Developmental Biology 430 (2017) 142–155

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Original research article

Wnt signaling positively regulates endothelial cell fate specification in the Fli1a-positive progenitor population via Lef1

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ARTICLE INFO

Keywords:
Primitive hematopoiesis
Vasculogenesis
Wnt reporter
Mesoderm
Zebrafish

ABSTRACT

During vertebrate embryogenesis, vascular endothelial cells (ECs) and primitive erythrocytes become specified within close proximity in the posterior lateral plate mesoderm (LPM) from a common progenitor. However, the signaling cascades regulating the specification into either lineage remain largely elusive. Here, we analyze the contribution of β-catenin dependent Wnt signaling to EC and erythrocyte specification during zebrafish embryogenesis.

We generated novel β-catenin dependent Wnt signaling reporters which, by using destabilized fluorophores (Venus-Pest, dGFP), specifically allow us to detect Wnt signaling responses in narrow time windows as well as in spatially restricted domains, defined by Cre recombinase expression (*Tg(axin2$_{BAC}$:Venus-Pest)^{mu288}*; *Tg(14TCF:loxP-STOP-loxP-dGFP)^{mu202}*). We therefore can detect β-catenin dependent Wnt signaling activity in a subset of the Fli1a-positive progenitor population. Additionally, we show that mesodermal Wnt3a-mediated signaling via the transcription factor Lef1 positively regulates EC specification (defined by *kdrl* expression) at the expense of primitive erythrocyte specification (defined by *gata1* expression) in zebrafish embryos.

Using mesoderm derived from human embryonic stem cells, we identified the same principle of Wnt signaling dependent EC specification in conjunction with auto-upregulation of LEF1.

Our data indicate a novel role of β-catenin dependent Wnt signaling in regulating EC specification during vasculogenesis.

1. Introduction

To supply the body with oxygen and nutrients, vertebrates developed the cardiovascular system consisting of the heart, the vasculature, and the blood cells. All these components are mesoderm-derived structures, and their development is tightly linked to each other. Especially vascular endothelial cells (ECs) and primitive hematopoietic lineages arise in close proximity to each other during the first stages of embryonic development (Palis and Yoder, 2001; Risau and Flamme, 1995). The existence of a progenitor cell population with the capability to give rise to either of the lineages has been shown *in vitro* and *in vivo* (Choi et al., 1998; Haferkamp et al., 2004; Vogeli et al., 2006). Furthermore, ECs and hematopoietic cells initially share expression of many genes, including *Etv2/ets1-related protein* (Sumanas and Lin, 2006), *fli1a*, *lmo2*, *gata2* (Thompson et al., 1998) and *tal1* (Gering et al., 1998). Additionally, some mouse gene knockouts (e.g. Flk1 (Shalaby et al., 1995), Etv-2 (Lee et al., 2008)) as well as zebrafish mutants *cloche* (Reischauer et al., 2016; Stainier et al., 1995) and *mirinay* (Jin et al., 2007) result in disappearance of ECs and hematopoietic cells.

In the zebrafish embryo, ECs and primitive hematopoietic cells become specified within the lateral plate mesoderm (LPM), with the anterior LPM giving rise to ECs and macrophages and the posterior LPM to ECs and primitive erythrocytes. ECs are specified once they express the *vascular endothelial growth factor receptor (vegfr) 2* ortholog *kdrl*, while primitive hematopoiesis is regulated and indicated

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http://dx.doi.org/10.1016/j.ydbio.2017.08.004
Received 22 February 2017; Received in revised form 3 August 2017; Accepted 3 August 2017
Available online 12 August 2017

by the expression of *pu.1* in macrophages (anterior LPM) and of *gata1* in erythrocytes (posterior LPM) (Jagannathan-Bogdan and Zon, 2013). Within the posterior LPM, specification of *kdrl*-positive ECs and *gata1*-positive erythrocytes can be observed from 11.5 h post fertilization (hpf) or 4–5 somite stage (ss) onwards in two parallel stripes along the anterior-posterior axis (Long et al., 1997). From 14 hpf both cell populations migrate in close association towards the midline, where ECs form the major axial vessels (Helker et al., 2015). It was suggested previously, that Notch signaling negatively regulates the EC number while promoting generation of primitive erythrocytes and thereby acts as molecular switch during cell fate specification (Lee et al., 2009). However, regulators promoting EC specification from multipotent progenitors have not been identified.

β-catenin dependent Wnt signaling regulates numerous processes in the developing vertebrate embryo (Clevers and Nusse, 2012; Loh et al., 2016). During gastrulation, it has been shown to be necessary for mesoderm induction (Liu et al., 1999; Martin and Kimelman, 2012). For β-catenin dependent signaling, binding of a Wnt ligand to Frizzled receptors results in disassembly of the Axin/Gsk3β/CkI/Apc destruction complex and thereby enables accumulation of β-catenin and translocation into the nucleus. As a result, β-catenin can bind to transcription factors of the Tcf/Lef family (TCF1/Tcf7, TCF3a/Tcf7l1a, TCF3b/Tcf7l1b, TCF4/Tcf7l2 and LEF1/Lef1) and induce target gene transcription (Angers and Moon, 2009; Behrens et al., 1996). One of the universal Wnt signaling target genes is *axin2* (Leung et al., 2002). Axin2 acts in a negative Wnt signaling feedback loop, as it stabilizes the destruction complex and therefore mediates β-catenin degradation (Behrens et al., 1998). Wnt signaling has been shown to regulate angiogenic blood vessel growth and remodeling (Franco et al., 2009; Reis and Liebner, 2013; Vanhollebeke et al., 2015). But until today, the commitment of Wnt signaling to vasculogenesis has not been elucidated.

In this study we have investigated the role of β-catenin dependent Wnt signaling during EC specification in the developing zebrafish embryo and human embryonic stem (ES) cell differentiation. Our results show for the first time, that ECs specify from Fli1a-positive progenitor population in a Wnt3a-dependent manner via Lef1.

2. Materials and methods

2.1. Zebrafish husbandry and transgenic lines

Zebrafish (*Danio rerio*) embryos were maintained under standard husbandry conditions at 28.5 °C (Westerfield, 1993). Zebrafish lines used were: *Tg(etv2:GFP)*^{ci1} (Proulx et al., 2010), *Tg(fli1a:EGFP)*^{y1} (Lawson and Weinstein, 2002), *Tg(fli1a:dsRed)*^{um13} (Covassin et al., 2009), *Tg(gata1:EGFP)*^{la781} (Long et al., 1997), *Tg(gata1:dsRed)*^{sd2} (Traver et al., 2003), *Tg(hsp70l:dkk1-GFP)*^{w32} (Stoick-Cooper et al., 2007) (referred as *hs:dkk1*), *Tg(hsp70l:wnt8-GFP)*^{w34} (Weidinger et al., 2005) (referred as *hs:wnt8*), *Tg(kdrl:EGFP)*^{s843} (Jin et al., 2005), *Tg(kdrl:mCherry)*^{s896} (Chi et al., 2008) and zebrafish mutants used were *lef1*^{u767} (Valdivia et al., 2011) and *clo*^{m39} (Stainier et al., 1995).

2.1.1. Generation of transgenic fish

Tg(axin2$_{BAC}$*:Venus-Pest)*^{mu288} is based on recombining a Kozak sequence and the Venus-Pest sequence (Aulehla et al., 2008) at the start codon of the *axin2* gene into the BAC clone CH211-66B14 (196 kb) according to the previously described protocol (Bussmann and Schulte-Merker, 2011). For *Tg(14TCF:loxP-STOP-loxP-dGFP)*^{mu202}, two tandem sequences of the 7TCF-BAT promoter motif (Maretto et al., 2003) were cloned upstream of a floxed STOP cassette (loxP-STOP-loxP (Hesselson et al., 2009)) followed by destabilized GFP (dGFP (Dorsky et al., 2002)) in pTol2Dest (Villefranc et al., 2007). The *Tg(fli1a:cre)*^{mu225} line was generated by cloning the Cre recombinase gene downstream of the *fli1a* promoter using pTol2fli1epDest (Villefranc et al., 2007). Specificity of Cre expression and recombination efficiency was tested by *in situ* hybridization and mating to recombination reporter lines. Transgenesis was performed as previously described (Helker et al., 2013).

2.2. Microinjections

Morpholino (MO) - microinjections were performed as previously described (Nasevicius et al., 2000). Translation blocking MOs used in this study: *lef1* 5'-CTCCTCCACCTGACAACTGCGGCAT (Dorsky et al., 2002), 2.5 ng injected a single-cell stage; *tcf7* 5'-TGCGGCATGATCCAAACTTTCTCAA, 8 ng injected, *tcf7l1a* 5'-CCTCCTCCGTTTAACTGAGGCATGT, 8 ng injected; *tcf7l1b* 5'-GGGCTTGTCTTGCAAACGGTCACAA, 5 ng injected; *tcf7l2* 5'-CTGCGGCATTTTTCCCGAGGAGCGC, 8 ng injected; *wnt3* 5'-GATCTCTTACCATTCGTCCTGC (Mattes et al., 2012), 8 ng injected; *wnt3a* 5'-GTTAGGCTTAAACTGACACGCACAC (Buckles et al., 2004), 8 ng injected; *wnt8* 5'-ACGCAAAAATCTGGCAAGGGTTCAT and 5'-GCCCAACGGAAGAAGTAAGCCATTA (Lekven et al., 2001), co-injection of 8 ng.

For Cre recombinase expression, the *cre* coding sequence (Hesselson et al., 2009) was cloned into pCS2+ and transcribed into mRNA using the SP6 mMessage mMachine Kit (Ambion). 2 nL of *cre* mRNA (200 ng/μL) were injected at single cell stage.

For transient CRISPR-Cas9 mediated knockdown, annealed template oligonucleotides were transcribed into gRNAs using MEGAshortscript T7 Kit (Ambion)**:** *wnt3a* 5'-AAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGC-CTTATTTTAACTTGCTATTTCTAGCTCTAAAACCCGCCAGAGACCTG-CAGACACCCTATAGTGAGTCGTATTACGC, *cldn5b* (used as control) 5'-AAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGC-CTTATTTTAACTTGCTATTTCTAGCTCTAAAACGGTGACGGTCAAGC-AGAGTCCTATAGTGAGTCGTATTACGC. 2 nL of 500 ng/μL gRNA and 300 ng/μL *nls-zCas9-nls* mRNA (Jao et al., 2013) were injected at single cell stage. Genotyping was performed by PCR amplification using *wnt3a* fwd 5'-CCTGTTCCTTTTTTGTGGG, *wnt3a* rev 5'-GAAACTGATGCTGACACTCCTG and consecutive PstI digest (ON, 37 °C). Digested PCR fragments were analyzed by gel electrophoresis on a 4% TBE-agarose gel. We used NCBI blast to check specificity of the gRNAs and controlled efficiency of the cutting by genotyping 10 pooled embryos.

2.3. Pharmacological and heatshock treatments

Dechorionated zebrafish embryos were incubated from 9 hpf to 18.5 hpf in either 20 μM IWR-1 (Chen et al., 2009), 1 μM BIO (Gore et al., 2011; Meijer et al., 2003) or a combination of 150 μM Aphidicolin and 20 mM Hydroxyurea (AHU). For heatshock induced gene expression, embryos were incubated for 45 min in 39 °C E3 medium at 11 and 13 hpf and analyzed at 18.5 hpf.

2.4. Immunohistochemistry

Immunohistochemistry was performed as described previously (Blum et al., 2008) using rabbit anti–red fluorescent protein (ABIN129578), rabbit anti-GFP-Alexa Fluor 488 conjugated (Invitrogen) and goat anti-rabbit Atto-594 (Sigma-Aldrich).

2.5. Image acquisition and quantification of fluorescent reporter expression (volume) and cell numbers

Fluorescence images were acquired as 3D confocal stacks of the whole tail region of GFP (Leica Sp5 DM 6000 upright) and dGFP or Venus-Pest (ZEISS LSM780 inverted) expressing embryos as previously described (Hamm et al., 2016). Volume quantification of the