of the blood is an aqueous fluid, also known as plasma and it allows the cells (solid component of the blood) to circulate in all the vessels in the body. Main functions of blood are transporting different substances, such as nutrition and oxygen, to and from the cells and to provide immunity against infectious agents.

Like mentioned before, blood is formed from several elements. Major components are plasma that makes around 55% of blood and cellular elements that takes 45% of blood. Cellular elements of blood are red blood cells, white blood cells and platelets. The red blood cells (erythrocytes) are in charge of the oxygen and dioxide carbon transport. The white blood cells (leukocytes) play an important role in innate and adaptive immune responses. And finally, the platelets (thrombocytes) play a very important role in clotting.

Nearly 20 years ago, a classical hematopoietic hierarchy has been proposed (Y. Zhang et al., 2018). To define the lineage relationships between HSCs and their progenies, a hematopoietic hierarchy was proposed as a tree-like branched roadmap that describes the differentiation process from HSCs to the different blood cell types (Akashi et al., 2000; Morrison & Weissman, 1994). HSC differentiation occurs through a stepwise process from multilineage production to lineage-restricted production to single lineage production of progenitors to mature blood cells (Morrison, Uchida, et al., 1995).

In this “tree” model, HSCs give rise to multipotent progenitors discriminated from stem cells by their lack of self-renewal ability, their commitment and their expression of lineage-specific markers. Progenitors will then produce precursor cells with distinctive differentiation potential. The hematopoietic system is thus organized into different cell compartments e.g., long-term HSCs (LT-HSCs), short-term HSCs (ST-HSCs), multipotent progenitor (MPPs), common myeloid progenitor (CMP) and common lymphoid progenitor (CLP). Cells within the compartments express homogeneously one or several feature(s) allowing the clear-cut discrimination from the adjacent compartments.

Different experiments have recently challenged the hierarchical model of hematopoiesis both in mice and human. Single cell barcoding tracing and refined functional assays have shown a degree of heterogeneity within cell compartments and unexpected cellular outputs. For example, a population of bone marrow  $c\text{-kit}^+\text{Sca-1}^+\text{Lin}^-\text{Flt3}^+$  HSCs that exhibit lymphomyeloid but not erythro-megakaryocytic potential has been identified (Adolfsson et al., 2005). Single cell lineage tracing has highlighted the heterogeneity of CMP and showed that most individual CMPs produce either erythroid or myeloid cells (Perié et al., 2015). Additionally, in humans, erythro-megakaryocyte lineages may directly emerge from BM HSCs and a shift in the architecture of the hematopoietic system has been proposed from the fetal to the adult stages (Notta et al., 2016) (Figure 1).



**Figure 1: Classical and redefined hematopoietic hierarchy**

Left: Classical hematopoietic hierarchy starting from HSC to different type of progenitors and precursors

Right: The redefined model that suggest a developmental shift in the architecture of progenitor cell from fetus to adult which changes in potency going from multipotent to unipotent cells (From Notta et al. 2016)

## 1.2. The biology of HSCs

HSCs are at the foundation of the hematopoietic system. These cells are the most immature cells of the entire hematopoietic system. In the adult they reside in BM where they are responsible for the constant production of all blood cell types during the lifespan of an organism (Dzierzak & Speck, 2008; Medvinsky et al., 2011). This long-term hematopoietic production is possible due to two fundamental properties that characterize adult stem cells – multipotency and self-renewal. Multipotency allows single HSCs to differentiate into committed progenitors and precursor cells that will then proliferate to produce all mature blood cell lineages while self-renewal allows them to sustain long-term blood production (Bryder et al., 2006). These two properties of HSCs were thought to be two independent fate choices (Yanai et al., 1995). Upon each division HSCs undergo only one of the three mutually exclusive cell divisions (symmetric self-renewal, asymmetric self-renewal, symmetric differentiation and asymmetric differentiation (Morrison & Kimble, 2006; Wu et al., 2007) and as such indicating that the regulation of HSC self-renewal cannot be separated from that of differentiation (Will et al.,

2013; Wilson et al., 2004). Recently it was published that the key epigenetic regulator of DNA methylation Uhrf1 specifically controls this process by DNA methylation mediated repression of the expression of certain erythroid-specific genes, and like that modulates cell fate decision of HSCs (J. Zhao et al., 2017). However, what are the key factors regulating the decision of self-renewal vs. differentiation within the HSC compartment is still missing.

### **1.3. Identification of HSCs**

In any organism, all cells are expressing surface membrane proteins and many of those are specific of the cells of interest (lineage, maturation stage) as they mediate particular functions (for example cell to cell adhesion, signaling transduction, transporters).

In the mouse BM two major cell populations can be distinguished:

1. Cells expressing markers of mature lineages (CD3, CD4, CD8, CD19, NK1.1, Gr1, CD11b and Ter119; defined as lineage positive cells,  $\text{Lin}^+$ ) containing committed progenitors and precursor cells of the lymphoid, myeloid and erythroid lineages.
2. Cells negative for these markers ( $\text{Lin}^-$ ) containing HSCs and immature progenitor cells.

In mice, HSCs and multipotent progenitors are enriched in the  $\text{Lin}^-$  cell fraction and express high levels of the stem cell antigen-1 (Sca-1) and c-Kit receptor expression (thus defined as  $\text{Lin}^- \text{Sca-1}^{\text{hi}} \text{c-Kit}^{\text{hi}}$  cells or LSK) (Okada et al., 1992). The LSK subset represents 0.5% of total BM cellularity but less than 10% LSK cells are capable of repopulating the hematopoietic system of lethally irradiated mice, indicating high degree heterogeneity within the LSK compartment (Okada et al., 1992). Enriched HSC population was defined in C57BL/Ka-Thy1.1 mice where  $\text{Thy1.1}^{\text{lo}} \text{Lin}^- \text{Sca-1}^+$  cells were shown to express low level of the Thy1.1 allele (also named CD90) (Uchida & Weissman, 1992). HSC frequency obtained after transplantation of  $\text{Thy1.1}^{\text{lo}} \text{Lin}^- \text{Sca-1}^+$  cells at limiting dilution in lethally irradiated mice, represent up to 18% of this cell fraction (Uchida & Weissman, 1992). LT-HSCs and ST-HSCs could be

distinguished based on the differential expression of CD34 and Flt3 cell surface expression: HSCs with long-term reconstitution capacity (LT-HSCs LSK CD34<sup>-</sup>Flt3<sup>-</sup>), can be distinguished from ST HSCs (LSK CD34<sup>+</sup>Flt3<sup>-</sup>) with a limited (up to 8 weeks) repopulation activity in lethally irradiated mice. The group of Sean Morrison using Signaling Lymphocytic Activation Molecule (SLAM), which are cell surface glycoproteins from the immunoglobulin super family, showed that HSCs could be further enriched as LSK CD150<sup>+</sup>CD48<sup>-</sup> cells of which 50% are LT-HSCs (Kiel et al., 2005). 30% of them do not express CD34 suggesting that the LSK CD150<sup>+</sup>CD48<sup>-</sup>CD34<sup>-</sup> cell population might be even more enriched in HSC (Wilson et al., 2008). Combining SLAM markers with absence of CD34 expression is the most commonly used strategy to isolate mouse BM HSCs (Cabezas-Wallscheid et al., 2014, 2017).

Additionally, based on their cell cycling capacity, two reservoirs of HSCs could be identified: “dormant” HSCs that divide every 145 days and “activated” HSCs dividing once a month (Cabezas-Wallscheid et al., 2014; Wilson et al., 2008; J. Zhang et al., 2003). Recently, using RNA sequencing, dormant HSCs were found to express specifically Gprc5 (G protein-coupled receptor, class C, group 5, member of the vitamin A/retinoic acid signaling pathway) (Cabezas-Wallscheid et al., 2017), therefore allowing dormant HSCs to be distinguished from activated HSCs using antibody-labeling strategy and not cell cycle-specific dyes.

In human, HSCs could also be isolated based on combination of surface markers expression. The most commonly used surface phenotype to track human hematopoietic stem and progenitor cells (HSPCs) is CD34 (adhesion-mediated glycoprotein) (Civin et al., 1996). Importantly, CD34 expression is not exclusive to HSPCs as other BM resident cells, including endothelial cells, also bear surface CD34 expression (Fina et al., 1990). Interestingly, several works including recent publications have shown that HSCs can be found among CD34<sup>-</sup> Lin<sup>-</sup> cells (Anjos-Afonso et al., 2013; Bhatia et al., 1998; M. Takahashi et al., 2014; Wang et al., 2003). The frequency of *in vivo* reconstituting cells remains very low in human cord blood CD34<sup>-</sup>



CD38<sup>-</sup>CD45<sup>+</sup>CD93<sup>+</sup> cells. Even though the existence of CD34<sup>-</sup> human HSPC has been suggested, all the data from the clinic, including the gene therapy protocols, show that human HSC, with long term multilineage hematopoietic reconstitution potentials, express CD34 marker (Ferrari et al., 2021).

Additional markers are to be combined to further enrich for more immature cells. The mainly used ones are CD38 (Terstappen et al., 1991), Thy1/CD90 (Baum et al., 1992; Majeti et al., 2007), CD45RA and CD133/prominin1 (Gallacher et al., 2000). Thus, future work should focus on understanding CD34 expression in human HSCs keeping in mind that CD34<sup>-</sup> cells may be quiescent ancestor of CD34<sup>+</sup> cells (Anjos-Afonso et al., 2013).

Finally, all this together should enable us to capture the heterogeneity of HSPC compartment in respect to their proliferative and differentiation potentials and their cell cycle status.

#### **1.4. Functional assays for identifying hematopoietic progenitors and HSCs**

##### **1.4.1. Colony forming cells and long-term culture-initiating cells culture systems**

HSCs and progenitor cells are characterized by their functional properties: self-renewal, specific for HSCs, and/or multilineage differentiation. Most of experimental assays used to characterized immature cells are retrospective as they rely on cell proliferation and differentiation.

The **Colony forming cells (CFCs)** assay is used to study the proliferation and differentiation template of hematopoietic progenitors through their ability to form colonies in a semisolid medium. CFCs assay is one of the first experimental assays developed to measure immature cells (Pluznik & Sachs, 1965). This is a clonal assay which means that every colony derives from a single progenitor. In mice and human, it is mainly oriented toward myeloid and erythroid differentiation (Paige et al., 1984), and they can be detected in one (mouse) to two (human) weeks following culture initiation. Counting the number of colonies is showing us the

frequency and the total number of committed progenitors of a given cell suspension. CFC assay also provides the quality (lineage, multi- vs. mono-potent clones and size: small/mature or large/immature colonies) of committed progenitor.

The **Long-term culture-initiating cells (LTC-IC)** assay measures primitive hematopoietic stem cells based on their capacity to produce myeloid progeny for at least 5 weeks. While culturing the total BM cell suspension, a stromal layer developed together with hematopoietic cells and these mesenchymal cells supported immature and mature cells (Dexter et al., 1977). Stromal cell lines were isolated and the LTC-IC assay is placed on co-culture of enriched immature cells with pre-established BM-derived adherent cells, such as MS-5 mouse stromal cells (Issaad et al., 1993). Importantly, LTC-IC assay can provide quantitative data, using limiting dilution of cells and data analyzed with Poisson statistics (Paige et al., 1984).

In humans, LTC-ICs are typically defined as cells that are able to give rise to CFCs after being co-cultured for 5 weeks with a feeder cell layer (Coulombel, 2004).

Both CFC/LC-IC assay can be extended in time, using, for example, serial replating of primary CFC or extended LTC culture period over 10 weeks. In both cases, it is used as a way to test the self-renewal capacity of immature cells and/or to reveal even more immature cells (Hao et al., 1996; Henry et al., 2020; Petzer et al., 1996).

#### **1.4.2. Hematopoietic transplantation assay**

One of the mandatory assays to study *in vivo* the functions of HSCs is the transplantation assay. Most commonly used approach is to myeloablate a recipient mouse, for instance with irradiation or chemical-busulfan- injection, to provoke BM aplasia and destroy hematopoietic activity. This assay is allowing us to follow the ability of transplanted HSCs to differentiate into different blood cell types and also their capacity to self-renewal through serial

transplantations. In human hematopoietic reconstitution studies, immune-deficient mice, such as NSG (NOD Scid Gamma) mice, are necessary.

This experimental approach was first used by Till and McCulloch in 1960s in which BM cells capable of clonal generation of multiple hematopoietic lineages and with self-renewal properties were firstly discovered (Till & McCulloch, 1961) .

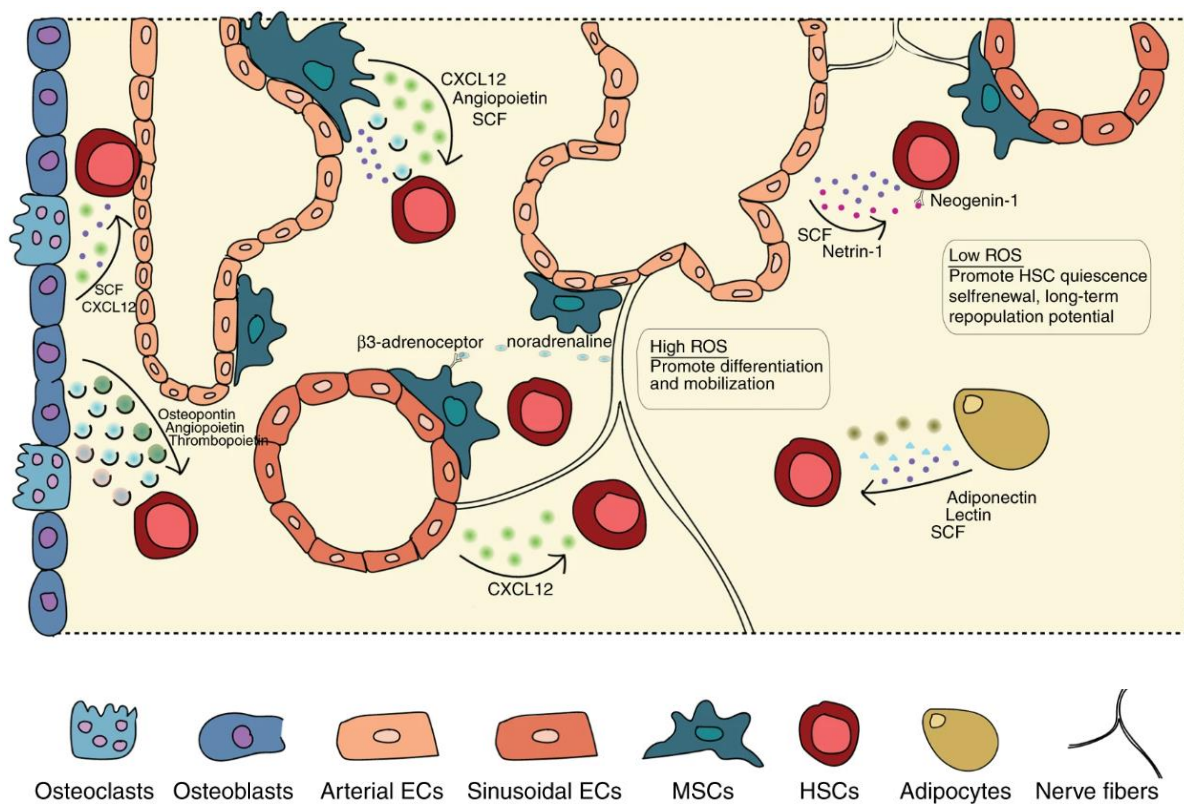
In overall, this analysis is based on the expression of different markers, especially CD45 since it has different alleles in mouse, and like that allows distinguishing cells deriving from the donor and recipient. The first reconstitution is called primary reconstitution and reveals the multipotency of injected cells. Secondary transplantation is used to assess the long-term self-renewal potential.

Immune-deficient (ID) mice were used as a recipient of either human BM cells or fetal hematopoietic tissues with the goal of avoiding rejection by the host immune system (Kamel-Reid & Dick, 1988; Lapidot et al., 1992; Spangrude et al., 1988). Following the reconstruction of human hierarchy in the BM of the ID mice it was shown that human immature CD34<sup>+</sup>CD38<sup>lo</sup> and/or CD90<sup>+</sup> cells recovered from mouse BM all the progenitors found in normal BM cells (Pflumio et al., 1996; Robin et al., 1999). After the secondary transplantation into ID mice hematopoietic development confirmed the engraftment of human HSCs in the mouse BM. Human hematopoietic reconstitution of ID mice however doesn't totally mimic the full human BM mature cell diversity (Doulatov et al., 2012), and of the recent improvements have been made in terms of humanization of ID mice that could produce human growth factor or recreate a human BM niche *in vivo* (Dupard et al., 2020).

## **2. The bone marrow: the primary niche for adult HSC maintenance**

During mammalian postnatal life, the BM is the primary site of hematopoiesis and HSC maintenance. The BM niche is an essential microenvironment, which evolves and responds

to the physiological demands of HSCs. The inner bone surface is called “endosteum” and it was the first anatomical niche of the BM to be functionally described (Arai et al., 2004; Calvi et al., 2003; J. Zhang et al., 2003). The central marrow emerged as another important niche of HSCs (Acar et al., 2015). Illustration of distinct coexisting BM niches which are important in orchestrating the fate of HSCs and regulation of the processes that occur in the BM including self-renewal, quiescence, engraftment and lineage differentiation is represented in Figure 2.



**Figure 2: The BM HSC niche**

HSC are closely attached to the small blood vessels in the BM. They are in contact with niche cells such as osteoblasts and perivascular mesenchymal stromal cells (MSCs).

SCF- Stem Cell Factor; CXCL12-C-X-C Motif Chemokine Ligand 12' ROS-Reactive Oxygen Species, MSCs- Mesenchymal Stromal Cells, ECs-Endothelial Cells (From Peci et al. 2020).

Here I will briefly present the most important components of the BM niche.

## **2.1. Vascular niche component**

To be able to provide nutrients and oxygen the BM needs to be highly vascularized. The nutrient artery and vein infiltrate the compact bone and subsequently branch to form small arterioles. These arterioles connect through transition zone vessels to the venous sinusoids near the endosteum. BM sinusoids form a complex network. The bone marrow sinusoids are unique and are not comparable with regular veins since the sinusoidal wall consists of a single layer of endothelial cells and is deprived of supporting cells (Tavassoli, 1981).

They are mainly found in the central marrow, operating as the site where HSCs and leukocytes enter systemic circulation due to their permeability (Itkin et al., 2016; Kopp et al., 2005). Recently, transcortical vessels crossing the bone have been suggested to carry up to 80% of arterial blood and 60% of venous blood and might therefore be important routes for cell trafficking in and out of BM (Grüneboom et al., 2019).

## **2.2. Mesenchymal stem/stromal cells**

Mesenchymal stem/stromal (MSCs) cells are a key cellular niche component that produces key niche factors that are directly affecting HSPCs. In 2010 Mendez-Ferrer and colleagues demonstrated that MSCs are an essential HSC niche component, and that BM perivascular cells with MSCs activity can be identified by the expression of Nestin (Méndez-Ferrer et al., 2010). They are divided into two subsets according to GFP expression in *Nestin-Gfp* transgenic mice: Nes-GFP<sup>bright</sup> and Nes-GFP<sup>dim</sup>. Nes-GFP<sup>dim</sup> cells are subendothelial cells in sinusoids while Nes-GFP<sup>bright</sup> cells include endothelial and perivascular cells in the arterioles and the transition zone vessels (Kunisaki et al., 2013; Isern et al., 2014; Itkin et al., 2016). Importantly, Nestin<sup>+</sup> MSCs are containing all the BM colony-forming-unit fibroblastic activity (Méndez-Ferrer et al., 2010). MSCs can be further divided to neuron-glial antigen (Ng2) positive cells, Cxcl12 abundant reticular (CAR) cells and leptin receptor (LepR)-expressing cells. All of these cell

types overlap with Nes-GFP<sup>+</sup> cells to varying degrees (Ding et al., 2012; Kunisaki et al., 2013; Méndez-Ferrer, 2019; Omatsu et al., 2010; Sugiyama et al., 2006).

Importantly, the existence of the balanced regulation of hematopoietic and mesenchymal lineages at the stem cell level was described as a cooperation of homeostatic (exp. SNS) and hormonal (exp. Parathormone) mechanisms that cooperate together to regulate HSC maintenance and also MSC proliferation and differentiation (Méndez-Ferrer et al., 2010).

### **2.3. Bone-associated cells**

Over a decade ago, in a same time two research groups discovered that in the mutant mice who exhibit an increased number of osteoblasts the number of HSPCs in the BM was also increased. Interestingly, one group used mutant mice where BMP receptor type IA was conditionally inactivated, while in the other mutant mice PTH/PTHrP receptors were constitutively activated (Calvi et al., 2003; J. Zhang et al., 2003). These discoveries provided strong evidence that osteoblasts could have a regulatory role on HSCs number and function within the BM niche.

In 2007, Bianco et al published that cells expressing MCAM/CD146 in human BM stroma were capable of transferring upon transplantation (Sacchetti et al., 2007). They showed that establishment of subendothelial stromal cells occurs through specific dynamic interaction with developing sinusoids, and that subendothelial stromal cells located on the sinusoidal wall are major producers of Angiopoietin-1 which is a pivotal molecule of HSC niche involved in vascular remodeling (Sacchetti et al., 2007).

Interestingly, it was also demonstrated that upon conditional deletion of two key cytokines C-X-C motif chemokine ligand 12 (CXCL12) and stem cell factor (SCF) from osteoblasts there was no significant alterations of HSC number in the BM (Ding et al., 2012). This inconsistency of the osteoblastic niche could be due to the lack of specificity of the genetic promoters that mark subsets of mesenchymal lineages.

Osteolineage cells that line the BM surface are expressing a higher level of Embigin and Angiogenin mRNAs and deletion of these molecules was shown to reduce HSC quiescence (Silberstein et al., 2016). This additionally highlights the relevance of the endosteal HSC niche in keeping HSC quiescent under proliferative stress.

## **2.4. Adipocytes**

With aging in the human BM active hematopoietic tissues are being progressively replaced by adipocytes. If the adipocytes have a role as an HSC niche has been debated. The group of George Daley demonstrated that the abundance of adipose tissue was negatively correlated with the number of HSCs (Naveiras et al., 2009). This process was accelerated in fatless A-ZIP/F1 mice or mice treated with peroxisome proliferator-activated receptor  $\gamma$  (PPAR  $\gamma$ ) inhibitor bisphenol A diglycidyl ether (BADGE) which is known inhibitor of adipogenesis (Naveiras et al., 2009). To explore the heterogeneity of the BM niche, single cell RNA sequencing was done using mouse models. It showed that adipocytic primed LepR<sup>+</sup> cells are a major reservoir of pro-hematopoietic factors in the BM niche (Baryawno et al., 2019; Tikhonova et al., 2019). They have been further refined as LepR<sup>+</sup>Osteolectin<sup>-</sup> (Shen et al., 2021). Baccin et al identified a specific CAR subpopulation expressing an adipocytic-lineage gene signature and they found it to be a critical component of the perivascular niche (Baccin et al., 2020).

However, the adipocytes trajectories and subpopulations of the hematopoietic niches in the BM are still the object of identification and further work on these characterizations is still needed.

## 2.5. Nerve cells

The BM is highly innervated by various types of nerves, while the autonomic branch is predominant (del Toro & Mendez-Ferrer, 2013). Sympathetic nerve fibres enter the BM through the nutrient foramen and they are closely associated with the blood vessels (Calvo, 1968).

Sympathetic nerves are covered by non-myelinating Schwann cells, which have been reported to maintain HSC quiescence via secreting Tgf- $\beta$  activator molecules and inducing Tgf- $\beta$ -Smad signaling in HSCs (Yamazaki et al., 2011). The sympathetic nervous system (SNS) regulates hematopoiesis directly and indirectly. Most of the regulation by SNS is done through the stromal cells, mediated by neurotransmitters binding to adrenergic receptors. The SNS plays a major role in regulating the proliferation and differentiation of HSPCs and the migration of HSPCs and leukocytes between the BM and extramedullary sites (Méndez-Ferrer et al., 2008). Spiegel et al reported that immature human CD34<sup>+</sup> cells expressed dopamine and beta2-adrenergic receptors, with higher expression in the primitive CD34<sup>+</sup>CD38<sup>(lo)</sup> population and that treatment with catecholamines enhanced human CD34<sup>+</sup> cell engraftment of NOD-SCID mice and increased cell mobilization and bone marrow Sca-1<sup>+</sup>c-Kit<sup>+</sup>Lin<sup>-</sup> cell numbers (Spiegel et al., 2007).

The parasympathetic nervous system (PNS) uses acetylcholine (Ach) as the main postsynaptic neurotransmitter, which binds to either muscarinic or nicotinic receptors. One study suggested that the PNS may innervate the distal femoral metaphysis (Bajayo et al., 2012) and another study supported the presence of cholinergic innervation within BM of rats (Artico et al., 2002). It was suggested that the bone anabolic effect of the PNS is indirectly mediated through the inhibition of central sympathetic tone through muscarinic receptors in the brain (Shi et al., 2010), which also regulate G-CSF-induced HSC mobilization (Pierce et al., 2017).



A recent study has shown that parasympathetic cholinergic signals coordinate with sympathetic signals to regulate the egress and homing of HSPCs and leukocytes. At night, the PNS acts to dampen the noradrenergic sympathetic branch and decrease BM egress of HSPCs and leukocytes (García-García et al., 2019). In contrast, epinephrine released at night in circulation can stimulate B2-adrenergic receptor and increase vascular adhesion and subsequent BM homing (Scheiermann et al., 2012). During the day, sympathetic noradrenergic fibres decrease Cxc112-mediated BM retention of HSCs and leukocytes, whereas sympathetic cholinergic signals inhibit vascular adhesion to allow cells to migrate into circulation (García-García et al., 2019).

## **2.6. Mature hematopoietic cells**

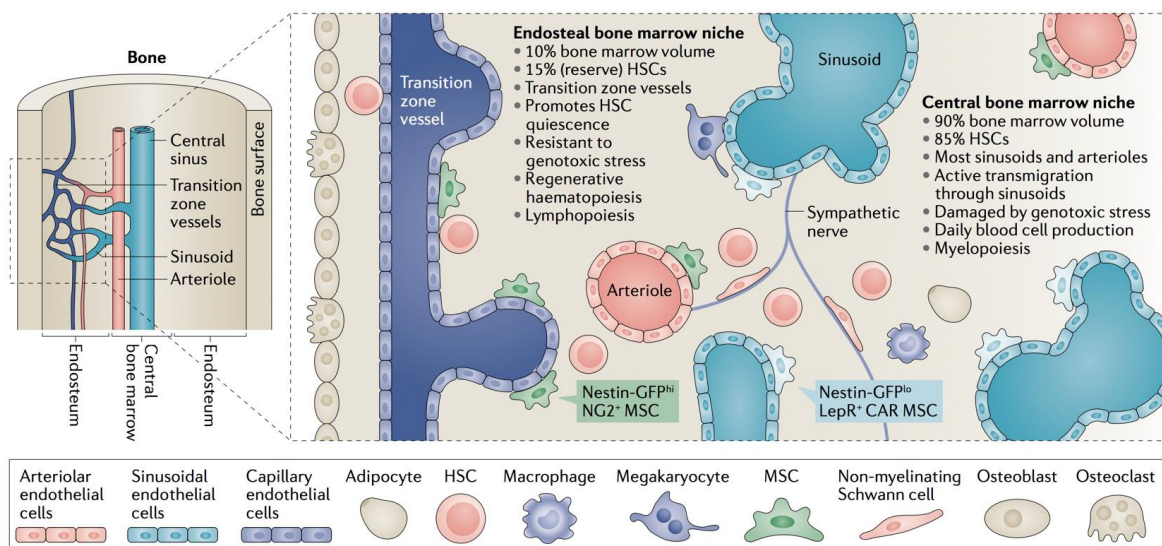
It has been suggested that HSC functions could be regulated partially by their progeny. This was based on the fact that tissue homeostasis demands regulatory feedback.

One of the first examples includes macrophages. Macrophages are located throughout the BM, and they act on perivascular MSCs to modulate Cxcl12 production, in turn influencing HSC release from the niche (Chow et al., 2013; Christopher et al., 2011; Winkler et al., 2010). This is supported by the fact that depletion of CD169<sup>+</sup> macrophages and subsequent reduction in *Cxcl12* expression in perivascular niche cells, led to increased HSC mobilization (Chow et al., 2013; Winkler et al., 2010).

In addition, it has been shown that hematopoietic cells of the myeloid lineage can also regulate HSC quiescence. Imaging studies indicated that around 35% of HSCs are located next to megakaryocytes and exhibited a ROS<sup>low</sup> (reactive oxygen species) quiescent state (Itkin et al., 2016). Other studies have indicated that megakaryocytes were able to maintain HSC quiescence through transforming growth factor b (TGFb), the chemokine Cxc14/platelet factor 4, and the C-type lectin domain family 2 (Clec-2) (Bruns et al., 2014; Nakamura-Ishizu et al., 2015; M. Zhao et al., 2014).

Recently, regulatory T cells (Tregs) were also identified as important BM niche cells. Tregs are a non-redundant sub-population of CD4<sup>+</sup> T cells, and they are enriched in within the BM having dual function as immunosuppression and maintenance of HSCs (Ashman et al., 2022). BM Tregs were found closely associated with HSCs and may regulate their quiescence and repopulation activity through the release of extrinsic molecules such as IL-10 and adenosine (Hirata et al., 2018). Whether and how Tregs provide an immune privilege for HSCs is a very interesting question.

Figure 3 recapitulates all the different cell types that constitute the BM HSC niche.



**Figure 3: Schematic representation of the BM HSCs**

The HSCs niche is constituted of different cell types such as vascular cells (arterioles, sinusoids), mesenchymal stem cells (MSCs), adipocytes, nerve cells and hematopoietic cells to regulate maintenance of the HSCs (From Mendez-Ferrer et al, 2020).

## 2.7. Extrinsic regulators

All these different cell types have to cooperate together to regulate the maintenance of the HSCs within the adult BM. Finally, it is important to highlight the roles of two major niche factors: the C-X-C motif chemokine ligand 12 (CXCL12) and stem cell factor (SCF).

Cxcl12 and its receptor Cxcr4 regulate the homing of HSCs and their downstream progenitors, and are critical for BM colonization and engraftment (Ara et al., 2003; Janssens et al., 2018; Peled et al., 1999). Stromal cells are characterized by the expression of Cxcl12 (Gomariz et al., 2018; Tavassoli, 1976).

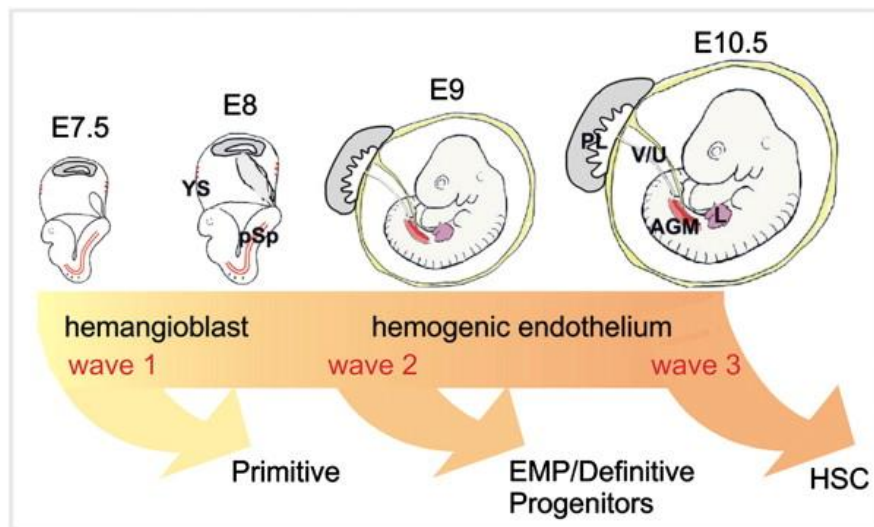
Scf (Kit ligand) is also produced by perivascular stromal cells for the maintenance of HSCs which express the cKit receptor (Ding et al., 2012). Scf is also produced by adipocytes and this secretion is important for regeneration of hematopoiesis after myeloablation (Zhou et al., 2017).

On top of these factors, other have been shown to play an important role on HSCs such as integrins and adhesion molecules. One of the most important integrin members is VLA-4 which mediates homing and adhesion of HSCs through VCAM-1, fibronectin and osteopontin expression by stromal cells (Papayannopoulou, 2003). Recently, it was shown that connexin 43 and 45 gap junction is involved in CXCL12 secretion and as such in BM retention of HSCs (Schajnovitz et al., 2011).

To extract the molecular core of the HSPC support and gene networks active in different supportive BM niche cell population, my team took advantage of systems biology approaches and of stromal lines with differing capacity to support HSPCs *ex vivo* (Charbord et al., 2014; Desterke et al., 2020). These studies also led to the identification of novel HSPC niche factors.

### 3. The embryonic hematopoietic waves

It is now well accepted that hematopoietic development occurs in three separate waves. In the mouse embryo, the first one takes place in the yolk sac (YS) at embryonic day (E) 7.5 (Palis et al., 1999), 19-22 hours in the chicken embryo (Minko et al., 2003), 21 days in the human embryo and 5-somite-stage in the zebrafish embryo. This wave gives rise to primitive nucleated erythroid cells, macrophages, and megakaryocytes (Palis et al., 1999). Shortly after the primitive wave, the second or pro-definitive wave occurs in the YS, the embryo proper and the allantois. It produces erythro-myeloid progenitors, immune-restricted progenitors and multilineage neonatal repopulating progenitors (Dzierzak and Bigas, 2018). Third wave occurs in the embryo in a specific region named aorta-gonad-mesonephros (AGM) and is characterized by the production of the first adult-type HSCs defined by their capability to reconstitute the entire hematopoietic system of adult irradiated recipients upon transplantation (A. Medvinsky & Dzierzak, 1996; Müller et al., 1994) (Figure 4).



**Figure 4: Schematic representation of hematopoietic waves in the mouse embryo.**

Different waves of embryonic hematopoiesis. First wave; YS: primitive erythroid cells, macrophages, and megakaryocytes. Second wave; YS, definitive EMPs and immune-restricted progenitors. Third wave; AGM: HSCs (From Imanirad and Dzierzak 2013).

### **3.1. Mouse dorsal aorta**

It has been shown that the dorsal aorta is a major site of HSC emergence in mice (de Bruijn, 2000). Prior to the formation of the single aorta harbouring HSCs, the presumptive aortic territory is contained within the splanchnopleural mesoderm located in the caudal region of the embryo (Cumano, 1996). At E8.5 splanchnopleural mesoderm will give rise to a structure containing the paired aortas, the sub-aortic mesenchyme and the endoderm of the digestive tract. This structure is called para-aortic splanchnopleure (P-Sp) (Godin et al., 1995). From E9.5 to E11.5 the P-Sp evolves to form the AGM region comprising the dorsal aorta and its underlying mesenchyme and the urogenital system (Medvinsky et al., 1993). Intra-aortic hematopoietic clusters (IAHCs) are formed within the AGM, precisely in the aortic lumen. (Yokomizo & Dzierzak, 2010a).

Experiments of P-Sp or YS culture isolated prior to the establishment of circulation between the YS and the embryo provided evidence that definitive hematopoiesis emerge in the aorta (Cumano, 1996). Authors were able to conclude that by grafting P-Sp or YS cells into adult immunodeficient mice only aortic region allowed reconstruction of the lymphoid lineage (Godin et al., 1993). At the same time, Alexander Medvinsky and colleagues showed that AGM carries a CFU-S activity initiated from E9, which peaks at E10 and then declines, then CFU-S activity increases in the fetal liver suggesting that hematopoietic progenitors emerging from the aorta subsequently colonize the fetal liver (Medvinsky et al., 1993).

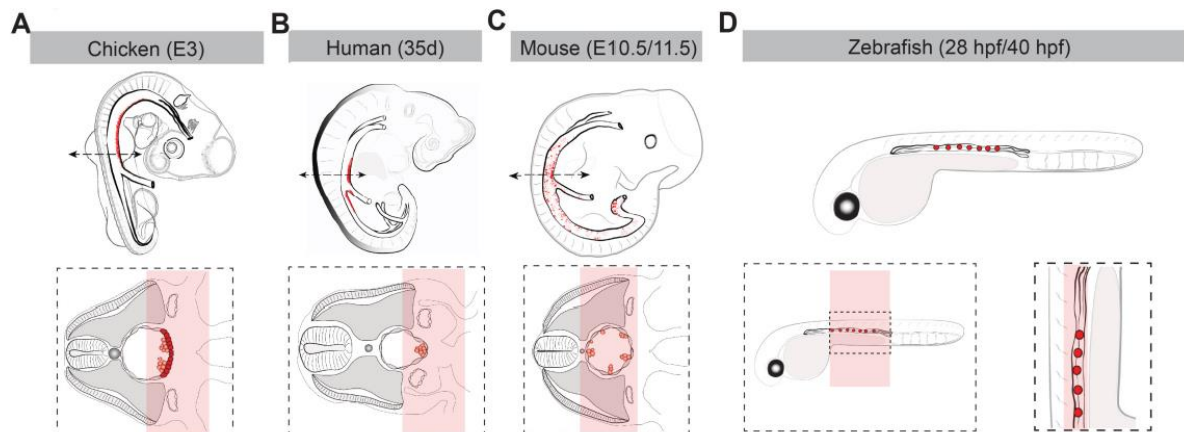
Medvinsky and Dzierzak showed that cells from E11 AGM could reconstitute the different hematopoietic blood lineages when injected into primary and secondary irradiated adult mice, testifying the presence of bona fide HSCs (Medvinsky and Dzierzak, 1996).

### **3.2. Intra-aortic hematopoietic clusters (IAHCs)**

It has been shown that HSCs are generated from endothelial cells via an endothelial to hematopoietic transition (EHT) (described in the next paragraph) in the AGM region of vertebrate embryos and notably in the dorsal aorta (Bertrand et al., 2010a; Boisset et al., 2010; Ivanovs et al., 2011; Jaffredo et al., 1998; Kissa & Herbomel, 2010b; Lam et al., 2010; Taviani et al., 2001; Oberlin et al., 2002). The process of HSC emergence is highly conserved and it is regulated both in time and space albeit species-specific differences exist (Klaus & Robin, 2017) (Figure 5). For instance, HSC generation is polarized and restricted to the ventral side of the aorta in the chicken, zebrafish and human embryos (Bertrand et al., 2010; Kissa & Herbomel, 2010; Lam et al., 2010; Taviani et al., 2001; Yvernogeu & Robin, 2017) while in the mouse embryo, EHT is also occurring in the dorsal part of the aorta (Taoudi & Medvinsky, 2007; Yokomizo & Dzierzak, 2010b). HSCs are produced via the formation of IAHCs in chicken, mouse and human embryos but not in zebrafish where single cells emerge underneath the aorta, along the elongated YS and are immediately released into the blood stream through the underlying cardinal vein (Kissa & Herbomel, 2010b; Yokomizo & Dzierzak, 2010b; Yvernogeu & Robin, 2017).

IAHC generation is also regulated in time. In zebrafish embryos its generation starts at around 26 hpf with a peak at 40hpf and totally ceases after 72hpf (Bertrand et al., 2010). Using whole-mount immunostaining combined with 3D reconstruction confocal imaging system, the distribution and number of IAHCs generated in the mouse and chicken aorta had been precisely determined (Yokomizo & Dzierzak, 2010b; Yvernogeu & Robin, 2017). In the mouse embryo IAHC emergence starts at E9.5 to reach a peak of about 700 IAHCs cells at E10.5. Then IAHC numbers progressively decrease and cease after E14.5 (Yokomizo & Dzierzak, 2010b). In the chicken embryo, IAHC emergence occurs only in anterior portion of the aorta and starts at E2.25 to reach peak of about 1500 IAHC cells at E3. IAHC numbers then decrease and are no

longer observed after E5.5 (Yvernogeu & Robin, 2017). Finally in the human embryo IAHC/HSC emergence occurs in the middle part of the aorta from day 27 to 42. This emergence is maximum at day 35 and generate an estimated around 1000 IAHC cells per embryo (Ivanovs et al., 2017).



**Figure 5: Localization of IAHC cells in different species**

A: In chicken IAHCs are restricted to anterior and ventral side of the dorsal aorta.

B: In human IAHCs are present in the middle part of the aorta, restricted to the ventral side of the dorsal aorta.

C: In the mouse embryo IAHCs are mainly observed in the ventral side of the dorsal aorta, but they could be found on the dorsal part as well.

D: In the zebrafish embryo, HCs emerge as a single cell in the aorta.

(From Yvernogeu et al. 2020).

### 3.3. Placenta and fetal liver (FL) as HSPC niches

Two research groups independently in 2005 found placenta as an extra embryonic niche for HSC development and expansion (Gekas et al., 2005; L. Li, 2005; Ottersbach & Dzierzak, 2005). These independent studies showed that the emergence of HSCs with repopulating capacity in the placenta occurs in parallel with that of the AGM starting at E10.5 to E11, and that this HSC pool expands until E12.5-E13.5. However, transplantation of cultured E10 mouse placenta, at a time point at which contains no detectable HSCs, did not repopulate irradiated

mice (Robin et al., 2006). Whether the placenta has the capacity to generate *de novo* HSCs or only supports HSC expansion remains debated and will need further investigation.

After their emergence, HSCs will migrate and colonize the FL in mammals where they will undergo multiplication and amplification until E16. Unlike the adult BM HSCs, which are mostly quiescent, FL HSCs is highly proliferative (Bowie et al., 2006, 2007; Morrison, Hemmati, et al., 1995). A comparison of the quiescent adult BM HSC niche (Arai & Suda, 2007; Wilson et al., 2008) with the highly proliferative FL HSC niche might provide mechanistic insight into how to achieve HSC expansion *ex vivo*, and reveal the key factors involved.

So far, little is known about the FL HSPC niche. Ihor Lemischka and colleagues used a panel of immortalized stromal cell lines from the murine FL and showed that these lines displayed extensive heterogeneity in their capacity to support HSPCs *ex vivo* (Moore et al., 2012).

In 2010, Chou and Lodish by performing competitive repopulating experiments showed that Scf<sup>+</sup>Dlk<sup>+</sup> hepatic progenitors support the maintenance of HSCs *ex vivo*. These cells express critical HSPC niche factors such as angiopoietin-like 3, Igf2, Scf and Cxcl12 (Chou & Lodish, 2010). More recently, Khan et al have evaluated the presence of perivascular nestin-GFP<sup>+</sup> cells in this tissue. Rare nestin-GFP<sup>+</sup> cells, marked by Ng2 (Cpsg4) expression, exist in the developing liver in association with portal vessels. They showed that the depletion of Ng2<sup>+</sup> cells, significantly reduced HSCs in the E14.5 FL. A comparison of the transcriptome of BM nestin-GFP<sup>+</sup> cells with FL nestin-GFP<sup>+</sup> cells revealed they share similar levels of expression of all major factors known to influence niche activity. However, FL nestin-GFP<sup>+</sup> cells differentially expressed cell cycle genes and were highly proliferative relative to nestin-GFP<sup>+</sup> cells from adult BM. Importantly, between E12 and E14.4 the number of niche cells expanded at the same scale as HSCs and the vasculature according to fractal geometries, highlighting the notion that during the fetal liver stage, expansion of the vascular tree, rather than a change in



the expression profile of niche cells, might drive HSC expansion (J. A. Khan et al., 2016). Additionally, Lee and colleagues explored HSC niche in the developing liver, and showed that endothelial and stellate cells are two major cellular sources of SCF in the FL niche, and as such present critical component of the niche. They demonstrated that combined deletion of *scf* in both cell types leads to HSC depletion (Lee et al., 2021).

Interestingly, the current dogma that the FL is an expansion niche for HSCs has been challenged. Rybtsov et al show that between E9.5 and E11.5 the pool of HSC precursors gradually matures and expands mostly in the AGM region, where it reaches the size that matches the number of definitive HSCs in the E12.5 fetal liver (Rybtsov et al., 2016). Ganuza et al used a non-invasive multi-colour lineage tracing strategy to follow the embryonic expansion of murine hematopoietic progenitors. They showed that this pool of fated progenitors expands actually only two-fold during FL ontogeny and that FL-HSCs are biased towards differentiation rather than symmetric self-renewal (Ganuza et al., 2022). They finally proposed that HSC pool fated to contribute to life-long hematopoiesis expands only modestly during FL ontogeny.

These and other studies have showed that indeed FL represents an important niche to support HSC expansion before transiting into the definitive hematopoietic organs (BM, thymus, spleen) while the amount of contribution for HSCs expansion is still to be confirmed.

### **3.4. Hemogenic endothelium and endothelial to hematopoietic transition**

Hemogenic endothelium represents a small, specialized subset of vascular endothelium that by acquiring hematopoietic potential is able to give rise to multilineage HSPCs in a short period of time, within distinct tissues (Jaffredo et al., 1998; Marcelo et al., 2013; Zambidis et al., 2006).

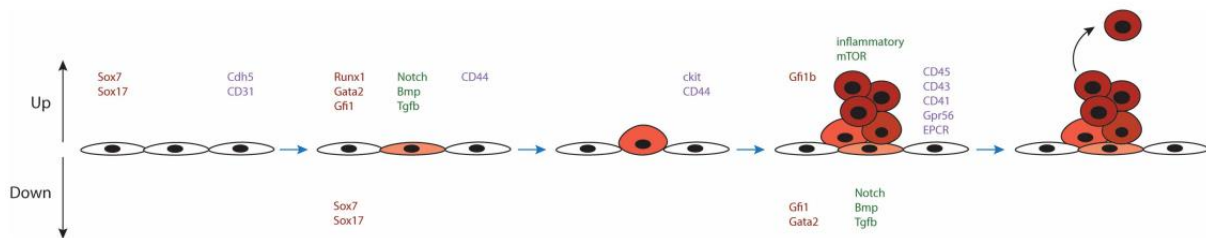
Subsequent *in vivo* fate tracing studies in murine embryos have demonstrated the emergence of HSCs from VE-cad<sup>+</sup> endothelial cells in the dorsal aorta that will go on to colonize intra-embryonic hematopoietic sites (Zovein et al., 2008).

Multiple real-time imaging studies has demonstrated hematopoietic cell emergence from vascular endothelium in both mice and zebrafish (Bertrand et al., 2010; Boisset et al., 2010; Kissa & Herbomel, 2010). Single cell lineage tracing studies have demonstrated multipotent HSPCs with cell surface markers CD31 (endothelial cell marker), CD41 (blood cell marker), c-Kit (stem cell growth factor receptor) and Sca-1 (stem cell antigen, also known as Ly6A) arising directly from the ventral wall of the murine dorsal aorta that can be traced to FL (Boisset et al., 2010) , and ultimately to the adult hematopoietic system in mice (Godin et al., 1993) , and zebrafish (Bertrand et al., 2010; Kissa & Herbomel, 2010).

De Bruijn et al demonstrated using a Ly6A (Sca-1)-GFP transgenic mice model that all HSCs within the developing aorta are Ly6A-GFP<sup>+</sup> and that these cells localize to the endothelial layer of the dorsal aorta, but not to the underlying mesenchyme (de Bruijn et al., 2002). To support the assertion that HSPCs are generated from an endothelial intermediate and not the underlying mesenchyme, it has been demonstrated that key hematopoietic regulators such as the transcription factor Runx1 are expressed at high levels by endothelial cells in hemogenic vascular sites in vertebrates (North et al., 1999). North et al demonstrated that while Runx1-expressing HSCs are found in both endothelial and mesenchymal cell fractions in the mouse embryo, the presence of two functional *Runx1* alleles segregates HSC to the endothelial fraction only (North et al., 2002). More recently, it has been shown that *Runx1* deletion in VE-cadherin expressing endothelial cells results in loss of IAHCs formation, again corroborating the link between cells of endothelial origin giving rise to hematopoietic clusters via the action of known hematopoietic mediators such as Runx1 (M. J. Chen et al., 2009a).

However, much less is known about the mechanisms underlying hemogenic specification and their subsequent generation of HSPCs.

As mentioned, definitive HSCs emerge during the third hematopoietic wave in the AGM. This process is known as endothelial to hematopoietic transition (EHT). EHT occurs in a short developmental window that begins around E9.5 in mice embryo (Yokomizo & Dzierzak, 2010a). A subset of endothelial cells (ECs) in the wall of the dorsal aorta acquires a hematopoietic transcriptional program that allows them to transform into hemogenic endothelial cells (HECs) that can ultimately give rise to hematopoietic stem and progenitor cells (HSPCs). These transcriptional changes are brought on by numerous signals coming from both inside the cells as well as the surrounding environment, which further encourage the formation of the IAHCs by the HECs, which are aggregates of cell attached to the aortic wall co-expressing endothelial and hematopoietic markers (Figure 6).



**Figure 6: Steps of endothelial to hematopoietic transition**

Morphological events that take place in the dorsal aorta of the mouse embryo during HSPCs formation. Upregulation of a hematopoietic transcriptional program in endothelial cells during the specification of HECs is marked by light red colour. In red, transcription factor trough EHT; in green, signaling pathways in each step of EHT; in purple, cell surface markers (From Ottersbach, 2019).

The maturation of definitive HSCs from VE-cadherin-expressing endothelial cells has been further divided into four major steps that are defined by differences in the temporal emergence and by the sequential upregulation of hematopoietic markers beginning with VE-

cad<sup>+</sup>CD41<sup>+</sup>CD43<sup>-</sup>CD45<sup>-</sup> pro-HSCs, which go on to become VE-cad<sup>+</sup>CD41<sup>+</sup>CD43<sup>+</sup>CD45<sup>-</sup> type I pre-HSCs followed by VE-cad<sup>+</sup>CD41<sup>+</sup>CD43<sup>+</sup>CD45<sup>+</sup> type II pre-HSCs and finally definitive HSCs. Type II pre-HSCs are phenotypically the same as the matured definitive HSCs but can be discerned using transplantation experiments as only definitive HSCs are able to fully repopulate the blood system long term when transplanted directly into irradiated mice (Rybtsov et al., 2011, 2014).

EHT is highly controlled by a network of transcription factors that are reliably up or downregulated at the correct time. The most critical transcription factors of the EHT are Runx1 and Gata2 (M. J. Chen et al., 2009a; de Pater et al., 2013): Runx1 knockout in mice, same as knockout of Runx1 cofactor CBF $\beta$ , results in embryonic lethality during midgestation, while Runx1 haploinsufficiency displays a dose-dependent effect on HSC activity in the AGM (Cai et al., 2000).

Runx1 expression in the aortic endothelium is thought to be the main driving force behind the transition from hemogenic endothelium to hematopoietic progenitor cell (M. J. Chen et al., 2009b; Kissa & Herbomel, 2010), but it is no longer required after the EHT for definitive HSC maintenance (Tober et al., 2013). Genome wide analysis of transcription factor interaction revealed that Runx1 interacts with other known hematopoietic factors such as SC1/Tal1 and Fli1 in HECs in order to initiate hematopoietic fate-associated transcription (Lichtinger et al., 2012). Other direct targets of Runx1 include Gfi1 and Gfi1b, which are responsible for the downregulation of the endothelial program and initiation of the hematopoietic program during the onset of hematopoiesis (Lancrin et al., 2012).

### **3.5. BM endothelial cells can generate a transient wave of HSPCs**

Recently, Laurent Yvernogeu and colleagues showed that the late fetal/young adult BM ECs were capable to generate, *de novo*, cohort of HSPCs through EHT similar to the one observed

in the aorta (Yvernogeu et al., 2019). By transplanting presomitic mesoderm (PSM) from GFP<sup>+</sup> transgenic embryo into wild-type chicken at the level of the limb, they could follow the formation of the vascularization of limb. They showed that limb vascularization was entirely derived from PSM and identified by flow cytometry and time-lapse confocal imaging that ECs were generating HSPCs using the same EHT as the one observed in the aorta. The newly-emerged HSPCs are capable of colonizing secondary hematopoietic organs and are long-lived since they could identify these cells in adult transplanted chicken reflecting their self-renewal capacity. The hemogenic potential of the BM ECs is also found in mammals. Using inducible VE-Cadherin-Cre transgenic mice, they could confirm the observation made in birds. This breakthrough discovery reveals that the microenvironment of the BM can also trigger ECs to become hemogenic and to ultimately generate HSPCs. The identification of the molecular signals originating from the BM allowing EHT to occur is of major interest and may have therapeutic potential.

#### **4. The AGM HSC microenvironment**

The AGM is an embryonic tissue derived from the mesodermal germ layer that contains the dorsal aorta, the genital ridge (that will give rise to the gonads), and the mesonephros (that will give rise to the kidneys), and that extends from the anterior limbs to the posterior limbs of the E9.5 to E12.5 mouse embryo (de Bruijn, 2000b; Medvinsky et al., 2011).

The AGM niche is made of several cell types including endothelial, macrophages, mesenchymal and neural cells, which cooperate together to regulate the development of the first HSCs. Many groups are interested in understanding how the AGM hematopoietic niche is shaped. This knowledge will be of great importance for mimicking the embryonic HSC niche with final goal to increase the efficiency of current protocols aiming at producing transplantable HSCs *ex vivo*. Niche signals are key in inducing HSC formation and maintaining their long-

term function, so efforts to derive HSCs *ex vivo* or modulate function *in vivo* depend upon recapitulating elements of the niche.

Here I will focus on different components of the AGM niche with a particular emphasis on the subaortic mesenchyme, the sympathetic nervous system and pro-inflammatory signaling.

#### **4.1. The subaortic mesenchyme and surrounding aortic tissues**

As for the FL HSPC microenvironment, the establishment of a large panel of AGM-derived stromal cell lines has been instrumental for studying the AGM hematopoietic niche (Oostendorp, Harvey, et al., 2002; Oostendorp, Medvinsky, et al., 2002). These stromal lines were shown to exhibit a myofibroblastic morphology and to follow the vascular smooth muscle differentiation pathway (Hackney et al., 2002). Additionally, they could give rise to other mesenchymal cell types including osteoblasts and adipocytes when cultured into appropriate conditions (Durand et al., 2006). The existence of mesenchymal progenitors in the AGM was also demonstrated *in vivo* both in murine and human embryos (Mendes et al., 2005; Wang et al., 2008). These findings thus highlighted the close association between HSPCs and mesenchymal progenitors in developmental hematopoietic niches. Molecularly, the comparison of the gene expression profiles of supportive and less-supportive stromal lines revealed novel HSPC niche factors (Charbord et al., 2014; Durand et al., 2007; Oostendorp et al., 2005). *Bmp4* was shown to be expressed in the subaortic tissue in several species and to regulate HSPC activity (Crisan & Dzierzak, 2016; Durand et al., 2007; McGarvey et al., 2017a). Studies in mice and zebrafish demonstrated that the Bmp signaling is necessary and integrates with the *Scl/Tal1* transcriptional network to regulate *Runx1* expression in the dorsal aorta (Pimanda et al., 2007; Wilkinson et al., 2009). AGM explant culture in the presence of gremlin (a Bmp antagonist) diminished HSC repopulating potential (Durand et al., 2007) whereas gain-of-

function for gremlin reduced *runx1* expression in the dorsal aorta of developing zebrafish embryos (Charbord et al., 2014). Interestingly, Souihol et al showed that the Bmp signaling was down-regulated in AGM HSCs and that BMP antagonists such as Noggin may be involved in that process (Souilhol et al., 2016), revealing a complex role for Bmp during AGM HSPC development. Importantly, AGM HSPC activity is also controlled by the surrounding embryonic tissues. At E10, dorsal (neural tube) and ventral (gut) tissues associated with AGM explant induced a decrease and an increase in HSPC activity, respectively (Peeters et al., 2009). The urogenital ridges were also shown to increase HSPC potential in reaggregation experiments (Souilhol et al., 2016). Hedgehog protein and Scf produced by the gut, and the urogenital ridges and subaortic mesenchyme respectively have been proposed as critical inductive signals (Peeters et al., 2009; Souilhol et al., 2016).

Recently, *Pdgfra* was identified as a marker of perivascular cells around the dorsal aorta (Sá da Bandeira et al., 2022). *In vivo* transplantation assays and co-culture experiments revealed that in absence of *Pdgfra* AGM HSPC support was impaired, suggesting that perivascular cells also contribute to the AGM hematopoietic microenvironment (Sá da Bandeira et al., 2022). Using RNA tomography on zebrafish, chick, mouse and human embryos, Yvernogeu et al explored the transcriptional landscape of the AGM HSPC niche. These authors identified biological processes conserved between species and ADM and its receptor RAMP2 (expressed in the subaortic mesenchyme and in endothelial cells and IAHCs respectively) as a novel HSPC regulators (Yvernogeu et al., 2020). In line with this study, Crosse et al identified through spatial transcriptomics on human embryos endothelins as potential niche factors. Endothelins were indeed detected close to IAHCs and support hematopoiesis both in mouse and human (Crosse et al., 2020).

## **4.2. The sympathetic nervous system and neural crest cells**

In a paper published in 2012, Katrin Ottersbach and colleagues provided the first evidence that the sympathetic nervous system (SNS) was a critical constituent of the AGM HSPC niche.

This observation took its origin in a transcriptomics analysis showing that the transcription factor Gata3 which was upregulated in the AGM at the time of HSC emergence (Mascarenhas et al., 2009b). Gata3 is a member of the GATA family and it is expressed in different tissues including the SNS, the kidneys, skin, hair and the hematopoietic system (Zaidan & Ottersbach, 2018). Gata3-deficient mouse embryos die by E12 due to severe abnormalities in the nervous system, but also at the hematopoietic sites (Pandolfi et al., 1995). Fitch et al demonstrated that the deletion of Gata3 was responsible for a reduction of HSPCs and IAHC numbers in the AGM at midgestation (Fitch et al., 2012). Importantly, these authors showed that Gata3 mediated its effects on HSPCs in a non-cell autonomous manner through the release of catecholamines from the SNS (Fitch et al., 2012). This study thus extended to the AGM context previous reports illustrating the roles of the SNS on BM HSPCs (Katayama et al., 2006; Méndez-Ferrer et al., 2008). Interestingly, catecholamines may have a direct effect on AGM HSPCs since these cells express adrenergic receptors (Fitch et al., 2012).

Recently, the group of Katrin Ottersbach showed that the cell cycle inhibitor p57Kip2/Cdkn1c regulates HSPCs by restricting the size of the SNS and the amount catecholamines released by these cells (Kapeni et al., 2022). Using single-cell RNA-Sequencing these authors explored the differentiation pathways of neural crest cells into SNS cells in the AGM and revealed that they were able to take an alternative differentiation pathway, giving rise to a subset of mesenchymal cells expressing HSPC-supportive factors. These interesting findings thus suggested that neural crest cells may contribute to the AGM HSC microenvironment via two possible mechanisms: through the release of catecholamine by the SNS and the contribution of a subset of HSPC-supportive mesenchymal cells (Kapeni et al., 2022).



A recent paper from Chandrakanthan and colleagues has reported that mesoderm-derived *Pdgfra*<sup>+</sup> stromal cells, exhibiting mesenchymal lineage potentials, were located around the dorsal aorta and in the subaortic tissue at E11.5 (Chandrakanthan et al., 2022). Interestingly, at E9.4, these cells were shown to contribute to hemogenic endothelial cells and HSCs. At E13.5, at a time where no IAHCs are detected in the dorsal aorta, *Pdgfra*<sup>+</sup> stromal cells were replaced by neural crest-derived cells (Chandrakanthan et al., 2022).

Interestingly, studies on zebrafish showed that trunk neural crest cell migration to the dorsal aorta was regulated by *Pdgf* signalling and that it was required for HSC specification (Damm & Clements, 2017). Altogether, these findings support an important role for neural crest cells on developing HSPCs.

#### **4.3. Pro-inflammatory signals and innate immune cells**

These last few years, accumulating evidence supported a critical role for pro-inflammatory factors on AGM HSPCs. In zebrafish, *Tnf $\alpha$*  signaling through *Tnfr2* was shown to be required, in a cell-autonomous manner, for HSPC development (Espín-Palazón et al., 2014). Mechanistically, *Tnf $\alpha$*  secreted by primitive neutrophils acts upstream the Notch signaling and activates the pro-inflammatory transcription factor NF- $\kappa$ B in HSCs. In the same line, Didier Stainier and colleagues demonstrated that the *Ifn $\gamma$*  factor and its receptor *Crfb17* positively influenced HSPCs in the developing zebrafish embryo (Sawamiphak et al., 2014). The *Ifn $\gamma$*  signaling was also shown to be connected to Notch pathway and blood flow to regulate HSPCs (Sawamiphak et al., 2014). In mice, AGM HSPCs and endothelial cells expressed genes involved in inflammation and innate immune responses (Y. Li et al., 2014). Moreover, using *Ifn $\gamma$* <sup>-/-</sup> and *Ifn $\gamma$ 1*<sup>-/-</sup> mutant mice, these authors revealed that the *Ifn $\gamma$*  pathway was required for AGM hematopoiesis (Li et al., 2014). The IL1 receptor was found to be expressed in the ventral

mesenchyme and dorsal aorta and its deletion resulted in decreased HSC activity (Orelia et al., 2008).

Macrophages have been shown to be an important part of the AGM HSPC niche. CyTOF mass spectrometry analysis demonstrated that macrophages are the most abundant hematopoietic cell types in the AGM (Mariani et al, 2019). Using lineage tracing and confocal imaging, these authors could show that macrophages accumulated in the AGM and were associated with IAHCs (Mariani et al, 2019). At the functional level, chemical depletion of macrophages resulted in a strong decrease of AGM HSC activity, demonstrating a critical role for macrophages on emerging HSCs (Mariani et al, 2019). In zebrafish, using transgenic embryos to label HSPCs and macrophages, Travnickova and colleagues also confirmed the presence of primitive macrophages in the AGM at the time of definitive HSC emergence (Travnickova et al., 2015). When macrophages were chemically or genetically inhibited, definitive hematopoiesis was impaired. Interestingly, macrophages may regulate AGM hematopoiesis through the release of metalloproteases (MMPs) such as MMP-9. This enzyme by degrading the ECM around HSPCs may indeed regulate HSPC mobilization and migration towards hematopoietic organs (Travnickova et al., 2015).

#### **4.4. Blood flow**

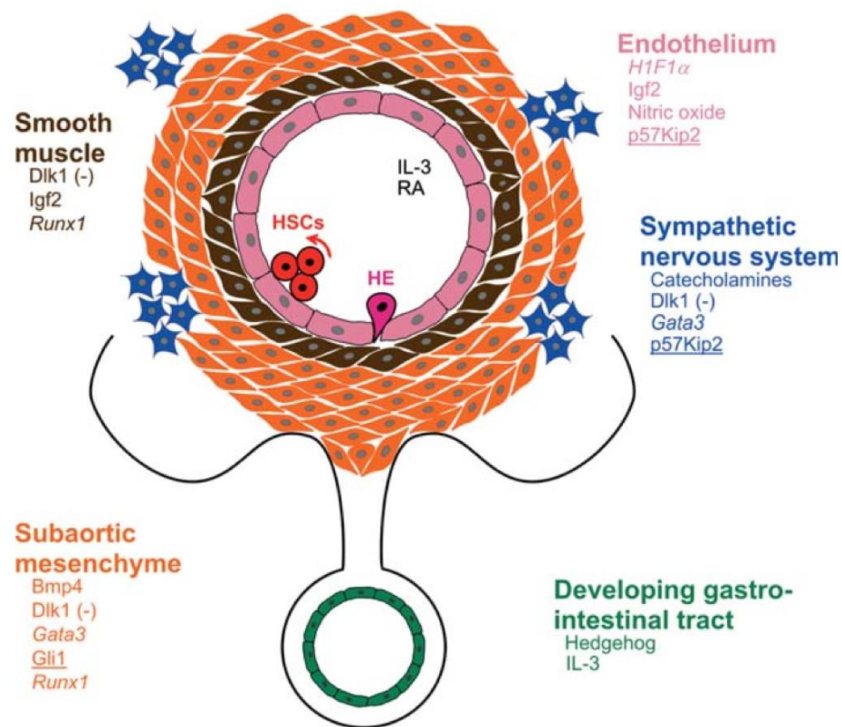
Using chemical blood flow modulators and zebrafish embryos lacking heart beating and blood flow, Trista North and colleagues documented the critical role for blood flow on HSPC development (North et al., 2009). This effect was shown to be mediated by the nitric oxide signaling acting cell autonomously (North et al., 2009). In the same line, shear stress was shown to promote ESC hematopoietic differentiation and to regulate runx1 expression and AGM hematopoiesis (Adamo et al., 2009). This last study confirmed the critical role of the nitric oxide pathway on embryonic hematopoiesis (Adamo et al., 2009).

#### **4.5. Hormones and hormone signalling**

It has been reported that endocrine signaling plays an important role as HSPC regulators. One of the hormones described to have a role is Vitamin D, who is known to be involved in many physiological functions. In 2015 Cortes and colleagues demonstrated in zebrafish embryo that increased levels of the inactive D3 in zebrafish embryos led to impaired HSPC production and niche specification of hemogenic endothelium through Hh axis inhibition (Cortes et al., 2015). Following year, same group, through gene expression and epistasis analysis showed that CXCL8 is functional target of D3-mediated regulation (Cortes et al., 2016).

Additionally, Kwan and colleagues through knockdown zebrafish studies revealed the function of the Central nervous system (CNS) in the control of embryonic HSPCs formation through activation of the hypothalamic-pituitary/internal (HPA/I) stress response axis (Kwan et al., 2016). This and other studies show that serotonin produced exclusively in the CNS/serotonergic neurons has a sustained role in actively modulating HSPCs number throughout embryogenesis (Kwan et al., 2016; Lv et al., 2017).

Finally, figure 7 recapitulates the complexity of the AGM microenvironment, with indication of some of the key signals active in different cell types.



**Figure 7: Schematic recapitulation of the AGM hematopoietic microenvironment**

The AGM niche comprises numerous different cell types including endothelial, mesenchymal and nervous system cells, which cooperate together as a network of signals to enable the emergence of the first HSCs (From Mirshekar-Syahkal et al 2014).

## 5. Zebrafish in hematopoiesis research

Over the past few decades, the zebrafish module has become a very used model to study hematopoietic niche and it has enabled discovery of novel cell-cell interaction as well as chemical and genetic regulator of HSCs.

Precisely describing and perturbing hematopoietic niche has been challenging in mammals because of the inaccessibility of bone marrow and live embryonic tissue. Zebrafish has proven to be an invaluable tool for studying hematopoiesis and the hematopoietic niche development. A paired mating can produce hundreds of transparent, externally fertilized embryos that are amenable to chemical and genetic screens, as well as high-resolution time-lapse imaging.

These animals develop rapidly, and blood formation and niche colonization can be directly observed within first few days of development. By 12 hours post fertilization (hpf), the “primitive” wave of hematopoiesis produces erythrocytes and primitive myeloid cells to oxygenate and support the early embryo (Thompson et al., 1998). Definitive hematopoiesis and stem cell production initiates at 26hpf. Because of the accessibility and visibility of the embryos, these processes can be analyzed by knockdown or knockout through injection of morpholino antisense oligonucleotides or CRISPR/Cas9 components. Moreover, processes, pathways and cell-cell interaction discovered in zebrafish are highly conserved in rapid translation of discoveries in fish to treatments in humans.

Here I will briefly present the different sites of hematopoiesis in the zebrafish embryo and their equivalents in the mammals.

### **5.1. Sites of definitive hematopoiesis across zebrafish embryo**

Beginning at around 26hpf, zebrafish initiate definitive hematopoiesis with the emergence of HSCs that will sustain blood production for life.

The ventral dorsal aorta is the functional equivalent to mammalian AGM and comprises what may be termed the initiating hematopoietic niche, both conferring stem cell identity and supporting early maturation. Although only a temporary site of blood production, HSPCs emerging from the ventral part of the dorsal aorta interact with a complex milieu of local signals and cell types that occupy the subaortic mesenchyme for several hours, and divide before entering circulation. Nearby vascular endothelial cells, somite-derived endothelial cells, as well as primitive macrophages and neutrophils give signals to induce HSPC formation and facilitate their exit into circulation.

After emerging from the ventral dorsal aorta, the nascent HSPCs travel to the primary site of embryonic hematopoiesis – caudal hematopoietic tissue (CHT). Analogous to the mammalian

fetal liver, the CHT is a vascular plexus in the ventral region of the tail between caudal artery and cardinal vein (Murayama et al., 2006). From approximately 48 to 96 hpf nascent HSPCs following cues from resident endothelial and stromal cells, exit circulation by transmigrating through the vascular endothelium and settle on the abluminal face of the vasculature. HSPCs interface with a series of tissue-resident endothelial cells, stromal cells, and myeloid cells for several hours, expand and exit in to the circulation.

Finally, beginning at around 4dpf, HSCs begin to seed the adult niche, the kidney. The zebrafish kidney is a highly vascularized flat structure extending along the dorsal body cavity wall of the animal, formed from ventral mesoderm specified in gastrulation (Kimmel et al., 1990). The kidney is the primary adult hematopoietic niche of zebrafish, corresponding to the mammalian bone marrow. Here, HSCs are regulated by cytoarchitecture highly similar to the CHT and are protected from irradiation by an overlying layer of melanocytes (Kapp et al., 2018; Murayama et al., 2006). In the kidney, HSCs reside, self-renew and differentiate to produce blood for the lifetime of the animal (Traver et al., 2003).

### **Aim of my PhD**

As described above, the AGM hematopoietic niche represents a very complex environment that helps and leads HSPC formation in the embryo. To investigate in more details the molecular landscape of the AGM HSPC niche, my team designed a laser capture strategy to isolate, in the mouse embryo, the dorsal and ventral aortic tissues at three developmental stages and analyzed their gene expression profiling at the bulk and the single cell population level.

As part of this project, my PhD thesis aims at providing new insights on revealing the molecular identity of the AGM hematopoietic microenvironment and reveal novel regulators of the first emerging HSCs in the embryo. Together with my supervisor and Dr. Estelle Hirsinger (CNRS

UMR7622), I have introduced the zebrafish model in the Jaffredo/Durand team, and established all needed tools and protocols for the model. My main contribution was to conduct loss of function studies utilizing morpholino and genetic approach. I have in particular investigated the potential of Decorin, an extracellular matrix protein on HSPC formation in zebrafish embryo. In addition, together with Dr. Laurent Yvernogeu and Pierre-Yves Canto, I conducted experiments based on AGM subdissections and single cell transcriptomics to investigate further the heterogeneity of the aortic mesenchyme and the cross talks between dorsal and ventral aortic tissues.

For the paper presented in the next section I performed the zebrafish study and contributed together with Pierre-Yves and Laurent the subdissected AGM experiments and single cell transcriptomics. Part of the zebrafish study (S5 figure) was done in collaboration with Dr. David Traver at UCSD. Pierre-Yves Canto performed laser capture microdissection, 2D and 3D imaging. Bioinformatic analysis was done by Pierre Charbord, Olivier Piau and Charles Durand. Taken together, my PhD within this project should provide new insight on the molecular identity of the AGM hematopoietic microenvironment and reveal novel regulations of the first emerging HSPCs in the embryo.

## **RESULTS**



## **A neuro-mesenchymal cell population characterizes the subaortic hematopoietic stem cell niche**

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Short running title: Molecular identity of the HSC subaortic niche

## **Abstract**

The first hematopoietic stem and progenitor cells (HSPCs) emerge in the embryonic dorsal aorta of midgestation mouse embryo but the precise nature of the supportive microenvironment remains largely unexplored. To address this question, we designed a laser microdissection-based approach to precisely isolate the dorsal and ventral tissues of the aorta at three distinct developmental stages harboring HSPCs. Bulk and single cell transcriptomics combined with computational analyses disclose a unique mesenchymal cell population in the E11.5 subaortic tissue. These cells express a molecular signature with interconnected genes involved in adhesive (e.g extracellular matrix ‘ECM’ and cell adhesion) and neuronal (e.g synapse and ion channel) functions. *In vivo* lineage tracing showed that these subaortic cells do not derive from neural crest cells. Histological and functional studies validated our strategy and revealed that Decorin, and Tenascin C, two components of the ECM, are essential for HSPC development. Together, our study identifies a yet unrecognized subaortic mesenchymal cell population and underscores the critical role of the ECM on embryonic HSPCs.

## **Key points**

A subaortic mesenchymal cell population, not derived from neural crest cells, exhibits adhesive and neuronal molecular features.

Decorin and Tenascin C regulate HSPCs in the dorsal aorta.

## Introduction

The first adult-type hematopoietic stem cells (HSCs) are generated in the Aorta-Gonad-Mesonephros (AGM) region of midgestation mouse embryo<sup>1,2</sup>. Intra-aortic hematopoietic clusters (IAHCs), containing hematopoietic stem and progenitor cells (HSPCs) closely attached to the ventral side of the dorsal aorta<sup>3-5</sup>, originate from hemogenic endothelial cells through a process of endothelial-to-hematopoietic transition (EHT)<sup>5-12</sup>. In the mouse, IAHCs are also found on the dorsal side of the aorta<sup>13</sup>, however HSC activity is preferentially detected in the ventral aortic domain<sup>13,14</sup>. Moreover, preventing the migration of the subaortic mesenchyme in the embryo was shown to inhibit IAHC production<sup>15</sup>. Collectively, these data indicate that the aortic hematopoietic activity is polarized along the dorsoventral axis and that the ventral domain constitutes a supportive HSPC microenvironment. This region contains diverse cell types including endothelial cells, macrophages and neural crest cells<sup>16-22</sup> intermingled with a mesenchyme. Since the subaortic mesenchyme is known to provide critical HSPC niche factors<sup>14,23-25</sup> extracting its precise molecular identity thus remains a technical and biological challenge. Recent studies also highlighted the heterogeneity of the AGM subaortic niche in terms of phenotype, gene expression and lineage history<sup>26-29</sup>. Molecularly, extrinsic factors including cytokines<sup>14,30,31</sup>, signaling pathways<sup>14,23,24,32-36</sup> and shear stress mediators, proinflammatory and sympathetic nervous systems<sup>16-18,20,21,37,38</sup> participate in HSPC regulation. However, how these pathways cooperate to shape the AGM hematopoietic microenvironment is still unknown.

We previously reported systems biology approaches for extracting the molecular core of HSPC niches<sup>39, 40</sup>. We present here an original approach combining precise laser capture, bulk and single cell transcriptomics and functional validation to capture the molecular identity of the AGM subaortic mesenchyme. The E11.5 subaortic mesenchyme exhibited a unique molecular

signature with strongly interconnected neuronal and adhesion-related genes. Lineage tracing experiments indicated that this singular cell population was not derived from neural crest cells. Using the zebrafish model, we functionally validated Decorin and Tenascin C as critical AGM HSPC regulators. Altogether, this study provides an unexpected insight onto the molecular identity of the AGM HSPC niche.

## **Methods**

### **Mouse and human embryos**

C57BL/6 mice were bred at Janvier (Le Genest, France) and maintained in the animal facility of the Laboratory of Developmental Biology according to institutional guidelines. Human embryonic tissues were obtained following voluntary, spontaneous or therapeutic abortions. Informed consent for the use of the embryo in research was obtained from the patient, and tissue collection and use were performed according to the guidelines and with the approval of the French National Ethics Committee.

### **Laser microdissection**

We used the Arcturus Laser Capture Microdissection System to isolate from mouse embryos the ventral and dorsal aortic tissues surrounding the dorsal aorta. Briefly, unfixed embryos were embedded in OCT medium in presence of isopentane cooled at  $-65^{\circ}\text{C}$ . Twenty  $\mu\text{m}$  cryostat sections were then prepared and stained before laser micro-dissection. The dorsal tissue localized in between the notochord and the roof of the aorta and the ventral tissue underneath the aorta were isolated from the same embryos. Three to eight microdissected tissues, corresponding to a total surface in between 84431 and 412076  $\mu\text{m}^2$ , were collected on appropriate caps and then proceeded for RNA extraction using Acturus Picopure RNA isolation kit. RNAs were eluted in a final volume of 13 $\mu\text{l}$  and their concentrations and integrity quantified

on a bioanalyzer (Agilent). For each developmental stage, 3 to 4 RNA samples corresponding to DT and VT were prepared from independent embryos.

### **Microarray, single cell screening and data analysis**

cDNA from microdissected tissues was hybridized to GeneChip® MouseGene 2.0 ST (Affymetrix). Normalization and functional analyses of the datasets were performed using Partek Genomics Suite software. E11.5 AGMs were dissected and submitted to scRNAseq using 10X Genomics Chromium next GEM single cell 3' kit version 3.1.

### **Zebrafish studies**

Zebrafish were maintained according to the University of California, San Diego, International Animal Care and Use Committee guidelines and the aquatic animal guidelines of Sorbonne Université. Wild-type or transgenic zygotes were injected with a morpholino solution and incubated at 28.5°C until they reached the stage of interest. Embryos were collected, staged, fixed, and processed for *in situ* hybridization as previously described<sup>41</sup>.

### **Statistics**

Statistical analysis was performed using GraphPad Prism software. Unpaired t or Mann and Whitney tests were used to evaluate statistical significance. Data were considered statistically significant for  $p < 0.05$ .

Detailed protocols and computational analyses are in supplemental Methods.

## Results

### **The dorsal and ventral aortic tissues exhibit dynamic and distinct molecular signatures**

We used a laser microdissection approach to isolate the ventral (VT) and dorsal (DT) aortic tissues from E10.5, E11.5 and E12.5 mouse embryos (Supplemental Table 1 and Video 1). The strategy is schematized in Figure 1 and developed below.

ANOVA with a low stringency p-value ( $p < 0.06$ ) was used to extract the lists of differentially expressed genes (DEGs) between VT and DT. There were 2704, 1838 and 1873 DEGs at E10.5, E11.5 and E12.5, respectively. VT and DT were characterized by different gene ontology (GO) profiles (Figure 2A). VT profiles (top panels), either peaked at E11.5 (e.g., 'ECM', 'cell junction', 'synapse'), or declined from E10.5 to E11.5 (e.g., 'transcription', 'cell cycle'). DT profiles (bottom panels) either peaked at E12.5 (e.g., 'developmental protein', 'transcription', 'cell cycle'), or declined from E10.5 onwards ('synapse', 'cytokine activity', 'cell junction'). The contrast of VT vs. DT was confirmed using Gene Set Enrichment Analysis (GSEA, Figures 2B and C)<sup>42</sup>. The gene sets 'Complement', 'IL6 Jak3 signaling' and 'Interferon a response' were significantly enriched in VT, in agreement with recent studies demonstrating the role of proinflammation on AGM HSCs<sup>17,18,20,21</sup>. Moreover, the 'Epithelial-to-Mesenchymal Transition' gene set was also significantly enriched in VT, including numerous highly ranked genes coding for ECM proteins such as *Dcn* and *Tnc*.

### **Modules including neuronal- and adhesion-related genes characterize the subaortic tissue**

Our further study focused on E11.5 since the expression profiles were most contrasted at this time point, and it is known that HSC numbers increase in the AGM between E11 and E12<sup>43</sup>.

To enrich the database and disclose the mesenchyme-specific genes, we merged our E11.5 transcriptomes with two other expression sets. The first one corresponded to AGM stromal lines with differing capacities to support HSPCs (AM14-1C4, AM30-3F4, UG26-1B6 and UG26-3B5) <sup>39,44</sup>. Using Principal Component Analysis (PCA), VT and DT were not discriminated using the entire transcriptome (Supplemental Figure 1A, top panel), but was evidenced after selection of the 3635 DEGs (gene set 1) (Supplemental Figure 1A, bottom panel). The second one corresponded to flow cytometry AGM sorted CD144<sup>pos</sup>CD45<sup>neg</sup> endothelial cells (EC), CD34<sup>low</sup>Kit<sup>low</sup>CD45<sup>pos</sup> differentiated hematopoietic cells (HC), and CD34<sup>high</sup>Kit<sup>high</sup>CD45<sup>pos</sup> hematopoietic progenitors enriched in HSCs. Similar to the first expression set, the different cell populations were not segregated by PCA using the entire transcriptome (Supplemental Figure 1B, top panel). but segregated after selection of 3231 DEGs (gene set 2). Of note, DT discriminated from VT according to PC1, and EC from HC according to PC2 (Supplemental Figure 1B, bottom panel). To refine the mesenchyme-specific gene list, we intersected gene sets 1 and 2 providing a list of 2401 genes (gene set 3) listed in Supplemental Table 2. PCA still enabled discriminating DT from VT according to PC1 (34,2% of the variance) and EC from HC (19,1% of the variance) according to PC2 (Figure 3A).

To find out which genes were specific to each of the four discriminated cell populations (VT, DT, EC, HC) and to reveal their relationships, Weighed Gene Correlation Network Analysis (WGCNA) (Supplemental Table 2 and Supplemental Figure 1C) combined to PCA was applied to gene set 3 <sup>45</sup>. PC1 and PC2 were used to quantify the external traits (DT-to-VT and EC-to-HC contrasts), and the merged dynamic tree cut method was used to identify the modules (Figure 3B). Eigenvector correlations to PC1 indicated that two modules, designated blue and yellow, corresponded to VT and another two, brown and turquoise, to DT. Likewise, correlation to PC2 trait enabled identifying one major cyan module for EC and two modules, magenta and

salmon, for HC (Figure 3C). Gene analysis confirmed the endothelial and hematopoietic identities of the populations corresponding to the EC and HC modules, respectively (Supplemental Table 2). Taken together these results indicate that our *in-silico* strategy has allowed excluding the contribution of genes expressed by endothelial and hematopoietic cells from those expressed by DT and VT cells.

The brown DT module included 151 genes (Supplemental Table 2) with significant categories of genes implicated in neurogenesis and/or the cell cycle (Supplemental Figure 2A). This module included in its central core many transcription factors essential for neuronal development and differentiation such as *Sox2*, *Pou3f3*, and *Pou3f2* (Supplemental Figure 2B). The turquoise module included 297 genes (Supplemental Table 2) many of which implicated in cartilage condensation or synapse formation (Supplemental Figure 2A). This module included in its central core the transcription factors *Pax1*, *Pax9* and *Gli1* required for skull skeleton, vertebral column and cranio-facial development, the cartilage proteoglycan *Acan* and the ECM cartilage components *Col9a1* and *Hapln1* (Supplemental Figure 2C).

The blue VT module included 295 genes (Supplemental Table 2) implicated in either neuronal-related processes (e.g., 'synapse', 'ion channel', 'GABA receptor activity') or adhesion-related ones (e.g., 'cell adhesion', 'basement membrane', 'ECM') (Figure 3D). The module central core included genes of the ECM component, *Dcn*, *Tnc* and *Col8a1*, the neurotrophin *Ntrk3*, GABA *Gabra3* and *Grid1* glutamate receptors genes and several soluble mediators implicated in the control of survival, growth or function of neural cells such as *Ntf4*, *Fgf1* and *Cntn4* (Figures 3E). The yellow VT module with 288 genes (Supplemental Table 2) included adhesion-related genes such as the ECM components *Fras1* and *Lama2*, and neuronal-related genes such as *Ntrk2*.



The VT modules thus exhibited a singular molecular identity with interconnected neuronal and adhesive genes. We hypothesized that this dual molecular signature could either be representative of a mix population of neuronal/neural crest cells and mesenchymal cells or of a unique 'neuro-mesenchymal' cell located underneath the dorsal aorta.

### **Single cell analyses identify a subaortic neuro-mesenchymal cell population**

To discriminate between these two hypotheses, we then performed single cell transcriptomics of the E11.5 AGM. After Quality Control (Supplemental Figure 3A) we retained 15154 out of 15862 cells, with 2698 genes detected on average per cell. After normalization and dimension reduction, the Louvain algorithm allowed defining 21 clusters projected on Uniform Manifold Approximation and Projection (UMAP) embedding (Figure 4A). Out of the 21 clusters, eight clearly corresponded to well-defined cell types (Supplemental Table 3). For example, C9 including 526 cells corresponded to EC according to GO categories (e.g., 'cell-cell adherens junction', 'sprouting angiogenesis'), and first-ranked genes (*Kdr*, *Cdh5*, *Emcn*, *Flt1*). The remaining Louvain clusters were part of a large central aggregate. C0 and C4 included genes characteristic of cartilage and smooth muscle, respectively. C6 and C8 included genes implicated in cell adhesion and neuronal processes, and C10 included genes involved in ECM organization. The remaining clusters included genes implicated in metabolic pathways (C1, C5), cell cycle and metabolism (C2), and developing tissues (C3, C12, C15, C17).

To find out whether the VT modules corresponded to distinct cell populations we studied the distribution of the corresponding genes among the different clusters. Genes of the blue VT module were predominantly expressed in the C6 and C8 clusters (Figure 4B and Supplemental Figure 3B), while the expression of the VT yellow module genes was less restricted, with

maxima in C4, C8 and C10 (Supplemental Figure 3C). To refine this analysis, we subdivided the blue VT module into two distinct gene subsets exhibited neural or mesenchymal characteristics (Supplemental Table 4). Genes of the neural subset were predominantly expressed in clusters 5, 6, 8 and 13 whereas the expression of mesenchymal genes was detected in clusters 1, 2, 4, 6, 8 and 10 (Figure 4C). Interestingly, 63.9% of cells within the clusters 6 and 8 were positive for both neural and mesenchymal gene expression (Figure 4D).

Analysis gene per gene showed that, among the mesenchymal subset, *Dcn* was expressed in C1, C2, C6, C8 and C10 whereas *Col8a1* or *Pitx2* were detected in C8 and C4 or C8 and C10, respectively (Figure 4E). In contrast, among the neural subset, *Ddc* and *Syt1* expression was predominantly detected in C6, C8 and C13 (Figure 4F). Although *Gabra3* was expressed at lower levels, its expression was detected in some cells of C11 and C13 as well as C3, C6 and C8 (Figure 4F). Finally, *Tnc* and *Ntrk3*, two genes harboring both mesenchymal and neural features were expressed in C6, C8 and C10 (*Tnc*), and in C4, C6, C8 and C13 (*Ntrk3*) (Figures 4E and F). UMAP profiles for additional genes are shown on Supplemental Figure 3. Analysis of fibroblast-specific single cell atlases<sup>46</sup> in steady and perturbed states confirmed the widespread expression of *Coll1a1* and *Dcn* in stem cell-like and ‘differentiated’ populations (Supplemental Figure 3G).

Importantly, the neural crest cell marker *Sox10* was expressed in C11 whereas the late markers of the sympathoadrenal compartment, *Phox2a* and *Th*, and the neuronal marker *Tubb3* were expressed in C13 (Figure 4G).

Finally, to get more insight on the organization of genes characterizing the neuro-mesenchyme and identify direct gene-to-gene interactions or potential causal links, we applied the multivariate information-based inductive causation (miic) algorithm<sup>47,48</sup> on the blue gene

module. After filtering out low confidence links, 89 nodes defining direct high-confidence paths were retained (Figure 4H). The resulting pruned network (as compared to that provided by WGCNA) included 4 major hubs with degree  $\geq 17$ : the netrin receptor *Unc5c*, the neural cell adhesion molecule *Ncam1*, the transcription factor *Zmiz1* and the collagen chain *Colla1*. *Dcn* was linked to *Colla1* and to *Ncam1*, while *Tnc* was linked to *Ncam1* and *Ntrk3*. Moreover, the miic algorithm uncovered a number of 'v-structures' (e.g., *Nr1h5* --> *Tnc* <-- *Ncam1*) suggesting cause-and-effect interactions, and the presence of one latent regulator upstream of *Mmp16* and *Nr1h5*. Search for protein-protein interaction using STRING<sup>49</sup> indicated a physical link for the following gene-to-gene interactions: *Dcn-Colla1*, *Ncam1-Mapk3*, *Ncam1-Ntrk3* and *Ncam1-Amph*. It further indicated correlated expression across a large number of experiments of *Dcn* to *Colla1* and *Colla1* to *Col8a1*. Finally, NCAM1 was reported co-expressed with UNC5A, a human ortholog of *Unc5c*. These data suggested that miic may uncover additional bona fide gene-gene associations beyond those already reported in the literature.

Taken together our findings reveal the heterogeneity of the subaortic mesenchyme that includes a cell population expressing genes organized in a network in which cell adhesion genes were directly linked to neuronal ones.

**Subaortic neuro-mesenchymal cells do not derive from neural crest cells and strongly express Decorin (Dcn), Tenascin C (Tnc) and the NT-3 growth factor receptor (Ntrk3) at E11.5**

To examine whether this cell population may derive from neural crest cells, we performed lineage tracing and immunodetection experiments for three molecules belonging to the blue module: *Dcn*<sup>50</sup> and *Tnc*<sup>51</sup>, encoding for two extracellular matrix genes and *Ntrk3* encoding a

neurotrophin receptor<sup>52</sup>. We took advantage of a Human tissue Plasminogen Activator (HtPA)-Cre mice labelling neural crest cells and their derivatives *in vivo*<sup>53</sup>. After crossing those mice with GT Rosa Tomato mice, E11.5 embryos were collected and analyzed for the expression of Tomato, Dcn, Tnc and Ntrk3. Tomato<sup>+</sup> cells were detected as expected on the dorsal, lateral and ventral sides of the aorta (Figures 5 A-C). No to little overlap was found between Tomato and Dcn, Tnc or Ntrk3 expression (Figures 5 A-C). Dcn was indeed detected at E11.5 around the notochord and in subaortic tissue, but not in aortic endothelial, vascular smooth muscle cells, or macrophages as shown by the absence of overlapping staining between Dcn and CD31,  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) or Allograft inflammatory factor 1 (Iba1) (Figures 5A, 5E and Supplemental Figure 4A). Dcn was expressed in a dynamic manner along the antero-posterior axis with strong expression in cells and associated ECM in large areas of the middle part of the AGM known to be enriched for HSC activity in mouse and human embryos<sup>54,55</sup> (Supplemental Figure 4E-H). Tnc was also detected in a dynamic manner and found also highly expressed in cells and associated ECM in large areas of the subaortic tissue (Figures 5B, 5F and Supplemental Video 2). In keeping with a previous report<sup>56</sup>, similar distribution of Dcn and Tnc were observed in human embryos at 30 days of gestation (Figures 5G and H). Ntrk3 was present not only as expected in the dorsal root ganglion cells and neurofilaments, but also in extensive areas in cells and associated ECM around and underneath the dorsal aorta (Figures 5C and 5I-K and Supplemental Video 3). Immunostaining for Tyrosine hydroxylase (Th), an enzyme involved in catecholamine biosynthesis, revealed discrete positive cells in the dorsal and ventral domains of the aorta, corresponding to sympathetic nerve cells (Figure 5J). Immunostaining for Tuj1, a neuron-specific class III beta-tubulin expressed in neurons of the peripheral and central nervous system, revealed a dense array of Tuj1<sup>+</sup> cells and neurofilaments on the lateral sides, and a looser cell distribution underneath the aorta (Figures 5D, 5K, 5L and Supplemental Figure 4D). Importantly, the distributions of Dcn, Tnc and Ntrk3 staining were quite similar in large

areas of the subaortic tissue, and clearly distinct from that of Th<sup>+</sup> cells and Tuj1<sup>+</sup> cells and neurofilaments (Figures 5I-L and Supplemental Figures 4B-D). Finally, transmission electron microscopy revealed the presence transverse and longitudinal ECM fibers in the intercellular space between subaortic cells at E11.5 (Figure 5N).

Thus, in line with our single cell transcriptomics, these results suggest that subaortic mesenchymal cells expressing *Dcn*, *Tnc* and *Ntrk3* were not derived from neural crest cells and were clearly distinct from neuronal Tuj1<sup>+</sup> cells. They also highlight the presence of a dense network of ECM molecules underneath the dorsal aorta.

### **Decorin and Tenascin C are essential for HSPC formation**

These observations prompted us to further examine the roles of *Dcn* and *Tnc* on AGM HSPCs. We performed knockdown experiments in zebrafish embryos using antisense morpholinos (MO). The formation of HSPCs in the dorsal aorta was monitored by whole mount *in situ* hybridization (WISH) for the expression of the HSPC markers *runx1* and *cmyb*. *dcn* morphants injected with start MO exhibited strong hematopoietic defects in the dorsal aorta, as shown by significant reduced expression of *runx1* and *cmyb* in morphants as compared to respective controls at 30 and 36 hours post-fertilization (hpf), respectively (Figures 6A-F). Similar phenotypes were observed with the injection of a splice MO (Supplemental Figures 5A and B). Since adult *dcn*<sup>-/-</sup> zebrafishes (*dcn*<sup>sa20247</sup> line), harboring a point mutation that induces a stop codon in the *dcn* coding sequence, were viable and fertile, we crossed *dcn*<sup>-/-</sup> females with *dcn*<sup>-/-</sup> males. Resulting embryos exhibited a significant decrease in the numbers of *runx1* and *cmyb* positive cells as compared to *dcn*<sup>+/+</sup> embryos from wild-type sibling crosses (Figures 6G-L), thus confirming the phenotypes of the *dcn* morphants.

*tnc* morphants also exhibited a decrease in the numbers of *runx1* and *cmyb*- expressing cells in the dorsal aorta as compared to respective controls (Supplemental Figures 5A and B). The reduced expression of the two HSC genes, *runx1* and *gata2b*, was also confirmed in *tnc* and *dcn* morphants as compared to uninjected embryos by qRT-PCR (Supplemental Figure 5A, right). Confocal microscopy using *kdlr:mCherry; gata2b:UAS; GAL4:eGFP* transgenic animals showed a strong reduction in the number of definitive HSPCs in the dorsal aorta in *dcn* and *tnc* morphants (Supplemental Figure 5C). These effects occurred in absence of any vascular defects as shown by confocal imaging using *flt1:dtomato; flt4:yfp* transgenic lines reporting vessel development (Supplemental Figure 5D). Collectively, these results suggest that *Dcn* and *Tnc* are critical HSPC regulators.

## **Discussion**

In this work we have uncovered the cell populations of dorsal (DT) and ventral (VT) aortic tissues at the critical times of HSPC formation in the mouse embryo. We focused on E11.5 as the time of HSC number increase in the AGM, and showing peak activity of a number of VT genes. By combining DT and VT transcriptomes with that of sorted hematopoietic and endothelial populations from age-matched AGM, we could exclude the specific contributions of the endothelial and hematopoietic tissues and focus on the other, yet less understood cell components, including the aortic mesenchyme. One major finding of our study was the identification of one VT 'blue' module in which neuronal genes were associated to genes implicated in cell adhesion. scRNAseq analysis revealed the extent of AGM tissue heterogeneity, and unraveled two ventral clusters corresponding to a yet unrecognized 'neuro-mesenchymal' cell population. We posited that the HSPC-supportive niche activity of the subaortic tissue was related to adhesion genes directly linked to neuronal ones. Therefore, the

tissue distribution and functionality of two ECM components, Tenascin C and Decorin, validated our hypothesis.

The functional association between the hematopoietic and neuronal systems has already been documented in the embryonic context. For example, it has been reported that Gata3 controls the numbers of AGM HSCs through the production of catecholamines, showing a strong influence of the sympathetic nervous system on AGM HSCs<sup>16</sup>. A transcriptomic analysis in the human embryo at the time of HSC development has highlighted a neuronal molecular signature associated with the ventrolateral domain of the dorsal aorta<sup>57</sup>. In zebrafish, trunk neural crest cells were shown to interact with precursors of aortic HSPCs and to contribute to their supportive microenvironment<sup>58</sup>. In mice, subaortic mesenchymal cells may have a dual origin (mesodermal and neural crest-derived)<sup>26,27</sup> with Pdgfra<sup>+</sup> mesodermal stromal cells being replaced by neural crest-derived cells at E13.5 coinciding with the loss of EHT<sup>26</sup>. Remarkably, in our present work we identified a mesenchymal cell population, not derived from neural crest cells, in which genes implicated in synapse formation, membrane channel activity, and neurotrophin signaling were linked to genes implicated in cell-to-cell or cell-to-ECM adhesion.

To evaluate the roles of Decorin and Tenascin C, we performed loss-of-function experiments in zebrafish embryos, which revealed that both proteins are necessary for HSPC formation and suggested an important role for ECM on AGM hematopoiesis. Interestingly, in BM HSPC-supportive stromal cells *Dcn* is not up- but down-regulated<sup>40</sup>, which is in agreement with its reported role as HSPC negative regulator in adult mice<sup>59</sup>. These data underscore the context-dependency of some of the mediators, activators in the embryonic niche but inhibitors in the adult one. Decorin and Tenascin C could exert their effects on AGM HSPCs as structural and/or signaling molecules by interacting with other ECM proteins (e.g., fibronectin, collagen,

periostin) and/or binding to cell surface receptors and soluble factors. For example, it has been reported that Tenascin C interacts with Wnt3a, Transforming growth factor b and Vascular endothelial growth factor<sup>51</sup>. As a consequence, Decorin and Tenascin C may play a critical role on cell-to-cell communication by regulating the half-life, local concentration and availability of critical growth factors, cytokines and morphogens that may modify the properties of the overlaying endothelial cell layer and /or act beyond this layer on HSPCs.

In conclusion, at E11.5 we have identified a unique mesenchymal cell population harboring both an adhesive and neuronal molecular identity within the subaortic tissue. Our data also highlight the role of specific ECM components as part of the HSPC-supportive niche in the embryo.

### **Accession numbers**

The NCBI GEO accession number for the microarrays presented in this study is GSE159592.

### **Author contributions**

PC, TJ and CD designed research, analyzed the data and wrote the manuscript. PYC, OM and CP performed experiments and analyzed the data. DT designed research on zebrafish, analyzed the data and wrote the manuscript. NR, PF, AM, SJ and EH performed experiments and analyzed the data. OP and HI analyzed data. SD and MS provided materials. TJ provided financial support.



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## Figure legends

### **Figure 1. A laser microdissection and systems biology strategy.**

Flow chart of the study.

### **Figure 2. The dorsal and ventral tissues surrounding the dorsal aorta exhibit dynamic and distinct molecular signatures.**

(A) GO analysis of DT and VT tissues at E10.5, E11.5 and E12.5.

(B) GSEA showing 4 gene sets significantly enriched in VT at E11.5. NES: normalized enrichment score, FDR: false discovery rate.

(C) Rank metric score of the 23 first genes from the 'Epithelial\_Mesenchymal\_Transition' gene set enriched in VT at E11.5.

### **Figure 3. Modules including neuronal- and adhesion-related genes characterize the subaortic mesenchyme.**

(A) PCA highlighting the different clusters using 2423 genes and 15 biological samples. The score plot of PC1 vs. PC2 is shown.

(B) Module detection using WGCNA. Modules named by colors correspond to squares along the diagonal of the topological overlap matrix plot shown below the clustering tree. DT and VT modules are highlighted.

(C) Module eigenvectors corresponding to the DT to VT (PC1 trait) and to the EC to HC contrasts (PC2 trait).

(D) Significant GO categories corresponding to genes in VT blue module.

(E) Connectivity plots of genes belonging to the VT blue module. Upper panel: Cytoscape connectivity plot of the 119 most connected genes (out of a total of 295 genes). Lower panel: eigengene-based connectivity bar plot of the Top-30 genes.

**Figure 4. Single cell level analyses identify a neuro-mesenchymal cell population in the subaortic tissue.**

(A) UMAP embedding of the Louvain clusters corresponding to E11.5 AGM single cell analysis.

(B) Distribution of genes of the blue WGCNA module and blue neuronal and mesenchymal subsets among the different clusters.

(C) Scatter plot of the blue neuronal and mesenchymal gene subsets.

(D) Expression pattern of *Dcn*, *Col8a1*, *Pitx2* and *Tnc*.

(E) Expression pattern of *Ddc*, *Gabra3*, *Syt1* and *Ntrk3*.

(F) Expression pattern of *Sox10*, *Phox2a*, *Th* and *Tubb3*.

(G) Miic plot of the central core genes of the blue module.

**Figure 5. Subaortic neuro-mesenchymal cells do not derive from neural crest cells and strongly express Decorin (Dcn), Tenascin C (Tnc) and the NT-3 growth factor receptor (Ntrk3) at E11.5.**

(A-C) Immunodetection for Dcn (A), Tnc (B) and Ntrk3 (C) on E11.5 sections of HtPA-Cre; R26R-tomato mouse embryos.

(D) Immunodetection for Tuj1 on E11.5 mouse embryo sections.

(E) Immunodetection for Dcn and CD31 on E11.5 mouse embryo sections.

(F) Immunodetection for Tnc and CD31 on E11.5 mouse embryo sections.

(G-H) Immunodetection for Dcn (G), Tnc (H), and CD105 on 30 days human embryo sections.

(I) Immunodetection for Ntrk3 and CD31 on E11.5 mouse embryo sections.

(J) Immunodetection for Ntrk3 and Th on E11.5 mouse embryo sections.

(K) Immunodetection for Ntrk3, Iba1 and Tuj1 on E11.5 mouse embryo sections.

(L) Immunodetection for Dcn, Iba1 and Tuj1 on E11.5 mouse embryo sections.

(M) Transmission electron microscopy of E11.5 subaortic tissue. N is a magnification of the square indicated in Figure 5M.

Scale bar for Figures 5A, B, C, D, E, F, I, J, K and Land O is 100µm. Scale bar for Figures 5G and H is 50µm. Scale bars for Figures 5 M and N are 10µm and 200 nm, respectively.

Nc: notochord, Ao: dorsal aorta.

### **Figure 6. Decorin is essential for HSPC formation.**

(A-B) WISH for *runx1* in 30 hpf *dcn* morphants and their respective uninjected controls.

(C) Numbers of *runx1*<sup>+</sup> cells in the dorsal aorta of control and *dcn* morphant embryos.

(D-E) WISH for *cmyb* in 36 hpf *dcn* morphants and their respective uninjected controls.

(F) Numbers of *cmyb*<sup>+</sup> cells in the dorsal aorta of control and *dcn* morphant embryos.

(G-H) WISH for *runx1* in 30 hpf *dcn*<sup>+/+</sup> and *dcn*<sup>-/-</sup> embryos.

(I) Numbers of *runx1*<sup>+</sup> cells in the dorsal aorta of *dcn*<sup>+/+</sup> and *dcn*<sup>-/-</sup> embryos.

(J and K) WISH for *cmyb* in 36 hpf *dcn*<sup>+/+</sup> and *dcn*<sup>-/-</sup> embryos.

(L) Numbers of *cmyb*<sup>+</sup> cells in the dorsal aorta of *dcn*<sup>+/+</sup> and *dcn*<sup>-/-</sup> embryos.

\*\*\*\* indicates p value < 0.0001 using either two-tailed t-test or Mann and Whitney test. 24 to 57 embryos were analyzed for each condition from 1 to 3 independent experiments.

### **Conflict-of-interest disclosure**

The authors declare no competing interests.

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Figure 01

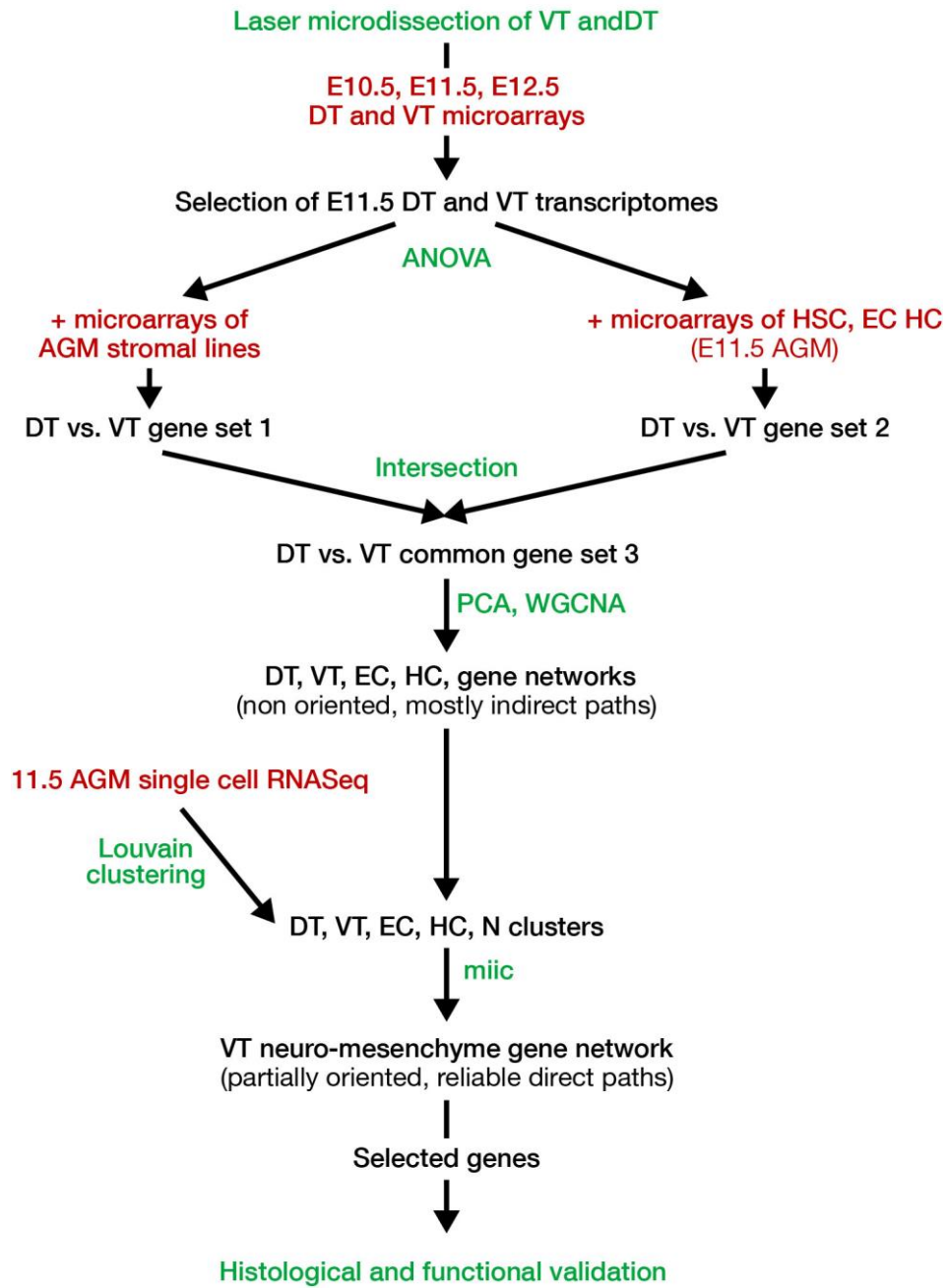




Figure 02

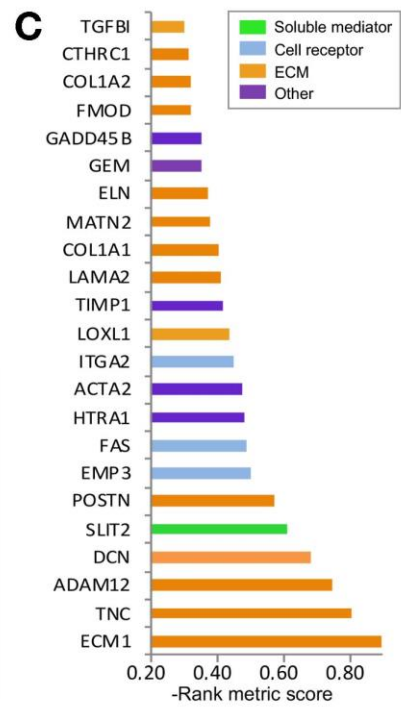
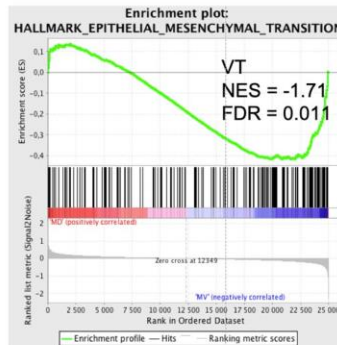
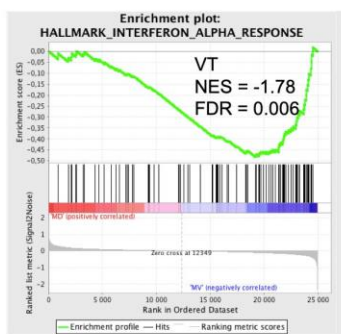
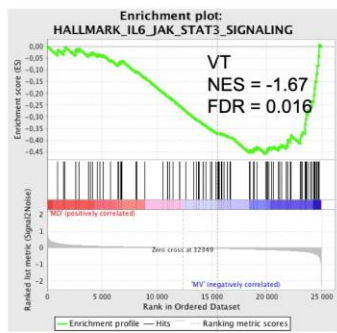
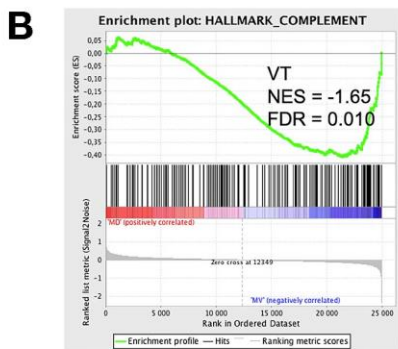
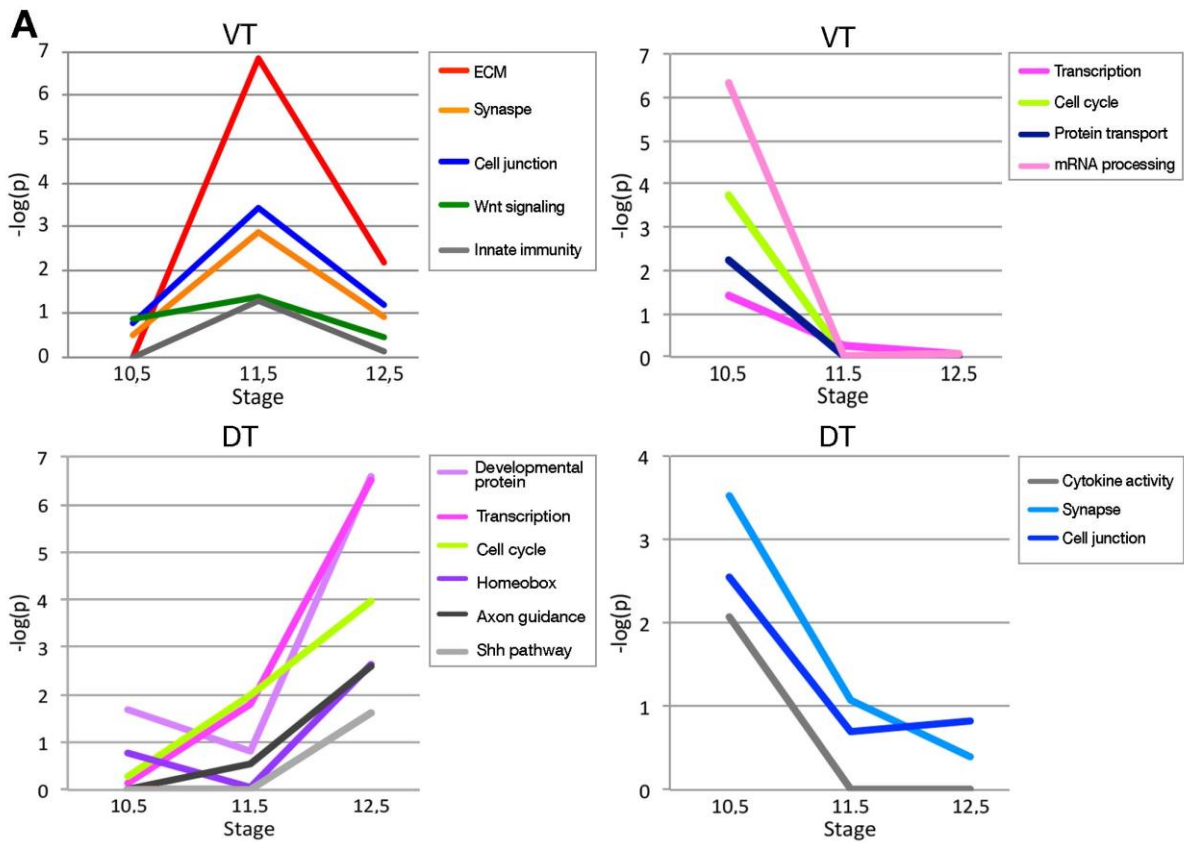


Figure 03

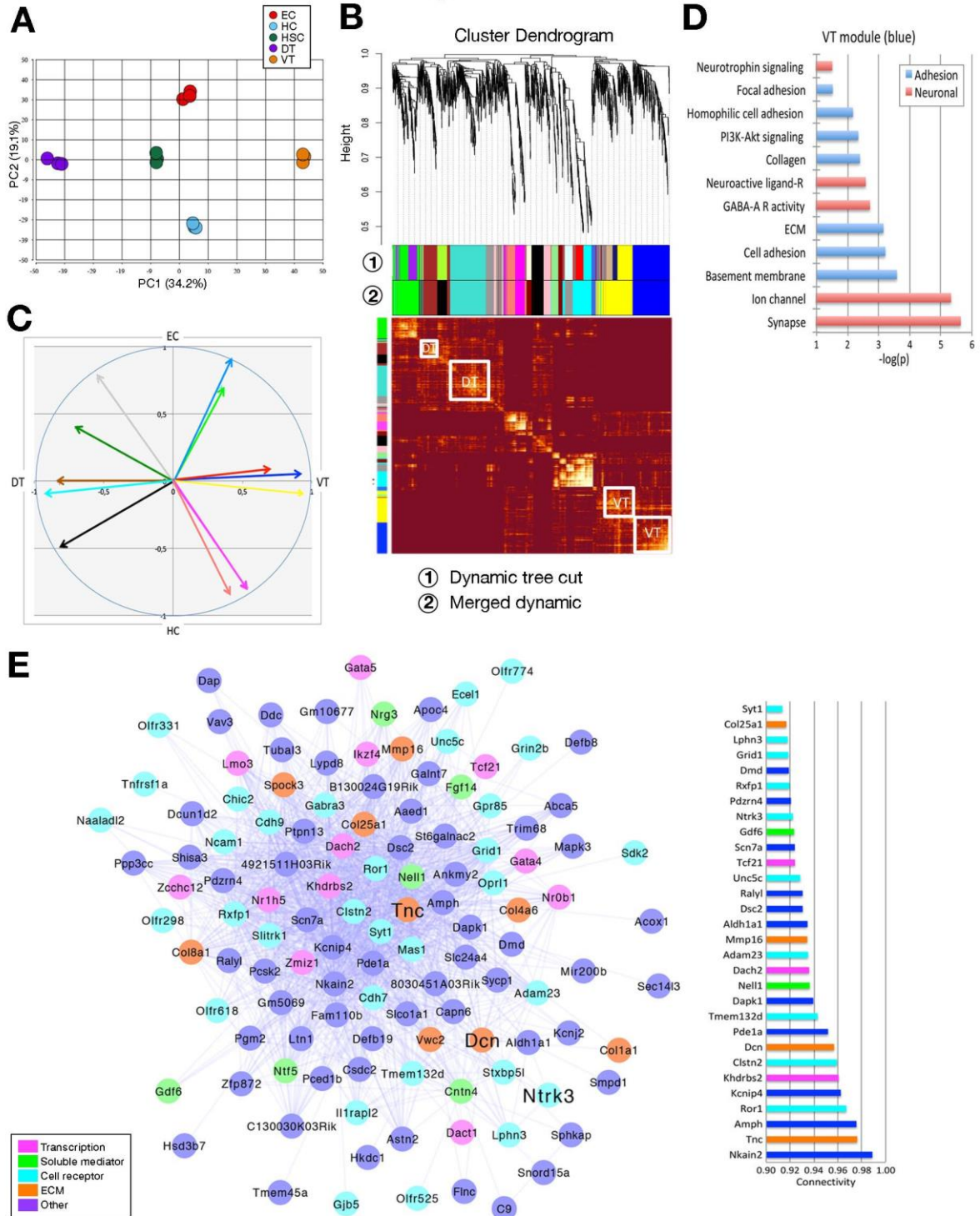


Figure 04

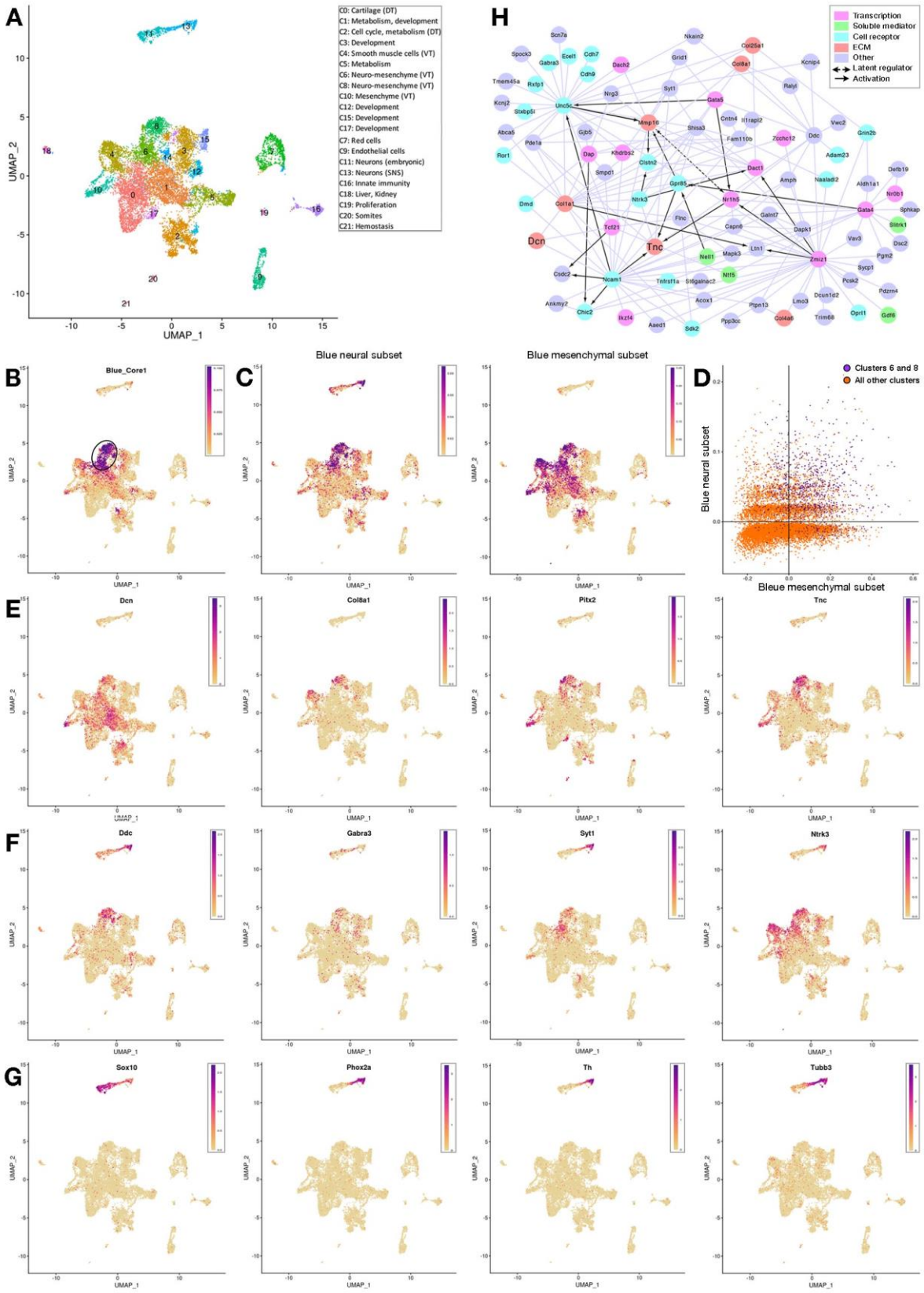




Figure 05

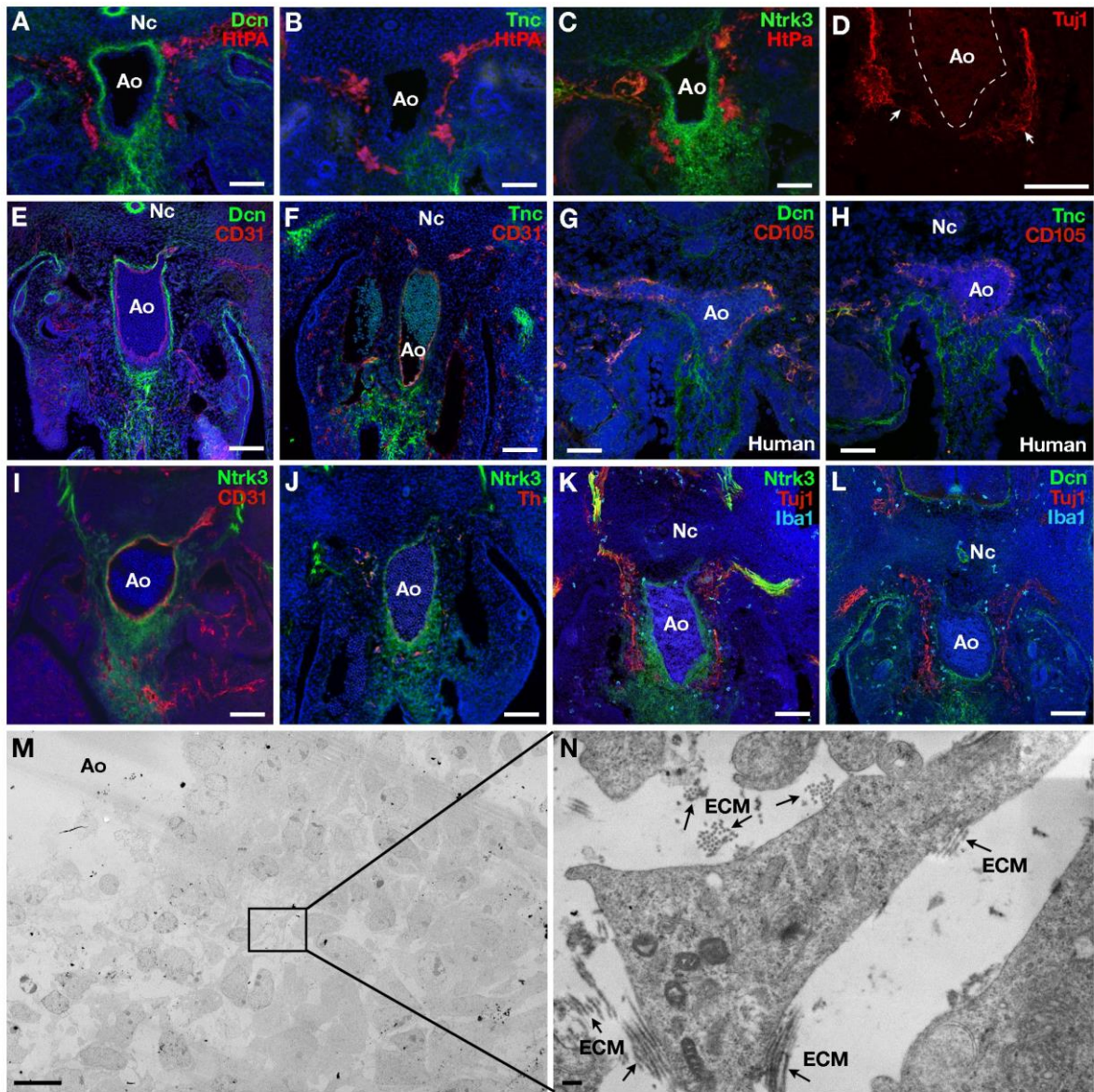


Figure 06

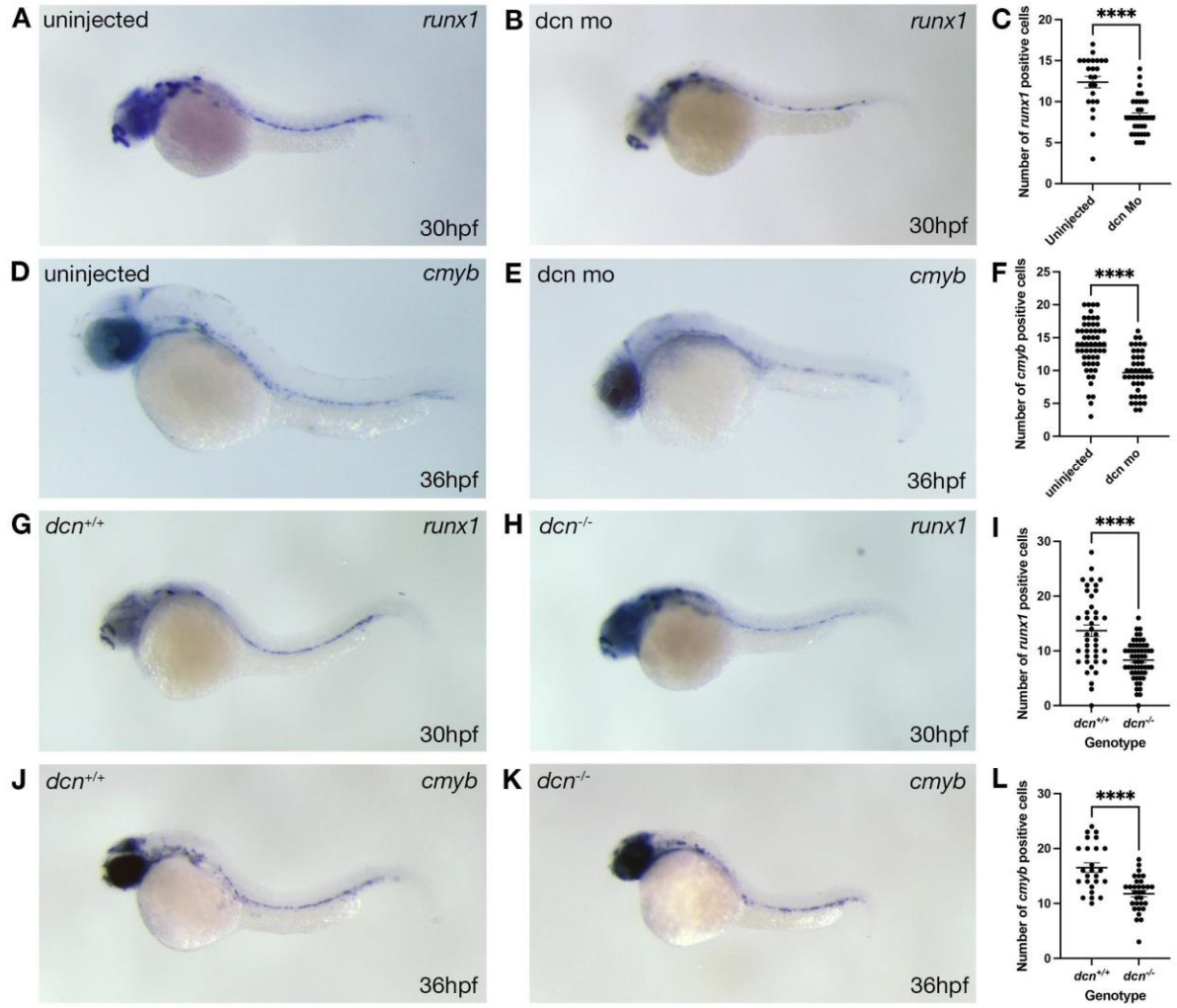


Figure S1

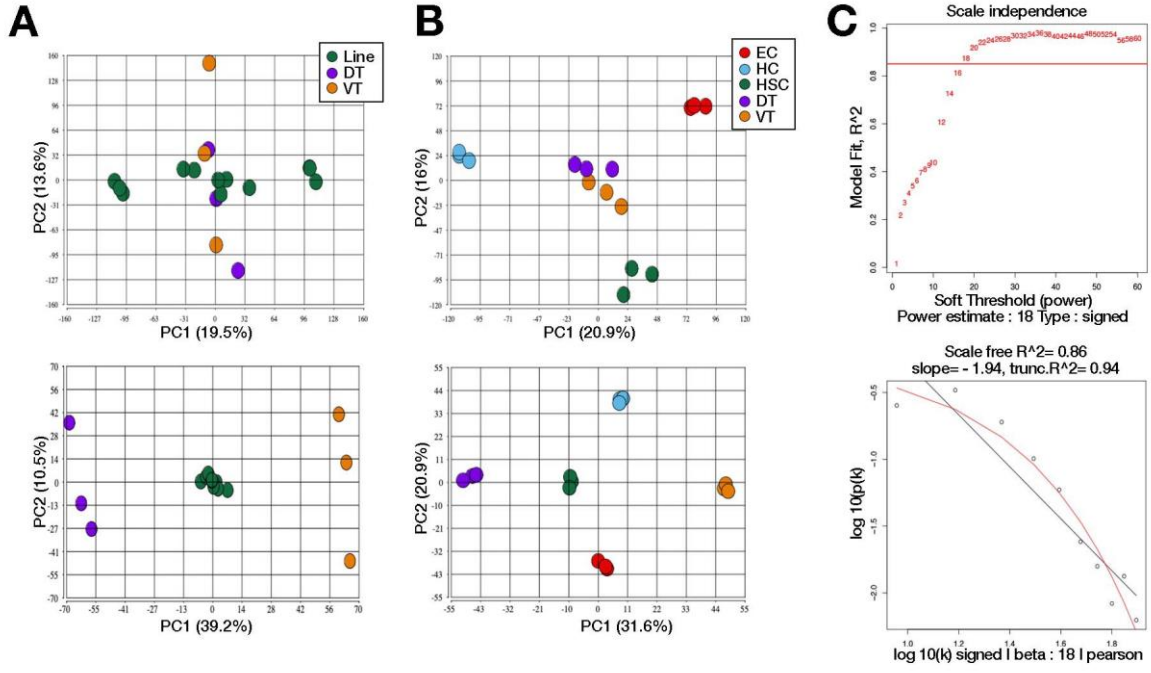




Figure S2

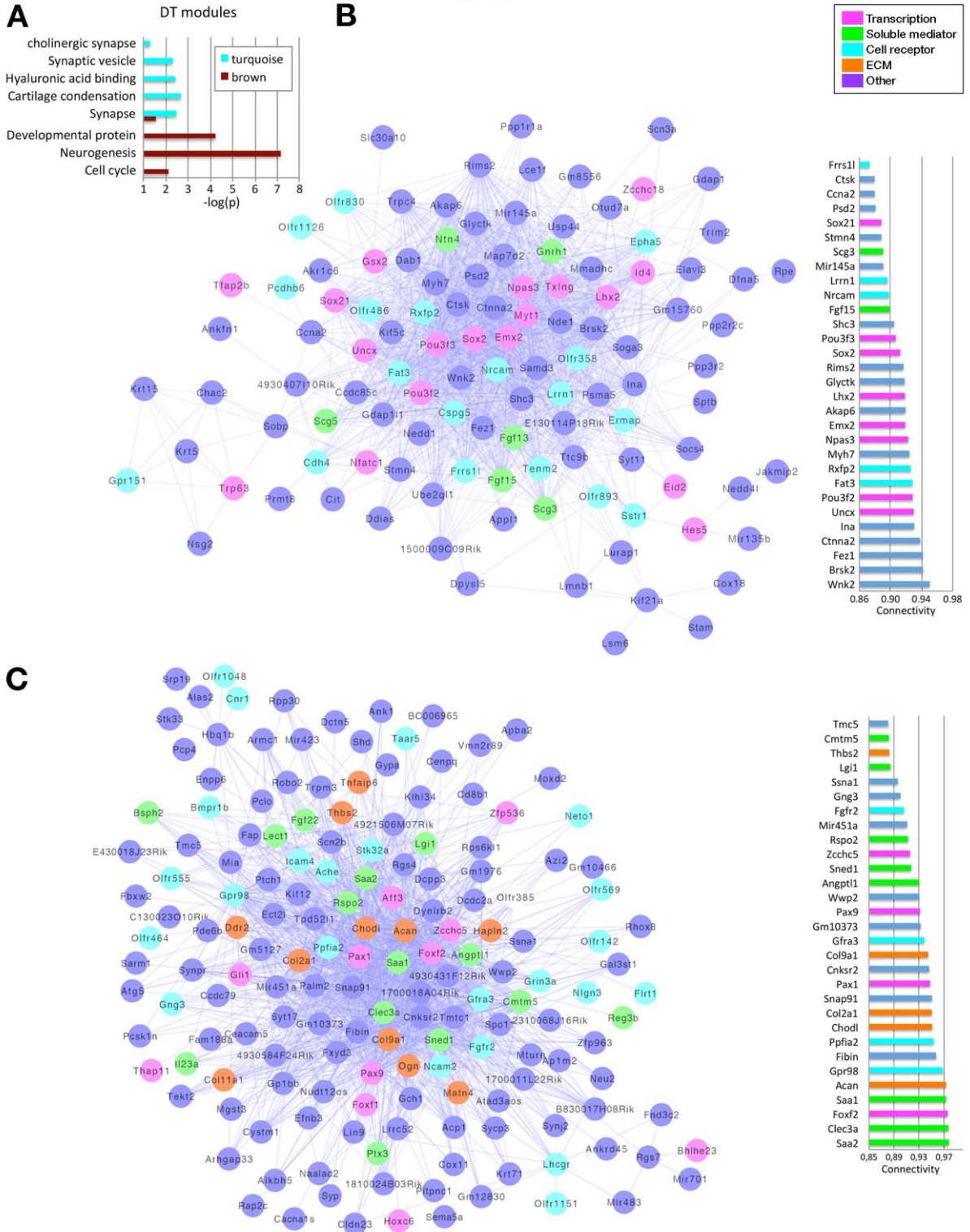


Figure S3

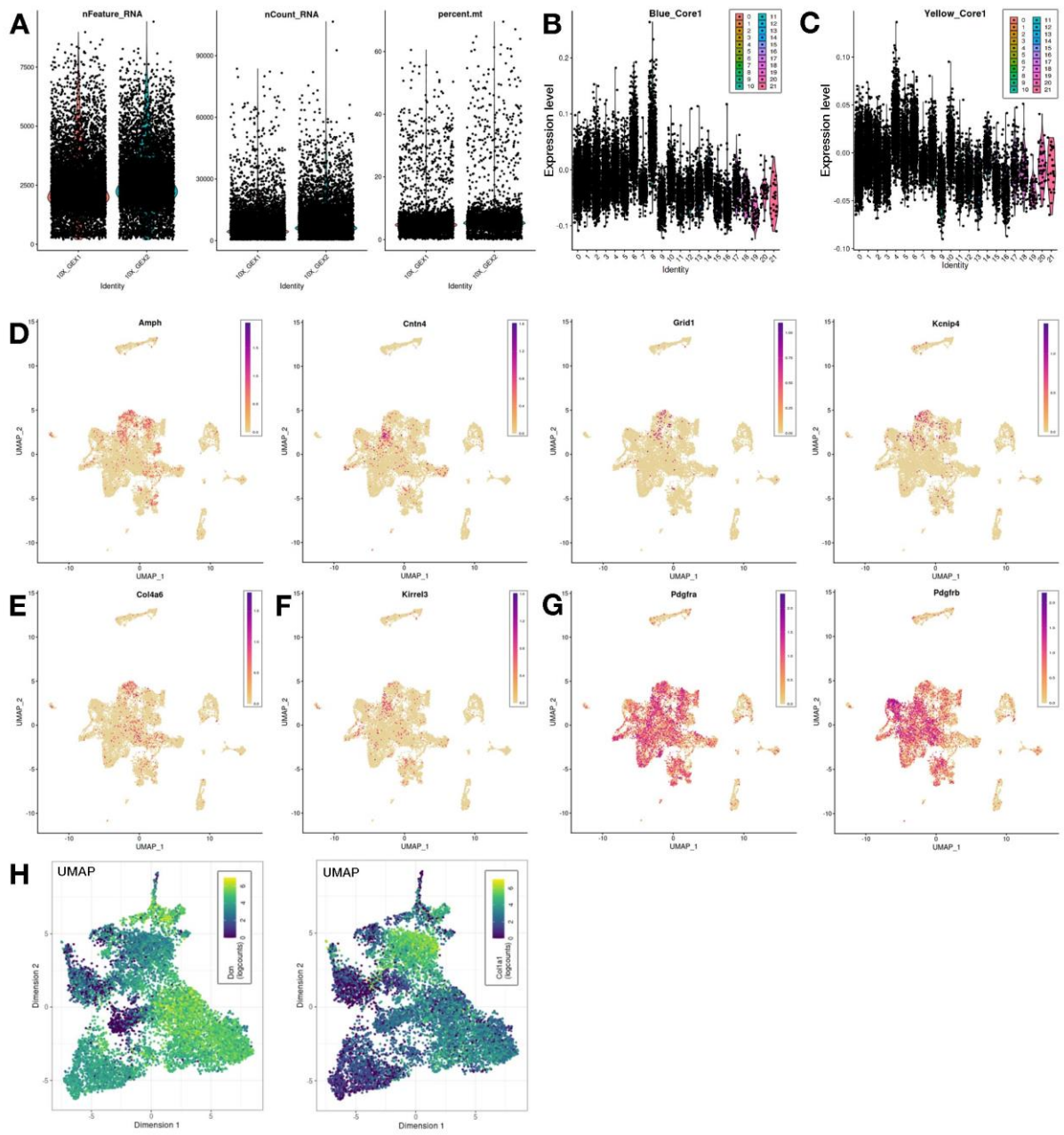




Figure S4

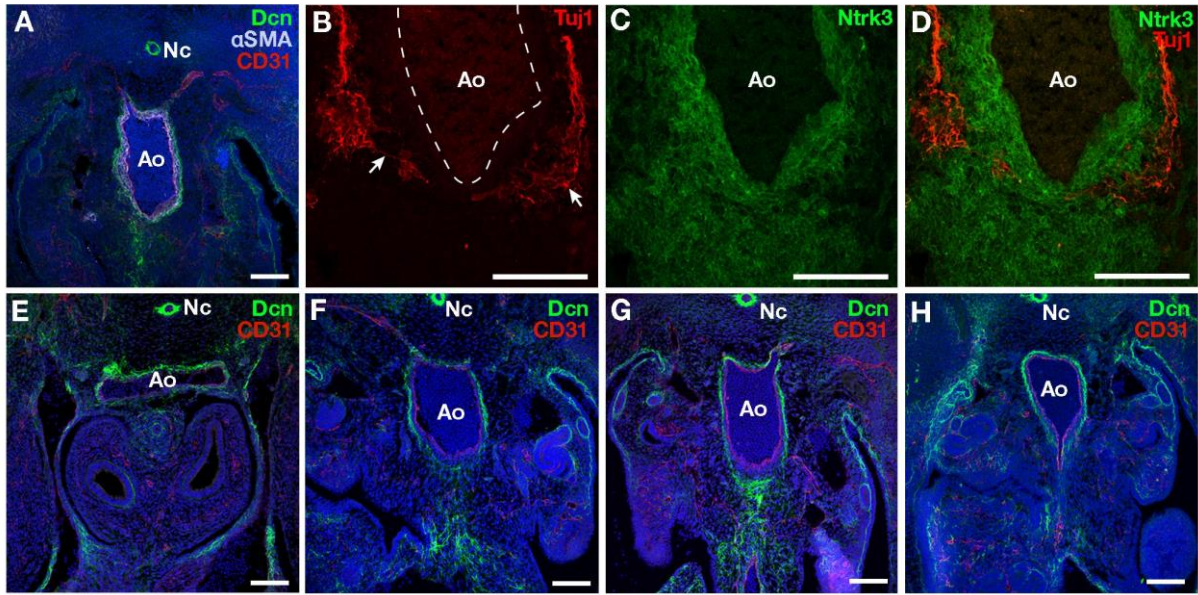
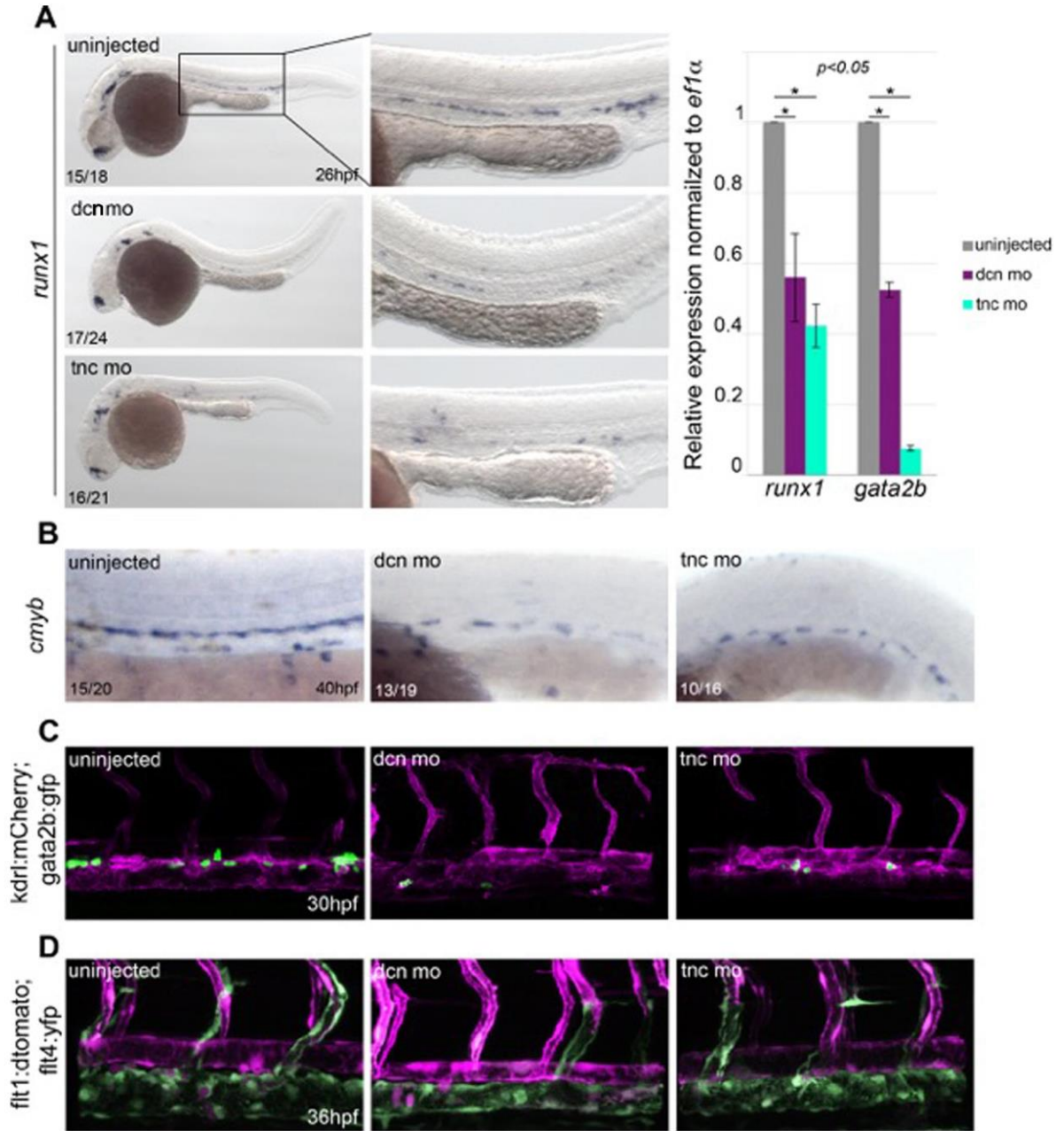


Figure S5



## **Supplemental Methods**

### **Mouse embryos**

Embryos were obtained from C57BL/6 mice. The day of vaginal plugging represents day 0.5 (E0.5). Pregnant females were killed by cervical dislocation at appropriate time of gestation. Uteri were taken and placed in 10% Fetal Bovine Serum/1% Penicillin - Streptomycin (Gibco)/phosphate-buffered saline (PBS) (Invitrogen). The embryonic stages were confirmed through morphological analysis.

### **Microarray screening and data analysis**

Between 200 and 1000 pg of total RNA was reverse transcribed following the Ovation Pico V2 system (Nugen) and the resulting double strand cDNA was used for amplification based on SPIA technology. After purification according to Nugen protocol, sens target DNA was fragmented and biotin labelled using Encore Biotin Module kit (Nugen). After control of fragmentation using Bioanalyzer 2100, cDNA was then hybridized to GeneChip® MouseGene 2.0 ST (Affymetrix).

*ANOVA*: DT vs. VT comparisons were made using 2-way ANOVA with a low stringency p-value ( $p < 0.06$ ) with ‘Polarity’ and ‘Embryo’ as grouping variables for E11.5 and E12.5 samples, or three-way ANOVA with ‘Polarity’, ‘Embryo’ and ‘Series’ (corresponding to two independent transcriptomic datasets) for E10.5 samples. After incorporation of additional transcriptomes (AGM stromal cell lines or AGM sorted cell populations) DT vs. VT comparisons were made using 2-way ANOVA with Cell identity and Series (the different batches) as grouping variables.

GO categories were analyzed using DAVID (<https://david.ncifcrf.gov>)<sup>1</sup>.

*GSEA*: GSEA was used to identify gene sets statistically enriched in the microdissected tissues with the curated, hallmark, computational and motif databases<sup>2</sup>.

*WGCNA*: To reconstruct gene network and identify specific modules for the different cell populations, we utilized WGCNA<sup>3</sup>. The  $\beta$  power of 18 was selected such that the connectivity distribution fitted a scale-free model (supplemental Figure 1C). Adjacencies were given according to signed Pearson's correlation. Modules were constructed with average linkage hierarchical clustering. Cytoscape (<https://cytoscape.org>) was used to visualize the connectivity plot of the most connected genes. Force-oriented layout was used for data representation.

### **Single Cell screening and data analysis**

E11.5 AGMs were dissected and submitted to scRNAseq using 10X Genomics (2 biological replicates). Only cells expressing between 200 and 7000 genes with a percentage of mitochondrial RNA under 15% were kept. Also, genes expressed in less than 3 cells also were not considered for the analysis. Since no batch effect was observed between the replicates, the datasets were merged without correction. ScTransform normalization<sup>4</sup> was performed with the Seurat package v3<sup>5</sup> with regression of the cell cycle. To regress the cell cycle genes, the default human cell cycle gene list given by Seurat was converted into mouse genes using the biomaRt package<sup>6</sup> and was used following the default recommendation for cell cycle regression with scTransform as described in the Seurat vignette. Clustering was performed with the Louvain algorithm with a resolution of 0,7 on the first 50 PCA components and the cells were projected on an Uniform Manifold Approximation and projection (UMAP) embedding based on the first 42 PCA components. Then, we identified differentially expressed genes by looking at the overexpressed genes with a fold-change superior to 0,25 in each cluster compared to the whole

dataset using the Wilcoxon's sum rank test. Only genes expressed in more than 25% of the cluster cells and with a q-value under 0,05 were kept. These genes were then ranked by p-value and used to determine each cluster identity. The expression of the WGCNA gene sets was performed using the Seurat AddModuleScore function with the gene list of each module. The cell identity of each cluster was assessed by considering the genes top-ranked by p-values, and by comparing the genes of a cluster to all others using Wilcoxon's sum rank test (p-value < 0,05). Multivariate information-based inductive causation (miic) algorithm<sup>7,8</sup> was used to analyze the genes of the blue module taking into account all cells belonging to the large central aggregate defined by the Louvain clustering (C0-6, C8, C10, C12, C14, C15, C17). The input matrix consisted of the raw counts for each gene of the blue module (12516 observations and 89 variables). To yield significant results the miic algorithm requires a large number of observations as compared to that of variables. We restricted therefore the analysis to the central core of the module including 98 genes and we considered all cells (N=12516) belonging to the large central aggregate defined by the Louvain clustering (C0-C6, C8, C10, C12, C14, C15, C17).

### **Immunodetection**

Embryos were fixed overnight in 4% paraformaldehyde (Sigma) at 4°C, transferred in a solution containing PBS/sucrose 15% for 48 hours, impregnated in PBS/15% sucrose/7.5% gelatin for 2 hours at 37°C, embedded at room temperature, frozen in isopentane at -65°C using liquid nitrogen and stored at -80°C until used for cryosectioning. Frozen sections (20 mm) of E11.5 embryos were cut on a cryostat (Leica). Antibodies used for staining the sections were: mouse anti-mouse/human Tnc (1/50, Thermofisher), goat anti-mouse Decorin (1/200, R&D Systems), mouse monoclonal anti-human Dcn (1/200, R&D Systems), goat anti-mouse Ntrk3 (1/200, R&D Systems), rabbit anti-mouse CD31(1/200, Abcam), mouse monoclonal anti-

mouse  $\alpha$ -smooth muscle actin (1/500, Sigma), rabbit polyclonal anti-mouse Iba1 (1/1000, Wako), mouse anti-mouse Tuj1 antibody (1/1000, BioLegend) and goat anti-human CD105 (1/100, R&D Systems). These primary antibodies were visualized with bovine anti-goat IgG-Alexa647 (1/400, Jackson), goat anti-mouse IgG2a-Alexa488 (Invitrogen, 1/200), donkey anti-mouse IgG-Alexa488 (1/400, Jackson) and donkey anti-rabbit Cy3 (1/400, Jackson). Fluorescent imaging was performed using fluorescence Leica microscope and confocal Leica TCS SP5 at the imaging facility, IBPS, Sorbonne Université.

### **Whole-mount immunostaining, tissue clearing and 3D imaging**

Embryos were incubated at RT for 3 hours in a solution (PBSGT) containing PBS 1X 0.2% gelatin, 0.5% Triton X100 (Sigma-Aldrich) and 0.01% thimerosal (Sigma-Aldrich). Embryos were then incubated with the first antibodies as described above in PBSGT at RT for 2-3 days. After 6 washes, samples were incubated with secondary antibodies in PBSGT overnight, washed and processed for 3DISCO tissue clearing as described by Belle et al <sup>9</sup>. Images were acquired using an ultramicroscope (LaVision BioTec). 3D imaging, stack images conversion and movies were generated using Vision 4D 3.0 software (Arivis).

## **Supplemental Figure legends**

### **Supplemental Figure 1: Modules including neuronal- and adhesion-related genes characterize the sub-aortic mesenchyme.**

(A) PCA on the set obtained by integrating AGM stromal cell lines. Top panel: PCA using the entire transcriptome of 20777 genes and 18 samples. Bottom panel: PCA using 3635 DEGs resulting from ANOVA comparing DT to VT gene sets.

(B) PCA on the set obtained by integrating sorted AGM cell populations (EC, HC, HSC). Top panel: PCA using the entire transcriptome of 20536 genes and 15 samples. Bottom panel: PCA using 2423 DEGs resulting from ANOVA comparing DT to VT gene sets.

(C) WGCNA selection parameters. Left panel: Plot for choosing the power  $\beta$  of the signed weighted correlation networks; the square of the correlation coefficient between the connectivity frequency and the binned connectivity is represented on the y-axis, while the different powers adjusting the adjacencies are given on the x-axis. Right panel: Connectivity distribution using  $\beta$  power of 18.

### **Supplemental Figure 2. Modules including neural crest and cartilage related genes characterize the dorsal aortic tissue at E11.5.**

(A) Significant GO categories corresponding to genes in DT modules.

(B) Connectivity plots of genes belonging to the DT brown module. Upper panel: Cytoscape connectivity plot of the 113 most connected genes (out of a total of 151 genes). Lower panel: eigengene-based connectivity bar plot of the Top-30 genes.

(C) Connectivity plots of genes belonging to the DT turquoise module. Left panel: Cytoscape connectivity plot of the 167 most connected genes (out of a total of 293 genes). Right panel: eigengene-based connectivity bar plot of the Top-30 genes.

**Supplemental Figure 3. Single cell level analyses identify neuro-mesenchymal cell populations in the subaortic tissue.**

(A) Number of genes, transcripts and percentage of mitochondrial RNA detected in each scRNAseq sample before filtering.

(B-C) Violin plots showing the distribution of the genes of the blue (B) and yellow (C) VT modules among the different cell clusters.

(D) Expression pattern of *Amph*, *Cntn4*, *Grid1* and *Kcnip4* neuronal genes.

(E) Expression pattern of the mesenchymal gene *Col4a6*.

(F) Expression pattern of *Kirrel3*.

(G) Expression pattern of *Pdgfra* and *Pdgfrb*.

(H) Expression pattern of *Dcn* and *Colla1* in the fibroblast-specific single cell atlas (perturbed state).

**Supplemental Figure 4. Immunodetection on E11.5 mouse sections.**

(A) Immunodetection for *Dcn*, CD31 and  $\alpha$ SMA.

(B-D) Immunodetection for *Tuj1* and *Ntrk3*. (D) represents the merge between *Tuj1* and *Ntrk3* staining. The dashed white line indicates the lumen of the dorsal aorta. Scale bar is 100  $\mu$ m.

(E-H) Immunodetection for *Dcn* and CD31 on several transverse sections along the antero-posterior axis of E11.5 mouse embryo.

Scale bar for Supplemental Figures 3A, E, F, G and H is 100  $\mu$ m. Scale bar for Supplemental Figures 3B, C and D is 100  $\mu$ m.

**Supplemental Figure 5. *Dcn* and *Tnc* are essential for HSPC development**

(A) Left panel: WISH for *runx1* in 26 hpf morphants embryos and their respective uninjected controls. Numbers of embryos with or without (out of total) *runx1* staining are indicated in the



bottom left corner. Right: qRT-PCR analysis for *runx1* and *gata2b* expression in morphants and uninjected embryos. \* indicates p value < 0.05.

(B) WISH for *cmyb* in 40 hpf morphants embryos and their respective uninjected controls. Numbers of embryos with or without (out of total) *cmyb* staining are indicated in the bottom left corner.

(D) Fluorescent microscopy of *kdlr :mCherry ;gata2b :gfp* in 30 hpf morphants embryos and their respective controls.

(E) Fluorescent microscopy of *flt1:dtomato;flt4:yfp* in 36 hpf morphants embryos and their respective controls.

#### Supplemental Tables

#### **Supplemental Table 1. Laser microdissection quantification at E10.5, E11.5 and E12.5.**

This table indicates for each developmental stage the numbers of biological samples for VT and DT, the numbers of micro-dissected tissues, their corresponding surfaces and RNA concentrations.

#### **Supplemental Table 2. The gene set accounting for the contrast between DT and VT tissues and the gene allocation to the different WGCNA modules.**

#### **Supplemental Table 3. Characteristics of Louvain clusters found by single cell RNA-Seq analysis.**

#### **Supplemental Table 4. Lists of genes within the blue WGCNA module with mesenchymal, neuronal and mesenchymal/neuronal characteristics.**

## **Supplemental Videos**

**Supplemental Video 1. Laser microdissection.** This movie illustrates the microdissection of the dorsal tissue located between the notochord and the roof of the dorsal aorta at E12.5. The green line indicates the trajectory of the ultraviolet laser used for cutting the tissue. The green spots indicate the position of the infrared laser used for capturing the microdissected tissue.

**Supplemental Video 2. 3D imaging of Tnc (in green) and CD31 (in red) expression at E11.5.** This video was generated using Arivis software after whole-mount immunostaining, 3DISCO clearing and imaging using an ultramicroscope (LaVision Bio Tec). The movie shows the expression profiling of Tnc and CD31 along the antero-posterior axis of the embryo.

**Supplemental Video 3. 3D analysis of Ntrk3 expression at E11.5.** This video was generated using Arivis software after whole-mount immunostaining, 3DISCO clearing and imaging using an ultramicroscope (LaVision Bio Tec). The movie shows the expression profiling of Ntrk3 along the antero-posterior axis of the embryo.

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## **DISCUSSION AND PERSPECTIVES**

During my PhD, I contributed to explore the molecular identity of the AGM hematopoietic microenvironment. I also implemented the zebrafish model in the team and identified through functional *in vivo* assays decorin (dcn) and the growth differentiation factor 6 (gdf6a) as potential novel HSPC regulators.

Deciphering the molecular framework of the AGM HSC niche is of particular importance for our basic understanding of HSC biology in the embryo and for increasing the efficiency of current protocols aiming at producing transplantable HSCs *ex vivo*. The AGM microenvironment is highly complex. It indeed contains diverse cell types and even though several cytokines, growth factors and signaling pathways have been identified as major extrinsic regulators of the first emerging HSCs a comprehensive understanding of the AGM HSC niche is still missing (Clements & Traver, 2013; Dzierzak & Bigas, 2018; Nitsche & Ottersbach, 2021; Stik et al., 2017). For example, studies in zebrafish and mouse embryos have shown that neural crest cells and the sympathetic nervous systems are key players of AGM hematopoiesis (Damm & Clements, 2017; Fitch et al., 2012; Kapeni et al., 2022). The subaortic mesenchyme is also known as a source of critical AGM niche factors such as Bmp4 (Crisan et al., 2015; Durand et al., 2007; McGarvey et al., 2017; Souihol et al., 2017). Preventing its installation under the dorsal aorta by ligature experiment prevented IAHC formation and *Runx1* expression (Richard et al., 2013). However, since endothelial cells, macrophages and neural crest cells are intermingled with the subaortic mesenchyme, extracting its molecular signature remains a technical and biological challenge.

In 2020, the group of Catherine Robin generated genome-wide dataset to identify spatial gene expression pattern along the developmental axis of four embryo species: chicken, human, mouse and zebrafish. By combining tomo-seq and RNA-seq and *in vivo* functional assays these authors described three new and conserved regulators: ADM and its receptor RAMP2 and SVEP1 (Yvernogeu et al., 2020).

Same year, the group of Alexander Medvinsky used a laser capture strategy coupled with RNA-seq, in the human embryo, to investigate dorso-ventral molecular difference across the dorsal aorta with a focus on cell layers close to IAHC formation. By exploring gene expression dynamic across EHT within the aortic niche at the population and single cell level they revealed a close link of emerging HSPCs with a specific endothelial subset in which arterial signature was downregulated. This study also revealed endothelin 1 as an important secreted regulator of human HSPC development (Crosse et al., 2020).

### **Evidence for a neuro-mesenchymal cell population located underneath the dorsal aorta**

To investigate further the molecular identity of the AGM HSPC microenvironment, we combined a laser microdissection strategy (to isolate the dorsal and ventral aortic tissues) with bulk transcriptomics and computational biology approaches. The originality of our experimental design was to exclude the genetic contribution of AGM endothelial and hematopoietic cells from the bulk transcriptomes of dorsal and ventral tissues. This led us to identify a unique molecular network (the blue and yellow VT modules) active in the subaortic tissue at E11.5. Intriguingly, the blue VT module contained both interconnected neural and mesenchymal genes involved in neurotrophin signaling (*Ntrk3*), synapse (*Syt1* and *Slitrk1*), neurotransmitter binding (*Gabra3* and *Grid1*), ion channels (*Kcnip4*, *Kcnj2* and *Kcnab1*), extracellular matrix (*Dcn*, *Tnc*, *Col8a1*, *Lox11* and *Mmp16*) and growth factors (*Bmp6* and *Csf1*).

The functional association between the hematopoietic and neuronal systems has already been reported in the embryonic and adult contexts. In the adult, the SNS nerves regulate G-CSF-induced HSC mobilization from the BM (Katayama et al., 2006). When adrenergic neurotransmission is ablated, the HSC migratory response to G-CSF stimulation is prevented, owing to the loss of *Cxcl12* downregulation (Katayama et al., 2006). The cyclical circadian

release of HSCs into the bloodstream under homeostasis is also orchestrated through the rhythmic release of adrenergic signals from sympathetic nerves in the BM which tightly controls Cxcl12 expression from stromal cells (Méndez-Ferrer et al., 2008). An additional link between brain and BM was also underscored by the role of the type-1 muscarine receptor (Chrm1) in the hypothalamus, which regulates G-CSF-mediated HSC mobilization from BM through a glucocorticoid signaling relay (Pierce et al., 2017).

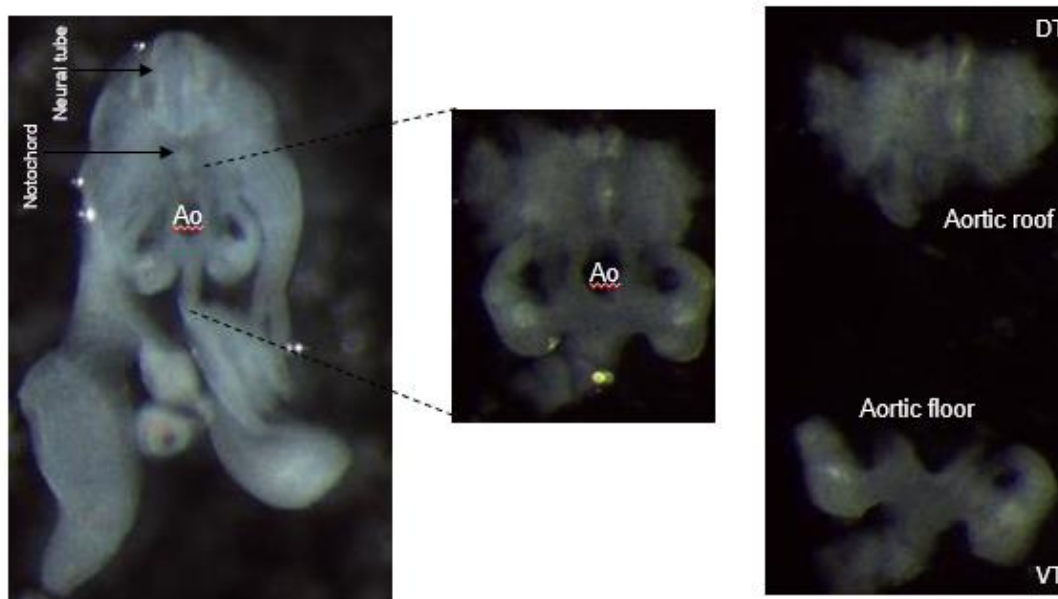
In the embryo, it has been reported that Gata3 controls the numbers of AGM HSCs through the production of catecholamines, showing a strong influence of the sympathetic nervous system on AGM HSCs (Fitch et al., 2012). The Neuronal growth factor increases HSC activity when added at high doses to AGM explant cultures (Durand et al., 2007) and in the human embryo a transcriptomic analysis at the time of HSC development has highlighted a neuronal molecular signature associated with the ventrolateral domain of the dorsal aorta (Crosse et al., 2020).

Two possibilities may account for our original observation. The blue VT module could be representative of a mix population of neuronal and mesenchymal cells captured together during the laser microdissection or of a ‘neuro-mesenchymal’ cell population located underneath the dorsal aorta. To discriminate between these two hypotheses, we then combined single cell transcriptomics, lineage tracing and immunodetection approaches. The single cell gene expression profiling revealed that the blue VT module predominantly corresponded to the mesenchymal clusters C6 and C8 clearly distinct from the neural/neural crest cell clusters (C11 and C13). Interestingly, by subdividing the blue module into a list of ‘mesenchymal’ genes and a list of ‘neuronal’ genes, we could see a significant overlap between the two gene signatures in C6 and C8. Analysis gene per gene showed that *Syt1* (encoding a calcium sensor active in synapse) was expressed in C6, C8 and C13 whereas *Ddc* (encoding an enzyme involved in dopamine and serotonin) was expressed in C8 and C13. Although expressed at a lower level, *Gabra3* and *Grid1* encoding for the alpha chain of the Gaba receptor and a glutamate receptor

respectively were also detected in some cells of C6 and C8. Collectively, these findings suggest that, at the single cell level, a population of subaortic cells exhibited both mesenchymal and neuronal gene identities. To investigate whether these cells may derive from neural crest cells, we took advantage of a Human tissue Plasminogen Activator (HtPA)-Cre mice labelling neural crest cells and their derivatives *in vivo* (Pietri et al., 2003). After crossing those mice with GT Rosa Tomato mice, E11.5 embryos were collected and analyzed for the expression of Tomato and the ‘neuro-mesenchymal’ markers Dcn, Tnc and Ntrk3. We did not observe any overlapping staining between Tomato and Dcn, Tnc or Ntrk3, suggesting that subaortic cells expressing those proteins were not derived from neural crest cells. As a perspective, it will be interesting to isolate by flow cytometry the different AGM cell populations based of Tomato and Ntrk3 expression to explore in more details their molecular identity and their capability to support HSPCs *ex vivo*.

With the help of Laurent Yvernogeu and Pierre-Yves Canto, I recently generated new single cell transcriptomics datasets after sub-dissecting E11.5 AGMs into their dorsal and ventral aortic domains. The aim consists in exploring how different types of niche cells may co-operate to regulate HSPCs according to their position along the dorsal-ventral axis. Our original approach was based on preparing fresh mouse embryo sections at E1.5. These sections were then manually dissected by cutting the dorsal aorta in half and collecting the dorsal domain including the aortic roof and the surrounding tissue, and the ventral domain, including the aortic floor potentially holding some IAHCs and the subaortic tissue (Figure 8).



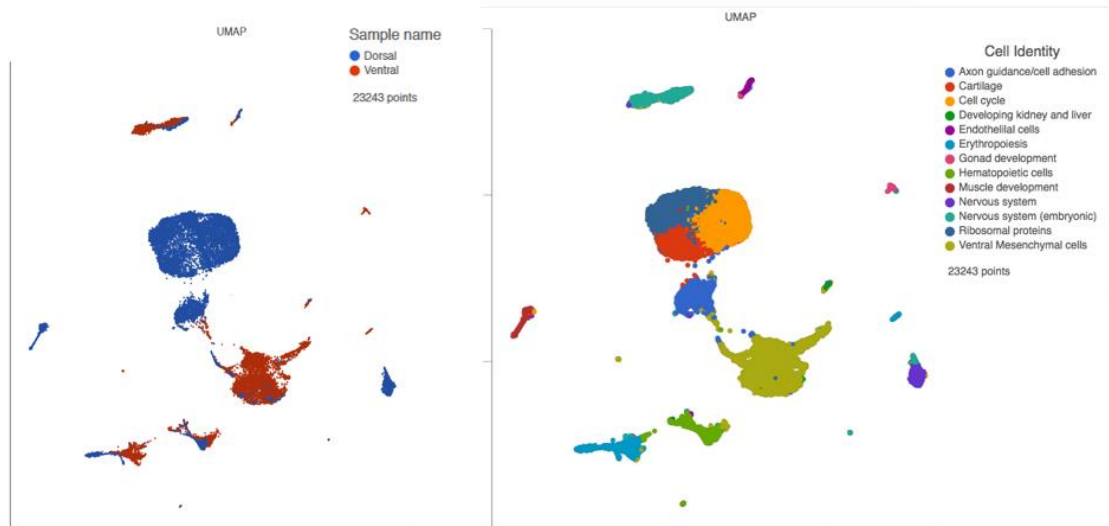


**Figure 8: Experimental design for microdissection of mouse embryo at E11.5**

Sections of E11.5 mouse embryo were made using tissue chopper. The region of interest – dorsal aorta and surrounding tissues were manually micro dissected. Right panel: Dissected section of dorsal (up) and ventral (bottom) aortic tissues

Around twenty dorsal and ventral sections were collected from 12 embryos, pooled and proceeded for 10X Genomics.

So far, we were able to recapitulate the results we gain by the single cell analysis using the whole AGM. We were able to see that most of the genes involved in cell cycle, ECM, cell adhesion was part of cluster differentiated from dorsal tissues. In the ventral aortic domain, we identified clusters for endothelial cells (C15), erythropoiesis (C9), hematopoietic cells (C8). Most importantly main cluster for ventral domain is for ventral mesenchymal cells, and two clusters for nervous system, C5 and C11 (Figure 9).



**Figure 9: Single cell plots**

Left: Differentiation of ventral (red) and dorsal (blue) tissue; Right: Clusters with cell identity

As mentioned above, the aim of this experiment consists in exploring how different types of niche cells may co-operate to regulate HSPCs according to their position along the dorso-ventral axis.

### **Dcn, Tnc and Gdf6a as potential novel AGM HSPC niche factors**

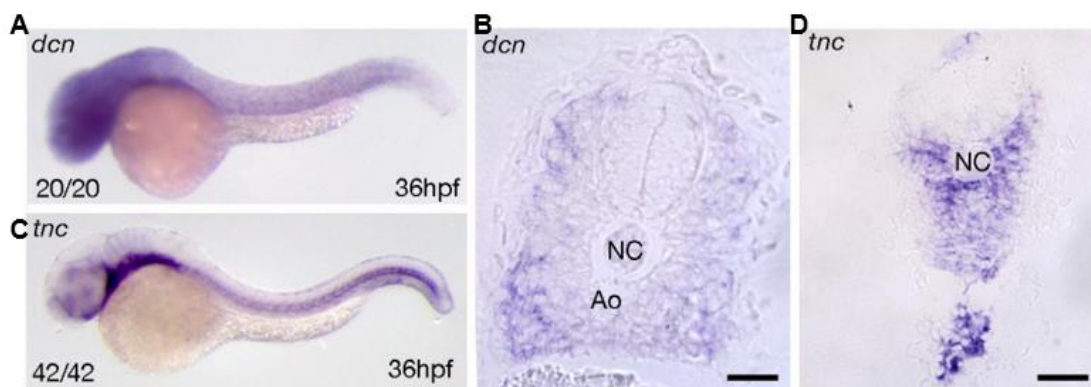
#### **Dcn and Tnc**

We selected Dcn and Tnc for functional studies since they were highly connected genes in the blue module and were expressed in the subaortic mesenchyme both in mouse and human embryos. Dcn is an ECM protein that belongs to the small leucine-rich proteoglycan family. Structurally, it has a domain of 12 leucine-rich repeats (LRRs) that form a curved solenoid fold, flanked by two cysteine-rich regions (Scott et al., 2004). Brown et al used an X-ray crystallography method to study the crystal structure of Dcn and found that its shape resembled a horseshoe comprising a convex region of L helix and a concave region of B sheets formed by leucine rich repeats that could interact with a variety of protein molecules (Brown et al., 2002). Each LRR is characterized by unique biological function that is specific to the known

bioactivities of Dcn: LRR V/VI assist in Dcn binding to Vegfr2 (G. A. Khan et al., 2011), LRR VII is the collagen binding sequence that is present in the inner surface of the solenoid (Kalamajski et al., 2007) and it directly mediates the interaction between Dcn and type I collagen. LRR XII binds to Ccn2/Ctgf (Vial et al., 2011) and LRR XI is known as the “ear” repeat whose mutation may cause congenital stromal corneal dystrophy (Chen et al., 2011; Chen and Birk, 2013). Mammalian Dcn, whose complex gene contains 8 exons, is located in chromosome 12q21-q22 and comprises a molecular weight of 92.5-kDa in human. Dcn has a coding gene that is 1080bp in length. The synthesis and secretion of Dcn mainly occurs in the rough endoplasmic reticula and Golgi apparatus of fibroblast, smooth muscle cells and macrophages.

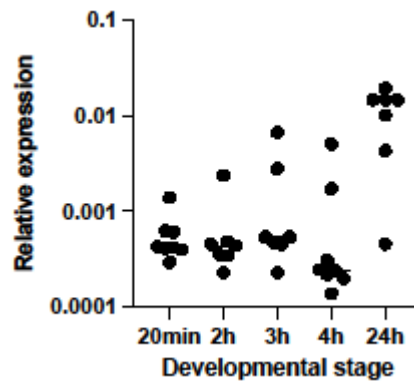
Tenascin-C (Tnc) is a large, extracellular matrix glycoprotein that exhibits a very restricted pattern of expression but an enormously diverse range of functions. Tnc consists of 4 distinct domains, which can interact with pathogenic components, matrix constituents, soluble factors and cell surface proteins. For example, the tenascin assembly domain forms inter-molecular hydrophobic interactions and disulfide bridges, the fibronectin (Fn) type III-like repeats interact with proteins as diverse as integrins, aggrecan and perlecan as well as binding to growth factors including members of the platelet derived growth factor (PDGF), fibroblast growth factor (Fgf) and transforming growth factor- $\beta$  (Tgf $\beta$ ) families (Giblin and Midwood, 2015). This enables Tnc to drive a range of process from oligomerization to induction of mitogenic responses, cell migration, cell attachment and many others. This collection of functions is reflected in the wide-ranging consequences of genetic deletion of Tnc in mice. Reported abnormalities included reduced Fn during dermal wound healing (Forsberg et al., 1996), hyperactivity (Fukamauchi et al., 1997), reduced kidney regeneration (Nakao et al., 1998), reduced hematopoiesis (Ohta et al., 1998).

Our data show that *Dcn* is expressed by the cells around the notochord (Nc), around the dorsal aorta following with expression in the subaortic mesenchyme in both mouse and human embryos. By performing double and triple staining's for labelling smooth muscle cells ( $\alpha$ SMA) and/or endothelial cells (CD31) we are showing that there is no overlapping between these markers. Our observation that *Dcn* is strongly enriched in the mesenchyme underneath the dorsal aorta not only in mouse but also in human is, to our knowledge, yet unreported. *Dcn* shows a dynamic expression along the antero-posterior axis with strong expression in cells and associated ECM in large areas of the middle part of the AGM known to be enriched for HSC activity in mouse and human embryos (Easterbrook et al., 2019; Mascarenhas et al., 2009a). In agreement with previous reports, in the zebrafish embryo, I observed *dcn* expression in the developing head, the lateral mesoderm, and at lower level in the somites at 36 hours post-fertilization (hpf) embryos (Figures 10 A and B) (Zoeller et al., 2009). I also investigated the relative expression of *dcn* by RT-qPCR, and in line with previous reports, *dcn* was showing a dynamic expression in the developing embryo (Figure 11).



**Figure 10: WISH for *dcn* and *tnc* in 36 hpf embryos**

(A-B) *dcn* expression in the developing head, the lateral mesoderm, and at lower level in the somites; whole mount and cross-section 20 out of 20 embryos showing this phenotype (C-D) *tnc* expression in the developing trunk; whole mount and cross section 42 out of 42 embryos showing this phenotype. Ao – aorta, NC – notochord; Scale bar is 25  $\mu$ m



**Figure 11: Zebrafish *dcn* expression analyzed by RT-qPCR**

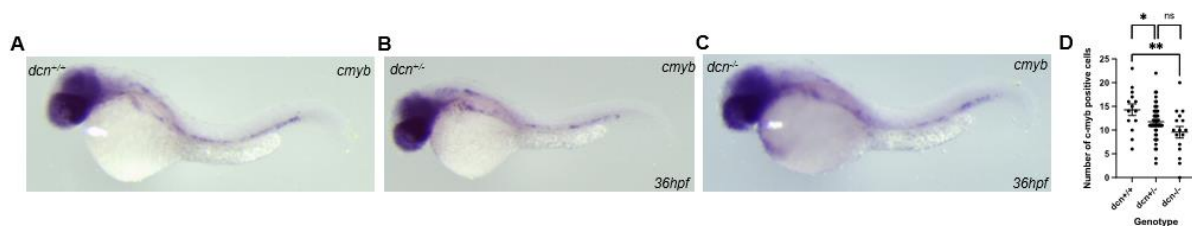
Dynamic expression of *dcn* in the developing zebrafish embryo starting at 20 minutes after fertilization up to 24hpf.

To investigate if Dcn has a role in the formation of HSPCs, I combined loss of function and genetic approach using the zebrafish model. To evaluate the dose effect of the morpholino, I first injected different dilutions of *dcn* morpholino going from high dose (17ng), medium dose (8.5ng) to low dose (4.25ng). High dose of *dcn* morpholino had a major effect on the morphology of the embryo, and recapitulated previously reported phenotype of shortening of the head-to-tail axis as well as compression and flattening of the embryo (Zoeller et al., 2009). Low dose didn't have any effect on injected embryo, while middle dose had an effect hematopoietic phenotype in the dorsal aorta, while keeping the morphology of the embryo unaltered. So, the next series of injections was done using middle dose of *dcn* morpholino.

Our results show that the expression of two HSPC reporter genes (*cmyb* and *runx1*) was decreased in *dcn* morphants compared to uninjected embryos in dorsal aorta in both 30 and 36 hpf, respectively.

To validate the phenotype of *dcn* morphants, I took advantage of the zebrafish mutant line *dcn<sup>sa20247</sup>*. These fishes have ENU-induced point mutation that is changing a base in *dcn* transcript by introducing the stop codon by A to T substitution in position 167 of the *dcn* coding sequence. First experimental approach was to analyse the phenotype of the embryos coming

from heterozygous parents. After WISH for *cmyb* and genotyping I couldn't see any statistically difference between *dcn*<sup>+/+</sup>, *dcn*<sup>+/-</sup> and *dcn*<sup>-/-</sup> embryos. This observation led me to consider a possible maternal effect for *dcn*. To investigate that question, I decided to inject a low dose of *dcn* morpholino (4.25ng) at one cell stage of all embryos coming from *dcn*<sup>+/-</sup> parents. After WISH, all embryos were genotyped, and evaluated for *cmyb* expression in the dorsal aorta. As shown on figure 12 I could see a significant decrease in the number of *cmyb* positive cells in *dcn*<sup>+/-</sup> and *dcn*<sup>-/-</sup> as compared to *dcn*<sup>+/+</sup>.



**Figure 12: *dcn*<sup>sa20247</sup> + *dcn* Mo**

(A-C) WISH for *cmyb* in 36 hpf *dcn*<sup>+/+</sup>, *dcn*<sup>+/-</sup> and *dcn*<sup>-/-</sup> embryos injected with 4.25ng *dcn* Mo (D) Numbers of *cmyb*<sup>+</sup> cells in the dorsal aorta of *dcn*<sup>+/+</sup>, *dcn*<sup>+/-</sup> and *dcn*<sup>-/-</sup> embryos.

Finally, since adult *dcn*<sup>-/-</sup> female and male were viable and fertile, I crossed them and observed a significant decrease in the numbers of *runx1*<sup>+</sup> and *cmyb*<sup>+</sup> cells in the dorsal aorta of *dcn*<sup>-/-</sup> embryos as compared to *dcn*<sup>+/+</sup> embryos. Taken together the genetic strategy recapitulated the phenotype of the *dcn* morphants.

In addition, thanks to our collaboration with David Traver (University of California San Diego), confocal microscopy using *kdlr:mCherry;gata2b:gfp* transgenic animals showed a reduction in the number of HSPCs in the dorsal aorta in the morphants. These effects occurred in absence of any vascular defects as shown by confocal imaging using *flt1:dtomato;flt4:yfp* transgenic lines reporting vessel development.

Taken together, these findings identify *dcn* a potential novel HSPC regulator in the developing embryo. Interestingly, in line with previous reports, (Marshall et al., 1999; Yvernogeu et al., 2020) we observed that Tenascin C (Tnc) is highly expressed in the subaortic mesenchyme both

in mouse and human embryos. In the zebrafish embryo at 36 $hpf$  *tnc* was highly expressed in the trunk of developing embryos (Figure 10C-D), notably in the somites and around the dorsal, as previously reported (Lin et al., 2013).

We showed that *tnc* knock-down in the zebrafish embryo led to a decrease in *runx1*<sup>+</sup> and *cmyb*<sup>+</sup> cells in the dorsal aorta. These results then suggest that the extracellular matrix may be a major constituent of the AGM HSPC niche. In line with these findings, the group of Trista North identified a distinct role for metalloproteases Mmp2 and Mmp9 in regulation of developmental HSPC production, migration and niche localizations (Theodore et al., 2017). They showed that during zebrafish development, Mmp2 is required to remodel fibronectin-rich ECM in the ventral dorsal aorta to facilitate HSPC egress from the AGM and subsequent migration to the CHT and thymus, and demonstrated that Mmp9 affects the structure of the vascular niche, altering HSPC localization, and promotes HSPC migration to subsequent hematopoietic niches (Theodore et al., 2017). They also showed that Mmp2 and Mmp9 reveal essential but discrete roles downstream of sterile inflammatory signaling during embryonic hematopoiesis, and that each can affect HSPC migration and localization in subsequent niche (Theodore et al., 2017). ECM restricting by MMPs affects development of lungs, mammary glands and salivary glands, but also MMP2 is required for local ECM digestion during epithelial branching (Bonnans et al., 2014). Interestingly, Ellis et al in 2013 reported the role Tnc in lineage commitment of thymic T cell progenitors and peripheral T cell migration. They showed that mice devoid of Tnc were developing normally, but that the absence of Tnc although it didn't not make an effect on the size of the BM stem cell pool it decreased thymic T cell progenitors with redistribution of other lymphoid organs, suggesting an anchoring role for Tnc (Ellis et al., 2013).

The ability to produce hemogenic endothelial cells (HE) from hiPSCs is considered a critical step toward the *de novo* generation of blood progenitors and stem cells. Uenishi et al established a new protocol where after planting hiPSCs from a single cell suspension in a completely

chemically defined medium that was free of serum components and xenogeneic proteins, they were able to support hematopoietic differentiation. They identified and showed the positive effect of Tnc on all stages of differentiation including the enrichment of hematovascular mesoderm, HE and CD34<sup>+</sup> hematopoietic progenitors (Uenishi et al., 2014). Importantly, they were able to support the development of definitive hematopoietic cells with T lymphoid potential.

Several signaling mechanisms implicated in cell interaction with Tnc have been identified, including suppression of fibronectin-activated focal adhesion kinase signaling, Rho-mediated kinase signaling, and stimulation of Wnt signaling pathways (Orend, 2005). Further studies to identify the mechanism of Tnc signaling and determination of the developmental stages that are most affected by Tnc would help elucidate the role of this matrix protein during development.

It is now important to explore in more details how Dcn (and Tnc) mediates its effect on HSPCs and whether it influences the specification, emergence and/or survival of HSPCs during development.

Since Dcn interacts with other ECM proteins (e.g., fibronectin, collagen) it could play a structural role by controlling the stiffness of the AGM hematopoietic microenvironment. Dcn could also play an important functional role since it can bind to cell surface receptors and soluble factors, and as a consequence Dcn may be involved in cell-to-cell communication by regulation the half-life, local concentration and availability of critical growth factors, cytokines and morphogens that may modify the properties of the overlaying endothelial cell layer and/or act beyond this layer on HSC within IAHCs. Interestingly, David Traver and colleagues through single cell analysis identified molecular signatures of somite-derived endothelial cells (SDECs), brain endothelial cells (ECs), kidney ECs and endocardium in 24 hpf zebrafish embryos. Their analysis suggested that Notch signaling is dispensable for the acquisition of



arterial cell fate but it is required for the specification of hemogenic endothelium. Among new findings, they identified *dcn* as one of differentially expressed genes in analysis of SDECs versus endothelial clusters (Sahai-Hernandez et al., 2020).

Together with previous reports (Khoshdel Rad et al., 2020; Ichii et al., 2012) we could hypothesize the Bmp and/or canonical Wnt could be associated with Dcn. Different studies have reordered the roles of BMPs in kidney organogenesis and indicated that Bmp2/4 signaling has a critical role in size determination and patterning of the nephrogenic field (Oxburgh et al., 2014), and that the Bmp7/Smad1/5 signaling pathway promotes susceptibility of nephron progenitor cells (NPCs) to differentiation signal of Wnt9b/b-catenin (Brown et al., 2013; Muthukrishnan et al., 2015). In this case, Dcn produced by stromal cells could accumulate in the ECM surrounding NPCs, antagonize Bmp7/Smad signaling and represses the differentiation signal of the canonical Wnt9b/b-catenin pathway (Ohmori et al., 2015).

To test these hypotheses, we could perform gain and/or loss of function experiments using zebrafish model for different Bmp or Wnt receptors and follow the expression of HSPC reporter genes by WISH and/or RT-qPCR.

In addition, Clements et al reported an important role of the Pdgf signaling pathway in HSC emergence in the zebrafish. They showed that trunk neural crest cell migration to the DA, regulated by Pdgf signaling is required for HSPC specification (Damm and Clements, 2017). It was reported by Jarvelainen et al. that Dcn indirectly acts against angiogenesis via interaction with cell surface receptors, signaling molecules and angiogenic growth factors, one of them being platelet-derived growth factor – PDGF (Järveläinen et al., 2015). Based on these finding we could then hypothesize that Dcn may interact with Pdgf to regulate of HSPC development. I am currently examining the relative expression of different inflammation molecules (such as TLR2/4) and signaling pathways molecules (e.g., *wnt3a* and *bmp7*) previously described to interact with Dcn, and *pdgf* receptors by RT-qPCR in *dcn* mutant and control zebrafish.

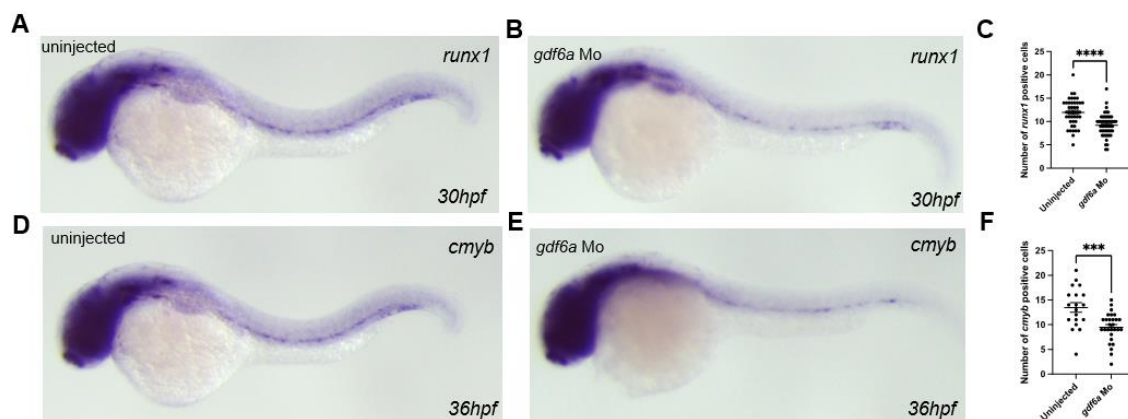
## **Gdf6a**

Growth differentiation factor 6 (Gdf6) is a Bmp family member (Bmp13). The amino acid sequence for Gdf6 is highly conserved across vertebrates, with sequence homology concentrated in the active C – terminal domain (Krispin et al., 2018). Even the most divergent of BMP13-homologues, zebrafish *gdf6a* (radar) and *gdf6b* (dynamo), display greater than 90% homology in this domain (Williams et al., 2008).

The role of Gdf6 is probably best understood in regulating bone and ocular development across a number of species, including humans (Asai-Coakwell et al., 2009; Tassabehji et al., 2008). Patients with mutation in *Gdf6* gene, such as those noted in Klippel-Feil syndrome, can phenotypically present with fusion of the vertebrae and limited mobility in addition to having many ocular disorders (Asai-Coakwell et al., 2009; Tassabehji et al., 2008). Ocular disorders known to be linked to mutations in Gdf6 include microphthalmia, coloboma and age-related macular degeneration (AMD) (Asai-Coakwell et al., 2007; den Hollander et al., 2010; Gosse and Baier, 2009; Pant et al., 2013; Zhang et al., 2012). An AMD “risk allele” linked to Gdf6 is also associated with increased expression of serin protease *Htra1*, and *Htra1* knockout mice show decreased vascular development in the retina together with increased *Gdf6* and decreased *Vegfa* expression (L. Zhang et al., 2012).

The expression of *gdf6a* was shown by Thisse et al in 2001 as part of Zfin database (Expression of the zebrafish genome during embryogenesis) but also by Crosier et al. in 2002 (Crosier et al., 2002). Interestingly, at 24 hpf *gdf6a* is expressed around the dorsal aorta and in the gut of the zebrafish embryo. While a Gdf6 deficient mouse line does exist and homozygous mutants are present in Mendelian ratios prenatally, there is a dramatic increase in mortality postnatally, as the number of *Gdf6*<sup>-/-</sup> mice at the time of weaning is significantly less than 25% (Settle et al., 2003).

In a recent work, Krispin et al showed that in the developing zebrafish embryo *gdf6a* promotes vascular integrity by counteracting Vegf activity (Krispin et al., 2018). In zebrafish genome, *gdf6* is duplicated having *gdf6a* (radar) and *gdf6b* (dynamo), however whole mount *in situ* hybridization showed that *gdf6a* is the sole player expressed in the zebrafish trunk at 48 hpf, while *gdf6b* was not expressed in the zebrafish trunk at this time (Krispin et al., 2018). It has been reported that *gdf6b* has a supporting role as a master sex-determining gene, and that the Pachon cavefish *gdf6b* gene is expressed specifically in differentiating male gonads, and that its knockout induces male-to-female sex reversal in B-carrying males (Imarazene et al., 2021). Since *Gdf6* is within the blue VT module and is expressed in the AGM, I decided to examine its role on HSPC development using knock-down loss of function experiments. Our data clearly show that in 30 hpf and 36 hpf morphants there is a significant decrease of the *runx1* and *cmyb* positive cells in the dorsal aorta respectively, as compared to uninjected controls (Figure 13).



**Figure 13: *gdf6a* a potential novel regulator of HSCs development**

(A-B) WISH for *runx1* in 30 hpf *gdf6a* morphants and their respective uninjected controls. (C) Numbers of *runx1*<sup>+</sup> cells in the dorsal aorta of control and *gdf6a* morphant embryos. (D-E) WISH for *cmyb*<sup>+</sup> in 36 hpf *gdf6a* morphants and their respective uninjected controls. (F) Numbers of *cmyb*<sup>+</sup> cells in the dorsal aorta of control and *gdf6a* morphant embryos.

These results could be confirmed by utilizing zebrafish mutant line, for example *gdf6a*<sup>s327</sup> which results in a single C to A substitution in position 164 of the *gdf6a* coding sequence, introducing

a stop codon early in the *gdf6a* open reading frame (Gosse & Baier, 2009) or the Crispr/Cas9 strategy.

It was first mentioned by Crosier et al in 2002 that *gdf6a* has a promising role in maintenance of vascular integrity. When *gdf6a* function was depleted by an antisense morpholino it resulted in vascular leakiness (Crosier et al., 2002). Interestingly, embryos generated by morpholino-knock down of VEGF-A show many features in common with *gdf6a* depleted embryos (Nasevicius et al., 2000). In 2018 Krispin et al published that *gdf6a* functions to antagonize *vegfr2* mediated phosphorylation of *vascular endothelial-cadherin (vecdn)* by reducing *vegfr2* receptors, preventing destabilization, internalization and degradation of *vecdn* and promoting junctional stability and vascular integrity (Krispin et al., 2018).

The link between Gdf6 and modulation of Vegf receptors still remains to be determined, since results obtained so far from different groups suggest a role of *gdf6a* not only in vasculogenesis but in hematopoiesis too. Our results indeed suggest that *gdf6* could influence HSPC development in a direct or indirect manner.

Overall, my PhD project should provide new insights on the molecular identity of the AGM hematopoietic microenvironment and reveal novel regulators of the HSCs emergence in the developmental hematopoiesis.

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## **ANNEX**

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## Future Outlook

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### **Introduction**

The second edition of the book “Stem Cell Biology and Regenerative Medicine” aims at providing the readers with a cutting-edge knowledge on stem cell biology. The first section was dedicated to introduce important concepts and methods used in the field (chapters 1 to 6). These included the biology of embryonic and adult stem cells, the procedures for enriching cell populations with stem cell activity and testing their functionalities, the modes of stem cell proliferation and differentiation, the existence of stem cell niches, and the molecular mechanisms involved in gene expression regulation and DNA repair. Computational approaches and models to investigate stemness were also discussed in details (chapters 5 and 6). The second section continued the discussion on pluripotency with a particular emphasis on reprogramming and disease modeling using hepatic cells as an example, X chromosome inactivation and regeneration in several model organisms such as plants, *Hydra* and anamniotic vertebrates (chapters 7 to 11). The third section covered the biology of diverse adult stem cells including hematopoietic, neural, dental, mesenchymal, epithelial, mammary, and intestinal

stem cells (chapters 12 to 21). The fourth section explored some aspects related to cancer stem cells and cell therapy (chapters 22 to 25). Finally, the last section addressed important issues on stem cell biology and regenerative medicine with an epistemological and regulatory perspective (chapters 26 and 27). Collectively, all these contributions invited the readers in understanding what are stem cells, how are they regulated during development and diseases and how could they be used in therapeutical settings. We also believe they provide opening and challenging questions on stem cell research both on the fundamental and translational points of view. Some of these issues are briefly discussed in this concluding chapter.

### **Understanding and Capturing Pluripotency**

We have seen that embryonic and adult stem cells represent two distinct cellular entities differing in terms of origin and functional properties. Pluripotency defines a unique differentiation potential (i.e., the ability to give rise to all tissues of an organism including the germ line) restricted to a very short period of time during development, the blastocyst stage prior implantation. From the naive epiblast, embryonic stem (ES) cell lines can be isolated and maintained undifferentiated in culture for long periods of time (Nichols and Smith, 2009). While the development of ES cell lines has enabled considerable progress on the identification of the molecular mechanisms and gene networks involved in pluripotency, whether these lines account for the full potential of the cells of the inner cell mass of pre-implantation embryos and capture their genetic and functional heterogeneity remains uncertain. Furthermore, it is unclear how pluripotent stem cells *in vivo* segregate and contribute to the germ line and the diverse somatic cell lineages representative of the three germ layers during early development. Studying pluripotency constitutes an exceptional opportunity to explore in depth the cascade of molecular and cellular mechanisms involved in cell differentiation during early development. It has also major impacts on increasing the efficiency of

reprogramming technologies. This field is under intensive investigations and raises major conceptual and methodological issues. For example, if a major gene regulatory network involving *Nanog*, *Sox2*, and *Oct4* has been shown to regulate pluripotency, how this network is connected with external signaling pathways, epigenetic modifications, and chromatin remodeling remains poorly understood. Moreover, different degrees of pluripotency (naive, primed, and alternative) have been identified and have to be further characterized at the molecular level and also between species (interestingly, human ES cells share many similarities with rodent primed ES cells) (Li and Izpisua Belmonte, 2018; Nichols and Smith, 2009). Contrasting with pluripotency as a transient state *in vivo*, several chapters in this book explored the fascinating issue related to the reprogramming of somatic cells into pluripotent stem cells. Reprogramming and differentiation of pluripotent stem cells into specific cell lineages are also instrumental for modeling human diseases, testing the toxicity and efficacy of novel medicine, and eventually producing cell derivatives that could be used in clinical applications to restore physiological functions. This is a very challenging issue. It requires to properly recapitulate in a petri dish the coordinated molecular and cellular pathways that are active in the developing embryo and that control in time and space complex cell specification processes. *Ex vivo*, defining the appropriate combination of cytokines, growth factors and signaling molecules at precise time points and concentrations is a prerequisite for efficiently support cell survival, proliferation and differentiation of specific cell types. Physical parameters such as the elasticity of the extracellular matrix, oxygen tension, blood flow and mechanical forces also play a crucial role and need to be adapted for any cell differentiation pathways. Moreover, as highlighted by chapters 8, 17, 21, and 23, novel 3D technologies and biomaterials are emerging as important strategies for mimicking the structure, mechanical properties and functions of specific tissues. Among the challenges that face stem cell biologists in this field are the availability of specific markers to select from the cell cultures

the cell types of interest, the exploration of their molecular and functional features (as compared to their physiological counterparts) and the refinement, in case of cell transplantation protocols, of their mode of delivery.

### **Integrating Signals of Intrinsic, Extrinsic and Systemic Origins**

Several chapters of this book addressed the fundamental question of where stem cells are and how stemness is controlled. It is now clear that, in a variety of experimental models and stem cell systems, both cell autonomous (active in stem cells themselves) and non-cell autonomous (relying on the functions of surrounding cells) mechanisms are major regulators of stem cell biology. Stem cells coexist and interact in a very dynamic manner with niche cells that constitute a supportive microenvironment for stem cell survival, proliferation and differentiation. To the variety of adult stem cell systems is associated a variety of niche cells. For example, specific somatic cells constitute the major niche cell components of germ stem cells whereas hematopoietic stem cells (HSCs) are closely associated with mesenchymal stem cells (MSCs) and their derivatives in the adult bone marrow. Cell-to-cell communication, cell adhesion and extracellular matrix remodeling are then major cellular processes active at the interplay between stem cells and their niches. What are the similarities and differences in the molecular pathways utilized by niche cells to regulate stemness in different tissues is still unclear. Moreover, accumulating evidence supports the notion that this crosstalk is bidirectional. Niche cells indeed receive signals from stem cells that influence their supporting capacities, phenotypes and molecular identities. How these dialogs are established during development, have emerged during evolution, and are eventually perturbed during diseases and ageing is a major issue, both in fundamental biology and regenerative medicine. Importantly, on top of these intrinsic and local extrinsic factors released by niche cells, systemic factors active at the level of the organisms or provided by the environment

are now appearing as critical regulators of stem cell functions. Hormones, neurotransmitters, the circadian clock, and infections are among those factors. In models as diverse as *Hydra*, the *Drosophila* ovarioles or the vertebrate intestine, it has been demonstrated that microbes are an essential component of stem cell ecosystems. Changes in the composition of the microbiota and in its metabolic activities may also play a critical role in the process of tumor formation. For example, elegant experiments in *Hydra* have revealed that *Fox0*, a gene encoding for a transcription factor highly expressed in *Hydra* stem cells, is involved in the maintenance of stem cells, in the control of stemness genes and antimicrobial peptides expression as well as in bacteria colonization (Boehm et al., 2012; Mortzfeld et al., 2018). In *Drosophila* females, *Wolbachia* (maternally transmitted intracellular bacteria) infect germ stem cells, the germ stem cell niche and brain cells secreting insulin-like peptides. Infected females produce more eggs than uninfected females. Thus, by controlling germ stem cell activity through cell autonomous, extrinsic and/or systemic regulation, *Wolbachia* promotes its own propagation (Fast et al., 2011), raising particularly interesting questions in evolutionary biology. How microbes are integrated within the immune system and the machinery of stemness is a fascinating biological question. Further investigations on the crosstalk between microbes, immune cells and stem cells are really promising and should bring major novel findings on the mechanisms that drive stem cell regulation and tumor formation. Collectively, one of the major challenges in the field of stem cell biology will be to understand how single stem cell integrates such variety of signals to survive and commit into self-renewal or differentiation pathways.

### **Stem Cell Heterogeneity and Plasticity**

Recent advances and novel technologies such as single cell transcriptomics, lineage tracing and refined functional assays revealed that stem cells are highly heterogeneous. This

heterogeneity occurs at multiple levels including cell cycle status, metabolism, gene expression, surface phenotype, niche cell association, responses to radiation and functional characteristics. It has been documented in several stem cell systems such as HSCs, intestinal and skin stem cells (Goodell et al., 2015). In the intestine, crypt basal columnar cells and +4 cells constitute two distinct stem cell compartments that can eventually interconvert, the former highly proliferative and providing robust cell turnover whereas the latter more quiescent and recruited in case of injuries. The classical view of hematopoiesis supports the existence of long-term HSCs that give rise to short-term HSCs and then multipotent progenitor cells, at the origin of the common myeloid and lymphoid progenitors. Single cell transplantation assays, lineage barcoding and transcriptomic approaches revealed that HSCs are lineage-biased, exhibit transcriptional lineage priming and thus are highly heterogeneous (Haas et al., 2018). Several mechanisms may cooperate for establishing cell heterogeneity. These may involve the preferential association of stem cells with specific niche cells, stochastic cellular events, and genetic and epigenetic heterogeneity within single stem cells (Haas et al., 2018). Interestingly, such cell heterogeneity and plasticity have also been reported for both liquid and solid tumors (Meacham and Morrison, 2013; Nassar and Blanpain, 2016). This is well exemplified by the seminal study of Gupta and colleagues based on the isolation by flow cytometry of three distinct epithelial state (luminal, basal and stem-like) from human breast cancer lines (Gupta et al., 2011). After culture, the three cell populations could separately reconstitute tumor heterogeneity at similar proportions to the parental population, demonstrating interconversion and equilibrium between cell states (Gupta et al., 2011). Together, these findings have profound impacts on how tissue homeostasis is maintained *in vivo*, how tissues are regenerated after injuries and how tumors are initiated and propagated. They also provide stem cell biologists with new concepts and methods for better understanding tumor resistance and for elaborating more efficient

treatment protocols. Finally, they provide a unique framework for elucidating what stem cells are. As underscored in this book (chapters 1 and 27), stem cells may be referred as either ‘entities’ or ‘states’ (Zipori, 2004). According to the ‘entity’ ontology, a stem cell would be a predetermined cell type with intrinsic stemness ability to self-renew and differentiate in relation to predefined supportive niche cells. By contrast, the ‘state’ ontology favors the hypothesis that stemness is a property of a cell population transiently acquired following stochastic or deterministic events. According to this hypothesis, stemness may be induced in fully differentiated cells exposed to specific niche factors. Non-hierarchical stem cell models are being developed and should be further refined to account for the ‘state’ ontology. Lucie Laplane proposed four distinct stem cell properties (categorical, dispositional, relational, and systemic) depending on niche-dependent and dedifferentiation characteristics (Laplane and Solary, 2019).

### **Interface Between Stem Cell and Computational Biology**

The advent of high throughput technologies and computational approaches are providing researchers with a unique methodological framework for interrogating the biology of stem cells. For example, bulk and single cell transcriptomics have considerably increased our knowledge on the molecular identities of pluripotent and adult stem cells, stem cell heterogeneity, and cell differentiation trajectories (Cahan et al., 2021; Kester and van Oudenaarden, 2018; Li and Izpisua Belmonte, 2018). They are also instrumental for reconstructing molecular networks representative and eventually predictive of a particular cell state or cell behavior (Cahan et al., 2021). This knowledge has also major implications in our understanding of human diseases such as cancer and for recapitulating developmental programs *ex vivo* for producing specific cell types from precursor cells. We have to admit that these fast technological advances are also very challenging since they are accompanied by



the development of multiple algorithms and statistical analyses. For example, in the field of single cell transcriptomics, several computational approaches have recently been developed for reducing inherent noise related to low level of gene expression and for improving the identification of cell clusters, extraction of sets of differentially expressed genes and data visualization (Kester and van Oudenaarden, 2018). On our side, we took advantage of some of these concepts and methods for exploring the molecular identity of stromal cells that provide a supportive microenvironment for HSCs. By analyzing the transcriptomes of stromal lines with differing capacity to support HSCs and by integrating numerous gene datasets produced by other laboratories on HSC niche cells, we uncovered a molecular core representative of the HSC support and identified gene networks active in discrete bone marrow niche cell populations (Charbord et al., 2014; Desterke et al., 2020). By combining a subtractive strategy with dimensionality reduction methods and network concepts, our analyses also revealed unexpected novel HSC niche factors. We also believe that computational biology provides stem cell biologists with unique resources such as gene expression datasets that can be utilized to improve the robustness of the *in silico* analyses, to compare and refine molecular signatures and to validate experimental designs. Major perspectives in the field will consist in integrating multiscale datasets (e.g., genomic, epigenomic, transcriptomic, and proteomic) to incorporate transcriptional informations with epigenetic regulation and chromatin accessibility. Since stem cells are strictly controlled by supportive niche cells, integrating the molecular identities of stem cells and surrounding cells also constitutes an immense challenge. We are confident that systems biology approaches using mathematical, statistical and physical tools and concepts to analyze the experimental data will be instrumental to provide the dynamics of the crosstalk between stem cells and their niches in development and diseases, to establish predictive models of stem cell behavior, and to point out molecular hubs that may be promising targets for drug development.

## **Therapeutic Applications of Stem Cells**

Clinical applications of stem cells have been, and still are, a major boost for stem cell research. Many aspects have been treated throughout this book. We will in this last chapter try and outline the main challenges that therapy using stem cells have to face today.

Transplantation of HSCs has been a remarkable success story. Over time the sources of HSCs have evolved: firstly bone marrow, then mobilized circulating blood and more recently cord (placental) blood. The indications have increased, from treatment of hematopoietic malignancies to treatment of solid tumors. In any case the procedure has been to induce an aplastic condition through chemo and radiotherapy (conditioning), then to provide the HSCs, via the intra-venous route, to reconstitute the blood system of the recipient. Grafts are either allogeneic, HSCs being recovered from an HLA-compatible donor (intra-familial or unrelated), or autologous, when HSCs are collected from the patient himself before conditioning. The major challenges today are to amplify *ex vivo* HSCs, a desirable procedure for cord blood transplantation in the adult, and to generate HSCs from pluripotent stem cells, which would be highly beneficial in patients without HLA-compatible familial donor since the clinical outcome of patients having received unrelated grafts remains of relatively poor prognosis. There is no satisfactory, and therefore standardized, procedure to amplify by culture a cell population containing substantial amount of repopulating HSCs with maintained self-renewal capacity. Although major achievements have been made through cell reprogramming (Lis et al., 2017; Sugimura et al., 2017), generating functional HSCs from ES cells or induced pluripotent stem (iPS) cells still constitutes a major issue.

The second success story of stem cell-based therapy has been the transplantation of autologous skin grafts in heavily burnt patients. One problem is to recover enough intact skin for culture in patients with extensively burnt skin surface. One way to circumvent this problem is to generate HLA-compatible skin equivalents from ES or iPS cells. This is presently feasible but still represents a major biological and logistical challenge.

Whether therapy using MSCs will be comparably successful, and in which indications, has to be definitively demonstrated. As already emphasized in chapters 1 and 23, there are many indications for such cell therapy, in orthopedics and rheumatology (e.g., bone fractures with wide bone defect, osteoarthritic cartilage lesions in the elderly), in cardio-vascular diseases (e.g., heart infarct, ischemia of the lower limbs), in hematology (e.g., double HSC and MSC transplantation in aplastic patients with poor engraftment prognosis), in immunological diseases (e.g., Crohn's bowel disease, graft-versus-host disease [GvHD] post-allogeneic transplantation, systemic sclerosis). Some of the indications (such large fractures, osteoarthritis, and GvHD), are beyond proof of concept and demonstration of feasibility, and await phase 2 and 3 trials for proof of efficacy in well-defined patient populations. However, besides efficacy, which may be due to MSC properties unrelated to stemness, there are many logistical problems to solve. The cost of the culture procedure is also a major issue due to the absolute requirement of Good Manufacturing Practices with all required controls of bacterial and viral non-contamination and of lack of potentially malignant clone selection. One solution would be to use a universal allogeneic donor, which would alleviate the procedure and its cost, MSCs being ready off-the-shelf when necessary. This is possible due to the relative immune tolerance of, if not immune suppression induced by MSCs. Another possibility would be to recover bioactive material from the culture supernatant since MSCs are known to release extracellular vesicles enriched in regulatory molecules such as microRNAs. The development of the different strategies will depend on

bio-technological improvements and, of course, proof of concept in pilot studies.

As underlined in chapter 1, the use of other adult tissue stem cells encounters many difficulties. Thus, two major strategies have been envisioned. The first is the generation of ES cell lines that could be differentiated to appropriate lineages before administration to the patient. This strategy is difficult to implement. The procurement of ES cells raises ethical and regulatory issues (see chapter 26). Allogeneic grafting implies the development of banks of cell lines with HLA group diversity representative of the general population. Cells from lines have to be completely differentiated lest residual pluripotent stem cells generate teratomas in the recipient. The second strategy is to use a population of autologous somatic cells reprogrammed into stem cells secondarily differentiated into appropriate lineage before infusion to the patient. The development of iPS cells renders such strategy an attractive possibility. However, as emphasized in chapters 8, 21, 23, and 24, indications have to be carefully selected and the number of hurdles that should be overcome remains considerable. Two situations appear more readily to lead to successful applications. As indicated in chapter 21, one situation is the generation of retinal cells from iPS cells. These cells can be locally implanted without fear of dissemination in the organism, and, most importantly, the clinical outcome of the graft can be closely monitored. As indicated in chapter 24, the other situation is the generation from iPS cells of red blood cells. The procedure has several advantages. The process of differentiation is well known and can be standardized. The graft is safe since consisting in anucleated terminally differentiated red blood cells. A large panel of red blood cell types can be generated, representing the diversity of red blood cell groups. However, whether it is possible to generate, at reasonable cost, amounts of cells large enough to satisfy the requirement of red blood cell transfusions in many patients remains to be demonstrated. A last point to mention is the direct transdifferentiation of a somatic cell type into another, which constitutes an alternative to the iPS cell strategy noticeably attractive due

to the generation of appropriate cells in short spans of time without the risk of intervening stem cells. However, the procedure is still far from being standardized and adapted to clinically-safe conditions. Finally, the reprogramming technologies and cellular differentiation strategies will also benefit from novel advances in the field of bioengineering, 3D cultures and biomaterials. These achievements should help stem cell biologists to better mimic the architecture and physical properties of a given tissue *ex vivo* and improve the production of specific functional cell types.

In conclusion, the reader should be aware of the remarkable clinical potential of stem cell-based therapy, but also of the no less remarkable difficulties that such therapy entails, since societal, ethical, and economical issues compound the biological hurdles. However, it may be hoped that a rigorous and perseverant approach, as exemplified by that of Donnal Thomas and collaborators, may lead to novel clinical applications as effective as the transplantation of HSCs.

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