Theriogenology 81 (2014) 1275–1285

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Heparan sulfate glycosaminoglycans modulate migration and survival in zebrafish primordial germ cells

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ARTICLE INFO

Article history:
Received 2 September 2013
Received in revised form 9 January 2014
Accepted 6 February 2014

Keywords:
Heparan sulfate proteoglycan
Primordial germ cell
Chemokine
Migration
Survival

ABSTRACT

Early in embryonic development, primordial germ cells (PGCs) are specified and migrate from the site of their origin to where the gonad develops, following a specific route. Heparan sulfate glycosaminoglycans (HS-GAGs) are ubiquitous in extracellular matrix and the cell surface and have long been speculated to play a role during the migration of PGCs. In line with this speculation, whole-mount immunohistochemistry revealed the existence of HS-GAGs in the vicinity of migrating PGCs in early zebrafish embryos. To examine the roles of HS-GAGs during PGC migration, zebrafish heparanase 1 (*hpse1*), which degrades HS-GAGs, was cloned and overexpressed specifically in PGCs. The guidance signal for the migration of PGCs was disrupted with the overexpression of *hpse1*, as cluster formation and marginal localization at the blastoderm were significantly perturbed at 6 hours postfertilization. Furthermore, the number of PGCs was significantly decreased with the lack of vicinal HS-GAGs, as observed in the whole-mount *in situ* hybridization and quantitative PCR of the PGC marker gene *vasa*. Terminal deoxynucleotidyl transferase dUTP nick-end labeling indicated significantly increased apoptosis in PGCs overexpressing hpse1, suggesting that HS-GAGs contribute to the maintenance of PGC survival. In conclusion, HS-GAGs play multifaceted roles in PGCs during migration and are required both for guidance signals and multiplication of PGCs.

1. Introduction

As the founders of the germ cells, primordial germ cells (PGCs) are specified early during embryonic development while retaining the genomic totipotency to pass the genetic materials from one generation to the next. In all vertebrates, PGCs migrate, following specific routes, from their sites of origin to the gonadal ridges where gonads develop soon after they are specified. In zebrafish, germplasm mRNA *vasa* can be detected in the edge of cleavage furrows at the two-cell stage, and four PGCs can be identified as early as the 32-cell stage [1]. Beginning at the 1000-cell stage, several rounds of mitosis result in the increase of PGCs and this multiplication is sustained slowly during the epiboly of the embryos, while the PGCs converge from ventral toward dorsal along the vegital border of the embryo and become aligned at the border of trunk mesoderm [1,2]. By the 20-somite stage, most of the PGCs reach the gonadal ridge where they later differentiate into germ cells [1,2].

It has long been an important and intriguing question as to the mechanisms that regulate PGC migration. Steel factor and its receptor c-kit were identified as important players in both PGC migration and survival [3,4], although their roles in PGCs are not conserved in zebrafish [5]. The interactions between cadherin, integrin, and extracellular matrix (ECM) were then confirmed as important regulators in both mouse and zebrafish [6,7]. Because the PGC migration route in avian is partially through the blood stream, chemoattractant cytokines were speculated to play a role as guidance cues. Accordingly, chemokine and chemokine receptors Cxcr4,

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0093-691X/$ – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2014.02.009

Cxcr7, and Sdf1 (Cxcl12) were found to play a key role as the common homing mechanism for PGC migration into the gonadal ridges throughout all examined vertebrate animals [8–13]. These data suggested that vertebrate animals share highly conserved molecular machineries in regulating PGC migration with few variations.

Before arriving at the gonadal ridge, PGCs are not present as independent tissue, but remain closely associated with surrounding somatic/extraembryonic cells, which implies that PGCs require nutrients and developmental signals for their totipotency, survival, and migration. Isolated PGCs cannot survive in cell culture long and undergo apoptosis [14]. It is therefore reasonable to speculate that extracellular matrix and cell surface signals play important roles in PGC development. Heparan sulfate proteoglycans (HSPGs) are glycoproteins comprising a core protein and one or more covalently attached heparan sulfate glycosaminoglycans (HS-GAGs). Heparan sulfate proteoglycans are found in ECM and the cell surface, and evidence showed that they are involved in a wide range of biological activities through their HS-GAGs chains. During biosynthesis, the monomers of HS-GAGs chains can be modified by epimerization, deacetylation, and various sulfations, which may result in a spectrum of sugar code sequences and various motifs; hence, HS-GAGs are able to interact with diverse ligands [15–18].

Previous study revealed the existence of GAGs and proteoglycans in the PGC migratory pathway [19]. Furthermore, *in vitro* studies suggested that Cxcl12/Cxcr4 signaling is regulated by HSPGs through the GAG side chains, and specific sugar motifs might play a role in this interaction [20,21]. In this study, we aimed to evaluate the role of HS-GAGs in PGCs during their migration, initially by providing correlative evidence that HS-GAGs do exist in the vicinity of migrating PGCs. In addition, by the overexpression of zebrafish heparanase 1 (*hpse1*) specifically in PGCs, we provide evidence indicating that HS-GAGs play roles in the localization, guidance, and survival of PGCs.

2. Materials and methods

2.1. Fish maintenance

The AB strain wild-type zebrafish and the transgenic zebrafish line Tg(kop:EGFP-F-nanos1-3'UTR) (a generous gift from Dr. Erez Raz) were housed at a density of two to four fish per 3-L tank in the aquatic facility with an automatic recirculation system. The system was maintained at 28.5 °C with a light/dark cycle of 14/10 hours, and the fish were fed with live adult brine shrimp twice a day [22]. Embryos were collected after spontaneous spawning, allowed to develop and staged by hour postfertilization (hpf) at 28.5 °C using morphological criteria [23]. All experimental procedures in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of National Taiwan University (NTU-101-EL-49).

2.2. Whole-mount immunohistochemistry

Whole-mount immunohistochemistry was performed as previously described [24]. Briefly, embryos were dechorionated, fixed in 4% paraformaldehyde (PFA; Sigma–Aldrich, St Louis, MO, USA) in PBS (Life Technologies, Carlsbad, CA, USA), and stored in 100% methanol (Sigma–Aldrich) at −20 °C for at least overnight. After serial washing with 0.1% Tween-20 in PBS (PBST; Sigma–Aldrich), embryos were blocked with 10% goat normal serum (Abcam, Cambridge, UK) in PBST for 1 hour at room temperature with agitation and then probed with monoclonal antibody 10E4 (1:50; US Biological, Salem, MA, USA) for 2 hours at room temperature. After washing five times with PBST, embryos were blocked and incubated with goat anti-mouse IgM antibody conjugated with Alexa-Flour 555 (1:400; Abcam) for 1 hour at room temperature. After washing five times with PBST, the embryos were documented analyzed using a fluorescent microscope (Leica DM2500).

2.3. In silico analysis of zebrafish hpse1

Zebrafish *hpse1* (ENSDART00000093155; Ch21) and orthologs from human (ENST00000405413; Ch4), chicken (ENSGALT00000038004; Ch4), mouse (ENSMUST00000045617; Ch5), and *Xenopus* (ENSXETT00000038861; Scaffold GL 172850.1) were compared for genomic structures. Amino acid sequence alignment and a phylogenetic tree were generated using neighbor-joining method with BLOSUM62 in the software Jalview2 [25].

2.4. Molecular cloning and microinjection

The full-length coding sequence of zebrafish *hpse1* was obtained by PCR with denaturation at 94 °C for 2 minutes, 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 60 °C for 1 second, extension at 72 °C for 30 seconds, and final extension at 72 °C for 7 minutes, and cloned into pJET1.2/blunt cloning vector (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instruction. The coding sequence of *hpse1* was then subcloned into the pT7-IRES2-EGFP vector to generate pT7-hpse1-IRES2-eGFP [26]. To specifically express *hpse1* in PGCs [27], the sequence of *nanos1* 3′-untranslated region (3′-UTR) was obtained by PCR with denaturation at 94 °C for 2 minutes, 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 60 °C for 1 second, extension at 72 °C for 50 seconds, and final extension at 72 °C for 7 minutes, and introduced into pT7-hpse1-IRES2-eGFP at NotI cutting site to generate pT7-hpse1-IRES2-eGFP-nanos1-3′UTR. The vectors without *hpse1* coding sequence, pT7-IRES2-eGFP and pT7-IRES2-eGFP-nanos1-3′UTR, served as empty vector controls.

To avoid the redundant fluorescent signal in Tg(Kop:EGFP-F-*nanos1*-3′UTR) transgenic line, the cistronically regulated cassette of IRES2-eGFP was removed from pT7-hpse1-IRES2-eGFP-nanos1-3′UTR plasmid to generate the pT7-hpse1-nanos1-3′UTR plasmid. All primer sequences used in this study are listed in Table 1.

To synthesize mRNA by T7 RNA polymerase, the plasmids were linearized by AflII (New England Biolabs, Ipswish, MA, USA), mRNA for microinjection was prepared using mMessage mMachine T7 kit (Life Technologies) according to the manufacturer's instruction. The synthesized mRNA