Sparc (Osteonectin): new insight into the function and regulation

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Tesis Doctoral

Eva Torres Núñez

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Memoria presentada por
Eva Torres Núñez

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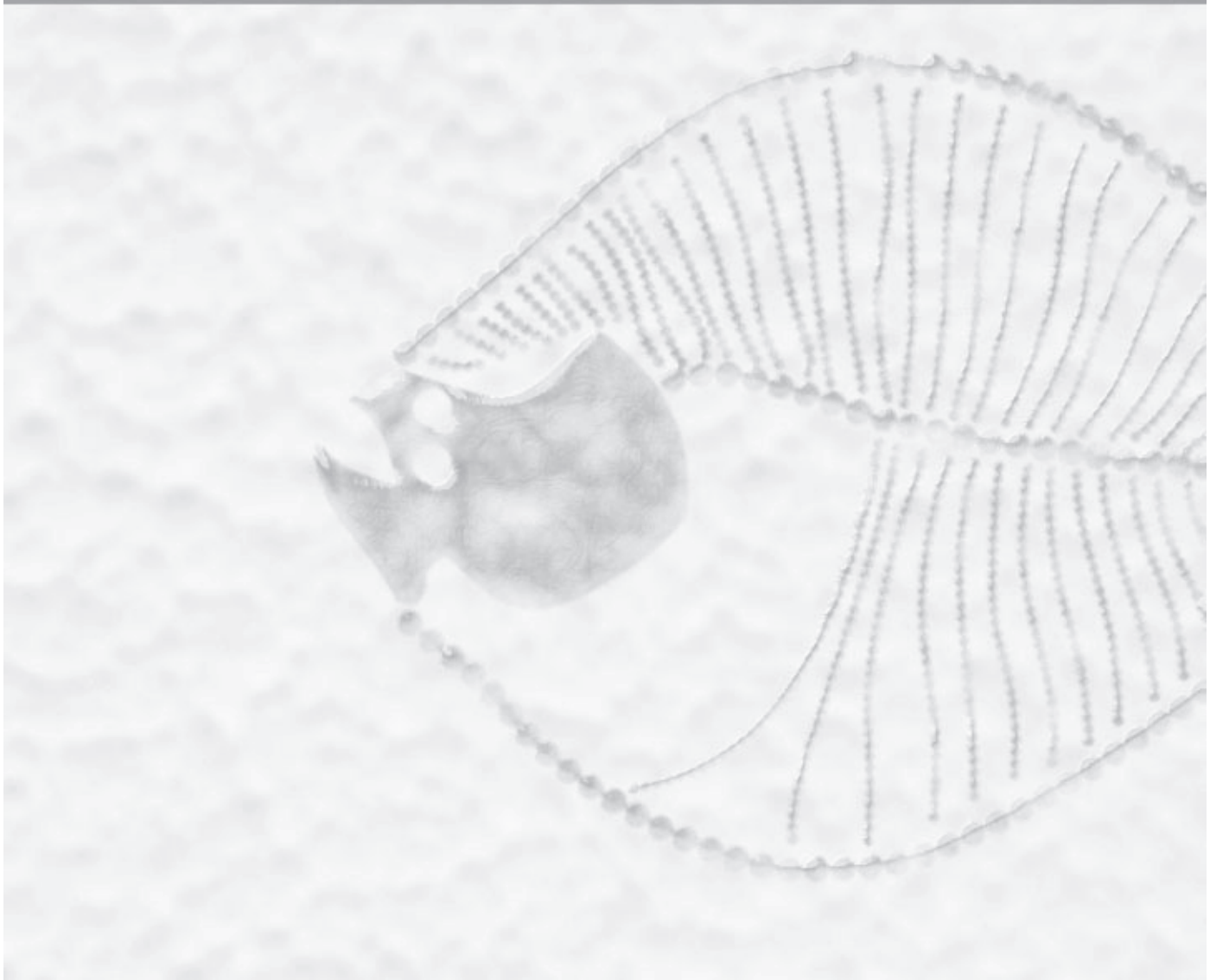

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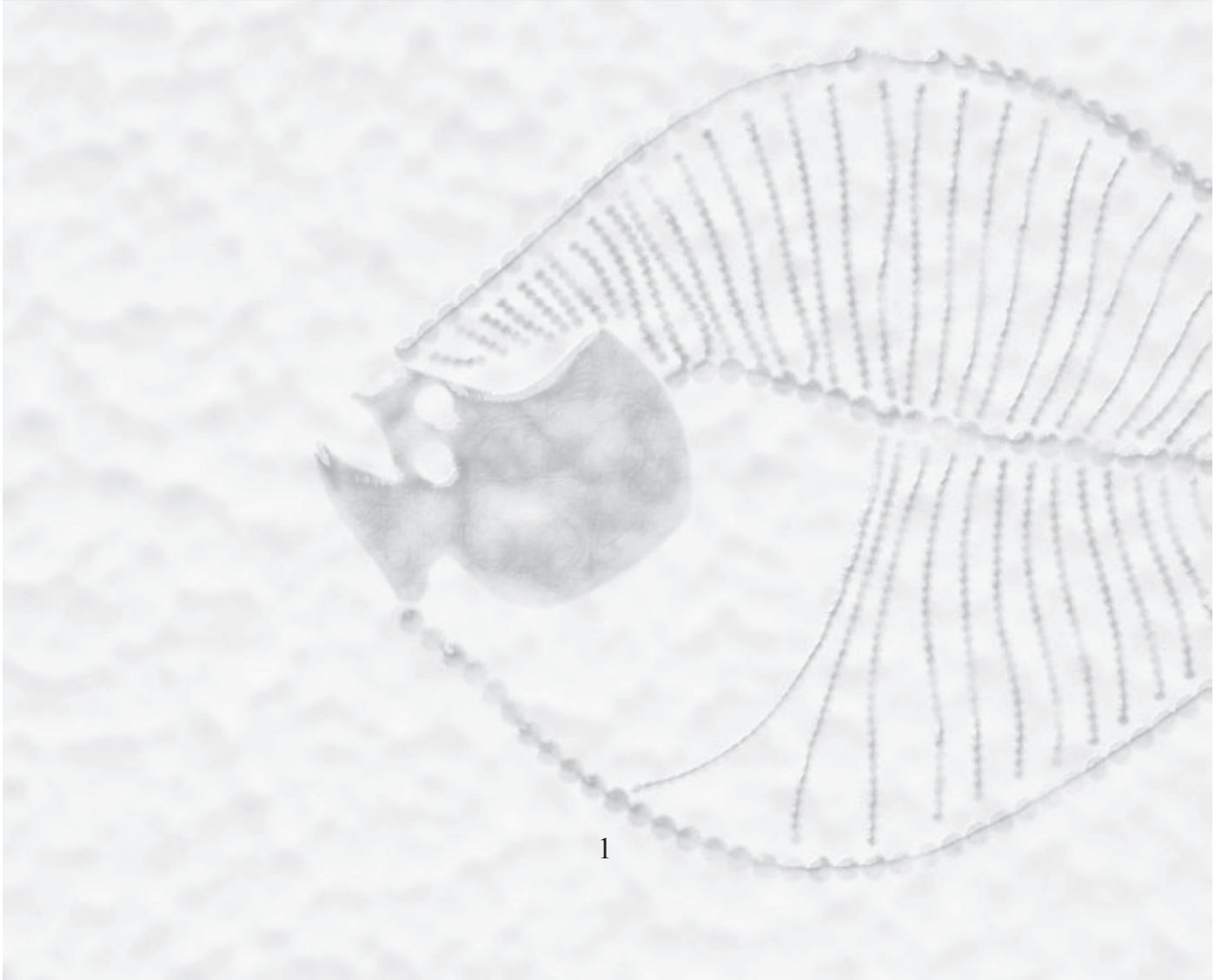
Prologue



This PhD research was conducted at the **Aquatic Molecular Pathobiology Laboratory,** Marine Research Institute (IIM-CSIC, Vigo), supervised by Dr. Josep Rotllant.
PhD thesis is ascribed to the Program of Aquaculture, Department of Physiology and Immunology, Faculty of Biology, University of Barcelona (UB) during the years 2009-2013.

The aim of this work was to characterize novel functions of Sparc (Osteonectin) and its transcriptional regulatory mechanisms in fish.

Therefore, the present PhD thesis is structured in four chapters that correspond to four different scientific papers:

- Chapter I: Ceinos, R.M., Torres-Núñez, E., Chamorro, R., Novoa, B., Figueras, A., Ruane, N.M. and Rotllant, J. (2013) **Critical role of the matricellular protein Sparc haematopoiesis in mediating erythroid progenitor cell development in zebrafish**. Cell Tissue Organs, 197: 196-208.

- Chapter II: Torres-Núñez, E, Sobrino, C., Neale, P.J., Ceinos, R.M., Du, S.J. and Rotllant J. (2012) **Molecular response to ultraviolet radiation exposure in fish embryos: implications for survival and morphological development.** Photochemistry and Photobiology, 88:701-707.

- Chapter III: Torres-Núñez, E., Cal-Delgado, L., Morán, P. and Rotllant, J. **5′-UTR intron is crucial for transcriptional regulation of the zebrafish *sparc* (*osteonectin*) gene.** In preparation.

- Chapter IV: Torres-Núñez, E., Ceinos, R.M., Cal, R., Cerdá-Reverter, J.M. and Rotllant, J. **Stage-specific expression of *sparc* during flatfish post-embryonic remodeling**. In preparation.

Part of results derived from the present thesis has been presented at the 9th International Congress on the Biology of Fish, Barcelona, Spain (July 2010).

A Research stay of four months (August 2011) at the California Institute of Technology was carried out during this thesis under the supervision of Dr. David Prober and several collaborations were made with the Smithsonian Environmental Research Center (Edgewater, MD), University of Vigo and Spanish Institute of Oceanography of Vigo.

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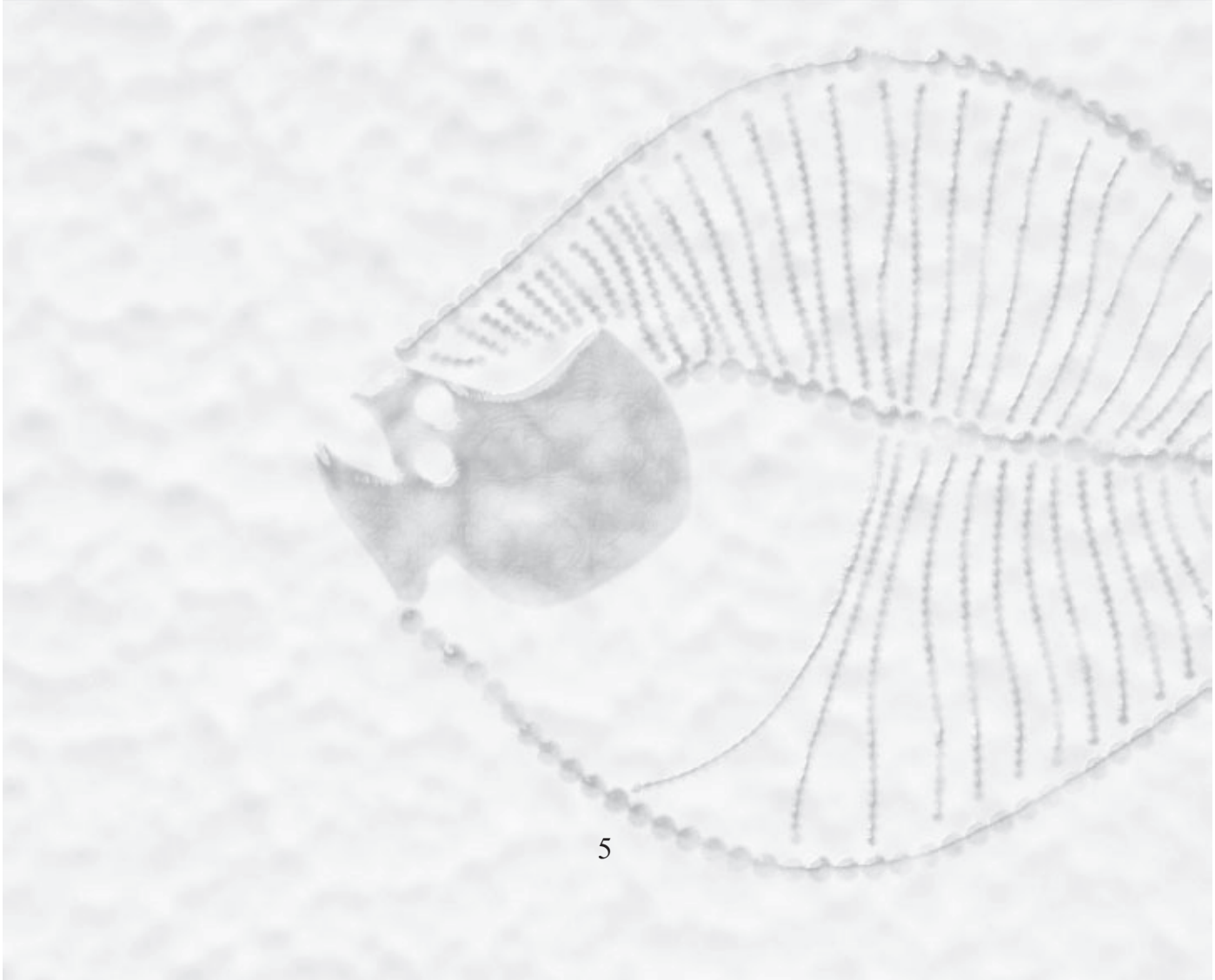
General Introduction



The Extracellular Matrix (ECM) is a complex network secreted by cells that serves as a structural element in tissues and also influences their development and physiology (Alberts *et al*., 2002). More specifically, the extracellular matrix helps cells to bind together and regulates several cellular functions such as adhesion, migration, proliferation and differentiation (Teti, 1992). It is composed of growth factors, proteoglycans, structural proteins and matricellular proteins.

Osteonectin, also named Sparc (Secreted Protein Acidic and Rich in Cysteine) or BM-40 (Membrane Protein-40), is a multifunctional glycoprotein that belongs to the matricellular protein family. This group modulates matrix-cellular interactions and takes part in several cell functions, rather than playing a role in the cell structure (Brekken and Sage, 2000). Sparc is known to have high affinity with calcium ions and was first discovered as the major component of ECM in mineral tissues, although it has since been located in many other tissues. Sparc expression is high during early development but remains low in adult life. However, it is expressed in tissues under renewal, tissue repair or tumorigenesis (Yan and Sage, 1999). Since Sparc is able to interact with multiple molecules, many important functions have been attributed to this protein, including counteradhesion, the regulation of cell proliferation and angiogenic activity (Yan and Sage, 1999).

STRUCTURE

In general, the structure of *sparc* gene has been conserved among species during evolution with some minor differences. In mammalian, *sparc* gene is composed of 10 exons (Lane and Sage, 1994). The first two exons contain the 5'UTR and the signal cleavage site while exon 10 encodes the last eight aminoacids of the Sparc protein and 3'UTR. This genomic structure is also shared with *Xenopus* and *Oryzias latipes* (Damjanovski *et al*., 1998; Renn *et al*., 2006), although the nematode *C.elegans* lacks exons 1, 3 and 10 while exons 6 and 7 are fused (Schwarzbauer and Spencer, 1993).

A table summarizing the variations in full transcript size in different species is shown below (Table 1).

Species	cDNA size	5'UTR (bp)	cds (bp)	3'UTR (bp)
H.sapiens	2133	57	912	1164
B.taurus	2141	54	915	1172
R.norvegicus	2019	68	906	1045
G.gallus	1009	43	897	69
X.laevis	1615	58	903	654
D.rerio	1359	138	876	345
O.mykiss	1431	85	903	443
D.melanogaster	1170	109	915	146
A.franciscana	1083	51	876	156
C.elegans	1109	18	795	296

Table 1. Comparison of *sparc* full transcripts among species (adapted from Redruello *et al.,* 2005)

Moreover, the mammalian *sparc* promoter lacks the classical TATA box but contains GCA boxes as well as cAMP, heat shock and glucocorticoid-response elements. However, a TATA box has been identified in *Xenopus* (Damjanoski *et al.,* 1998).

Although *sparc* is a single-copy gene in most species, four orthologs were found in the diploblastic *Nematostella vectensis* (nvSparc 1, 2, 3, and 4) (Koehler *et al*., 2009). In

addition, the only triploblastic organism with more than one copy is *Petromyzon marinus* (-A and –B) (Kawasaki *et al*., 2007).

Sparc is mapped to human chromosome 5 (Hsa5) and in linkage group (chromosome) 14 (LG14) in zebrafish. *Sparc* and two of the three closest neighbors on one side are arranged in the same order, thus demonstrating conservation of this chromosome segment in both lineages from the last common ancestor of zebrafish and humans (Fig.1). The human *ATOX1* gene does not appear to have an ortholog in the zebrafish genome (Zv7), a finding that demonstrates the interest of studying *sparc* in zebrafish due to the high degree of conservation between both species.

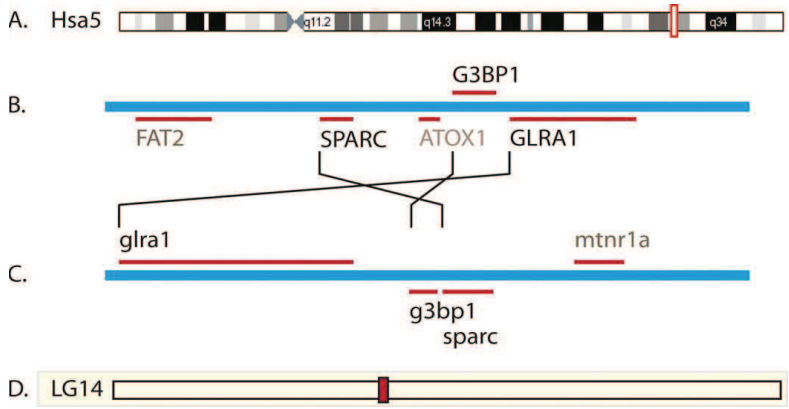


Figure 1. Conserved syntenies confirm the orthology of zebrafish *sparc* and human *sparc* genes. **A**. Human chromosome 5 (Hsa5) with the location of *sparc* boxed in red and expanded in **B**. **C**. the *sparc* containing region of the zebrafish genome, which resides in linkage group (LG) 14, in the red boxed region in **D** (Rotllant *et al*., 2008)

The human 32K-Da Sparc protein consists of a signal peptide containing 17 aminoacids, an N-terminal domain (I) that comprises 50 amino acid residues, 18 of which are negatively charged, followed by a Follistatin-like domain (II) with 10 cysteines in a typical pattern and an Extracellular calcium-binding domain (III) with two EF-hand calcium binding motifs, each with a bound calcium in the X-ray structure.

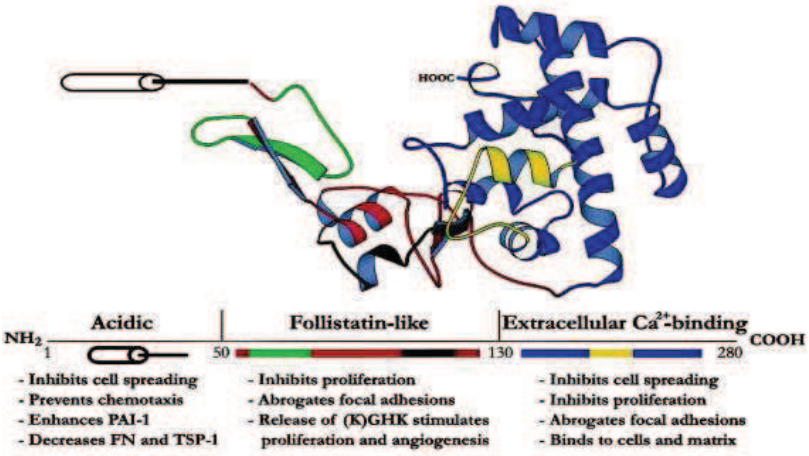


Figure 2. Structure of human Sparc. The Follistatin-like domain is shown in red except for peptide 2.1, aa 55-74, and the (K)GHK angiogenic peptide, aa 114-130, which are shown in green and black, respectively. The EC-module is shown in blue except for peptide 4.2, aa 255-274, which is shown in yellow. PAI-1: plasminogen activator inhibitor; FN: fibronectin; TSP-1: thrombospondin (Brekken and Sage, 2000)

In detail, 286 residues are divided into 3 regions:

- **Domain I/module I aa 3-51** is encoded by exons 3 and 4. It is highly acidic and sensitive to changes in calcium concentration. This NH_2-terminal binds to calcium with low affinity and interacts with hydroxyapatite regulating mineralization processes.

- **Domain II/module II aa 52-132** is encoded by exons 5 and 6. The Cys-rich sequence (10 cysteines) encodes a structure homologous to a repeated domain in follistatin (FS domain). The proteolysis of Sparc generates several bioactive peptides with different properties to the intact protein. In particular, peptide 2.1 inhibits the proliferation of endothelial cells, while peptide (K)GHK stimulates endothelial proliferation and angiogenesis.

- **Domain III/module III aa 133-285** is encoded by exons 7-9. It constitutes the extracellular Ca^{+2} binding (EC) part and contains two EF-hand motifs. Collagen I,

III, IV and V are also bound to this domain. This domain contains the peptide 4.2, which has the capacity to bind to endothelial cells and inhibit their proliferation.

The Sparc protein structure is a highly conserved among all vertebrates but the percent identity is lower in invertebrates (Table 2). The cause of this dissimilarity is to be found in the highly variable domain I. In ancient species such as *C. elegans,* where mineralized tissues do not exist, the number of acidic residues is approximately 35% lower than in mammalian Sparc.

	Ci	Bm	Dm	Dy*	Ce	Af	Ag	Mm	Rn	Cf	Mmu	Hs	Ss	Bt	Oc	Gg	Cc	Ts*	Cn*	Es*	Xt	Xl	Rc	Ga	Tr	Tn	Ol	Ip	Dr	Om1	Om2	Ssa1	Ssa2	Sa*	Ca*
Ci																																			
Bm	20																																		
Dm	22	44																																	
Dy*	20	31	96																																
Ce	22	29	33	31																															
Af	20	49	39	30	34																														
Ag	20	45	46	42	31	42																													
Mm	32	21	21	20	28	22	21																												
Rn	32	21	21	20	28	22	21	98																											
Cf	33	21	21	21	28	22	21	92	92																										
Mmu	33	21	21	20	28	22	21	92	91	97																									
Hs	33	21	21	20	28	22	21	92	92	97	100																								
Ss	33	21	21	20	28	22	21	92	91	96	96	96																							
Bt	33	22	21	21	28	22	21	92	92	98	98	99	97																						
Oc	33	22	21	21	28	22	21	90	90	94	94	94	93	95																					
Gg	32	20	21	19	27	21	20	83	84	86	85	85	87	85	84																				
Cc	33	21	21	19	28	22	21	84	85	88	87	87	88	88	86	97																			
Ts*	46	28	31	33	32	29	30	90	90	91	91	91	90	91	91	93	94																		
Cn*	45	28	29	31	31	28	30	90	90	91	91	91	91	91	90	96	96	93																	
Es*	46	29	29	31	30	28	30	89	89	90	90	90	89	89	88	91	91	90	93																
Xt	33	22	20	18	26	21	21	79	79	81	81	81	82	81	79	80	81	89	89	89															
Xl	32	22	20	19	27	22	21	79	79	81	81	81	82	81	80	80	82	89	88	88	96														
Rc	32	22	21	20	26	22	21	77	77	81	81	81	81	80	79	79	79	87	87	86	86	86													
Ga	33	21	20	18	26	20	21	75	76	76	75	75	75	75	74	73	73	83	83	83	77	78	76												
Tr	33	20	20	19	25	20	20	72	73	73	73	73	73	73	71	72	72	82	81	81	73	73	70	81											
Tn	33	21	20	19	26	20	20	74	75	75	74	74	74	75	73	74	74	83	81	81	75	75	73	81	93										
Ol	34	20	20	19	26	22	22	77	78	78	78	78	78	78	77	78	78	85	84	83	77	77	74	82	82	86									
Ip	33	21	22	21	27	23	22	74	74	74	74	74	75	74	73	74	75	83	82	80	75	76	72	78	76	77	82								
Dr	33	21	21	19	27	22	21	76	77	76	75	75	76	75	74	76	76	84	83	81	77	77	72	78	76	79	83	89							
Om1	32	22	21	19	27	20	22	74	75	75	74	74	75	74	73	73	73	82	82	81	74	74	70	78	76	77	79	78	80						
Om2	32	22	21	21	27	21	22	74	75	76	75	75	76	75	74	73	74	84	83	81	73	75	73	80	75	77	78	79	79	94					
Ssa1	32	22	21	20	27	21	22	74	74	74	74	74	74	74	74	73	74	84	83	81	73	74	72	78	76	77	79	78	79	98	94				
Ssa2	32	22	21	21	27	21	22	75	75	77	76	76	76	76	75	74	74	84	83	81	74	76	73	80	76	78	79	80	80	95	99	95			
Sa*	42	22	27	32	23	20	23	84	84	83	84	84	83	83	83	82	83	84	83	83	83	84	81	90	90	91	87	83	86	88	89	88	89		
Ca*	41	24	26	25	27	22	24	80	80	77	77	77	77	77	76	81	80	80	78	77	82	85	77	76	76	81	78	87	92	78	77	78	77	80	

Table 2. Pairwise percent identities among Sparc protein sequences. *White*, invertebrates; *dark grey*, vertebrates; * partial sequences. Ci, *C.intestinalis*; Bm, *B.mori*; Dm, *D.melanogaster*; Dy, *D.yakuba*; Ce, *C.elegans*; Af, *A.franciscana*; Ag, *A.gambiae*; Mm, *M.musculus*; Rn, *R.norvegicus*; Cf, *C.familiaris*; Mmu, *M.mulatta*; Hs, *H.sapiens*; Ss, *S.scrofa*; Bt, *B.taurus*; Oc, *O.cuniculus*; Gg, *G.gallus*; Cc, *C.coturnix*; Ts, *T.scripta*; Cn, *C.myloticus*; Es, *Elaphe sp.*; Xt, *X.tropicalis*; Xl, *X.laevis*; Rc, *R.catesbeiana*; Ga, *G.aculeatus*; Tr, *T.rubripes*; Tn, *T.nigroviridis*; Ol, *O.latipes*; Ip, *I.punctatus*; Dr, *D.rerio*; Om, *O.mykiss*; Ssa, *S.salar*; Sa, *S.aurata* ; Ca, *C.auratus* (Laizé *et al*., 2005)

Recently, a structural difference of Sparc has been discovered between radiata and bilateria. Although the trimodule structure is maintained in all bilaterian species, with minor variations in size (vertebrates *vs* invertebrates), alignment of Sparc including the cnidarian *Nemastotella vectensis* showed that domain I is absent from nvSparc1-4 (Koehler *et al*., 2009) suggesting that domain I was a later addition in Sparc evolution, after the emergence of bilaterians. Due to the variability in domain I among species (or its absence), a phylogenetic tree was created based in FS-EC domains (Fig.3). The Sparc phylogeny is consistent with the accepted taxonomic group, showing 3 divisions, each one of them corresponding to one of the 3 clades: cnidaria, protostomia and deuterostomia.

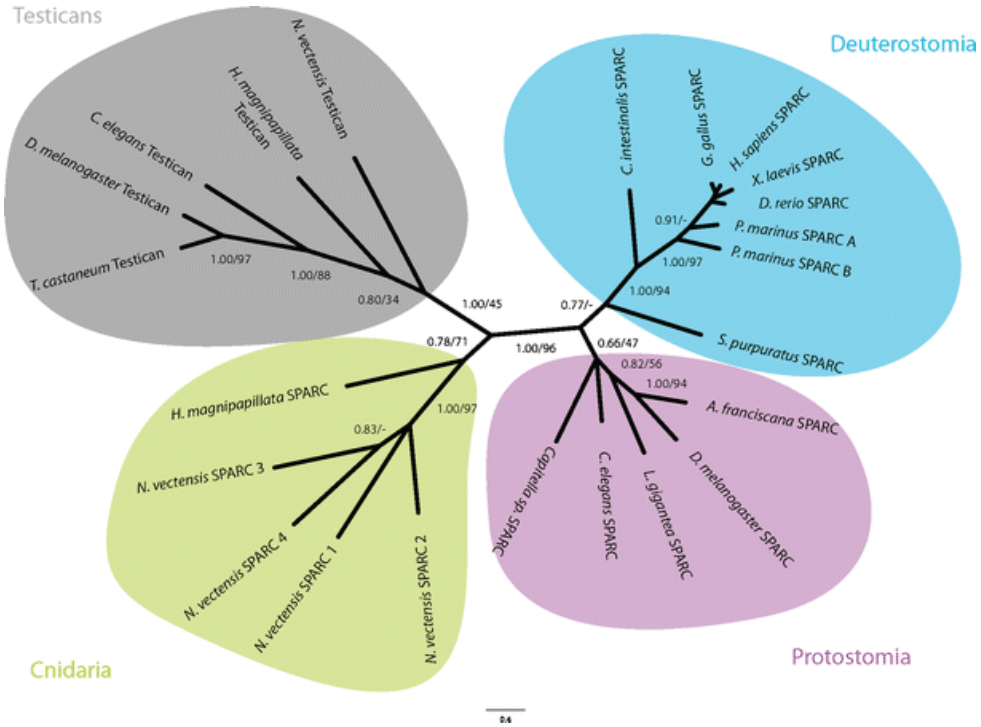


Figure 3. Bayesian phylogeny of metazoan Sparc FS-EC domains, with Testican FS-EC domains included as outgroup sequences (Koehler *et al*., 2009)

As regards the domain structure, Sparc has been included in the Sparc Family-related Proteins, which comprises five proteins that have been grouped together because they share FS and EC domains (Fig.4). These proteins are:

- Hevin/Sparc-like protein (SLP) shares the trimodule structure within Sparc but with an expanded N-terminal domain. Due to the high conservation of its collagen-binding site, hevin is, together with Sparc, the only Sparc-related protein able to bind and modify collagen. It is located mainly in the nervous system and has been proposed as a tumor suppressor and regulator of angiogenesis.

- The human testicular proteoglycan testican/SPOCKs contain a follistatin domain, one thyropin domain and an EF domain. It was originally found in testicles but the highest expression was in brain. It is associated with the regulation of protease activity.

- SMOC-1contains an EC domain common to the Sparc family members with an additional follistatin-like domain, two thyroglobulin-like domains and a novel domain. It was originally located in basement membranes and was also found in gonads and reproductive tract. It acts as a regulator of BMP signaling.

- SMOC-2 acts as an angiogenic stimulator through the binding to VEGF and bFGF and has the same structure as SMOC-1. However, it is found predominantly at non-basement membrane pattern such as heart, muscle, spleen and ovary.

- Fstl-1 (Follistatin like protein-1)/TSC-36. The EC domain is not functional. It acts as a novel pro-inflammatory protein, as a regulator of BMP signaling and as a regulator of homeostatic regulation of somatic sensation.

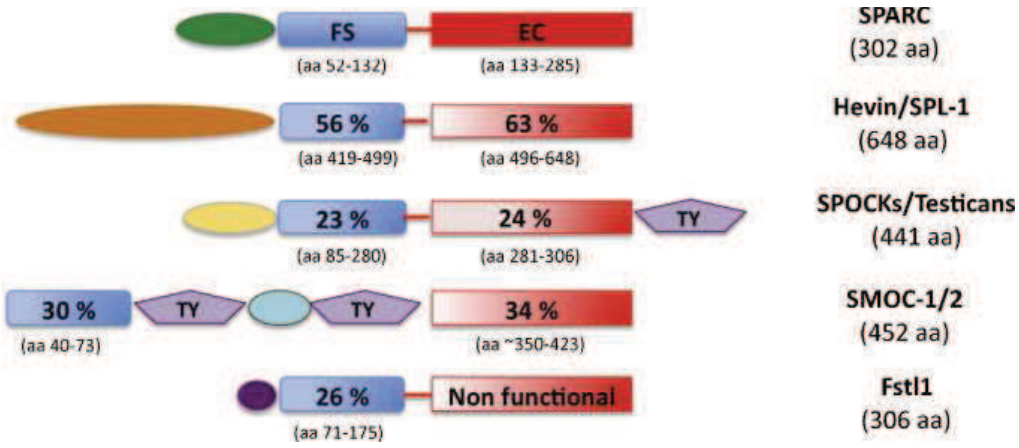


Figure 4. Schematic representation of the modular domain structure of the Sparc family of proteins (Bradshaw, 2012)

EXPRESSION

Sparc is one of the major non-collagenous components localized in the ECM. It was first found in the ECM of mineralized tissues but it is expressed in a variety of locations. Moreover, it is highly expressed during embryogenesis and restricted in the adult to tissues undergoing remodeling, tumorigenesis, wound healing/repair or angiogenesis.

In humans, *sparc* has been found in bone, cartilage, teeth, kidney, gonads, adrenal gland, lung, eye, vessels, liver, meninges and choroid plexys during embryonic and fetal development (Mundlos *et al*., 1992). In adults, *sparc* is also expressed in the intestine (Lussier *et al*., 2001), skin (Hunzelmann *et al*., 1998) and aorta (Hao *et al*., 2004).
In mice embryos, *sparc* was found in the alimentary tract (tongue, oral epithelium, esophagus and small intestine), thymus, skeletal muscle, somites, cartilage, bone, heart, lung and skin (Sage *et al*., 1989). In adults, it was identified in the alimentary tract (tongue, esophagus, stomach and small intestine), glandular tissue (submaxillary gland, parotid gland and mammary gland), marrow, reproductive system and skin.

Sparc is present in the notochord, somites and floor plate in *Xenopus* embryos, (Damjanovski *et al*., 1994). In zebrafish, *sparc* was found during pharyngeal morphogenesis and in the inner ear, notochord, floor plate, fin fold and otic vesicle (Rotllant *et al.,* 2008). Besides, during regeneration of the caudal fin, *sparc* is differentially expressed in this area (Padhi *et al*., 2004). In seabream, *sparc* is abundant in scales, intervertebral disc, vertebrae, caudal rays, branchial arches and opercular bone, whereas neurocranium, brain, gonad and liver have low levels (Redruello *et al.,* 2005; Estêvão *et al*., 2005). During embryogenesis in medaka, *sparc* is expressed in sclerotome, notochord and floor plate. However, in adult life it is present in organs like kidney, heart, gill, spleen, brain and eye (Renn *et al*., 2006). *Sparc* was identified predominantly in the mantle and at low levels in gills and mid-gut of the bivalve *Pinctada fucata* (Miyamoto and Asada, 2011). This location suggests *sparc* may have a role in the construction of the shell.

Sparc is expressed in body wall, pharynx and gonads in *C.elegans* (Fitzgerald and Schwarzbauer, 1998). Finally, the expression of *sparc* genes is restricted to the endoderm during post-gastrula *Nematostella vectensis* development (Koehler *et al*., 2009).

Taken together, these results indicate *sparc* expression is mainly found in skeletal tissues but also in many embryonic and adult tissues that undergo remodeling.

FUNCTION AND REGULATION

Sparc is a multifunctional protein with a high affinity for cations and hydroxyapatite; Sparc provides support to the extracellular matrix and mediates the activities of a wide range of growth factors (Brekken and Sage, 2000). Phenotypic abnormalities revealed by loss-of-function studies also support the interpretation that Sparc functions mainly in cell–matrix interactions (Gilmour *et al*., 1998; Delany *et al*., 2003; Bradshaw *et al*., 2003; Brekken *et al*., 2003; Eckfeldt *et al*., 2005).

Since Sparc binds a high number of different ECM components, growth factors and other molecules, different biological functions are attributed to this protein. From a cellular point of view, Sparc has a wide range of action in ECM organization, migration, proliferation, antiadhesion, differentiation and survival (Bradshaw and Sage, 2001; Delany *et al.,* 2003).

Here, the most important roles of Sparc are explained according to the type of interaction.

a) Interaction with ECM molecules

The binding of Sparc to collagens is the best characterized of these interactions. This binding is modulated by Ca^{+2} ions and implies an alteration in the conformation that leads to a reduction in the susceptibility to proteases and an alteration of its affinity for collagen (Fig.5) (Maurer *et al*., 1995; Bradshaw, 2009; McCurdy *et al*., 2010). Moreover, Martinek *et al*., 2007 suggested a possible intracellular role for Sparc as a conserved chaperone essential for collagen folding in the endoplasmatic reticulum.

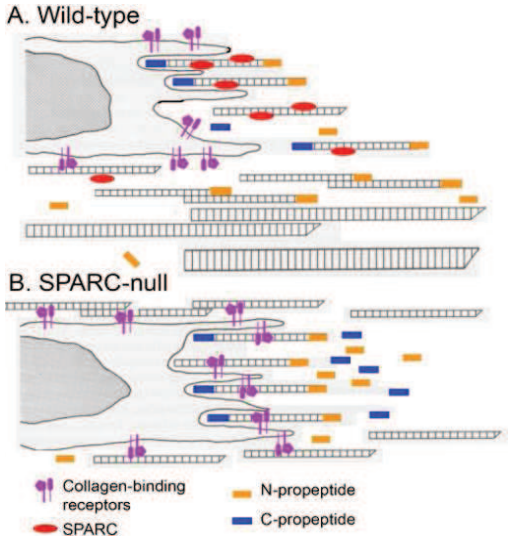


Figure 5. Sparc activity in modulating collagen cell interaction and procollagen processing. In **A**, procollagen fibrils are bound by Sparc, which diminishes collagen engagement by cell-surface receptors. In the absence of Sparc **B**, procollagen interacts with receptors to a greater degree and is tethered to cell surfaces. Sparc-null fibrils fail to aggregate as efficiently as fibrils on wild-type cells. (Rentz *et al.,* 2007; Bradshaw, 2009)

Different studies have showed the affinity between Sparc and collagen I, II, III, IV, V and VIII. The interaction between these two molecules is shown to protect collagen from degradation, for example, in periodontal ligament after LPS treatment (Trombetta and Bradshaw, 2010) but also results in the remodeling of extracellular matrix, which leads to different events such as morphogenesis processes. For example, Vincent *et al*., 2008 first detected *sparc* in regions of the murine developing brain undergoing neurogenesis, the central nervous system, spinal cord, developing blood vessels and in radial glia cells but it is also retained in the adult in places that require a high degree of plasticity/remodeling. In zebrafish, *sparc* is required for pharyngeal cartilage morphogenesis and in the inner ear (Rotllant *et al.,* 2008; Kang *et al.,* 2008). In medaka, the expression of *sparc* appears before ossification in the somites, notochord, floorplate and otic vesicle (Renn *et al*., 2006).

Several defects were observed by using Sparc-null mice like smaller collagen fibrils (Bradshaw *et al*., 2003), disc degeneration (Gruber *et al.*, 2005), cataracts (Gilmour *et al*., 1998), accelerated wound healing (Bradshaw *et al*., 2002), enhanced growth of tumors (Brekken *et al*., 2003), osteopenia (Delany *et al*., 2003) or an increment in adiposity (Bradshaw *et al*., 2003; Nie and Sage, 2009). These defects are associated with changes in the ECM, mainly involving a decrease in the amount of collagen or an incorrect cell differentiation (e.g. osteoblasts).

The overexpression of Sparc in *Xenopus* interferes with tissue morphogenesis through the modification of cell body shape, inhibition of cell migration, proliferation and the incapacity to form focal adhesions (Damjanovski *et al*., 1997; Huynh *et al*., 1999). In addition, diseases such as fibrosis or sclerosis are also caused by the overexpression of Sparc followed by an abnormally high rate of collagen deposition in the ECM (Trombetta and Bradshaw, 2012), which could be restore by inhibition of Sparc (Zhou *et al*., 2006; Atorrasagasti *et al*., 2013).

The Glu-rich sequence in domain I of Sparc was identified as a possible hydroxyapatite-binding site and therefore this site might be related to mineralization processes of different bone tissues. In fact, Fujisawa *et al*., 1996 found that Sparc enhanced the mineralization *in vitro*.

Sparc also regulates the activity of metalloproteinases (Bradshaw, 2012), a family of enzymes that mediate ECM proteolysis and turnover. In some cases, Sparc induces the activation of metalloproteinases, which leads to tumor invasion (Gilles *et al*., 1998; Shen *et al*., 2010). However, Said *et al*., 2007 observed the downregulation of metalloproteinases by Sparc in ovarian cancer. The functional role of Sparc in cancer is tumor- and tissue-dependent as Sparc has been shown to both promote and inhibit different types of tumor.

Copper has been shown to accumulate in tissues during an immune response. Hence, copper-binding proteins are necessary for tissue repair and have an angiogenic role *in*

vivo experiments. In *in vitro* assays using endothelial cells showed that the degradation of Sparc releases the bioactive peptide (K)GHK with copper-dependent angiogenic properties (Lane *et al.,* 1994).

The heparin binding site of the matricellular protein vitronectin is essential for interaction with the Ca^{+2} binding site on Sparc in vessel wall sections in kidney tissue **(**Rosenblatt *et al*., 1997). Since these two proteins have opposite effects on cell adhesion, the function of the interaction between both molecules might be the regulation of endothelial cell function during angiogenesis.

Finally, it was shown that another matricellular protein, thrombospondin**,** is able to form a complex with Sparc protein. Such binding is involved in the platelet aggregation process (Clezardin *et al*., 1991).

b) Interaction with growth factors

Binding to growth factors is another important characteristic of Sparc. Growth factor activity can influence cell proliferation, migration and differentiation (Taipale and Keski-Oja, 1997).

Sparc was shown to bind the vascular endothelial growth factor (VEGF) in human endothelial cells. It seems that a disagreement exists as regards the action mechanism. Meanwhile intact Sparc does not allow the binding between VEGF with its receptor inhibiting the mitogenic effect of VEGF, Sparc-derived peptide (K)GHK shows an angiogenic effect in endothelial cells (Kato *et al*., 2001). Therefore, Sparc appears to be a significant factor in the regulation of vascular growth.

The expression of Sparc and platelet-derived growth factor (PDGF) is minimal in most normal adult tissues but increases after injury. The interaction of Sparc with the B-chain of PDGF prevents the binding to its receptor to fibroblasts. As a consequence of this

binding, inhibition exists in endothelial cell cycle progression suggesting that Sparc may control proliferative repair processes (Raines *et al*., 1992).

Similarly, Sparc inhibits the migration of endothelial cells induced by fibroblast growth factor (bFGF) (Hasselaar and Sage, 1992). However, bFGF reciprocally downregulates Sparc synthesis in cultured osteoblasts (Delany and Canalis, 1998).
The capacity of Sparc to inhibit VEGF, PDGF and bFGF, factors which have been shown to improve healing, might contribute to the enhancement of wound closure in the absence of Sparc.

Sparc maintains the balance between matrix protein production and cellular proliferation in kidney. In fact, Sparc modulates the synthesis of collagen I and the activity of growth factors through a TGF-β1-dependent pathway (Fig.6) in response to injury (Francki and Sage, 2001).

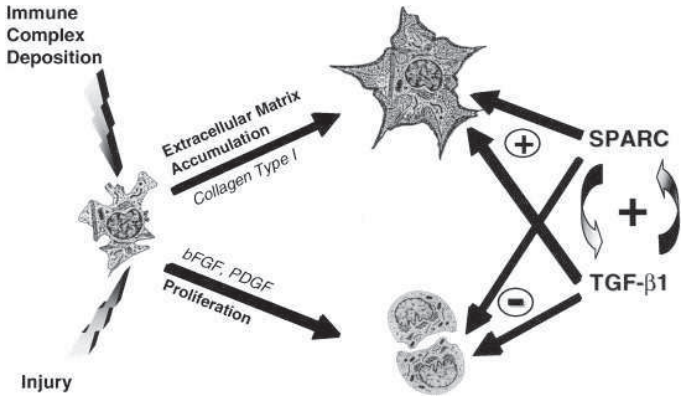


Figure 6. Proliferation and extracellular matrix accumulation in mesangial cells are regulated through Sparc and TGF-β1. Sparc modulates proliferation and ECM production, in particular the synthesis of collagen type I, partially through a TGF-β1-dependent pathway. Sparc induces the synthesis of TGF-β1, an anti-mitogenic factor for mesangial cells. TGF-β1 decreases the hyperproliferation of activated cells upon glomerular injury and drives the accumulation of ECM in the mesangium through the induction of the synthesis of collagen type I and Sparc (Francki and Sage, 2001)

Since Sparc is a marker of odontoblasts, several growth factors were tested in an attempt to elucidate the mechanisms of gene regulation. In human pulp cells, Sparc expression is upregulated by TGF-β in a dose-dependent manner before calcification while bFGF, TNF-α, PDGF and IL-1β downregulate its expression (Shiba *et al.,* 1998).

c) Interaction with chemicals

Retinoic acid stimulates chondrocyte maturation promoting the activation of certain genes which are related with this event such as *sparc*, collagen X, fibronectin or osteopontin (Iwamoto *et al*., 1994).

Dexamethasone is a glucocorticoid member of steroid drugs that acts as a cataractogenic agent. Treating cultured bovine lens epithelial cells with this reagent leads to an increase in levels of Sparc. Since Sparc binds to collagen IV, a major component of lens basement membrane, the upregulation of Sparc by dexamethasone may have a function in the deposition or assembly of ECM proteins in the lens epithelium (Sawhney, 2002).

Not only chemicals but also heat shock affects Sparc levels. Due to the presence of two heat-shock elements in *sparc* gene, exposing culture chick chondrocytes to high temperatures results in increased Sparc expression (Neri *et al*., 1992).

d) Interaction with other molecules

The copper domain of Sparc mediates cell survival *in vitro* via interaction with integrin β1 and the activation of integrin-linked kinase in lens epithelial cells after stress conditions (Weaver *et al*., 2008).
Arnold and Brekken, 2009 proposed that Sparc (SP) acts as an extracellular scaffolding protein (Fig.7), controlling the interactions between the extracellular matrix (ECM), integrins (α, β) and growth factor receptors (RTK). By modulating integrin clustering

and activation, as well as integrin communication with growth factor receptors, Sparc can function as a rheostat for signaling and cellular response.

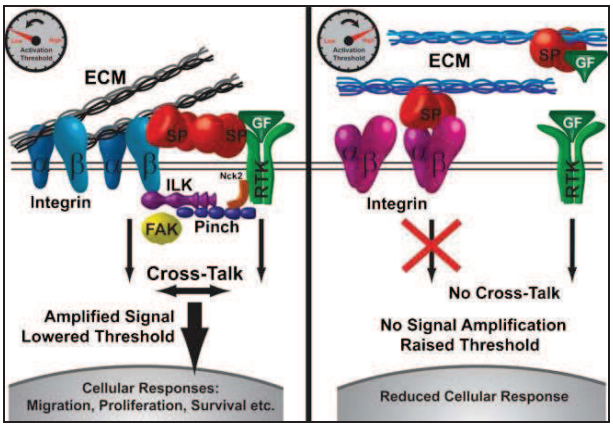


Figure 7. Sparc as an extracellular scaffolding protein and rheostat. **(Left)** Sparc may decrease the activating threshold of certain growth factors (GF) by enhancing complex formation and cross-talk between integrins and growth factor receptors. Integrin-linked kinase (ILK), Pinch, and Nck2 link integrins and growth factor receptors, intracellularly, to form localized signaling cascades, while Sparc acts as an extracellular scaffold to reinforce this complex. Focal adhesion kinase (FAK) is just one example of a signaling molecule located downstream of both integrins and growth factor receptors whose activation is influenced by SPARC. Ultimately, integrin-growth factor receptor cross-talk leads to signal amplification and enhanced cellular responses. **(Right)** Sparc may also increase the activating threshold of integrins and growth factors by inhibiting the binding of certain integrins to the ECM, opposing integrin-growth factor receptor clustering, and/or sequestering growth factors in the extracellular milieu. All of these effects result in a loss of communication and signal amplification of integrins and growth factor receptors, which reduces cellular responses. ECM composition, integrin profile, cytokine profile, cell-type and Sparc concentration/cell-surface localization are all factors dictating this differential response to SPARC (Arnold and Brekken, 2009)

In *Drosophila melanogaster*, Sparc increases the life of cells that are undergoing apoptosis by interaction with an unidentified secreted factor (KS=killer signal) and immobilizing it through integrin-linked kinase activity (Fig. 8) (Bradshaw, 2012).

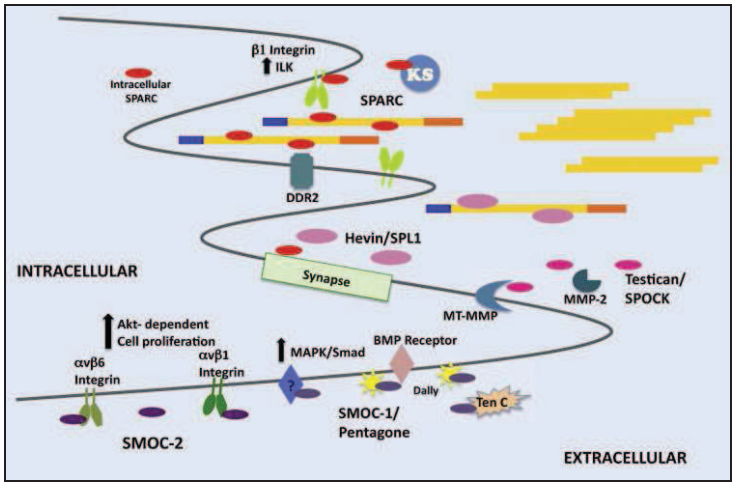


Figure 8. Sparc is regulating of synapse formation *via* as yet unidentified factors either in the ECM and/or on cell surfaces. Collagen fibrils are shown in yellow, with blue and orange rectangles representing the N and C-propeptides, respectively. KS: killer signal (secreted by "winning" cells in *Drosophila*), DDR2: discoidin domain receptor 2, MT-MMP: membrane-type metalloproteinase, BMP: bone morphogenic protein, Ten C: tenascin C, MAPK: mitogen-associated protein kinase (Bradshaw, 2012)

In contrast, Rahman *et al*., 2011 found that the N- terminus of Sparc appears to enhance apoptosis by interacting with caspase8.

The functions of albumin include the delivery of hormones and fatty acids. Sparc was proposed to be an albumin-receptor in epithelial tissues (Liddelow *et al*., 2011).

e) Sparc and methylation

Recently, epigenetics has been widely studied as a new regulation mechanism of Sparc expression and new functions are being explored. Epigenetics refers to modifications to the genome that do not involve a change in the nucleotide sequence. Examples of such modifications are DNA methylation and histone modification, both of which serve to regulate transcriptional gene expression without altering the underlying DNA sequence. Increases in DNA methylation are associated with many cases of gene silencing and

reductions in DNA methylation are often associated with gene activation. Changes on methylation pattern might be due to heredity but also in response to certain types of stress such as environmental, especially temperature (Varriale and Bernardi, 2006; Whittle, *et al*., 2009), nutritional (Feil and Fraga, 2012), pathogenic infections (Dowen *et al*., 2012) or exposure to reagents that interfere with DNA methylation.

CpG islands, regions rich in CG content, are a frequent target of methylation. It has been shown that methylation is a potent regulator of Sparc. Specifically, *sparc* promoter has been described as a frequent target of methylation events in mammals since different CpG islands have been detected. Most of the cases in which Sparc is associated with methylation events are described in a carcinogenic context since the ECM is responsible for several events that can lead to tumor cell differentiation, survival, proliferation, and migration (Larsen *et al*., 2006; Lu *et al.,* 2012).

Hypermethylation of *sparc* is associated with disc degeneration (Tajerian *et al*., 2011) and pancreatic, colorectal or ovarian cancers (Gao *et al*., 2010; Cheetham *et al.,* 2008; Socha *et al*., 2009). Moreover, demethylating the *sparc* CpG island reactivates Sparc expression and has been shown to attenuate invasiveness of lung and colorectal cancers, supporting the idea that Sparc acts as a tumor suppressor (Pan *et al*., 2008; Cheetham *et al*., 2008). However, an overexpression of Sparc leads to other types of cancer, pointing to a potential role for Sparc as a tumor inducer in brain, breast, colon, kidney or pancreas (Arnold and Brekken, 2009). Hence, the tumorigenic effect of Sparc is cell type-specific and may be dependent on the environment surrounding the tumor cell.

These seemingly contradictory actions of Sparc in many important biological events mean its regulation mechanisms should be studied. The scientific literature describing the roles of Sparc in tissues has seen a recent increase but there is still scarce information concerning the mechanisms that regulate Sparc itself.

In view of these gaps in our knowledge regarding the regulation of Sparc and its contradictory roles in different tissues, the overall objective of this thesis is to deepen our understanding of this extracellular matrix protein in teleost fish.

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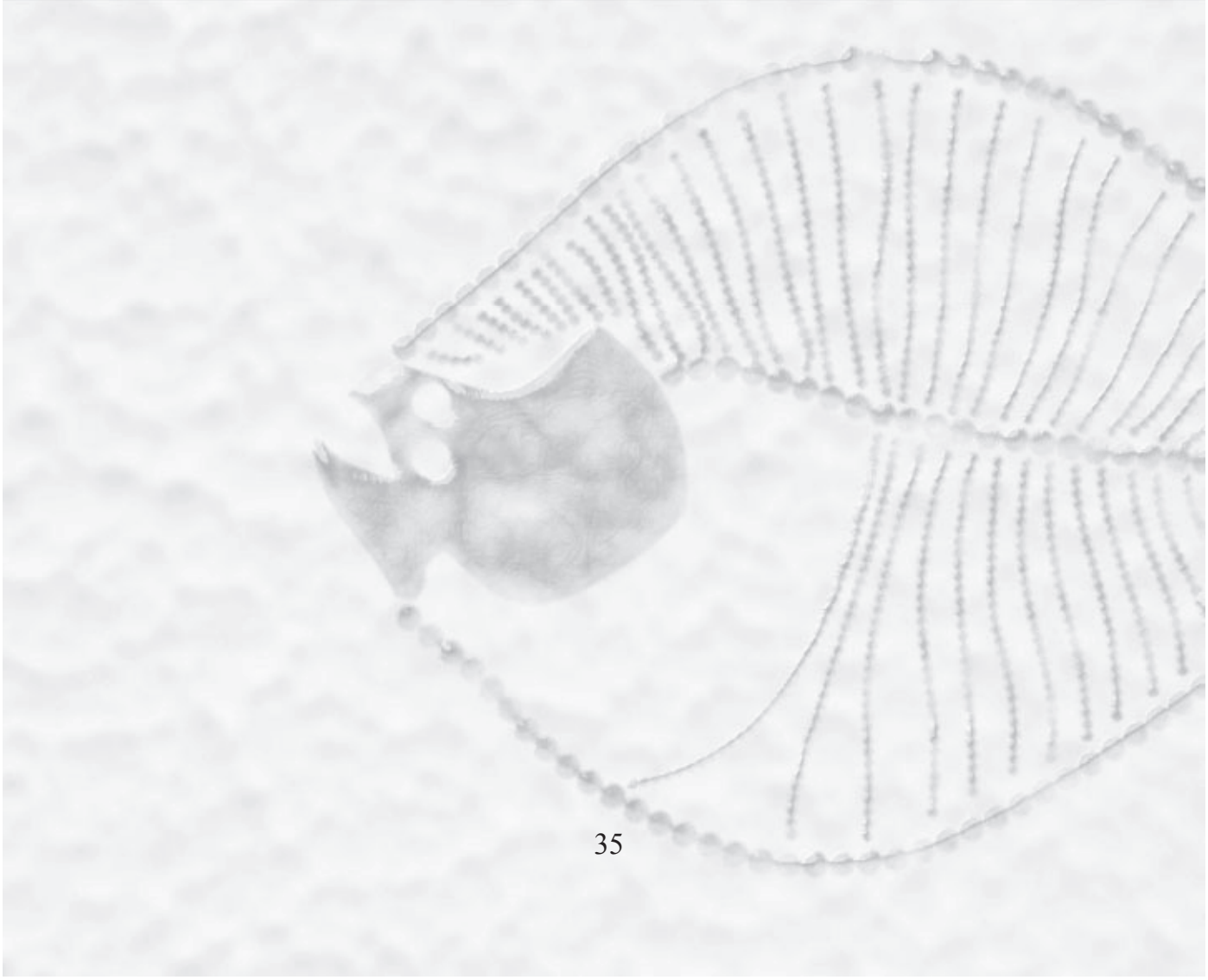
Objectives



Numerous studies indicate that Sparc has complex multiple functions during development. However, despite the knowledge gained from recent in vivo and in vitro studies, the precise morphogenetic functions of Sparc during development are still poorly understood.

The present thesis aims in general to clarify the precise functions of Sparc, in particular during early development of two teleost species and will focus on the following specific objectives:

1. Provide further evidence from gene-silencing studies of the involvement of the Sparc in the control of embryonic hematopoiesis in zebrafish (Chapter I).

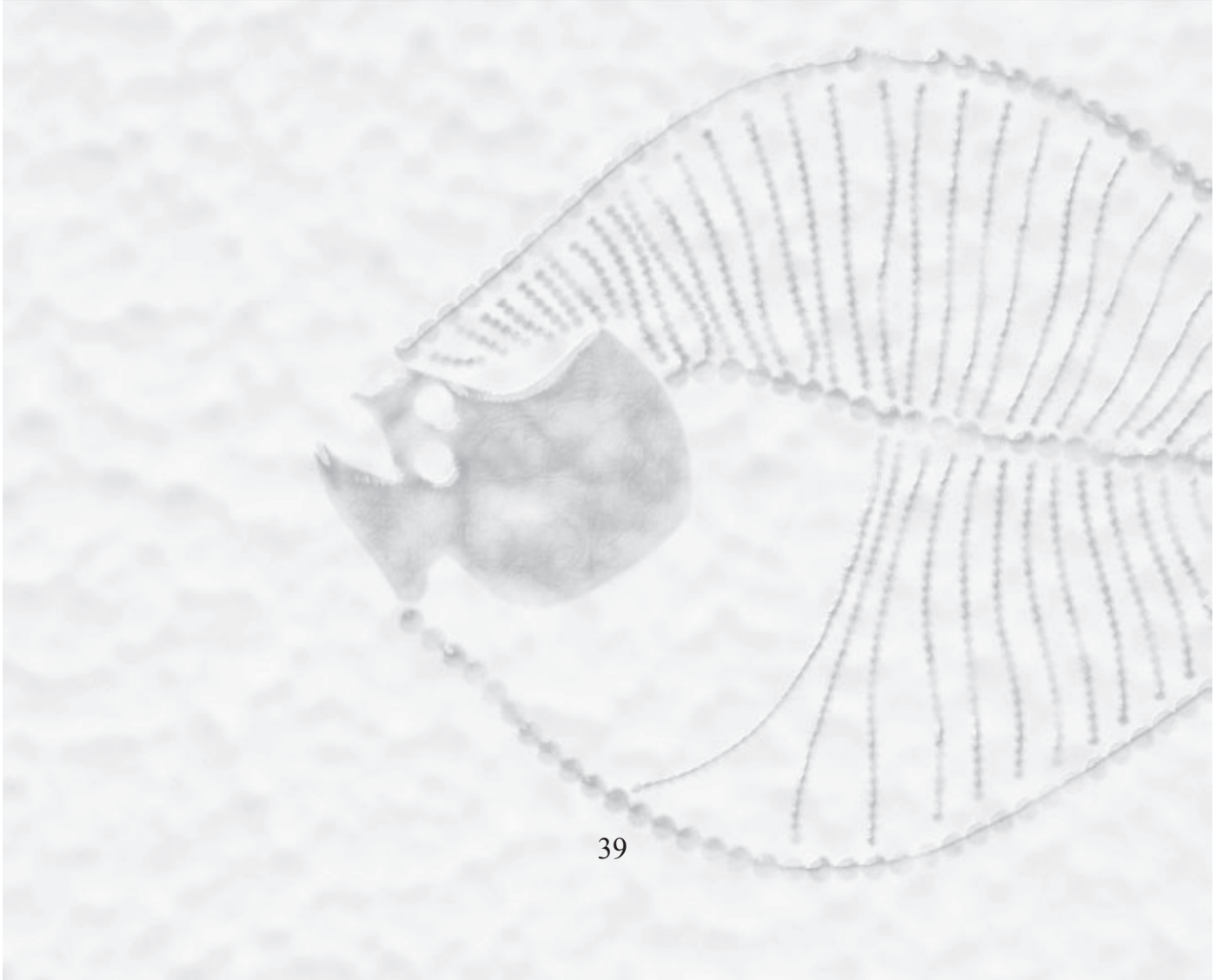
2. Establish possible mechanisms of Sparc regulation in zebrafish

   i. Determine the potential role of Sparc on the developmental abnormalities produced by solar UV radiation exposure in fish embryos. (Chapter II).

   ii. Transcriptional regulation and characterization of the promoter region of the *sparc* gene in zebrafish embryos using reporter gene expression (Chapter III)

3. Molecular cloning and characterization of turbot Sparc to unravel the spatio-temporal expression pattern in flatfish metamorphic remodeling. (Chapter IV)

Impact Factor



El Dr. Josep Rotllant Moragas, como director de la tesis titulada "Sparc (Osteonectin): nuevos conocimientos sobre sus funciones y regulación" realizada por Eva Torres Núñez, manifiesta la veracidad del factor de impacto y la implicación del doctorando en cada uno de los artículos científicos que se presentan en esta tesis doctoral:

Capítulo I/ Artículo I

Título: **Critical role of the matricellular protein Sparc haematopoiesis in mediating erythroid progenitor cell development in zebrafish**

Autores: Ceinos, R.M., Torres-Núñez, E., Chamorro, R., Novoa, B., Figueras, A., Ruane, N.M. y Rotllant, J.

Ref. revista: Cells Tissues Organs, 197: 196-208

Factor de impacto: 1.961

Participación: Eva Torres se ha encargado de realizar gran parte del trabajo experimental que compone esta publicación así como de redactar y corregir en coautoría las diferentes versiones del manuscrito.

Observaciones: Este trabajo ha sido hecho en coautoría con Ceinos, R.M. y no ha sido utilizado implícita o explícitamente para la elaboración de ninguna otra tesis anteriormente.

Capítulo II/ Artículo II

Título: **Molecular response to ultraviolet radiation exposure in fish embryos: implications for survival and morphological development.**

Autores: Torres-Núñez, E, Sobrino, C., Neale, P.J., Ceinos, R.M., Du, S.J. and Rotllant J.

Ref. revista: Photochemistry and Photobiology, 88:701-707.

Factor de impacto: 2.287

Participación: Eva Torres ha realizado la síntesis de ARNm de osteonectina, microinyección e hibridación in situ. Se ha encargado de generar los resultados y de redactar la primera versión del artículo.

Capítulo III/ Artículo III

Título: **5′-UTR intron is crucial for transcriptional regulation of the zebrafish *sparc* (*osteonectin*) gene.**

Autores: Torres-Núñez, E., Cal-Delgado, L., Morán, P. and Rotllant, J.

Ref. revista: en preparación

Factor de impacto: --

Participación: Eva Torres ha realizado la totalidad de la parte experimental, generado los resultados y redactado la primera versión del trabajo.

Capítulo IV/ Artículo IV

Título: **Stage-specific expression of *sparc* during flatfish post-embryonic remodeling**

Autores: Torres-Núñez, E., Ceinos, R.M., Cal, R., Cerdá-Reverter, J.M. and Rotllant, J

Ref. revista: en preparación

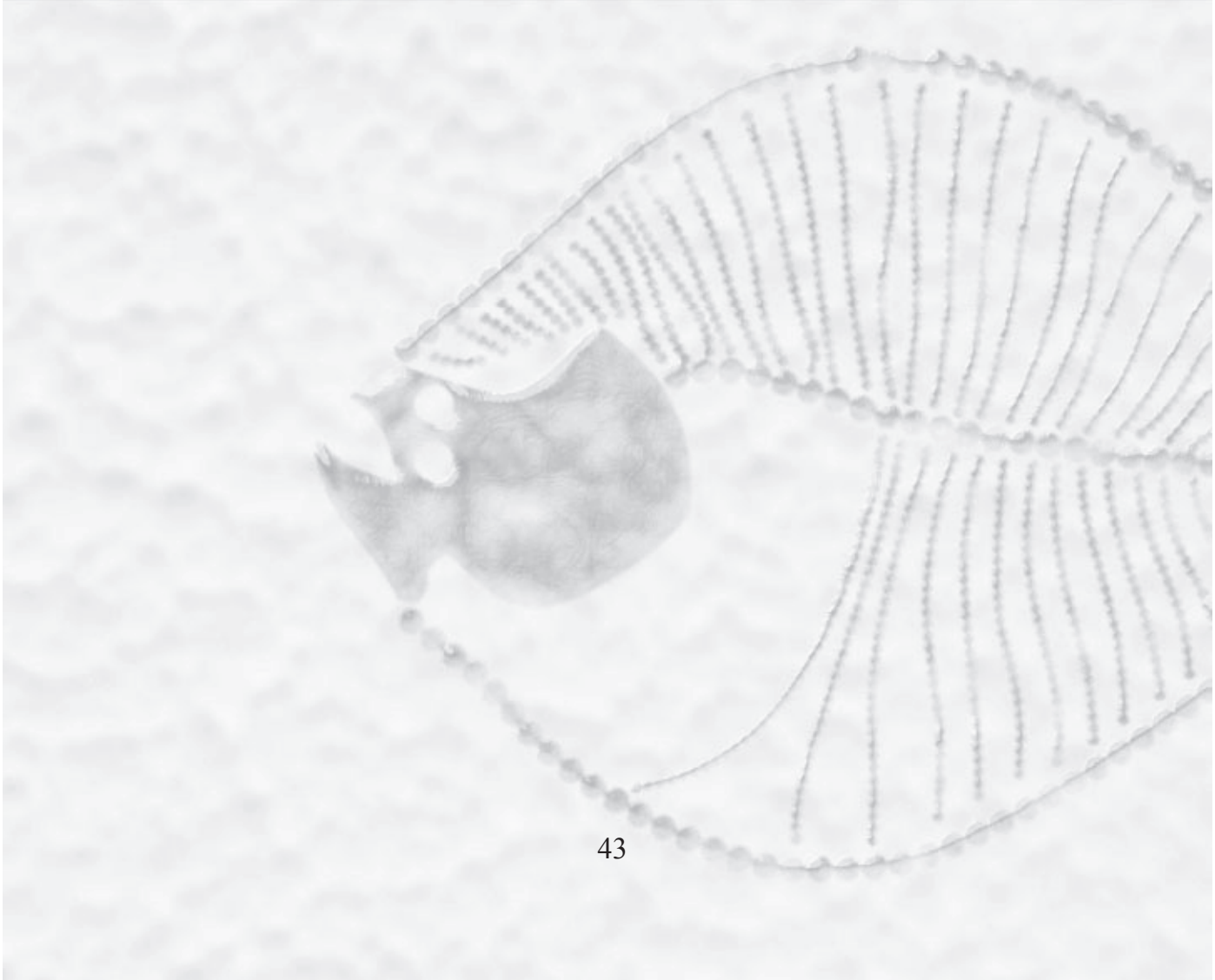
Factor de impacto: --

Participación: Eva Torres ha realizado la totalidad de la parte experimental, generado los resultados y redactado la primera versión del trabajo.

En Vigo, Septiembre, 2013

Josep Rotllant Moragas

Chapter I



Chapter I

Critical role of the matricellular protein Sparc in mediating erythroid progenitor cell development in zebrafish

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Cells Tissue Organs, 197: 196-208 (2013)

ABSTRACT

Sparc (osteonectin) is a multifunctional matricellular glycoprotein expressed by many differentiated cells. Members of this family mediate cell-matrix interactions rather than acting as structural components of the extracellular matrix (ECM); therefore, they can influence many remodeling events, including haematopoiesis. We have investigated the role of Sparc in embryonic haematopoiesis using a morpholino antisense oligonucleotide-based knockdown approach. Knockdown of *sparc* function resulted in specific erythroid progenitor cell differentiation defects that were highlighted by changes in gene expression and morphology, which could be rescued by injection of *sparc* mRNA. Furthermore, a comparison of blood phenotypes of *sparc* and *fgfs* knockdowns with similar defects and the *sparc* rescue of the *fgf21* blood phenotype places *sparc* downstream of *fgf21* in the genetic network regulating haematopoiesis in zebrafish.
These results establish a role for an ECM protein (Sparc) as an important regulator of embryonic haematopoiesis during early development in zebrafish.

Key words: ECM, *fgf21*, *gata1*, haematopoiesis, osteonectin, *sparc*, zebrafish

INTRODUCTION

Haematopoiesis is the biological process describing the formation and development of blood cellular components. Evolutionary comparisons have revealed that haematopoiesis is conserved within vertebrates, among whom the zebrafish *Danio rerio* has been shown to be a valuable model organism for the study of haematopoiesis (Albacker and Zon, 2009).

From zebrafish to mammals, haematopoiesis occurs in two principal successive waves: the first (or primitive) wave supports the developing embryo while the second (or definitive) wave provides the organism with long term haematopoietic stem cells (HSC) to last its entire lifetime. In zebrafish, the primitive wave takes place between 12 and 24 hours post-fertilization (hpf), producing erythrocytes and myeloid cells. Primitive myelopoiesis takes place in the anterior lateral mesoderm (ALM), whereas primitive erythropoiesis occurs in the posterior lateral mesoderm (PLM) which later forms the intermediate cell mass (ICM). The definitive wave produces long term HSCs which will support the generation of all blood lineages. These differentiated lineages include not only erythroid and myeloid cells like the primitive wave, but also lymphocytes, thrombocytes and a larger variety of myeloid cells. In addition, it has been shown that the definitive wave may first generate committed erythromyeloid progenitors in the posterior blood island between 1 and 2 days post-fertilization (dpf) before HSCs arise (Bertrand *et al*., 2007). Therefore, these erythromyeloid progenitors will serve as transient progenitors to initiate definitive haematopoiesis independently of HSCs and they represent a transient wave between primitive and definitive haematopoiesis.

Haematopoiesis is a complex developmental process controlled by a large number of factors that regulate stem cell renewal, lineage commitment and differentiation. These regulatory molecules include hematopoietic growth factors, hedgehog signalling molecules (Dyer *et al.,* 2001), vascular endothelial growth factors (Liang *et al*., 2001),

fibroblast growth factors (Fgfs) (Songhet *et al.,* 2007; Yamauchi *et al*., 2006) and bone morphometric proteins (Thisse and Zon, 2002) amongst others.

Furthermore, it is now known that activities of vascular endothelial growth factor A (Nozaki *et al*., 2006), FGFs (Taiple and Keski-Oja, 1997; Whitehead *et al*., 2005) and other regulatory molecules are influenced by the interaction of cells with the extra cellular matrix (ECM). Matricellular proteins regulate cell-ECM communication and therefore can influence many remodelling events, including haematopoiesis. A recent study on morpholino antisense oligonucleotide (MO)-based functional screening in zebrafish showed a potential hematopoietic function of 14 genes (Eckfeldt *et al*., 2005). Sparc, an ECM protein also termed osteonectin, was among them.

Sparc is a multifunctional protein that modulates cell-matrix interaction and cell function, but does not seem to have a direct structural role in the matrix (Brekken and Sage, 2001). Sparc is an evolutionary conserved matricellular protein (Laizé *et al*., 2005; Rotllant *et al*., 2008). Within all vertebrates, *sparc* is expressed in a temporally and spatially specific manner with strong expression during embryogenesis in developing tissue such as the notochord, somites and embryonic skeleton (Holland *et al*., 1987; Renn *et al.,* 2006; Rotllant *et al*., 2008) and a marked reduction in *sparc* expression occurs once adulthood is reached. However, it re-emerges in response to tissue injury, remodelling and inflammation (Bornstein and Sage, 2002). Therefore, its dynamic expression patterns during embryogenesis and its sequence homology with other vertebrates suggest a conserved function of *sparc* in vertebrates (Rotllant *et al*., 2008). However, the precise function of *sparc*, in particular during early embryogenesis, is largely unknown. Additionally, the apparent absence of other *sparc* functional homologs in teleost fish compared with mammals (Rotllant *et al*., 2008) may result in a greater understanding of the role of *sparc*, which may be applicable to all vertebrates.

In the present study, it was demonstrated that zebrafish *sparc* plays a critical role in mediating erythroid progenitor cell development and additionally that *sparc* interacts with genes, in known genetic networks, thus further unveiling its novel function in the regulation of haematopoiesis.

MATERIAL AND METHODS

Animals

Zebrafish embryos were cultured as previously described Westerfield, 2007 and staged according to Kimmel *et al*., 1995. Experiments were performed using standard wild type strain (AB, Zebrafish International Resource Center). To inhibit embryo pigmentation, embryo medium was supplemented with 0.003% (w/v) 2-phenylthiourea (Westerfield, 2007). Dechorionated embryos were collected for total RNA extraction and cell proliferation assays or fixed overnight at 4°C in 4% paraformaldehyde in 1XPBS, washed in PBS, dehydrated through a series of methanol and stored at -20°C in 100% methanol for *in situ* hybridization and TUNEL assay. Ethical approval (N011011) for all animal studies was obtained from the Institutional Animal Care and Use Committee of the IIM-CSIC Institute in accordance with the National Advisory Committee for Laboratory Animal Research Guidelines licensed by the Spanish Authority (1201/2005).

RNA isolation and quantitative real-time polymerase chain reaction

Embryos at 19, 24 and 30 hpf were de-chorionated and total RNA was extracted using Trizol reagent according to manufacturer's protocol (Invitrogen). cDNA was made from total RNA using superscript III (Invitrogen) according to manufacturer's recommendations. Primer sequences are given in Table 1. All expression levels were normalized to *actin* using the $2^{-\Delta\Delta T}$ method (Livak and Schmittgen, 2001). Real-time quantitative polymerase chain reactions (qPCRs) reactions were performed using an AB 7300 real-time PCR System and SYBR green incorporation (Applied Biosystems). The PCR cycles for all primer sets were: denaturation at 95ºC for 10 min, followed by 40 cycles of 95ºC for 15s and 60 ºC for 1 min. All samples were done in triplicate and each condition was repeated 3 times.

Gene	Forward primer sequence (5'- 3')	Reverse primer sequence (5' – 3')
gata1	TACTGCCACCCGTTGATG	ACTTGGCGAACTGGACTG
pu.1	CAGAGCTACAAAGCGTGCAG	GCAGAAGGTCAAGCAGGAAC
hbbe3	TTTCCGGCTGTTAGCGGACT	TTGCCTTCTGAGGGCTGACA
lcp1	CCTGACGGATGAAAAGAAGC	GTTTCAGGCGTATAATGGAG
actin	AGCACGGTATTGTGACTAACTG	TCGAACATGATCTGTGTCATC

Table 1. Real-time qPCR primer sequences

Morpholino knockdown

Two independent MOs, a translation blocker (ATG-MO: 5'GATCCAAACCCTCATCTTGAGTTTC3') and a splicing blocker at the exon 3-intron 3 (E3I3) junction (E3I3-MO: 5'GAAAAATGAACTCACTCTCAGCAAT3'), were used to target *sparc* (Rotllant *et al*., 2008). Additionally, MO specific for *fgf21* (*fgf21*-MO) (Yamauchi *et al*., 2006) and/or *p53* (*p53*-MO) (Robu *et al*., 2007) were also used to target *fgf21* and *p53* genes, respectively. A scrambled MO with no known target in zebrafish, *c*MO, 5'-CCTCTTACCTCAGTTACAATTTATA-3' was used as control. All antisense oligonucleotides were synthesized by GeneTools, LLC (Corvalis, Oreg., USA). The MOs were resuspended in 1x Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM $Ca(NO_3)_2$, 5 mM HEPES, pH 7.6) to a final concentration of 0.5 mM (ATG *sparc*-MO) or 1mM for splicing (E3I3-*sparc*-MO), 1 mM (*fgf21*-MO), 1mM (*p53*-MO) and 1mM (cMO). Subsequently, ~1 nl was injected into one- or two-cell stage embryos. To determine morpholino functional duration, the splicing blocker (E3I3) morpholino was used. To test for disruption of splicing, reverse transcriptase (RT)-PCR was performed (primers: exon 1 forward, 5'GCTGAAACTCAAGATGAG-3'; exon 4 reverse, 5'- TCCAATCGGAGACTTCGAGCA-3'). Total RNA from 2 pools of 10 uninjected (wild-type) 1-dpf embryos, 2 pools of 10 (1-dpf embryos) injected with 1nl of 1mM cMO 1dpf embryos and 2 pools of 10 (1-, 2-, 3- and 5-dpf embryos) injected

with 1 nl of 1 mM E3I3-MO were collected and the cDNA transcribed following the above protocol.

In situ hybridization, mRNA synthesis and rescue

Whole-amount in situ hybridization was performed using digoxigenin-labeled antisense probes as previously described Rotllant *et al*., 2008. *Sparc* antisense riboprobe was made from linearized partial length *D.rerio sparc* cDNA containing a 3'UTR fragment (GenBank Accession No: BC071436; primers: forward 5'-GATGAAGCCATTGAGGTCGT -3'; reverse 5'-AATCCACCACCAAAGAGTGC -3'). Other antisense RNA probes used in this study were *gata1*, *cmyb and βe3globin* (Gardiner *et al*., 2007); *runx1* (Murayama *et al*., 2006); *pu.1* and *l-plastin* (Bennet *et al.,* 2001) and *rag1* (Trede *et al*., 2008).

For *in vitro* mRNA synthesis, the pCS2+-*sparc* was linearized with EcoRI. Capped RNA was transcribed *in vitro* using the SP6 Message Machine Kit (Ambion). The $PCS2^{+}$-*sparc* construct used in the rescue experiments includes a Kozak sequence upstream of the ATG instead of the endogenous zebrafish sequence, resulting in five mismatches between the antisense sequences and the rescue mRNA. Thus, the capped mRNA rescue construct were not susceptible to the ATG-*sparc*-MO. Rescue mRNA was injected into 1- or 2-cell stage embryos either alone or in the presence of a MO. For each rescue experiment, the amount of mRNA injected was titrated for the maximal dose which could be injected (Rotllant *et al*., 2008). For *sparc* rescue experiments, approximately 1 nL of 0.5 mM (4µg/µl) ATG-*sparc*-MO or 1 mM (8µg/µl) E3I3-MO was injected together with 1 nl of two different *sparc* RNA concentrations (325 µg/mL or 750 µg/ml) per embryo. Approximately 150 embryos were used. For the *fgf21* morphant phenotype rescue, approximately 1 nl of 1 mM (8µg/µL) of *fgf21*-MO was injected together with 1 nl of *sparc* RNA (750 µg/ml) per embryo (approximately 200 embryos were used).

Detection of apoptotic and proliferating cells

Cell proliferation was measured with the FLUOS in situ cell proliferation kit (Roche, Mannheim, Germany) as described previously (Flores *et al*., 2008). Apoptotic cells were examined by TUNEL assay using the POD in situ cell death detection kit (Roche).

Statistics

Data are expressed as means ± SEM (calculated by dividing the standard deviation by the square root of the number of replicate experiments). Comparisons between numerical data were evaluated by paired Student t tests. A p-value < 0.05 was considered statistically significant.

RESULTS

Haematopoietic marker analysis suggests a specific role of Sparc in erythroid progenitor cell development

To examinate the spatial expression of zebrafish *sparc,* whole-amount mRNA in situ hybridization was performed on 22-, 25- and 35-hpf zebrafish embryos (Fig.1). By 22hpf, *sparc* transcripts were strongly expressed in the caudal fin fold, notochord, floor plate, somites and the PLM/ICM region (Fig.1A). At 25hpf, sparc messenger RNA was still detectable in the PLM/ICM region where *gata1* is strongly expressed (Fig.1B). At 35hpf, *sparc* was also expressed above the yolk extension at the aorta-gonad-mesonephros region (Fig.1C). Embryos treated with control sense probes did not show any signal (data not shown).

To investigate the role of *sparc* during embryonic haematopoiesis, we adopted a loss-of-function approach. Two independent MOs, a translation blocker (ATG-MO) and a splicing blocker (E3I3-MO) were used to target *sparc*. A scrambled MO with no known target in zebrafish, *c*MO, was used as control as described previously (Rotllant *et al*., 2008). The specificity and efficacy of the morpholinos were previously analyzed either by their ability to inhibit protein translation in an *in vitro* transcription-translation assay or their efficacy at inhibiting transcription processing *in vivo* in 24hpf embryos (Rotllant *et al*., 2008). To assess the functional duration of *sparc* inhibition *in vivo*, the test for disruption of *sparc* gene splicing by RT-PCR was performed. The splice junction morpholino E3I3-MO targets the third coding exon-intron boundary (Fig.1D). When injected into zebrafish embryos, the splicing morpholino induced the formation of a new transcript (401bp) due to the retention of the first 95 bp of intron 3 sequence (Fig.1D). This leads to premature termination, producing a peptide that lacks the highly conserved C-terminal collagen and calcium binding domains (Rotllant *et al*., 2008). Quantitative analysis showed that almost 100% of the *sparc* transcripts were incorrectly spliced in 1 nl of 1 mM E3I3-MO injected embryos up to 3 dpf, while at 5dpf almost all of the *sparc*

transcripts were correctly spliced compared with cMO injected or non-injected embryos (Fig.1D).

Therefore, in this study, we injected *sparc* and scrambled cMO into 1- to 2-cell stage embryos, and examined development for 3 days. Most injected embryos developed normally until 30 hpf, however they appeared smaller than cMO-injected embryos, as previously shown by Rotllant *et al*., 2008.

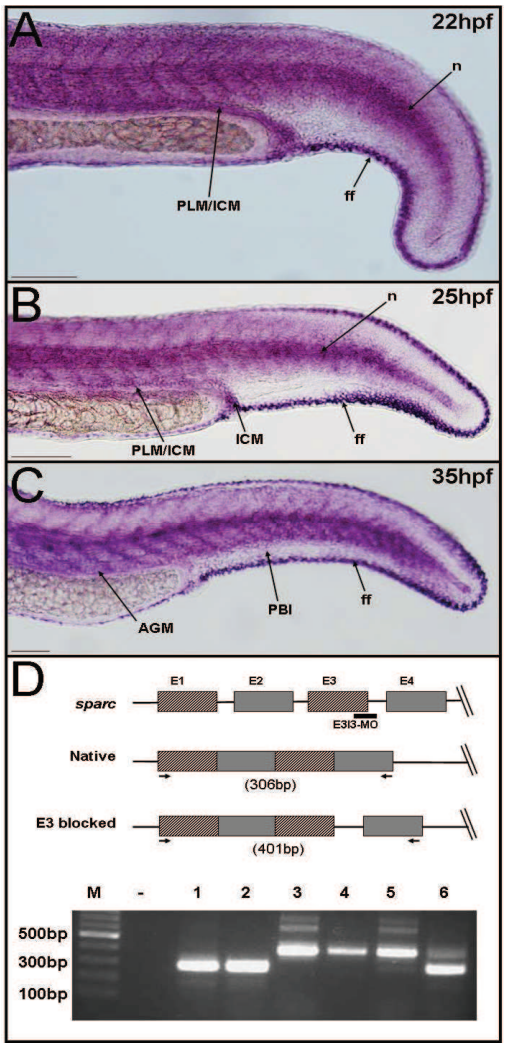

Figure 1. In situ hybridization showing the expression pattern of *sparc* in the trunk and tail region in zebrafish embryos at 22 **(A),** 25 (**B**) and 35hfp (**C**). (**D**) Efficacy and functional duration of *sparc* inhibition in vivo. Location of the splice blocker E3I3-MO. E3I3-MO blocks the splicing of *sparc* transcript. RT-PCR shows the defective splicing induced by the E3I3-MO. PCR results from non-injected and cMO injected embryos show a single band (306 bp) (lane 1 and 2). A single band (401bp) was also detected in 1mM E3I3-MO-injected embryos at 1 (lane 3), 2 (lane 4) and 3 dpf (lane 5). In 5-dpf (lane 6) 1mM E3I3-MO-injected embryos, two bands (306 bp and 401 bp) were detected. In lanes 3, 4 and 5, the 401-bp band, which is the major PCR product, is a result of defective splicing from using a cryptic splice donor located 95 bp 3'of the normal E3/I3 splice site in intron 3 as shown by DNA sequencing (data not shown). AGM= aorta-gonad-mesonephros; ff=fin fold; ICM=intermediate cell mass; n, notochord; PBI= posterior blood island; PLM= posterior lateral mesoderm (**A-C**) Anterior to the left, dorsal to the top. Scale bars: 100 µm.

Eckfeldt *et al.,* (2005) used a similar ATG-MO against *sparc* in a MO-based functional screen in zebrafish to determine the haematopoietic function of 61 genes and they reported a reduced blood cell production identified by *gata1:DsRed* transgenic fish in more than 70% of *sparc*-MO injected embryos. Taken together, these results suggest a potential role of Sparc in zebrafish haematopoiesis. The first 12 bases of their *sparc*-MO (5'ATCTTGAGTTTCAGCCTTCTGTCCG-3') were identical to the last 12 bases of our independently designed ATG-*sparc*-MO (5'GATCCAAACCCTCATCTTGAGTTTC3') (Rotllant *et al*., 2008).

To better describe the effects of loss of *sparc* on zebrafish hematopoiesis, molecular markers that specify distinct stages of haematopoietic differentiation were analyzed.
The initial embryonic wave of blood production, the primitive wave, takes place in two locations, namely the ALM and the ICM. Primitive erythropoiesis occurs in the ICM, and primitive myelopoiesis in the ALM. It has been shown that the zinc-finger transcription factor *gata1* is crucial for primitive erythropoiesis and the myeloid–specific transcription factor *pu.1* for primitive myelopoiesis. Furthermore, *gata1* is co-expressed with *pu.1* in the ICM from 16 hpf to 24 hpf and it is has been shown that the interplay of *pu.1* and *gata1* regulates the production of primitive erythroid and myeloid cells, respectively (Rhodes *et al*., 2008). Moreover, it has recently been shown that an additional transient wave exists between primitive and definitive haematopoiesis (24-48hpf) (Bertrand *et al*., 2007; Zon and Chen, 2009). This wave is also known as the first wave of the definitive haematopoiesis; however it produces erythromyeloid progenitors that arise independently of HSCs and they exhibit an immature, blastic morphology and express only erythroid and myeloid genes.
Therefore, *gata1* and *pu.1* were used as gene markers for primitive and transient wave characterization. Additionally, other specific lineage markers such as *βe3globin (hbbe3)* for erythroid cells, *l-plastin (lcp1)* for all myeloid cells and *rag1* for lymphoid cells were also used.

As shown in Fig. 2A,C, *gata1* mRNA is expressed in the embryo ICM at 19 and 24 hpf, a crucial signalling centre for zebrafish primitive haematopoiesis (Berman *et al*., 2005) and in the posterior blood island at 30 h (E) where the first hematopoietic progenitor cells with multilineage potential (erythromyeloid progenitors) are found. The *gata1* gene expression level in these regions is markedly reduced in *sparc* morphant embryos at 19, 24 and 30 hpf (Fig.2B, D, F). Unlike *gata 1*, the expression of *pu.1* a transcription factor that is necessary for myeloid progenitor cell development (Odenthal *et al*., 1996), was not significantly reduced in *sparc*-MO embryos at 19 and 30 hpf (Fig.2G-J). These results indicate that *sparc* is essential for mediating primitive and transient erythroid progenitor cell development but not the myeloid progenitor cells.

Blood defects identified were confirmed by in situ hybridization of the erythroid-specific *βe3globin,* the leukocyte specific *l-plastin* and the lymphoid-specific *rag1* in embryos injected *sparc*-MO compared to embryos injected scrambled cMO (Figs.2K-P).

A significant reduction in *βe3globin* was observed in *sparc*–MO injected embryos (Fig.2 K, L). No significant differences in *l-plastin* and *rag-1* expression in *sparc* morphants were observed (Fig.2 M-P) suggesting a critical role of *sparc* in erythropoiesis but not in myelopoiesis and lymphopoiesis. These results were also established by standard reverse qPCR (Fig.2Q).

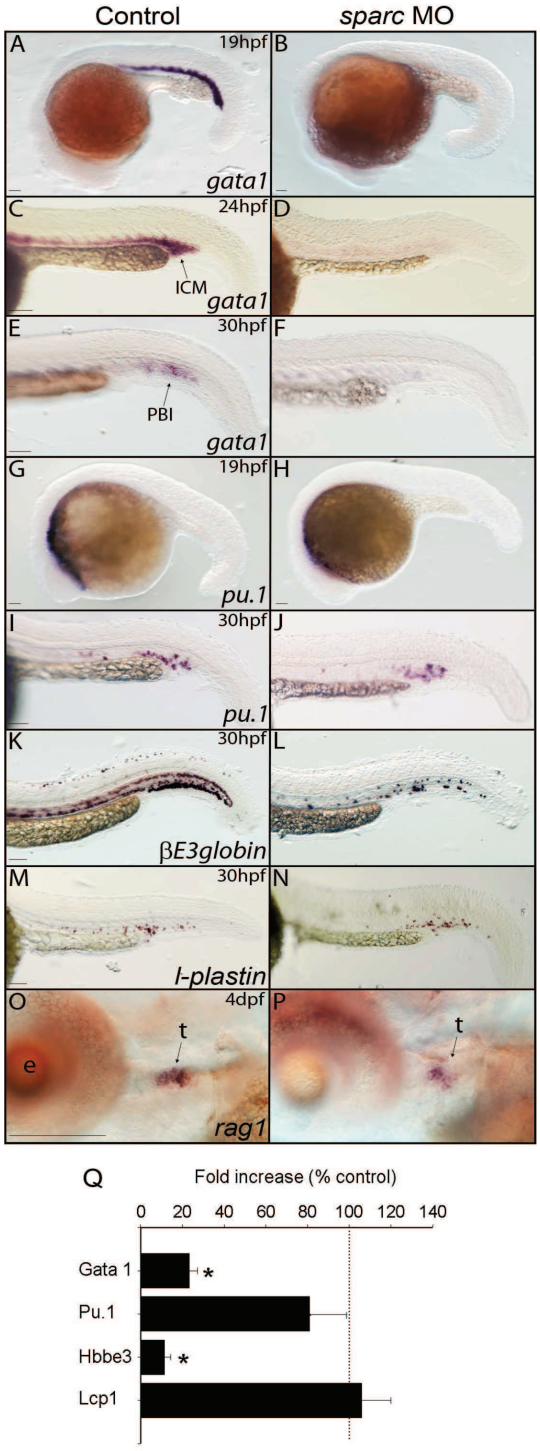

Figure 2. *Sparc* is required for normal erythroid progenitor cell development in zebrafish embryos. Expression of haematopoietic markers in embryos injected wild type (cMO; **A,C,E,G,I,K,M,O**) and *sparc*-MO (**B,D,F,H,J,L,N,P**). Reduced embryonic *gata1* and *βE3globin* gene expression but not *pu.1, l-plastin* and *rag1* in *sparc* morphants. The expression was examined by whole-mount *in situ* hybridization (**A-P**), and confirmed by real-time qPCR (**Q**) of *gata1, pu.1, βE3 globin (hbbe3)* and *l-plastin (lcp1)*. *gata 1* for erythroid progenitors; *pu.1* for myeloid progenitors; *l-plastin* for late myelomonocytic linages, *βE3 globin* for erythrocytes and *rag1* for lymphoid cells. e=eye ICM=intermediate cell mass; PBI= posterior blood island; t=timus. (**A-P**) lateral views, anterior to the left. Scale bars: 100 (**A-N**) and 150 μm (**O-P**).

Following the hematopoietic conserved gene program (Davidson and Zon, 2004), definitive wave specific markers such as *c-myb* and *runx1* were used for *in situ* hybridization. The expression patterns for specific HSC markers *c-myb* and *runx1* revealed non-significant changes at 48h and 72h, indicating that loss of *sparc* does not affect the emergence of HSC (Fig.3C,D; Fig.4A-D) and the proliferation and differentiation of HSC (Fig.3E,F; Fig.4E,F). The slightly difference found in *c-myb* expression at 24h (Fig.3A,B) is likely due to residual *c-myb* expression in primitive erythrocytes.

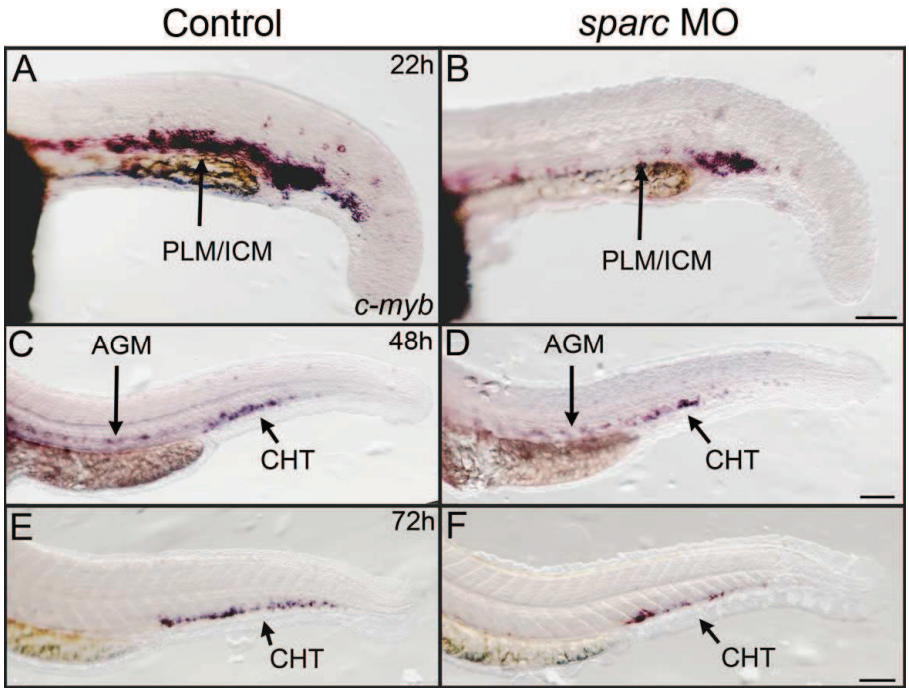

Figure 3. Knockdown of *sparc* does not affect genes associated with definitive haematopoiesis. Whole-mount mRNA *in situ* hybridization with *c-myb* antisense probe. Lateral views, anterior to the left. (**A,B**) 22 hpf. (**C,D**) 48 hpf. (**E,F**) 72hpf. AGM= aorta-gonad-mesonephros; CHT= caudal haematopoietic tissue; PLM=posterior lateral mesoderm; ICM=intermediate cell mass. Scale bars: 100 μm.

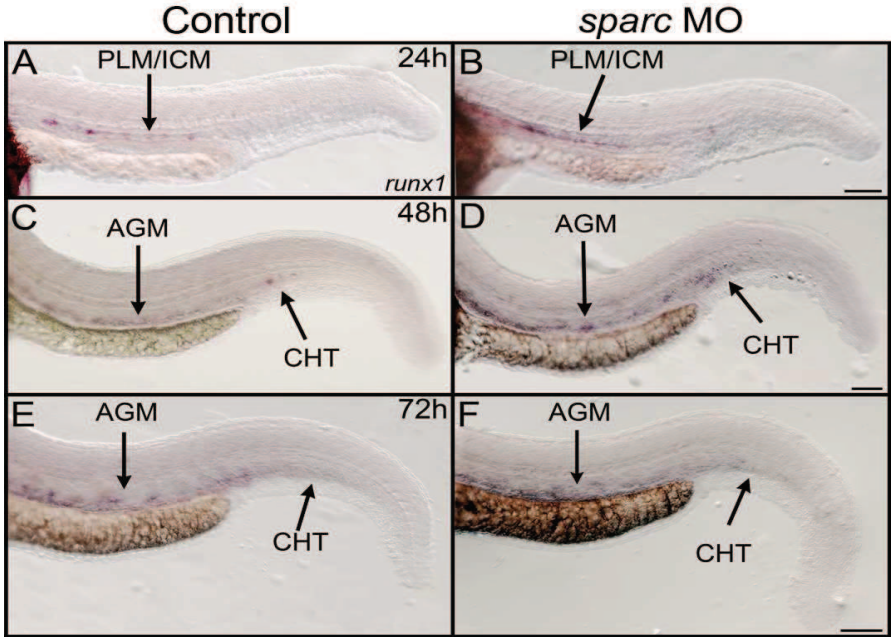

Figure 4. Knockdown of *sparc* does not affect genes associated with definitive haematopoiesis. Whole-mount mRNA *in situ* hybridization with *runx1* antisense probe. Lateral views, anterior to the left. (**A,B**) 24 hpf. (**C-D**) 48 hpf. (**E,F**) 72hpf. Abbreviations: PLM, posterior lateral mesoderm; ICM, intermediate cell mass; AGM, aorta-gonad-mesonephros; CHT, caudal haematopoietic tissue. Scale bars: 100 μm.

Knockdown of sparc has no effect on angiogenesis

To further confirm the *sparc* knockdown effect on haematopoiesis, we examined its effect on angiogenesis as both processes arise from a common precursor, the haemangioblast. Furthermore, we also examined vasculogenesis to rule out the possibility that the haematopoietic defect observed in the *sparc*-MO was secondary to a vascular defect.

We injected *sparc*-MO into *fli-EGFP* transgenic zebrafish. Allowing for the general dysmorphic appearance of the embryo at 48 h, neither vasculogenesis nor angiogenesis were affected, as shown by intact axial and intersegmental vessels (supplementary data, Fig.1,A-C).

Sparc morphant zebrafish embryos do not show altered cell death but do show altered cell proliferation in posterior haematopoietic tissues

To examine *sparc*-deficient ICM cell proliferation and apoptosis, wild-type and *sparc*-deficient embryos were analyzed. Fluorescent immunohistologic analysis of 22 hpf larvae for BrdU incorporation revealed an increase in the number of ICM region BrdU positive cells in morphant samples when compared with controls (Fig.5A, B, E). These results demonstrate that *sparc* could regulate cell proliferation in zebrafish, supporting the well-characterized role of *sparc* as a modulator of cell proliferation in other organism (Brekken and Sage, 2001).

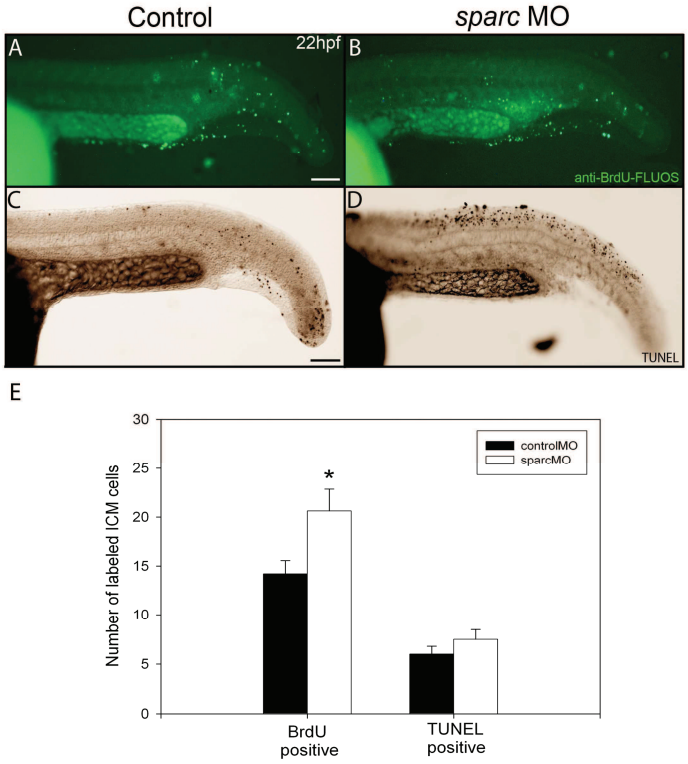

Figure 5. Proliferation and apoptosis in *sparc*-deficient 22 hpf embryos. (**A–D**) Lateral views (anterior to left) of trunk region of cMO-injected (**A** and **C**) and *Sparc*-MO injected embryos (**B** and **D**) processed for fluorescent labelling of BrdU incorporation (**A** and **B**) and TUNEL reaction (**C** and **D**). (**E**) Graph depicting a comparison of BrdU and TUNEL-positive cells in the ICM of uninjected (n=20) and morphant (n=26) embryos. Numbers represent average counts of labelled ICM cells per embryo. Means ±SEM, *p<0,05.

The frequency of apoptotic cells in the ICM region measured by TUNEL reaction was low and was unaltered between *sparc*-MO and scrambled cMO-injected embryos (Fig.5, C-E). However, apoptotic cells were significally increased in the trunk region of *sparc*-MO at 22 h (Fig.5D).

Sparc mRNA rescues defects caused by *sparc* MOs

To verify the specificity of the defects produced by *sparc* knockdown, we rescued ATG-*sparc*-MO-injected embryos by co-injecting synthetic *sparc* mRNA (Rotllant *et al*., 2008). Nearly complete rescue was achieved. The severe reduction in *gata1* ICM expression after *sparc* knockdown (Fig.6A, B) was rescued by co-injection of *sparc* mRNA (Fig. 6D, E) although the body shape was still somewhat abnormal. The rescue success was dose dependent with 27% (13 from 48 embryos) of the co-injected embryos (0.5 mM ATG-*sparc*MO plus 320 µg/ml *sparc* mRNA) showing some *gata1* expression (Fig. 6D) compared to 69% (33 from 48 embryos) of the co-injected embryos (0.5 mM ATG-*sparc*MO plus 750 µg/ml *sparc* mRNA) showing *gata1* expression (Fig.6E). These results demonstrate that exogenous *sparc* is sufficient to correct the blood defects caused by *sparc*-MO, consistent with the interpretation that these haematopoietic defects are due to reduced levels of *sparc* protein function.

To provide further evidence that *sparc* morphant phenotypes are *sparc*-specific and not due to non-specific off-target effects (e.g. *p53* mediated apoptosis), we also analyzed *gata-1* expression in *sparc* morphants that were coinjected with a *p53* morpholino (Robu *et al*., 2007). *gata1* mRNA expression was dramatically reduced in embryos co-injected with *sparc* and *p53* morpholinos (Fig.6C), with *p53* morpholino-injected embryos exhibiting gene expression patterns similar to control embryos (data not shown), indicating that *sparc* protein is critical for *gata1* expression.

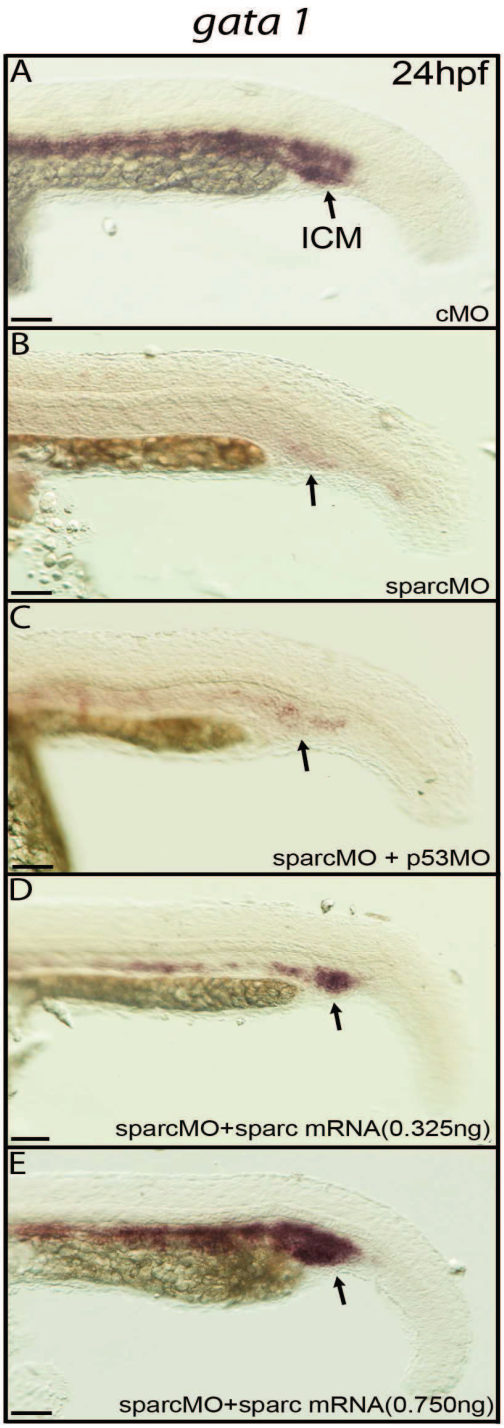

Figure 6. *Sparc* mRNA can rescue the haematopoietic defect (*gata1* expression) in *sparc* morphants. Sense *sparc* mRNA (1 nl of 350 or 750 µg/ml) was co-injected with 1-2 nl of 0.5 mM *Sparc*-MO and the embryos were fixed for *in situ* hybridization. (**A**) cMO-injected embryos at 24hpf, (**B**) *sparc*-MO-injected embryos at 24hpf, (**C**) *sparc*-MO plus *p53*-MO-(2 nl, 1mM) injected embryos, (**D**) *sparc*-MO plus *sparc* mRNA (0.325 ng) injected embryos and (**E**) *sparc*-MO plus *sparc* mRNA (0.750 ng) injected embryos. (**A-E**) Whole-mount *in situ* hybridization analysis of *gata 1* expression. (**C**) Knockdown of *sparc* causes haematopoietic developmental abnormalities independent of *p53* dependent apoptosis. (**A-E**) lateral views, anterior to the left. ICM, Intermediate Cell Mass. Scale bars: 100 µm.

Sparc expression is dependent on Fgf signalling

The regulation of *sparc* gene expression by members of the *fgf* family of signalling molecules (Taiple and Keski-Oja, 1997; Whitehead *et al*., 2005) and the resemblance between *sparc* and *fgf21* morphant blood phenotypes characterized by a severe disruption of erythroid progenitor cell development (Yamauchi *et al*., 2006) led us to investigate whether fgf signalling may mediate the decrease of *sparc* activity. This, in turn, acts by modulating the lineage-specific transcription factor *gata1* expression levels or activity. Injection of *fgf21*-MO (10ng embryo) (Yamauchi *et al*., 2006) induced a significant reduction in *sparc* expression in nearly 80 % of the injected 24-hour embryos (Fig. 7A, B).

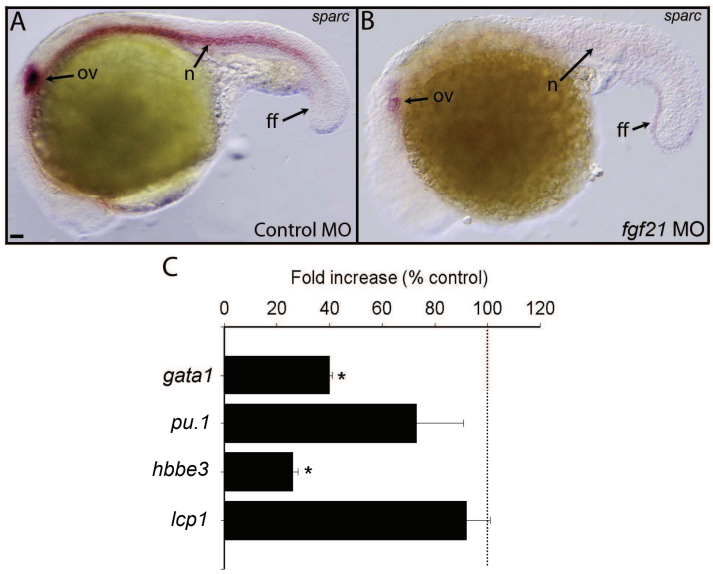

Figure 7. *Fgf21* morphants show altered *sparc*, *gata1* and *hbbe3* expression. (**A, B**) Early *sparc* expression (19hpf) is decreased in *fgf21* morphants; *sparc* expression was examined by whole-mount *in situ* hybridization. (**C**) haematopoietic defects were quantified by real-time qPCR for the expression of *gata1, pu.1, hbbe3 and lcp1* transcripts in *fgf21*-MO-targeted embryos relative to uninjected 19hpf embryos. Average fold change in expression calculated from 3 independent experiments, with samples (n=10 pools of 5 embryos each) analyzed each time in triplicate, is shown. Samples were normalized to *β-actin*, and control set to 1. Data are expressed as means ± SEM. Comparisons of numerical data were evaluated by paired Student t tests. *p<0.05. ff=fin fold; n=notochord; ov=otic vesicle. Scale bars: 100 µm.

In addition, erythroid-specific *gata 1* and *βe3globin* and the myeloid-specific *pu.1* and *l-plastin* were analyzed by qPCR in *fgf21* morphant embryos. A significant reduction of *gata 1* and *hbbe3* was seen on *fgf21*–MO injected embryos (Fig.8C). On the other hand, no significant differences in *pu.1* and *l-plastin (lcp1)* expression in *fgf21* morphants were observed (Fig.7C).

These results demonstrate the similarity between *fgf21* morphant blood phenotype (Fig. 7C) and *sparc* morphant blood phenotype (Fig. 2Q), which are characterized by a severe disruption of erythroid-specific cell makers. No differences in *sparc* gene expression were found when other fgf family members (*fgf3* and *fgf8*) were knocked-down (data not shown).

Sparc rescues the haematopoietic defect induced by *fgf21* knockdown

The disruption of expression of *gata-1* and *βe3globin* mRNA in *sparc* (Fig.2B,D,F,L,Q) and *fgf21* (Fig. 7C, 98B, 9B,) morphants together with the significant reduction in *sparc* in *fgf21* morphants (Fig.7B) raises the possibility that the effects of *sparc* on haematopoiesis may, in part, be due to perturbed *fgf21* signalling. Therefore, to test this possibility, we examined whether exogenous *sparc* can rescue *gata1* and *βe3globin* deficiency in gene-targeted *fgf21* zebrafish embryos. The severe reduction in *gata1* and *βe3globin* ICM expression after *fgf21* knockdown (10ng per embryo) (Fig.8B and Fig. 9B) was partially rescued by co-injection of *sparc* mRNA (~0.75 ng) (Fig. 8C and 9C).

The rescue success was 30.5 % (n=56) and 36.3 % (n=40) respectively, with an *fgf21*MO efficiency (the rate of embryos showing decreased *gata1* or *βe3globin* levels in the injected embryos) of 78% and 67%, respectively. No rescue was achieved when different mRNAs were co-injected with *fgf2*-MO (data not shown).

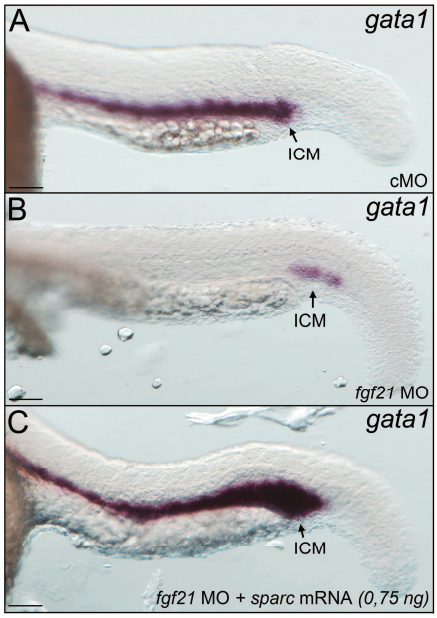

Figure 8. *Sparc* RNA partially rescues *gata1* expression in *fgf21* morphant haematopoietic phenotypes. Sense *sparc* mRNA (1 nl 750 µg/ml) was co-injected with 1 nl of 1mM *fgf21*-MO and the embryos were fixed for *in situ* hybridization. (**A**) cMO-injected embryos at 22hpf, (**B**) *fgf21*-MO injected embryos at 22hpf and (**C**) *fgf21*-MO plus sparc mRNA (0.750 ng). (**A-C**) Whole-mount *in situ* hybridization analysis of *gata1* expression. ICM= intermediate cell mass. Lateral views, anterior to the left. Scale bars: 100 µm.

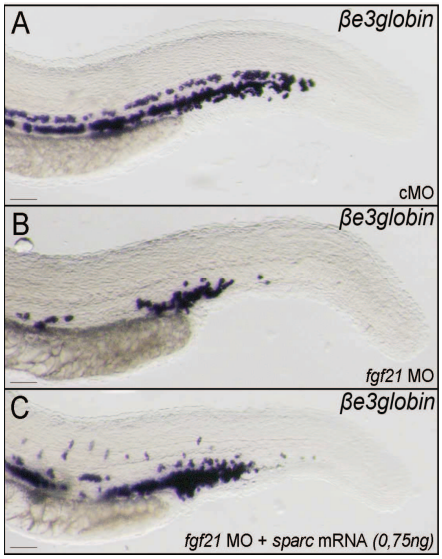

Figure 9. *Sparc* RNA partially rescues *βe3globin* expression in *fgf21* morphant haematopoietic phenotypes. Sense *sparc* mRNA (1 nl 750 µg/ml) was co-injected with 1 nl of 1mM *fgf21*-MO and the embryos were fixed for *in situ* hybridization. (**A**) cMO injected embryos at 30hpf, (**B**) *fgf21*-MO injected embryos at 30hpf and (**C**) *fgf21*-MO plus *sparc* mRNA (0.750 ng). (**A-C**) Whole-mount *in situ* hybridization analysis of *gata1* expression. Lateral views, anterior to the left. Scale bars: 100 µm.

DISCUSSION

In this study, we examined the role of Sparc during embryonic haematopoiesis in zebrafish. We have previously demonstrated that *sparc* is dynamically expressed in skeletal and non-skeletal tissues from early development to adulthood in zebrafish, suggesting a potentially wide range of action (Rotllant *et al*., 2008). While its specific role remains elusive, the high degree of similarity of zebrafish Sparc protein to Sparc protein of other vertebrates (Laizé *et al*., 2005) and analysis of conserved syntenies (Rotllant *et al*., 2008) suggests a strong evolutionary pressure to conserve this protein.
Injection of *sparc* morpholinos into 1-cell embryos resulted in specific inner ear (Rotllant *et al*., 2008), cartilage (Rotllant *et al*., 2008) and blood defects (Eckfeldt *et al*., 2005), suggesting a role for *sparc* in zebrafish development and haematopoiesis. We extended these studies to investigate the function of Sparc in zebrafish haematopoiesis in more detail.

Results demonstrated that *sparc* knockdown using MOs significantly reduced embryonic haematopoiesis at the lineage-committed cellular level. In particular, genes associated with primitive and transient erythroid progenitor cell development (*gata 1* and *βe3globin)* were down-regulated in the *sparc* morphants. Conversely, genes associated with primitive and transient myeloid progenitor cell development and genes associated with definitive haematopoiesis were not deregulated. This suggests a critical role of *sparc* in mediating erythroid progenitor cell development probably modulating the lineage-specific transcription factor *gata 1* expression levels or activity. Specificity of gene targeting was confirmed both *in vivo* by RT-PCR and *in vitro* by transcription-translation assay (Rotllant *et al*., 2008) as well as by successful *sparc* mRNA rescue (Fig.6).

We have also demonstrated that *sparc* knockdown had no effect on endothelial cell specification as shown by the intact vasculature in *fli-EGFP* transgenic zebrafish

embryos injected with *sparc*-MO. Proliferating and apoptotic cells were also examined in the ICM of control and *sparc* morphant zebrafish embryos. The rates of apoptotic cells in the ICM were not affected by injection of *sparc*-MO. In contrast, cell proliferation was increased in the ICM region of *sparc* morphant embryos. These results demonstrate that while *sparc* may not be essential for apoptosis, it could regulate cell proliferation, supporting the well-characterized role of *sparc* as a modulator of cell proliferation (Brekken *et al*., 2001).

We have previously shown that temporal expression of *sparc* during zebrafish embryonic development is initially detected by 14 hpf and the expression subsequently increased and persisted (Rotllant *et al*., 2008). Whole-mount mRNA *in situ* hybridization showed that by 22 and 25 hpf, *sparc* transcripts were strongly expressed in the notochord, fin fold and the PLM/ICM region where *gata1* is strongly expressed (Fig.1A,B). However, it has been shown that some notochordless mutants (*bozozok* (*boz*), *floating head* (*flh*) and *no tail* (*ntl*)) do not seem to have an apparent blood defect (Odenthal *et al*., 1996; Chin *et al*., 2000); consequently, the possible role of *sparc* in the notochord is unclear.

Our evidence suggests that *sparc* acts by modulating the lineage-specific transcription factor *gata 1* expression levels or activity. However, this assumption raises a puzzling question of how a matricellular protein can regulate expression of transcription factor genes. The role of *sparc* in cell-matrix interactions may hold the answer; *sparc* may mediate or trigger signal transduction pathways required for activation or maintenance of target genes transcription. This concept could be explored by identifying extracellular signalling molecules that act upstream of these genes encoding for *gata1* and *sparc*.
It is known that members of the Fgf family of signalling molecules can regulate *sparc* gene expression (Brekken and Sage, 2001; Whitehead *et al*., 2005). Furthermore, the expression of *gata1* transcription factor gene is regulated by Fgf signalling pathways (Nakazawa *et al*., 2006; Songhet *et al*., 2007), as altered *fgf* expression leads to

perturbation of expression of this gene (Yamauchi *et al*., 2006). The disruption of expression of *gata1* mRNA in *sparc* morphants raises the possibility that the effects of *sparc* on haematopoiesis may at least in part be due to perturbed *fgf* signalling. This hypothesis is supported by the fact that the *sparc* morphant blood phenotype is very similar to the *fgf21* morphant blood phenotype, which is characterized by a severe disruption of erythroid/myeloid progenitor cell development (Yamauchi *et al*., 2006).
Therefore, to test this hypothesis, we examined whether *sparc* gene expression was perturbed in *fgf21* morphants and if exogenous *sparc* could rescue *gata1* deficiency in gene targeted *fgf21* zebrafish embryos. We found that *sparc* expression was substantially reduced or missing in *fgf21* morphant embryos (Fig. 7B). Furthermore, injection of ~0.75 ng of synthetic *sparc* mRNA together with ~10ng *fgf21*-MO per embryo resulted in the partial rescue (30.5 %, n=56) of *gata1* expression in the ICM of *fgf21* morphant embryos (Fig. 8C). In addition, we also tested the *fgf21* gene expression in *sparc* morphants and found that *fgf21* mRNA expression was not altered (data not shown).
Our findings therefore suggest that *sparc*, at least in part, acting downstream of the *fgf21* signalling pathway, is critically required in mediating erythroid progenitor cell development in zebrafish.

In mammals, although it is well known that *sparc* gene expression is regulated by members of the *fgf* family and in turn the *fgf* pathway regulates primitive haematopoiesis by modulating *gata1* expression level and activity its function in haematopoiesis is not clear. Surprisingly, no mutations in *sparc* have been identified in humans, although mouse deficient in *sparc* have no severe developmental alterations including hematopoietic defects. It has been hypothesized that the presence of more *sparc* functional homologues in mammals functionally compensates for the lack of *sparc* expression, possibly leading to mild defects in *sparc*-null mice. However, studies carried out in other organisms such as *Caenorhabditis elegans* and zebrafish, where there is less redundancy, reduction in *sparc* produces much more significant defects. Consequently, our observations in zebrafish likely uncover the significant roles of Sparc.

In summary, our study shows that *sparc* has a critical role in embryonic haematopoiesis during zebrafish early development. In this process, it functions as a modulator of lineage-specific transcription factors *gata1* expression levels or activity. Our results also suggest that the effects of *sparc* on erythroid progenitor cell development may at least, in part, be due to a perturbed *fgf* signalling. However, the detailed mechanism on how *sparc* affects the lineage-specific transcription factors *gata1* (potentially via *fgf* signalling) and its functional conservation in other vertebrates remains to be elucidated.

SUPPLEMENTARY DATA

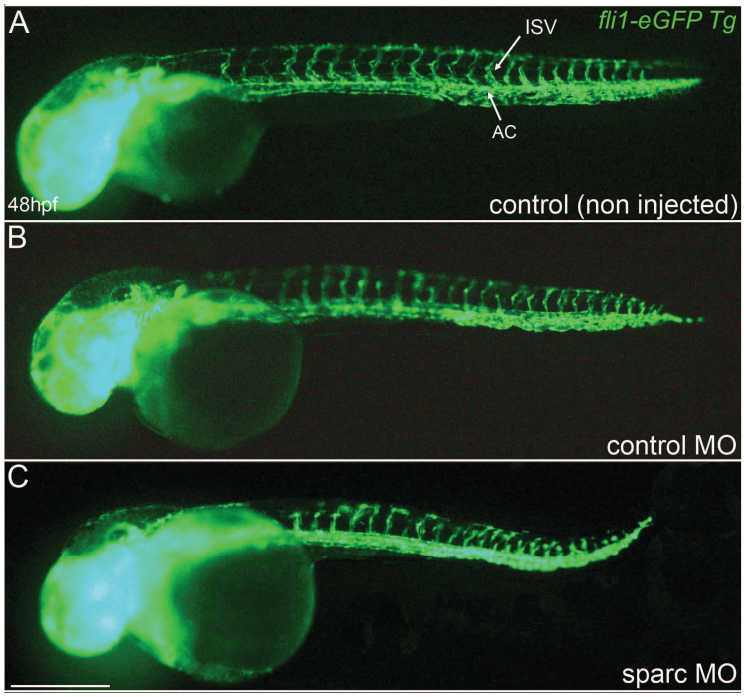

Figure 1. Phenotypes seen with wild type non-injected (A, control), injected cMO (B) and injected *sparc*-MO (C) embryos at 48h using *fli1-EGFP* transgenic fish (EGFP in vascular endothelial cells). The embryos do not display major vascular defects. AC, axial circulation; ISV, intersegmental vessels. Scale bar: 500 µm.

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Chapter II

Chapter II

Molecular response to ultraviolet radiation exposure in fish embryos: implications for survival and morphological development

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ABSTRACT

UVR exposure is known to cause developmental defects in a variety of organisms including aquatic species but little is known about the underlying molecular mechanisms. In this work we used zebrafish (*Danio rerio*) embryos as a model system to characterize the UVR effects on fish species. Larval viability was measured for embryos exposed to several UVR spectral treatments by using a solar stimulator lamp and an array of UV cutoff filters under controlled conditions in the laboratory. Survival rate and occurrence of development abnormalities, mainly caudal (posterior) notochord bending/torsion, were seriously affected in UV-exposed larvae reaching values of 53% and 72%, respectively, compared with non-UV-exposed larvae after 6 days postfertilization (dpf). In order to elucidate the molecular mechanisms involved, a matricellular glycoprotein named Sparc (Osteonectin) and the expression of a DNA-repair related gene, *p53*, were studied in relation to UVR exposure. The results indicate that *sparc* and *p53* expression were increased under UVR exposure due to wavelenghts shorter than 335 nm (i.e. mainly UVB) and 350 nm (i.e. short UVA and UVB), respectively. Furthermore, parallel experiments with microinjections of *sparc*-capped RNA showed that malformations induced by *sparc* overexpression were similar to those observed after a UVR exposure. Consequently this study shows a potential role of *sparc* in morphological deformities induced by solar UV radiation in zebrafish embryos.

INTRODUCTION

The ultraviolet (UV) region of the spectrum is generally classified into UVC (200–280 nm), UVB (280–315 nm) and UVA (315–400 nm) but only the UVB and UVA components reach the Earth's surface while the UVC radiation is completely absorbed by the stratospheric ozone layer. The UVB reaching the Earth's surface has increased during the last decades as a result of the stratospheric ozone depletion (Smith *et al*., 1992; Caldwell and Flint, 1994). After the Montreal Protocol there are some early signs of ozone recovery (http://www.esrl.noaa.gov/csd/assessments/2006/report.html); however, ozone is also affected by factors such as changes in the temperature and dynamics of the stratosphere which are, in turn, affected by climate change. This is delaying, perhaps indefinitely, a full recovery of ozone and consequent reduction in UVB.

Moreover, global change can also affect UVA and UVB in the aquatic environment through variations in cloud cover and the amount of the colored dissolved organic matter, among other factors (Mckenzie *et al*., 2003; Häder *et al*., 2003). While this increased UVB and the potential for long-term variation in UVR has motivated a variety of studies on their effects in both terrestrial and aquatic ecosystems, there are still major gaps in our understanding of the mechanisms involved. Several experiments have demonstrated significant alterations generated by UVR in organisms from different environments such as the induction of cutaneous malignant melanomas in mammals (Atillasoy *et al*., 1998; De Fabo *et al*., 2004), skeletal malformations and low hatching success in amphibians (Tietge *et al*. 2001; Häder *et al*., 2007; Marquis *et al*., 2008) and decreased survival and oxidative stress in different fish species (Charron *et al*., 2000; Zaragare and Williamson, 2001; Dahms and Lee, 2010). However, very little has been done to establish the molecular basis of the mentioned alterations produced by the exposure to UVR.

It is well known that DNA damage caused by UV radiation provokes adaptive cellular responses, which include DNA repair events, activation of several signaling cascades, and changes in transcription (Rastogi *et al*., 2010). The repair of UV-induced DNA lesions is launched during and immediately after a UV exposure. At the same time, a cellular response, either a replication arrest or apoptosis takes place (Rastogi *et al.,* 2010; Yabu *et al*., 2001).

p53 is an important transcription factor in vertebrates, expression of which acts as a protective mechanism after exposure to stress (*e.g.* UV radiation) (Latonen and Laiho, 2005). Multiple functions have been described for the *p53* activity. This gene can act like an effective inhibitor of cell cycle inducing a G1 arrest (Lin *et al*., 1992). Moreover, due to its 3′–5′ exonuclease activity (Janus *et al*., 1999), *p53* also is involved in DNA repair processes such as nucleotide excision repair (Ford, 2005; Zeng *et al*., 2009). However, the most described function of *p53* is activation of the apoptosis pathway after a severe cellular lesion. Recent studies have shown that apoptosis of cultured cells is led by *p53* gene after a DNA-damaging event. Cellular *p53* is normally maintained at a low expression level, but rapidly increases upon exposure to harmful agents such as UVR (Jhappan *et al*., 2003). For example, in zebrafish an enhanced rate of apoptosis associated with a high *p53* expression was observed after UV exposure (Zeng *et al*., 2009). Furthermore, a mutation in *p53* may inhibit the apoptotic process and trigger carcinogenesis (Li *et al*., 1996; Chen *et al*., 2008).

In skin, the cellular events are coupled with paracrine events and the following photoprotective responses, such as changes in the extracellular matrix. However, the role of matrix proteins in protective mechanisms after a UV exposure is still unclear. Three matricellular senescence-associated proteins, *i.e.* fibronectin, Sparc and SM22, were increased in human skin diploid fibroblasts 72 h after several exposures to UVB (Chainiaux *et al*., 2002). Furthermore, Sparc is also associated with an aggressive tumor phenotype in certain types of cancer such as melanomas (Tai and Tang, 2008). Multiple

biological functions have been associated with this protein as it was first described as the major noncollagenous constituent of vertebrate bones. In zebrafish, *sparc* expression appears early in development and it is required for skeletal development (Rotllant *et al*., 2008).

The purpose of this study was to characterize the potential molecular responses caused by UV radiation in the freshwater species zebrafish, *Danio rerio*. Zebrafish is a species widely used as a model organism in laboratories because of several properties that make this species simple to use. Some of these advantages are their small size, fast development and hundreds of embryos per spawning. Moreover, external development and their transparent embryos are important characteristics for an easy phenotype observation allowing an appropriate morphological monitoring.

In the present study, we investigated potential underlying molecular mechanisms of solar UV radiation induced musculo-skeletal deformities in fish embryos. First, different exposures of full spectrum irradiance including photosynthetic active radiation (PAR, *i.e.* visible radiation, 400–700 nm), UVA and UVB, as well as different spectral treatments using an array of several UV cutoff filters were used to determine the embryonic sensitivity to UVR. Expression of *p53*, a DNA–repair-related gene and a well-characterized marker of cellular damage caused by UV exposure (Zeng *et al*., 2009) and Sparc, an extracellular matrix protein involved in cell–matrix interactions and bone development (Tai and Tang, 2008; Rotllant *et al*., 2008) were measured under all the conditions. Second, survival and malformation percentages were assessed in non-UVR exposed (*i.e.* control) and UVR exposed embryos. Finally, *sparc* overexpression experiments were carried out in zebrafish embryos to determine the potential role of Sparc on developmental abnormalities produced by UVR exposure and how these affect performance, health and well-being of fish species.

MATERIAL AND METHODS

Fish husbandry

Zebrafish embryos of the standard wild type Tue (Tuebingen) strain were raised at 10 h light and 14 h dark photoperiod at 28°C. The procedures for zebrafish culture and embryo collection have been described previously (Westerfield *et al*., 2007). The designation of zebrafish developmental stages follows that of Kimmel *et al.* 1995.

Experimental setup and UVR exposure

Zebrafish embryos were exposed in a special polychromatic incubator, the "photoinhibitron." The incubator uses a 2500 W xenon lamp (Solar simulator lamp, Schoeffel Instrument Corp., Westwood, NJ), which, after appropriate filtration, provides PAR, UVA and UVB in similar proportions as solar irradiance (Fig. 1).

The beam passes through an array of eight long-pass filters constructed using Schott (Duryea, PA) WG filters (nominal 50% transmittance [*T*] at 280, 295, 305, 320 and 335 nm), a Schott GG filter (50%*T* at 395 nm) and Newport (Franklin, MA) LG filters (50%*T* at 350 and 370 nm). For convenience, we subsequently refer to each of these long-pass filters by their wavelength of 50%*T* or "cutoff" wavelength. In order to obtain treatments with varying irradiance, long-pass filters were combined with neutral density screens to produce up to 10 different irradiances for each filter for a total of 80 spots of varying spectral composition and irradiance.

For embryo exposures we selected several positions for each long-pass filter in which PAR irradiance was about 600 ± 50 μmol photons m^{-2} s^{-1} as measured with a QSL-2101 spherical sensor (Biospherical Instruments).

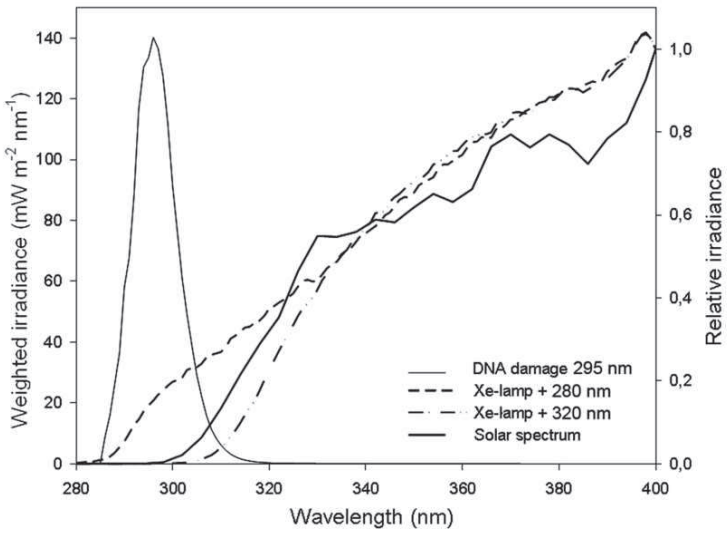

Figure 1. Spectral irradiance from the xenon lamp used to expose zebrafish embryos to UVR in the polychromatic incubator "photoinhibitron" and the solar irradiance. The thick lines show the unweighted irradiance normalized to 1 at 400nm to facilitate comparison among spectra from the most damaging treatment in the photoinhibitron (280 nm cutoff; dashed line); a treatment very similar to solar spectrum (320 nm cutoff; dashed-dotted line) and the solar spectrum (solid line). Weighted irradiance ($E_{eff}(\lambda)$, mW m^{-2} nm^{-1}) for the 295 nm cutoff using the Setlow action spectra for DNA damage (Setlow, 1974) normalized to 300 nm is also shown (thin solid line)

This PAR value is *ca* 30% of the maximum irradiance of a sunny day at mid latitudes. Unweighted UVR irradiance ranged from 45.8 W m^{-2} in the most damaging treatment (280 nm cutoff) to 1.5 W m^{-2} in the least damaging treatment (395 nm cutoff), with UVA irradiance being 40.9 and 1.5 W m^{-2}, and UVB irradiance 4.9 and 0.0 W m^{-2}, respectively. Weighted irradiance in the two most damaging treatments (280 and 295 nm cutoff) calculated using the action spectra for DNA damage of Setlow normalized to 300 nm (Setlow *et al*., 1974) was 4.74 and 1.68 W m^{-2} (Fig.1). Weighted irradiance in the next most damaging treatment, 320 nm cutoff, was 0.014 W m^{-2}. For comparison, solar exposures can reach up to 0.161 W m^{-2} at the equator (Cullen and Neale, 1997). The light treatments are directed to 1.8 cm diameter, flat-bottom quartz cuvettes that are mounted within a temperature-regulated block and that were filled with water and fish embryos for exposure. Temperature was maintained at 28°C and spectral irradiance was

measured with a scanning monochromator (SPG 300 Acton Research, Acton, MA) with a fiber optic and photomultiplier tube as previously described (Neale and Fritz, 2001).

To determine the effect of exposure duration, 4 h postfertilization (hpf) embryos were exposed to full spectrum irradiance (280 nm cutoff) in the photoinhibitron for 60, 120, 180 and 240 min and then sampled at 24 hpf for RNA extraction.

Subsequently, in order to establish the effect of different wavelengths of UV a second exposure experiment with 4 hpf embryos was carried out using different cutoff filters (280, 295, 320, 335, 350, 370 and 395 nm cutoff) for 150 min. Dark control group was also included as nonexposed reference sample. Samples were collected at 24 hpf for analyzing *sparc* and *p53* expression by quantitative real time PCR (qRT-PCR). In addition, fish exposed to UVR filtered through the 295 nm cutoff or the 395 nm cutoff filter (*i.e.* UVR excluded, control) were collected for *in situ* hybridization. Control and UV exposed 24 hpf embryos were fixed overnight at 4°C in 4% paraformaldehyde in 1XPBS, washed in PBS, and stored at −20°C in 100% methanol for *in situ* hybridization. Some embryos exposed to the 295 and 395 nm cutoff treatments were raised until 6 days postfertilization (dpf), sampling at 1, 2, 3 and 6 dpf, to test the larval viability and development abnormalities. Ethical approval for all animal studies was obtained from the Institutional Animal Care and Use Committee of the IIM-CSIC Institute in accordance with the National Advisory Committee for Laboratory Animal Research Guidelines licensed by the Spanish Authority. Results show the mean ± SEM of two independent experiments, with samples analyzed each time in triplicate.

RNA isolation and qRT-PCR

Control nonexposed and UV exposed 24 hpf embryos were collected and total RNA was extracted using Trizol reagent according to manufacturer's protocol (Invitrogen). cDNA was synthesized from total RNA using superscript III (Invitrogen) according to manufacturer's recommendations. The following primer sequences were used for qRT-

PCR: for *sparc* (5′primer / 3′primer): CCCTCTGCGTGCTCCTCTTA / GCATCGCACTGCTCAAAGAA, for *p53* (5′primer / 3′primer): GGATCCTTCTTGCAAAGCAATGGCGCA / CCGGTGAATAAGTGCAAGTTA and for 18S (5′primer / 3′primer): ACCACCCACAGAATCGAGAAA / GCCTGCGGCTTAATTTGACT. All expression levels were normalized to *18S* using the $2^{-\Delta\Delta T}$ method (Livak and Schmittgen, 2001). qRT-PCR reactions were performed using an AB 7300 real time PCR System and SYBR green incorporation (Applied Biosystems). The PCR cycles for all primer sets were: denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All samples were done in triplicate and each condition was repeated two times. Dark control group was used as reference sample.

Larval viability and developmental deformities percent

Survival and developmental deformities percentages were calculated in the control (395 nm cutoff) and UV (295 nm cutoff) exposed embryos groups. Larvae were exposed for 150 min in the photoinhibitron as explained previously and were subsequently transferred to 1 L tanks under optimal growth conditions.

Survival percent was calculated as the number of embryos survived within 1, 2, 3 and 6 dpf divided by the total number of embryos and multiplied by 100. Alterations in spinal curvature were used as marker for the calculation of developmental deformities percentage. Developmental abnormalities percent was calculated as the number of abnormal embryos survived within 1, 2, 3 and 6 dpf divided by the total number of surviving embryos and multiplied by 100.

mRNA synthesis, microinjection and in situ hybridization

For mRNA synthesis, the pCS2^{+}-*sparc* was linearized with Not I. Capped mRNA was transcribed *in vitro* using the SP6 Message Machine Kit (Ambion).

sparc-capped mRNA was injected into one- or two-cell stage embryos. The amount of mRNA injected was titrated for the maximal doses that could be injected (Rotllant *et al*., 2008). Approximately 1 nL of two different *sparc*-capped mRNA concentrations (200 or 800 μg mL^{-1}) was injected per embryo. Approximately 150 embryos were used. Whole-mount *in situ* hybridization was performed using digoxigenin-labeled antisense *sparc* probe as previously described (Rotllant *et al.,* 2008). Control embryos were injected with 1 nL of eGFP (Green Fluorescent Protein) capped mRNA (500 μg mL^{-1}).

Statistical analysis

Results are given as mean ± SEM. First one-way analysis of variance (ANOVA) was applied followed by the Student–Newman–Keuels (SNK) test to check differences between particular groups. Data were log-transformed when necessary to achieve normality and homogeneity of variance (INSTATtm; GraphPad Software, V2.04a). The level for accepted statistical significance was $P < 0.05$. Significant differences in the figures are indicated by asterisks. Exposure–response curves were fitted using a nonlinear data analysis program describing a sigmoid curve (Sigma Plot; Scientific Graphic Software, Version 9.0).

RESULTS

Time series of full spectrum exposure on the matricellular protein Sparc and the DNA repair-related gene *p53* expression

The expression levels of the matricellular protein Sparc and the DNA repair-related gene *p53* after different UVR exposure times (60, 120, 180 and 240 min) under full spectrum irradiance (280 nm cutoff) on 4 hpf embryos were determined by the qRT-PCR in 24 hpf embryos. Results showed a time-dependent increase of the expression of both genes in response to a full spectrum exposure (Fig.2). The maximum increase in expression was obtained after a 180 min exposure when *sparc* and *p53* expression levels reached a 2.2 ± 0.16 and 1.77 ± 0.15-fold increase, respectively. Furthermore, an exposure–response curve was fitted to the measured levels in each expression time series (Fig.2), from which the exposure time to reach 50% of the maximum was estimated to be 150 min for *sparc* and 140 min for *p53*.

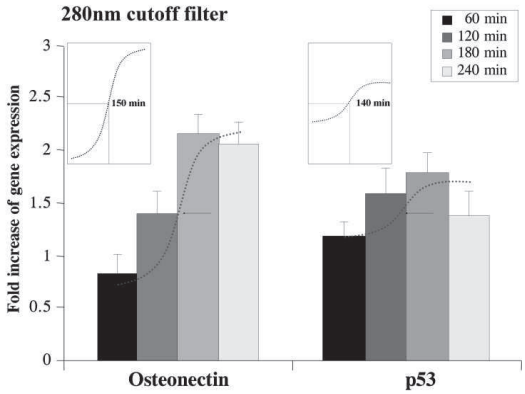

Figure 2. Effect of duration of exposure to full spectrum radiation (WG280) on *sparc* and *p53* gene expression. Sphere stage (4hpf) zebrafish embryos were exposed to full spectrum irradiance (280nm cutoff) in the polychromatic incubator photoinhibitron for 60, 120, 180 and 240 min and then sampled at 24hpf. qRT-PCR for *sparc* and *p53* was carried out on 24 hpf exposed and non-exposed embryos. Shown is the average forl change in *sparc* and *p53* gene expression calculated from two independent experiments, with samples analyzed each time in triplícate. Samples were normalized to *18S* and dark control reference group set to 1. Data are expressed as mean ± SEM. Exposure-response curves (insets) were fitted using a nonlinear data analysis program describing a sigmoid curve

UV action spectra for the matricellular protein Sparc and the DNA repair-related gene *p53* expression

Once the estimated exposure time to reach 50% of the maximum increase in gene expression in response to the full spectrum exposure was determined, we examined the effect of excluding various portions of the UV spectrum on the expression of the *sparc* and *p53*. For that purpose, a 150 min time exposure experiment with 4 hpf embryos using different cutoff filters (280, 295, 320, 335, 350, 370 and 395 nm) was carried out.

The increase in *sparc* and *p53* gene expression was higher when embryos were exposed to UVB than when UVB was excluded from the spectra, reaching values around a two-fold increase compared with those of non-UV exposed embryos (395 nm cutoff) (Fig.3).

Significant differences were also observed for the spectral response between *sparc* and *p53* gene expression. *sparc* levels increased significantly only when wavelengths shorter than 335 nm were included in the exposure spectra while *p53* expression was induced by longer wavelengths, when wavelengths shorter than 350 nm were included in the spectra. Moreover, spectral treatments including only longer wavelengths of the UVA region and PAR did not produce significant increases in *sparc* and *p53* gene expression compared with non-UV exposed embryos.

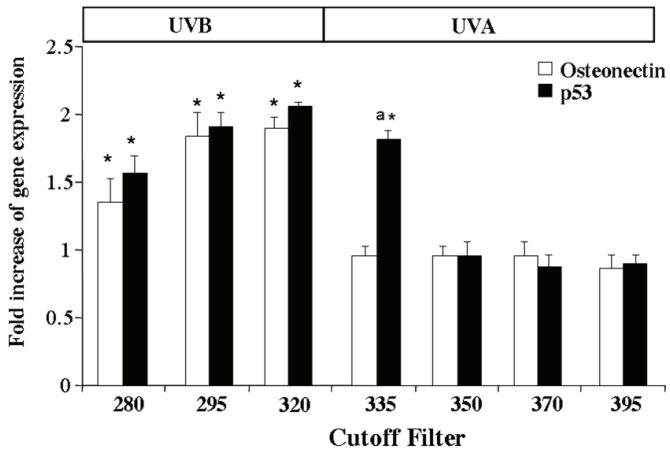

Figure 3. UVR wavelength exposure dependent expression of the *sparc* and *p53* genes in zebrafish embryos. Sphere stage (4hpf) zebrafish were exposed for 150 min to different UVR spectral treatments (280, 295, 320, 335, 350, 370 and 395 nm long-pass filters) then sampled at 24hpf. The figure shows the average fold change in *sparc* and *p53* gene expression calculated from two independent experiments, with samples analyzed each time in triplícate. Samples were normalized to 18S and dark control reference group set to 1. Data are expressed as mean ±SEM. Comparisons of numerical data were evaluated by one-way ANOVA followed by Student-Newman-Keuels (SNK) test (INSTATtm; GraphPad Software, V2.04a). Degree of freedom=95 (treatment, 15; residuals, 80). (a) denotes significant difference between both genes at that particular exposure treatment and (*) denotes significant differences with dark control reference group. The significant level was $P< 0.05$.

To verify the *sparc* expression increase in UV exposed embryos, *in situ* hybridization was carried out in UVR excluded (control, 395 nm cutoff) and UV-exposed embyros (295 nm cutoff). By 24 hpf, *sparc* transcripts were significantly increased in the caudal fin fold, notochord, somites and the otic vesicle of UV-treated embryos compared with embryos where UVR was excluded (Fig.4, arrow heads).

Alterations in spinal curvature were also identified in the UV-exposed group (see Fig.4, arrow). The results obtained by *in situ* hybridization agree with those observed by qRT-PCR, indicating a significant increase of *sparc* expression after UV exposure.

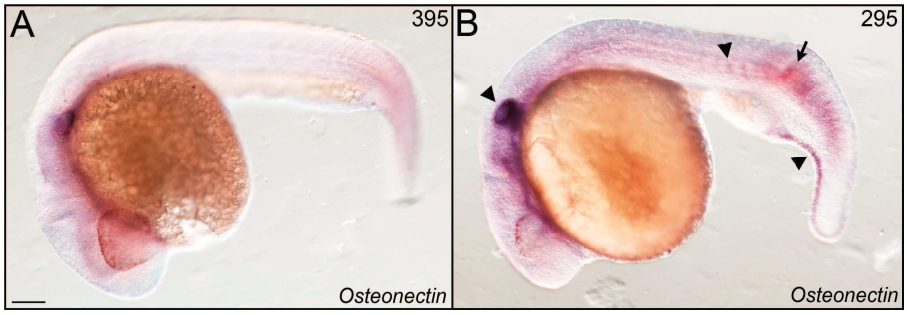

Figure 4. Increased *sparc* expression in UV-exposed (295nm cutoff) zebrafish embryos. Whole-mount in situ hybridization analysis of *sparc* expression in (**A**) UVR excluded control (395 nm cutoff) and (**B**) UV (295 nm cutoff) exposed zebrafish embryos. Sphere stage (4hpf) zebrafish were exposed for 150 min at 295 or 395 nm cutoff then sampled at 24 hpf. Arrow indicates phenotypic mallformation observed. (**A, B**) lateral views, anterior to the left. Scale bars: 100µm

Larval viability and morphological phenotypes

We examined the percent survival and incidence of development abnormalities for UVR excluded control (395 nm cutoff) and UV (295 nm cutoff) exposed embryos within 1, 2, 3 and 6 dpf. As indicated in Table 1, in the UVR excluded control groups, 94 ± 1.3% of the embryos survived up to 6 days. In contrast, only 53 ± 8.6% of embryos exposed to UV survived up to 6 days, which is significantly lower from the control treatment group. Significant reduction of survival rate in UV (295 nm cutoff) exposed embryos was also found at 1, 2 and 3 days after treatment. The incidence of fish with developmental abnormalities was also significantly higher in UV-exposed embryos in all developmental stages analyzed (Table 1). The number of abnormalities increased with time after exposure in the UV-exposed larvae from 5 ± 0.6% and 48 ± 10% in the control and UV-exposed embryos, respectively, at day 1 to 7 ± 1% and 72 ± 4.6%, respectively, at day 6.

	% Survival		Developmental abnormalities	
Day	Control	UV	Control	UV
1	92(0.8)	89(0.15)*	5(0.6)	48(10.2)*
2	98(0.7)	78(4.5)*	4(0.4)	54(8.3)*
3	96(1.6)	69(5.6)*	5(0.5)	56(7.9)*
6	94(1.3)	53(8.6)*	7(1.2)	72(4.6)*

Table 1. Mean survival and deformities (±SEM) of zebrafish embryos under full spectrum radiation (295nm cutoff) or UVR-excluded (395nm cutoff) exposures.

The type of developmental abnormalities were similar in all developmental stages analyzed, with caudal (posterior) notochord bending/torsion the most frequent developmental abnormalities recorded in the UV (295 nm cutoff) exposed embryos (Figs.4 and 5).

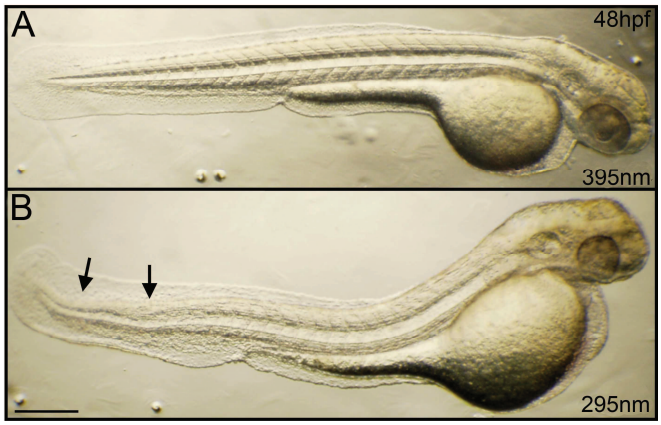

Figure 5. Exposure of zebrafish embryos to UVR (i.e 295nm cutoff) yielded high frequencies of morphological malformations. Sphere stage (4hpf) zebrafish were exposed to UVR for 150min then sampled at 48h. (**A**) UVR excluded control (395 nm cutoff) and (**B**), UV (295nm cutoff) exposed zebrafish embryos. Arrow indicates phenotypic malformation observed. (**A, B**) lateral views, anterior to right. Scale bars: 100µm

Sparc injection mimics the morphological phenotypes observed in embryos exposed to damaging UV radiation

To link the increase of *sparc* expression with the high incidence of developmental abnormalities found in UVR exposed embryos, 0.2 or 0.8 ng of *sparc*-capped mRNA were injected into one- or two-cell stage embryos. After injection the embryos were raised to 24 hpf and their phenotypes were scored. The expression of *sparc* was dose dependent, as it was higher when a larger amount of capped mRNA was injected (Fig.6). Phenotypic malformations observed, mainly caudal (posterior) notochord bending/torsion, were similar to those observed after a damaging UV exposure (Figs.5 and 6).

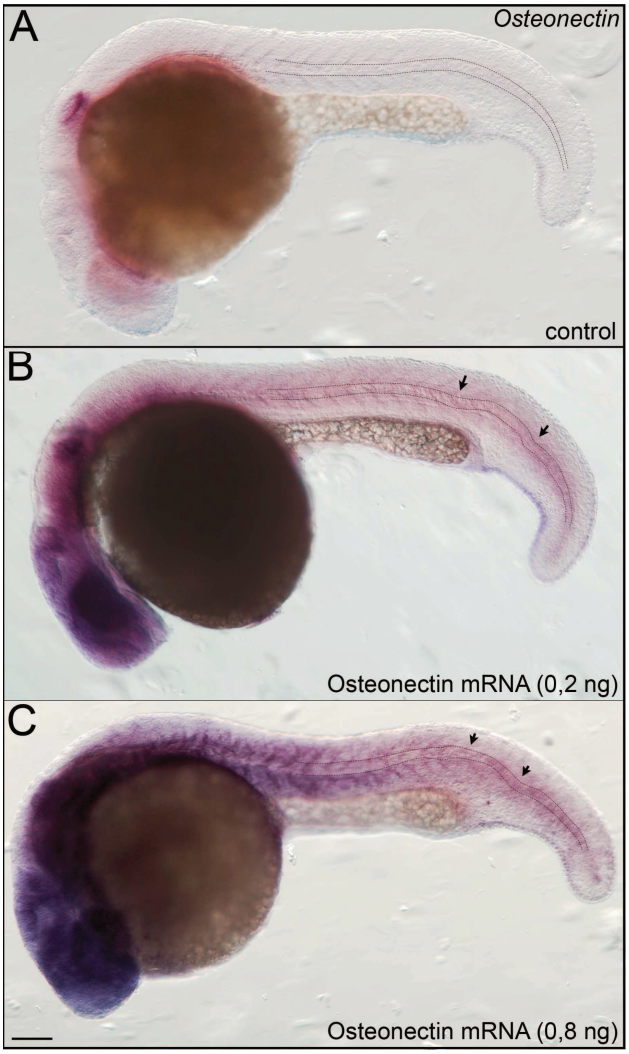

Figure 6. *Sparc* overexpression phenotype mimics the UVB exposure phenotype (295 nm cutoff) in 24hpf embryos. (**A**) Control sense EGFP-capped mRNA-injected embryos (0.5ng per embryo). (**B**) Sense *sparc*-capped mRNA-injected embryos (0,2 ng per embryo). (**C**) Sense *sparc*-capped mRNA-injected embryos (0,8 ng per embryo).Arrow indicates phenotypic malformation observed. (**A-C**) lateral views, anterior to the left. Scale bars: 100µm

DISCUSSION

Ultraviolet radiation is widely mentioned as a damaging environmental factor for organisms in both terrestrial and aquatic systems (Caldwell *et al*., 1998). The effects derived from a deleterious UV exposure are known to cause irreparable effects at different levels from organism survival and reproduction (Tietge *et al*., 2001; Häder *et al*., 2007; Marquis *et al.,* 2008; Charron *et al*., 2000) to cellular metabolism and viability (Dahms and Lee, 2010; Rastogi *et al*., 2010). However, the molecular responses triggered in an animal organism after a UV exposure are not yet understood. Previous studies have already established that the zebrafish system can be an important tool to investigate the biological effects of UV light in vertebrate development (Charron *et al*., 2000; Jhappan *et al*., 2003). Moreover, it has been demonstrated that zebrafish have a competent antioxidant response and photorepair system to repair UV induced DNA damage (Jhappan *et al*., 2003). This photorepair system includes upregulation of *p53* gene and cell cycle arrest (Sandrini *et al*., 2009).

In this study, UVR exposure was performed using a special polychromatic incubator, the "photoinhibitron," under controlled conditions in the laboratory. Under natural conditions the direct effects of UV radiation on specific molecular targets are difficult to assess due to the interaction with other environmental factors and changes in irradiance caused by the variability in cloud cover, atmospheric composition and/or the amount of the colored dissolved organic matter, among others. The incubator uses a solar simulator lamp which, after appropriate filtration, emits PAR, UVA and UVB in similar proportions as those observed under natural conditions (Neale and Fritz, 2001). This produces a reliable UVR-dependent response in zebrafish embryos where the damage induced by short UV wavelengths is counteracted by the repair mechanisms activated by longer wavelengths. Our results show that using this experimental setup, exposure to UV can cause a DNA damage response in zebrafish embryos (Fig.2).

We demonstrate that UV exposure can induce an exposure-dependent increase in the gene *p53* expression. Therefore, like mammalian and fish cells, zebrafish embryos do show an increase in *p53* gene expression in response to UVR. In analogy with these other organisms, *p53* expression in zebrafish is expected to be beneficial by increasing DNA repair, but other functions of *p53*, like apoptosis may have contributed to abnormal development. It has been shown that keeping *p53* at low levels during embyogenesis is critical to protect normal development (Zeng *et al.*, 2009).

In parallel with *p53* induction, we also demonstrated a UV exposure-dependent increase in the expression of the matricellular protein, Sparc. Sparc is a multifunctional protein that modulates cell–matrix interaction and cell function, but does not seem to have a direct structural role in the matrix (Brekken and Sage, 2001). Sparc is an evolutionary conserved matricellular protein (Rotllant *et al.*, 2008, Laizé *et al.,* 2005). Within all vertebrates, *sparc* is expressed in a temporally and spatially specific manner with strong expression during embryogenesis in developing tissue such as the notochord, somites and embryonic skeleton (Rotllant *et al*., 2008, Holland *et al.,* 1987, Renn *et al*., 2006). A marked reduction in *sparc* expression occurs once adulthood is reached, although it has been shown that it re-emerges in response to tissue injury, remodeling and inflammation (Bornstein and Sage, 2002). However, the precise function of Sparc, in particular during early embryogenesis, is largely unknown. Its dynamic expression patterns during embryogenesis and its sequence homology with other vertebrates, suggests a conserved function of Sparc in vertebrates (Rotllant *et al*., 2008). Consequently, Sparc potentially influences important physiological and pathobiological processes as a regulator of cell–matrix interactions.

To date, there is little information on the direct effect of UV on *sparc* gene expression regulation. Aycock *et al.*, 2004 showed that *sparc* was present in relatively high quantities in UV-induced squamous cell carcinoma, however, was undetectable in skin from the nonirradiated control group. In addition, Sparc null mice were tumor resistant,

developing no squamous cell carcinoma in response to UV radiation. Therefore, they suggested that Sparc had a critical role in mediating skin tumor formation in response to UV irradiation.

Regarding the spectral dependence of gene expression, exposure to a combination of UVB and UVA radiation produced a greater *sparc* and *p53* expression increase than UVA alone (Fig.3), thus probably indicating a higher capability of UVB to produce cellular damage in zebrafish. However, significant differences in the expression of both genes were observed in the shorter wavelengths of the UVA, in which *p53* was activated by less damaging spectral treatments than *sparc*. Longer wavelengths of UVA did not produce a significant expression increase of both genes compared with embryos exposed to nondamaging PAR. Interestingly, the highest expression of both genes occurred in the WG320 treatment, even though embryos received lower exposure to DNA damaging irradiance than the other treatments with UVB. This could have occurred because DNA damage was so high in these latter treatments that incipient apoptosis had already decreased embyronic capacity for gene expression by 24 hpf (20 h after exposure).

Previous work in zebrafish has demonstrated the capacity of UVA to activate a mechanism, the photoenzymatic repair (PER), which repairs the DNA damage caused by UVB exposure (Dong *et al*., 2007). The evidence of the mentioned repair system is the initial detection of photolyase enzyme in 3 hpf zebrafish embryos (Dong *et al.,* 2008). The induction of PER partially compensates for a considerable decrease in tolerance of UVB exposure at this developmental stage (Dong *et al*., 2008). It is suggested that the higher UVB tolerance at the egg stage may be related to other (dark) repair mechanisms as well as possible shielding by the chorion and other maternally derived photoprotective compounds. In conclusion, it has to be considered that sensitivity to UV radiation may vary between developmental stages.

In addition, a decrease in survival percent and an increase in developmental abnormalities were observed in UV-exposed embryos (Table 1). Decreased survival in

the WG295 treatment is expected given the very high exposure to DNA damaging irradiance (Fig.1). The increase of *sparc* expression detected by qRT-PCR and *in situ* hybridization could be an additional cause for these mentioned effects in UV-exposed embryos. The phenotypic abnormalities revealed by previous overexpression and loss-of-function studies (Damjanovski *et al*., 1997) also support this possibility. It has been shown that injection of *sparc* RNA into early blastomeres is associated with head and axis defects in xenopus. Histological analysis revealed somite malformations that corresponded with the kinked axis (Damjanovski *et al*., 1997).

In this study we also show that ectopic expression of *sparc* affects zebrafish development. Microinjection of capped and poly(a)-tailed full-length zebrafish *sparc* mRNA into 1–2 cell zebrafish embryos generated phenotypic malformations, with caudal (posterior) notochord bending/torsion as the most frequent deformity.

The fact that similar phenotypic malformations linked to an increase in *sparc* gene expression were found in UV-exposed and in ectopic *sparc* expression experiments therefore suggests *sparc* expression as one of the possible molecular mechanisms of UV-radiation induced phenotypic anomalies. The main features of these anomalies were reproduced by ectopic Sparc expression, suggesting a limited role of other stress induced genes like *p53* in this type of developmental abnormality. However, *p53* expression is known to have pervasive effects on a number of developmentally important processes (Latonen and Laiho, 2005) and so may also be affecting survival and morphological development in UV-exposed embryos.

In summary, the present study has demonstrated that zebrafish Sparc plays a critical role in mediating UV-radiation induced phenotypic developmental anomalies thus further unveiling its function in the regulation of embryonic development. However, the precise Sparc-mediated signal transduction mechanism remains to be determined. Moreover, the present results also support the previous demonstrated upregulation of *p53* gene in response to UV-radiation exposure in fish.

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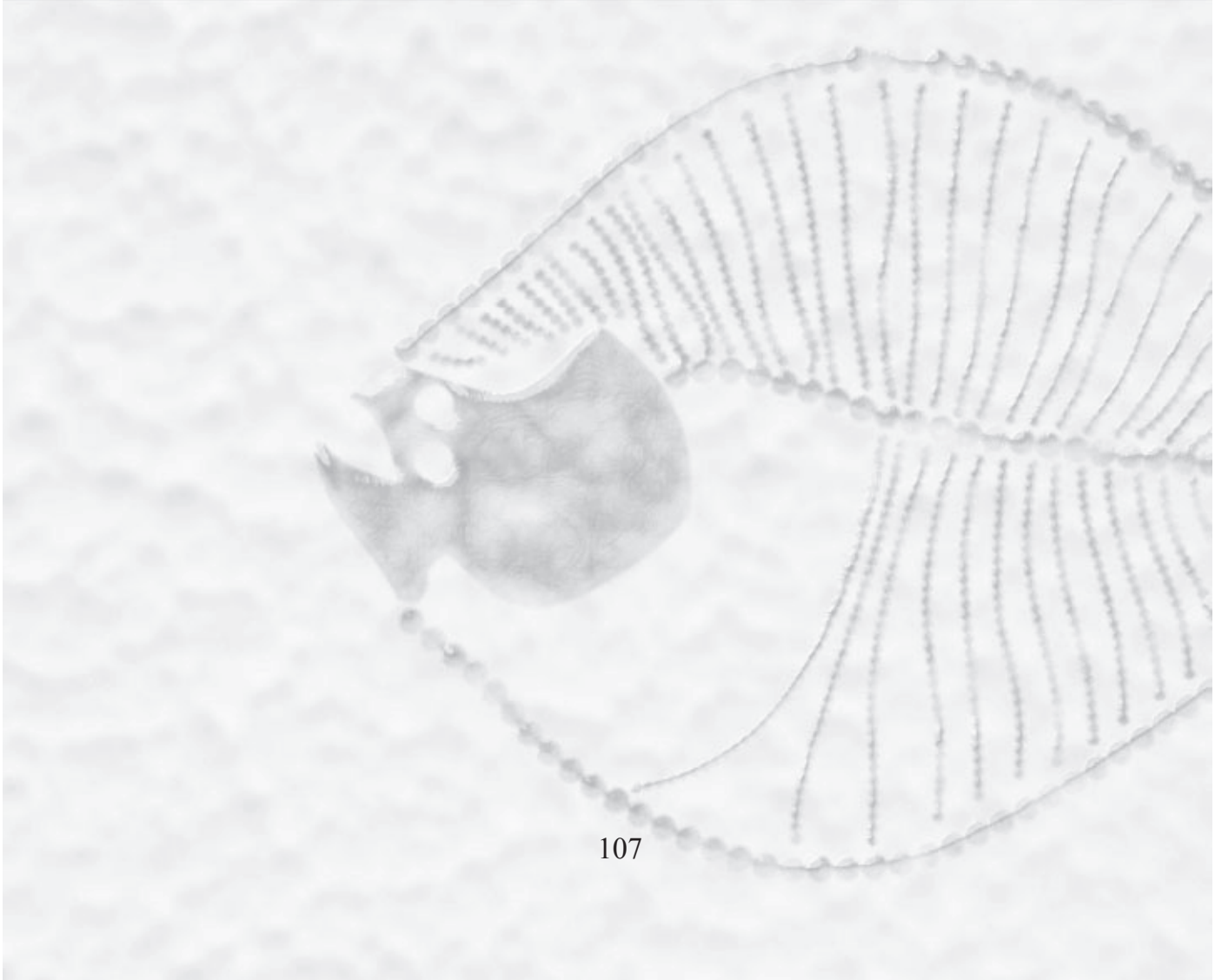
Chapter III



Chapter III

5'-UTR intron is crucial for transcriptional regulation of the zebrafish *sparc* (*osteonectin*) gene

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In preparation

ABSTRACT

Sparc (Osteonectin) is an evolutionarily conserved matricellular protein that modulates cell-matrix interaction and cell function. In all vertebrates, Sparc is expressed in a temporally and spatially specific manner, with strong expression during embryogenesis in developing tissues such as the notochord, somites and embryonic skeleton; a marked reduction in Sparc expression occurs once adulthood is reached. However, the precise function of Sparc and the regulatory elements required for its temporally and spatially specific expression in particular during early embryogenesis are largely unknown. We report here transient and stable expression analyses of *egfp* expression from 7,2kb-*sparc*-EGFP construct generated with the 0.2-kb *sparc* promoter and its 5' flanking sequence 7 kb upstream of the translated exon II. *Egfp* expression was found in the notochord, otic vesicle, fin fold, somites, intermediate cell mass, olfactory epithelium and skeletal and cardiac muscles of 7,2kb-*sparc*-EGFP injected embryos. In situ hybridization confirmed *sparc* mRNA expression in these tissues, suggesting that transient and stable expression of the 7,2kb-*sparc*-EGFP construct recapitulates that of the endogenous *sparc* gene. To understand the molecular mechanisms that regulate *sparc* gene expression, we functionally characterized the promoter activity of putative zebrafish 5' genomic fragment. Deletion analyses on the 5′ end of the −127/+7168 promoter region excluded the functional importance of nt -127/+125 in the transcriptional regulation of the gene, and intron removal (nt+126/+7168) resulted in complete reduction of promoter activity. Computer-based analysis revealed a number of *cis*-acting transcription factor binding sites and a CpG island immediately proximal to the translation start site within the intron sequence. DNA-specific methylation assays revealed that CpG dinucleotide-specific demethylation can increase *sparc* gene transcriptional activity three- to four fold.

INTRODUCTION

Sparc (Secreted Protein Acidic and Rich in Cysteine), also named Osteonectin or BM-40, is a non-structural component of the extracellular matrix that it is thought to modulate cell–matrix interactions, particularly during tissue remodelling and at sites of high cellular turnover during development, wound-healing and carcinogenesis. Sparc is spatially and temporally regulated during development and displays a high degree of sequence conservation (Laizé *et al*., 2005; Rotllant *et al*., 2008), indicating a conserved, essential functional role in vertebrates.

We have previously shown that the zebrafish *sparc* gene is also expressed in a temporally and spatially specific manner, with strong expression in the developing inner ear and pharyngeal cartilage (Rotllant *et al*., 2008). We showed further that *sparc* interacts with genes in known genetic networks, unveiling its novel functions in regulating pharyngeal cartilage and inner ear development. We also demonstrated a critical role of Sparc in embryonic haematopoiesis during early development of zebrafish (Ceinos *et al*., 2013). Specifically, we showed that *sparc* is a modulator of lineage-specific transcription factor *gata 1* expression levels or activity. Furthermore, we demonstrated a UV exposure-dependent increase in the expression of the matricellular protein osteonectin in zebrafish embryos (Torres-Núñez *et al*., 2012).

Therefore, because of its spatially and temporally regulated expression during development and its multifunctional role, *sparc* gene is expected to be tightly regulated. Although isolation of *sparc* promoter regions from different species has been reported (McVey *et al*., 1988; Young *et al*., 1989; Damjanovski *et al*., 1998), little is known about regulation of *sparc* gene expression at the transcriptional level during embryogenesis. It has been shown that the 5' flanking region of the *sparc* gene contains *cis*-regulatory elements that might be responsible for differential expression during normal development in different vertebrate species. Possible regulatory sequences found on *sparc* promoter include GATA factor binding sites, growth hormone consensus

sequences, heat shock factors, metal responsive elements, NF1 and SP1 binding and myogenic elements. Another important class of regulatory regions, the so-called *CpG islands*, has also been found in *sparc* promoter regions (Yang *et al*., 2007; Gao *et al*., 2010). CpG islands are specific regions of 200 base pairs (bp) with over 50% G+C content and a CpG frecuency of 0.6 (observed/expected ratio) susceptible to transcriptional gene regulation by DNA methylation. Therefore, aberrant methylation of *sparc* promoter has been associated with disc degeneration (Tajerian *et al*., 2011) and pancreatic, colorectal and ovarian cancers (Gao *et al*., 2010; Cheetham *et al.,* 2008; Socha *et al*., 2009). In summary, although numerous factors appear to be involved in transcriptional regulation of the *sparc* gene, detailed information on the molecular mechanisms regulating *sparc* gene activity is still lacking, and a consistent promoter study has not yet been performed in non-mammalian vertebrates, particularly teleosts, although it has been shown that they apparently have less Sparc or Sparcl1 functional homologues than mammals. Therefore, observations in non-mammalian vertebrates might reveal key functions of Sparc and its regulatory mechanisms.

The present study was undertaken to explore the molecular mechanisms that regulate *sparc* gene expression by *in-vivo* functional characterization of the *sparc* promoter and identification of the possible *cis–trans* regulatory elements that govern basal promoter activity. The results obtained demonstrate that the 5'UTR-intron (+126bp to +7168 bp) region is essential for transcriptional regulation of *sparc*. Furthermore, we characterized specific regulatory regions in the intron sequence that significantly influence *sparc* transcriptional regulation.

MATERIAL AND METHODS

Experimental animals

Zebrafish embryos were cultured as previously described (Westerfield, 2007) and staged by standard criteria (Kimmel *et al.,* 1995) or by hours (hpf) or days (dpf) post fertilization. Experiments were performed with the TU (Tuebingen) wild-type strain (Nüsslein-Volhard Laboratory). To inhibit embryo pigmentation, embryo medium was supplemented with 0.003% (w/v) 2-phenylthiourea (Westerfield, 2007). For histology, dechorionated embryos were fixed overnight at 4 °C in 4% paraformaldehyde in 1XPBS, washed in PBS, and either stored at 4 °C in 1XPBS for confocal imaging or dehydrated through a methanol series and stored at -20 °C in 100% methanol for in situ hybridization. Ethical approval (N011011) for all animal studies was obtained from the Institutional Animal Care and Use Committee of the IIM-CSIC Institute in accordance with the National Advisory Committee for Laboratory Animal Research Guidelines licensed by the Spanish Authority (1201/2005).

Determination of transcription start site by 5-RACE

RACE was carried out to determine the transcription start site according to the instructions provided by SMART RACE cDNA Amplification Kit (BD Bioscience, Clontech Laboratory). 5'-RACE-Ready cDNA was amplified with the adapter primer (see manual protocol) and a 5' gene specific primer (5'-GCCAGCGAGGCAGAACAGGAAGAAG -3'). The polymerase chain reaction (PCR) product was subcloned into the pGEM-T easy vector for sequencing.

Reporter DNA constructs

The zebrafish *sparc* promoter sequences (GenBank accession number: BX640507) for the reporter constructs used in this study were amplified from genomic DNA with KOD Xtreme^{tm} Hot Start DNA Polymerase (Novagen, 71975) Platinum. The PCR conditions were: 94 ºC for 2 min and 40 cycles with a 10-s denaturation step at 98 ºC, 55 ºC for 30 s and 68 ºC for 10 min. The primers used were 5'AAGCTTAGCACAATAGGATG -3' and 5'-TTTTGCTTAGGCTGAAACTCAAG-3'. The agarose band was extracted and purified with the QIAquick® Gel Extraction Kit. The PCR product was diluted 1:10, and 1 µl was ligated into 1 µl of P-ENTR^{Tm} /D-TOPO® Cloning Kit (Invitrogen) and transformed according to the protocol. Both fragments were then ligated by LR clonase into the destination vector containing the *egfp* sequence downstream, and sequenced. Construct 1 contains 127 nt upstream to the transcription start (herein referred to as the proximal promoter, PP), the 125 nt of exon 1, the 7043 nt of intron 1 and the 13 nt of exon 2, excluding the translation starting site (+7182). Construct 2 was similar to construct 1, except that it lacked the UTR intron (+126/+7168) (Figure 1).

cis-acting transcription factor binding sites located in the zebrafish *sparc* promoter sequence were identified with MatInspector software (Cartharius *et al*., 2005). CpG island predictor analyses were done with Methprimer software (Li and Dahiya, 2002).

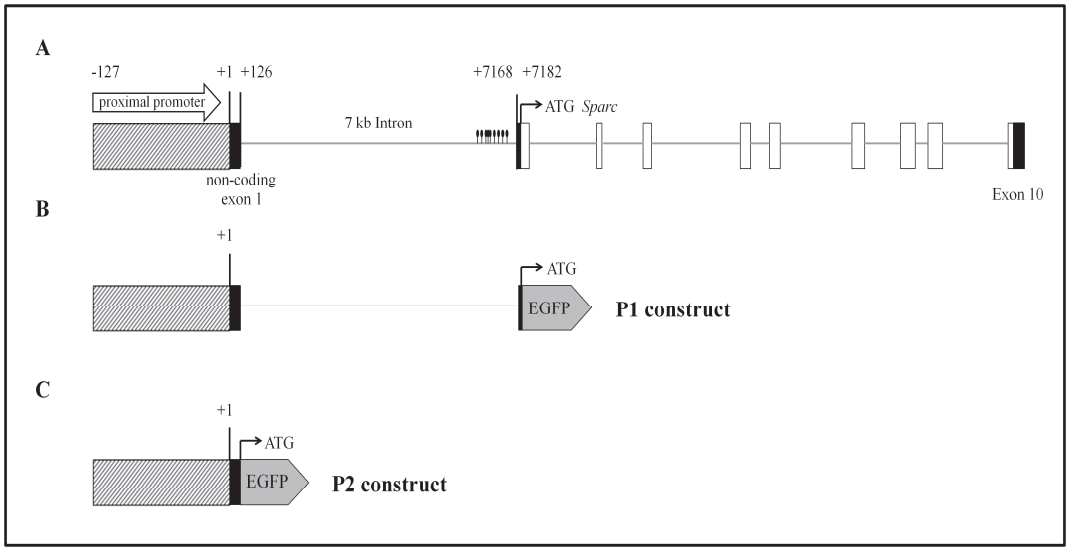

Figure 1. Zebrafish *sparc* promoter region investigated. **A)** Promoter region spanning nt −127 to the transcription start site (+1) and the genomic organization of *sparc*, with 10 exons. **B)** Tol2-EGFP reporter construct (P1) including the proximal promoter (PP), the first exon, the unique intron of the 5′-UTR of the gene and a small part of the second exon. **C)** The promoter fragment devoid of the intron (nt −371/+64), cloned into the Tol2-EGFP vector (P2), is highlighted. Black boxes indicate untranslated regions; open boxes indicate coding exons; lollipops indicate CpG island detected

Transient and stable expression assays

Reporter constructs were dissolved in distilled H_2O to a final concentration of 50 μg/ml. Approximately 2 nl of DNA solution with transposase mRNA (60 μg ml^{-1}) were microinjected into the cytoplasm of zebrafish embryos at the one- or two-cell stage. Microinjection was carried out under a dissection microscope (MZ8, Leica) fitted with a MPPI-2 pressure injector (ASI systems). EGFP expression in the injected and transgenic zebrafish embryos was analyzed at 24, 48 and 72 h post fertilization (hpf). EGFP expression was analyzed by direct observation of EGFP expression under a fluorescence microscope. The number of embryos showing EGFP expression was determined, and different reporter constructs were compared to score activity and tissue specificity.

In situ hybridization

Whole-mount in situ hybridization was performed with digoxigenin-labelled antisense probes as previously described (Rotllant *et al*., 2008). Antisense riboprobes were made from linearized full-length *Danio rerio sparc* cDNA (GenBank Accession number: BC071436) (primers: forward 5'-TGCTTAGGCTGAAACTCAAGATGAG-3'; reverse 5'-GCATCAATGGAAGACGTCCTTAGAT-3').

5'Azacytidine treatment

5'Azacitydine was purchased from Sigma Aldrich, Spain. A concentration of 50 μM was used. The powder was applied directly to the zebrafish water and changed every 2 days to prevent degradation. The application was initiated at 11 dpf and finished at 40 dpf.

Sampling of genomic DNA and RNA

DNA and total RNA were extracted at 40 dpf from control and Aza-treated zebrafish larvae. Additionally, individuals for each treatment were collected, anaesthetized with MS-222 (Sigma–Aldrich, Madrid, Spain), and photographs were taken with a Leica DFC310 FX camera and Leica M165FC stereomicroscope.

gDNA was extracted with the NucleoSpin® Tissue Kit (BD Biosciences). DNA quality was verified by electrophoresis on 1% agarose gels. Total RNA was extracted from zebrafish larvae with TrizolReagent (Ambion), and first-strand cDNA was synthesized according to the Maxima First Strand cDNA Synthesis Kit (Fermentas) protocol with 1 μg RNA.

Methylation-sensitive amplification polymorphism (MSAP) genotyping

A modification of the MSAP method described by Reyna-Lopez *et al.*, (1997) and Xu *et al.*, (2000) was used. In short, genomic DNA was digested with two methylation-sensitive isoschizomers (MspI and HpaII) as frequent cutters, each in combination with the same rare cutter (EcoRI) in parallel batches, ligation of adaptors and selective PCR amplification with primers complementary to the adaptors but with unique 3' overhangs. The two isoschizomers recognize the same sequence (5'-CCGG) but differ in their sensitivity to DNA methylation. Comparison of the two profiles for each individual allowed assessment of the methylation state of the restriction sites. Methylated CpG are restricted by MspI, and hemiMethylated CpCpG sites are restricted by HpaII (REBASE). Sites that are hypermethylated (i.e. at both the internal and external Cs) and sites that are fully methylated at the external Cs (i.e. on both strands) are not cut by either enzyme, whereas sites that are free from methylation are restricted by both.

Two primer combinations (EcoRI-AAG- HpaII -TC, EcoRI -ACT- HpaII -TC) were used for selective amplifications Primer sequences and PCR details are available in Morán and Pérez-Figueroa (2011). HpaII primers were end-labelled with a 6-FAM reporter molecule. PCR products were loaded simultaneously with a GeneScan 500 ROX size standard into an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Fragment analysis and scoring was performed with GeneMapper v.3.7 software (Applied Biosystems). DNA fragments shorter than 100 bp, longer than 500 bp or less than 70 relative fluorescent units were excluded from the analysis.

Methylation analyses of *Sparc* CpG island by bisulfite-mediated genomic sequencing

One microgram of genomic DNA was used for bisulfited DNA conversion, according to the manufacturer's protocol (EZ DNA Methylation-Direct™ Kit, Zymo Research). Primers were designed to amplify a 300-bp fragment in the *sparc* intron 1, where a CpG island was detected with Methprimer software (Li and Dahiya, 2002). Primer F: 5'-AATTAAAAGGAAGAGAGATTTTTGG-3' and primer R: 5' -TCAAACCACCAAACCTACTCTA-3' were used. Two microlitres of bisulfited DNA were taken for the PCR reaction. DreamTaq MasterMix (Fermentas) was used to amplify the fragment. Bands were gel-purified and cloned into PGem-Teasy (Promega). Five individuals and 10 clones of each individual per treatment were taken and sequenced with SP6. A total of 100 clones were analysed. Using the program BDPC DNA methylation analysis platform (available at: http://biochem.jacobs-university.de/BDPC/) (Rohde *et al*., 2010), different methylation levels of CpG dinucleotides were computed.

Relative quantification of *sparc* gene expression by real-time PCR

cDNA was made from total RNA with superscript III (Invitrogen) according to the manufacturer's recommendations. The primers designed to detect *sparc* transcripts were: OsteoRT (F): 5'-CCCTCTGCGTGCTCCTCTTA-3' and OsteoRT (R): 5'-GCATCGCACTGCTCAAAGAA-3'. Expression levels were standardized to *18S* by the $2^{-\Delta\Delta T}$ method (Livak and Schmittgen, 2001). Dilutions of 1:10 of cDNAs were made for quantifying the number of *sparc* transcripts. Real-time quantitative PCR (qPCR) reactions were performed in an AB 7300 real-time PCR System (Applied Biosystems) and incorporation of Maxima® SYBR Green/ROX qPCR Master Mix (2X) (Fermentas). The two-step cycling conditions for the two primer sets were: denaturation at 95 ºC for

10 min, followed by 40 cycles at 95 ºC for 15 s and 60 ºC for 1 min. All samples were done in triplicate.

Finally, a melting-curve analysis was carried out at 95 ºC for 15 s, 60 ºC for 30 s and 95 ºC for 15 s for testing the specificity of the primers.

Statistical analysis

MSAP profiles, pooled from both primer combinations, were analyzed using the R package msap (Pérez-Figueroa, 2013. http://msap.r-forge.r-project.org). We scored the MSAP fragments as follows: fragments present in both *Eco*RI-*Hpa*II and *Eco*RI-*Msp*I products (1/1), denoting a non-methylated state; those fragments present only in either *Eco*RI-*Hpa*II (1/0) or *Eco*RI-*MspI* (0/1) products, corresponding to a methylated state; or absent from both *Eco*RI-*Hpa*II and *Eco*RI-*Msp*I products (0/0), which we considered as an hyper-methylation of the target. Individual fragments (loci) were, therefore, classified into 'methylation-susceptible loci' (MSL) if the observed proportion of methylated scores (1/0, 0/1 and 0/0) exceeded a 5%, and "methylation-susceptible fragments" if the methylated state was the dominant marker (1 for the methylated state and 0 for the non-methylated state). Epigenetic differentiation among treatments was assessed by means of principal coordinates analysis (PCoA) followed by analyses of molecular variance (AMOVA; Excoffier *et al.*, 1992).

Student's t test was used to test differences in methylation levels on *sparc* CpG islands and also to test the significant significance of differences in *sparc* levels between treatments. Statistical software of Statistical Package for the Social Sciences (IBM SPSS Statistics 21) was used for statistical analyses. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Analysis of 5'-untranslated region of *sparc* sequence in zebrafish

The 5'UTR of *sparc* was characterized by RACE. The start of transcription was found to be separated from the start of translation by a 7-kb intron (Fig. 1A). The whole 5'UTR sequence comprises 138 nucleotides, of which 13 correspond to the region proximal to the *sparc* ATG in exon 2. Therefore, the non-coding exon I contains 125 bp, while exon II contains 70 bp (13 pb from the untranslated region plus 57 bp from the coding sequence).

Characterization of transient and stable expression of 7,2kb-*sparc* –EGFP

The promoter activity of putative zebrafish 5' genomic fragment was explored through expression of *egfp*. The 0.2-kb *sparc* promoter and its 5'-flanking sequence 7 kb upstream of the translated exon were linked with the *egfp* reporter gene (Fig. 1B). The resulting gene construct, *Tol2-7,2kb-sparc*-EGFP, was microinjected into zebrafish embryos for transient and stable expression analysis. *Egfp* expression in injected embryos was monitored by direct observation under a confocal fluorescence microscope. As shown in Figure 2 (A-E), injected embryos predominantly displayed *egfp* expression initially at 24 hpf in the notochord (90%), intermediate cell mass (70%), otic vesicle (60%), olfactory epithelium (60%) and muscle fibres (100%). At 48 hpf, fluorescence was detected moderately in the notochord (15%), heart (30%) and mandibular structures (5%) but at a higher incidence in the intermediate cell mass (70%), otic vesicle (40%), olfactory bulb (60%) and muscles (100%). At 72 hpf, no expression was detected in the notochord but was seen in the intermediate cell mass (100%), otic vesicle (20%), olfactory epithelium (60%), muscle fibres (100%), heart (50%), mandibular structures (40%) and dorsal fin fold (10%).

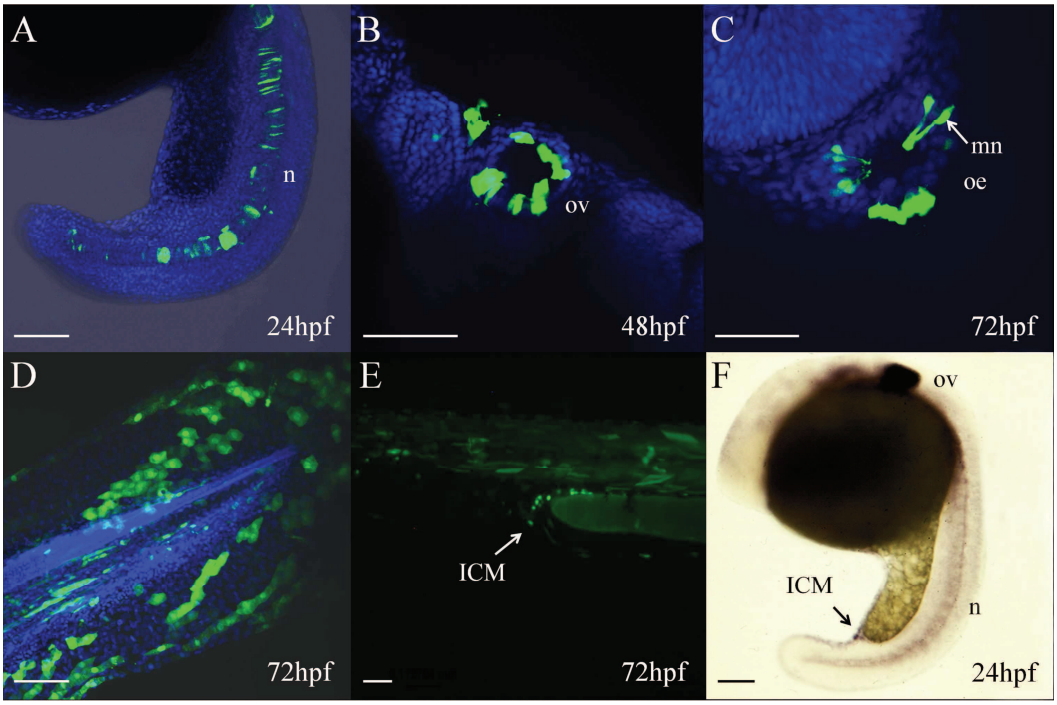

Figure 2. EGFP fluorescent under the control of 0.2-kb *sparc* promoter and its 5' flanking sequence 7 kb upstream of the translated exon II were visualized in live zebrafish embryos at 24 hpf, 48 hpf and 72 hpf. Observed fluorescence from the stable 7,2kb-*sparc*-EGFP transgenic lines and transient expression from 7,2Kb-*sparc*-EGFP construct were equivalent. Expression of *egfp* are localize in (**A)** notochord at 24 hpf; otic vesicle at 48 hpf (**B**); microvillous neurons in the olfactory epithelium at 72 hpf (**C**); caudal fin at 72 hpf (**D**) and intermediate cell mass at 72 hpf (**E**). (**F**) Whole-mount *in situ* hybridization of *sparc* at 24 hpf establishing that observed fluorescence accurately track the endogenous *sparc* expression. mn= microvillous neurons; n=notochord, ov=otic vesicle; oe=olfactory epithelium; ICM=intermediate cell mass. Scale bar: (**A,B,D,E,F**)= 100µm; (**C**)=20µm

Observed fluorescence from the stable 7,2kb-*sparc*-EGFP transgenic lines and transient expression from 7,2kb-*sparc*-EGFP construct were equivalent. To determine whether the endogenous *sparc* gene was specifically expressed in the same domains, *in situ* hybridization was performed with a *sparc* antisense probe (Rotllant *et al*., 2008) in zebrafish embryos. *sparc* mRNA was indeed expressed in same domains as in transgenic 7,2kb-*sparc*-EGFP fish (Fig. 2F). Therefore, *egfp* expression in the 7,2kb-*sparc*-EGFP

transgenic line recapitulated the endogenous expression pattern of *sparc* mRNA (Rotllant *et al*., 2008).

These data indicated that the 0.2-kb *sparc* promoter and its 5'-flanking sequence 7 kb upstream of the translated exon contained the regulatory element(s) that temporally and dynamically drive tissue-specific expression in the notochord, intermediate cell mass, otic vesicle, microvilliuos neurons in the olfactory epithelium, muscle fibres, heart, mandibular structures and dorsal fin fold.

To identify the key regulatory region within the P1 construct sequence responsible for *sparc* expression, a second construct similar to construct 1 but lacking the UTR intron (+126/+7168) (Fig.1C) was generated. The P2 construct containing only the proximal promoter and the 5' untranslated region of exon 1 (-127bp/+125bp) did not express green fluorescent protein (EGFP), indicating that removal of the 5'UTR-intron (nt+126/+7168) resulted in complete reduction of promoter activity.

On the basis of our finding that the 5'UTR-intron +126bp to +7168 bp region is key to the transcriptional regulation of *sparc,* we sought to identify transcription factors that could be involved in this regulation. Using the MatInspector database, we identified several putative transcription factor binding sequences in the (+126/+7168) region, including sites for heat shock elements, cAMP responsive element binding proteins, gata factors, sox factors, myoblasts factors, glucocorticoid response elements, retinoic acid receptors and activating protein-1. These putative regulators have been shown to be involved in *sparc* transcriptional regulation in other species, but most were located upstream the initial transcription site instead of the intronic sequence as we found in zebrafish (Young *et al*., 1989; Damjanovski *et al*., 1998). Additionally, zebrafish *sparc* promoter lacks both the consensus CAAT box and TATA box, elements usually associated with developmentally regulated genes. Moreover, a CpG island was identified in the +6614 to +6921 region where nine CpG dinucleotides were susceptible to methylation.

Experimentally induced 5'-azacytidine DNA hypomethylation

As we detected a CpG island immediately proximal to the translation start site in the intron sequence, we hypothesized that *sparc* is one of the genes controlled by epigenetic regulation in fish. Therefore, to investigate the relationship between *sparc* transcriptional level and DNA methylation, we treated developing zebrafish larvae with 50 µM 5'-azacytidine (aza) starting 11 dpf. Larvae were examined at the end of treatment, at 40 dpf. Approximately 40% of aza-treated larvae showed distinctive phenotypic abnormalities, with a shortened tail, torsion of the spinal cord, head malformations and depigmentation (Fig. 3).

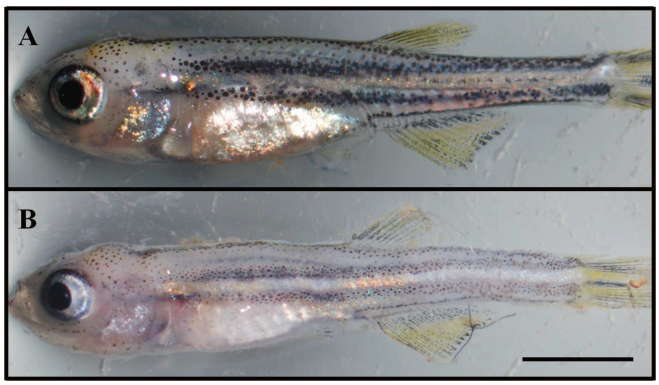

Figure 3. Phenotypic malformations observed in control (**A**) and 5'-azacytidine-treated (**B**) juvenile zebrafish at 40 dpf. Scale bar= 250 µm.

In order to verify the experimentally induced 5'-azacytidine hypomethylation, samples from untreated and aza-treated fish were MSAP genotyped. The number of loci obtained for the primer combination used in this experiment was 423, of which 355 were classified as methylation-susceptible loci. The percentage of polymorphic methylation-susceptible loci was 63%, and the mean Shannon's diversity index was 0.598 standard error ± 0.101).

The proportions of the four methylation states (unmethylated, hemi-methylated, internal cytosine methylation and full methylation) are shown in Table 1. The major difference between groups, 25%, was detected for the unmethylated state, the aza-treated fish showing 41.6% demethylation and the control fish 16.6%. The percentage internal cytosine methylation and full methylation states were higher in the control group, and little difference was seen for the hemi-methylated state.

	HPA+/MSP+ (Unmethylated)	HPA+/MSP- (Hemi-methylated)	HPA-/MSP+ (Internal C methylation)	HPA-/MSP- (Full methylation)
Control	16.6	9.4	31	43
5'-Aza	41.6	10.2	17.1	31.1

Table 1. Percentage of each methylation state in control and 5'-azacytidine groups

The differences between groups in genome-wide methylation were statistically significant (AMOVA; Φ_{st}= 0.5123, $p < 0.0001$). In the principal coordinates analysis, the control group was clearly separated from the aza-treated group along the first coordinate (44.1% of variance explained) (Fig. 4). The two groups were also differentiated along the second coordinate, with 10.1% of variance explained. These data support the use of 5'-azacitydine as a demethylating agent in zebrafish larvae.

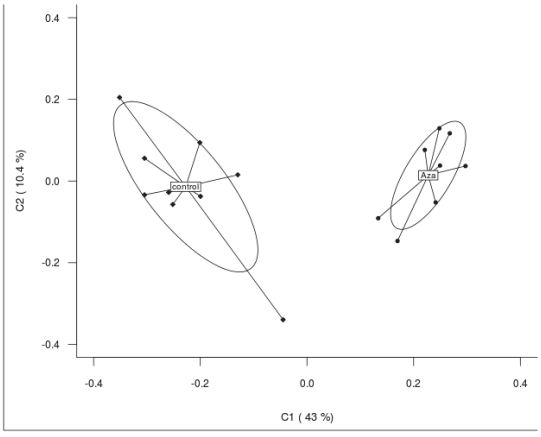

Figure 4. Genoma-wide DNA methylation changes in control and 5'-azacytidine-treated juvenile 40 dpf zebrafish. Principal coordinates analysis (PCoA) results for epigenetic differentiation between control and 5'-azacytidine-treated juvenile 40 dpf zebrafish. The two coordinates (C1 and C2) show the percentage variance in parentheses. Each circle represents an azacytidine-treated individual, and each square represents a control fish. Ellipses represent the dispersion of each group around its centre. The long axis represents the direction of maximum dispersion, and the short axis the minimum dispersion.

Sparc CpG island hypomethylation and its association with transcriptional gene activation

To determine more clearly the methylation status of the CpG sites in the +6614 to +6921 nt region upstream of the SPARC translation starting site and included in intron 1, bisulfite-treated DNA from control and aza-treated fish was sequenced

Exposure to aza decreased *sparc* CpG island methylation from 44.2±2.4 to 22.7±4.5 % in control and treated fish, respectively (t = 4.153, p = 0.001) (Fig. 5). Significant differences in eight of nine positions were detected between treated and untreated fish, positions +6835 and +6873 showing the most significant differences (t = 2.381, p = 0.004 and t = 4.394; p = 0.003, respectively).

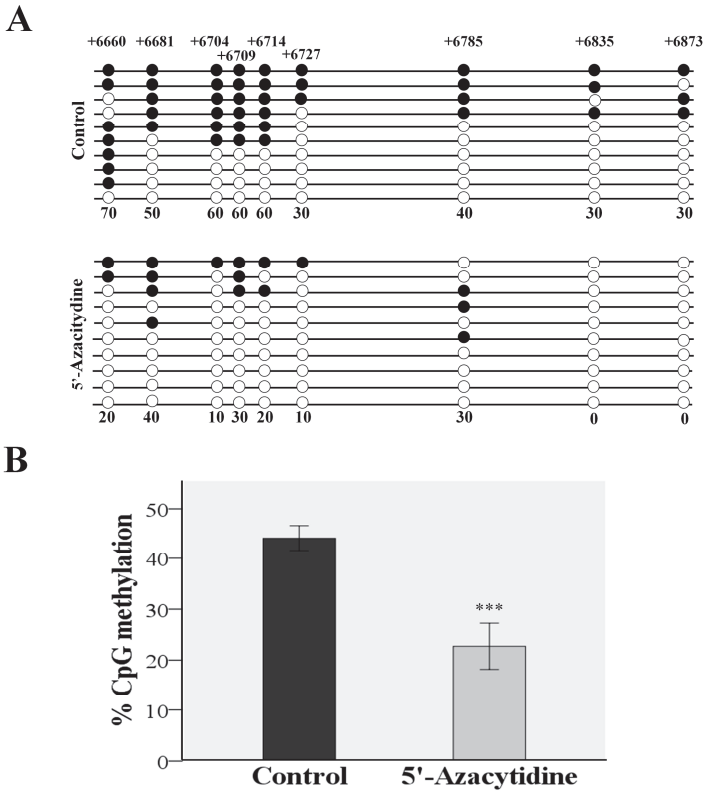

Figure 5. Methylation patterns of zebrafish *Sparc* promoter at 40dpf. **(A)** Methylated (filled circles) and demethylated (open circles) CpG positions in control and 5'-azacytidine-treated fish. Numbers with a positive sign indicate CpG positions in respect to the transcription starting site. **(B)** Percentage of methylated CpGs in both groups. Data are expressed as mean ± SEM. Statistically significant differences (p = 0.001) are indicated by asterisks (***).

Significant differences in *sparc* gene expression were also seen according to treatment. *sparc* levels were increased in fish treated with aza by up to threefold in respect of control fish (t = -4.86; p = 0.001) (Fig. 6).

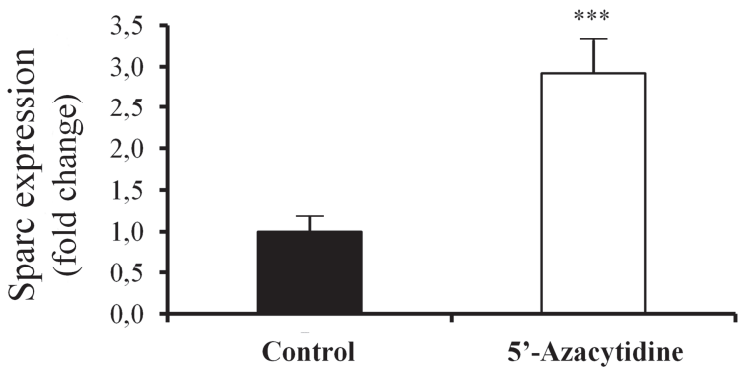

Figure 6. Expression of *Sparc* in response to 5'-azacytidine treatment. The relative expression of *Sparc* was determined by real-time PCR and standardized to 18S. The results are expressed as mean ± SEM with respect to the control, which was set at 1. Statistically significant differences (p = 0.001) are indicated by asterisks (***)

DISCUSSION

Sparc is a highly conserved extracellular matrix protein which is actively involved in many cellular processes including development, wound healing, angiogenesis, tumorigenesis and inflammation. This critical role of Sparc in a variety of different biological processes imposes a tight regulation of its transcriptional regulation. Although isolation of *sparc* promoter regions from different species has been reported (McVey *et al*., 1988; Young *et al*., 1989; Damjanovski *et al*., 1998) and numerous factors appear to be involved in its transcriptional regulation, detailed information on the molecular mechanisms regulating *sparc* gene activity is still lacking, and a consistent promoter study has not yet been performed in non-mammalian vertebrates.

In this study, we investigated *sparc* gene expression and the regulatory elements required for its temporally and spatially specific expression in particular during early embryogenesis by using transient and transgenic expression analyses in zebrafish embryos. Comparative molecular analysis of *sparc* promoter and its 5'-flanking sequence between zebrafish and other vertebrate species showed no nucleotide homology at the 5' ends (Damjanovski *et al*., 1998). However, a number of similarities in their overall organization were found (Young *et al.,* 1989). Thus, the molecular organization of the first and second exons is conserved. Exon I, containing 125 bp in zebrafish, represents the majority of the 5' untranslated region in zebrafish and other vertebrate species (McVey *et al*., 1988; Young *et al*., 1989; Damjanovski *et al*., 1998), while exon II, which comprises 70bp in zebrafish, contains the remainder of the 5' untranslated region and encodes the entire signal peptide like other vertebrates species. Another characteristic found in zebrafish and other vertebrate species is the presence of the 5'CCTG3' motif in the *sparc* promoter and its 5'-flanking sequence. The function of this conserved sequence has been shown to be important in either regulation on the gene or the stability of RNA (McVey *et al.,* 1988). A common characteristic of *sparc* gene organization in all vertebrates species studied is the presence of an intronic sequence between the first non-

coding and the second coding exon. However, the size of the first intron seems to be species-specific, being 7 kb in zebrafish, 10 kb in humans and 2 kb in *Xenopus* (Damjanovski *et al.,* 1998).Similar to mammalian vertebrates, the promoter of *sparc* gene in zebrafish lacks the classical CAAT and TATA box motifs found in many eukaryotic promoters. We found that the 0.2-kb *sparc* promoter and its 5' flanking sequence 7 kb upstream of the translated exon drive *egfp* expression in the notochord, otic vesicle, fin fold, somites, intermediate cell mass, skeletal and cardiac muscles, which mimicked the already well described expression pattern of the endogenous *sparc* mRNAs (Rotllant *at al*., 2008; Ceinos *et al*., 2013). Similar results were found in mice, where *sparc* transcripts were detected in developing tissues, such as the otic vesicle (Mothe and Brown, 2001), notochord, somites and embryonic skeleton (Holland *et al*., 1987; Mason *et al*., 1986). In addition, the 0.2-kb *sparc* promoter and its 5'-flanking sequence 7 kb upstream of the translated exon drove the *egfp* expression in the olfactory epithelium. Although specific expression of *sparc* in the olfactory bulb of mice has already been reported (Mendis and Brown, 1994), this is the first demonstration of the expression of *sparc* in the microvilliuos neurons of the olfactory epithelium in non-mammalian vertebrate. We were unable to detect *sparc* expression in the olfactory epithelium by whole-mount in-situ hybridization. One possible explanation may be the limited sensitivity of our assay to detect faint expression of *sparc* in some regions. It should also be noted that, although the conclusion was based on transient and stable expression analysis, it is unlikely that the tissue-specific spatial expression pattern of the *egfp* was due to a position effect of the integration site, because the pattern of *egfp* expression mimicked endogenous *sparc* expression in many ways. However, we cannot exclude the possibility that there might be a position effect on the activity of the promoter, which might explain the specific *egfp* expression in the olfactory epithelium in transgenic and mosaic fish.

Transient expression analyses in zebrafish embryos demonstrated that promoter activity resides in the unique 5′-UTR intronic region (nt+126/+7168). Specific deletion of this

region resulted in a complete reduction of promoter activity. Transcriptional regulation of other genes (such as ubiquitine C) has also been shown to be exclusively regulated by the 5'UTR intron sequence (Bianchi *et al.,* 2009). Therefore, the 5′-UTR unique intronic region (nt+126/+7168) provides the regulatory elements required for expression of a reporter gene in a subset of tissues that normally express the endogenous *sparc* gene in zebrafish embryos.

Sequence analyses of the zebrafish *sparc* 5′-UTR intron 1 region revealed a number of transcription factor binding sites. Because of the similarities in the overall organization of the *sparc* promoter and *sparc* expression domains and the highly conserved amino acid sequence in diverse vertebrate species (Laizé *et al.,* 2005), we compared conservation of *cis*-acting genetic elements that regulate *sparc* expression in the zebrafish 5′-UTR intron 1 sequence. Several transcription factors in zebrafish were common to other *sparc* promoter sequences, including heat shock elements, cAMP-responsive element binding proteins, myoblast factors, gata binding factors, activating protein 1, retinoic acid receptor and glucocorticoid elements. All these factors have been shown to regulate *sparc* expression *in vitro* (Brekken and Sage, 2000). Additionally, transcription factor binding sites belonging to the Sox family were identified in the zebrafish 5′-UTR intron 1 region. This finding is in agreement with several other studies showing the role of SOX elements in *sparc* transcriptional regulation (Huang *et al.*, 2008; Rotllant *et al*., 2008).

A CpG-rich sequence (CpG island) was also identified in the zebrafish 5′-UTR intron 1 region. It has been shown that *sparc* is transcriptionally regulated by DNA methylation, and CpG-rich sequences were also identified in mammalian *sparc* promoter sequences (Rodríguez-Jiménez *et al*., 2007; Gao *et al.,* 2010; Tajerian *et al*., 2011). In order to obtain insights into the transcriptional regulation of *sparc* expression, we investigated the role of DNA methylation in the expression of *sparc* in zebrafish embryos. 5'-Azacytidine was used to artificially induce DNA hypomethylation. This method has already been used to induce aberrant DNA hypomethylation in zebrafish embryos (Martin *et al*., 1999; Christman, 2002). Our results show that (i) exposure to 5'-azacytidine produces

distinctive phenotypic abnormalities in zebrafish larvae, including shortened tail, torsion of spinal cord, head malformations and depigmentation; (ii) exposure to 5'-azacytidine produces significant global DNA demethylation in zebrafish larvae; (iii) exposure to 5'-azacytidine specifically reduced CpG-rich sequence (CpG island) methylation in the zebrafish *sparc* 5′-UTR intron 1 region; and (iv) *sparc* is highly expressed in 5'-azacytidine-treated zebrafish larvae. These results suggest that *sparc* is transcriptionally regulated by DNA methylation.

In summary, our study provides the first evidence that the 5′-UTR intron of zebrafish *sparc* gene contains the functional and regulatory elements required for its temporally and spatially specific expression, in particular during early embryogenesis. We also provide evidence that *sparc* is transcriptionally regulated by DNA methylation.

Our findings should provide a basis for further studies to characterize critical *cis*-regulatory elements and to elucidate the molecular mechanisms underlying transcriptional regulation of the *sparc* gene under both physiological and pathological conditions.

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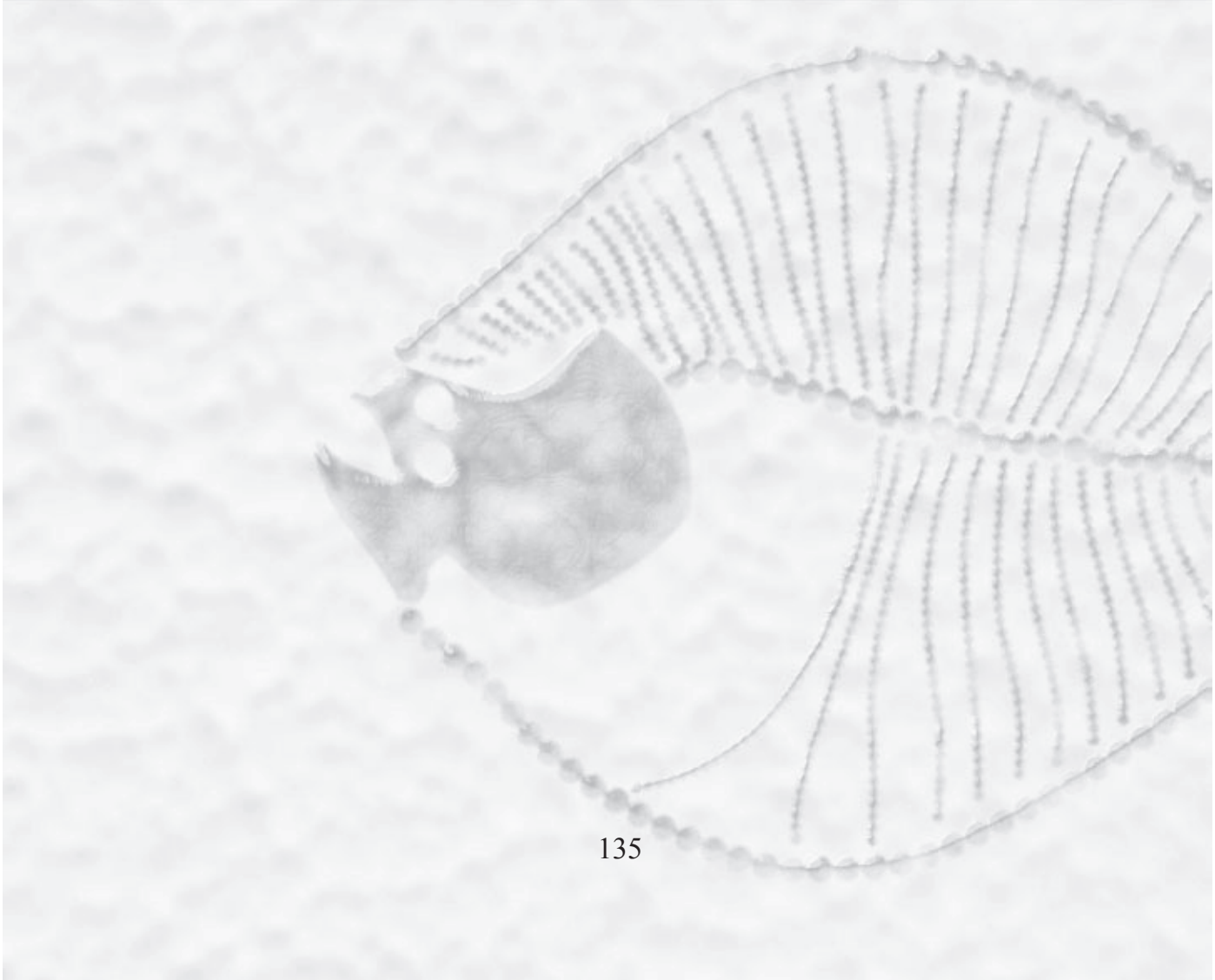
Chapter IV



Chapter IV

Stage-specific expression of *sparc* during flatfish post-embryonic remodeling

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In preparation

ABSTRACT

Sparc (Osteonectin) is a multifunctional matricellular glycoprotein expressed in embryonic and adult tissues that undergo active proliferation and dynamic morphogenesis. Recent studies indicate that *sparc* expression appears early in development, although its function and regulation during development are largely unknown. In this report, we describe the isolation, characterization and post-embryonic development expression of *sparc* in turbot. The full-length turbot *sparc* cDNA contains 930 bp encoding a protein of 310 amino acids which shares 77, 75, 80 and 34 % overall identity with human, *Xenopus*, zebrafish, and *C.elegans* respectively. Results of whole-mount in situ hybridization reveal a dynamic expression profile during post-embryonic development. *Sparc* is expressed differentially in cranioencephalic region mainly in jaws, pectoral fin, branchial arches and pterigiophores of caudal, dorsal and anal fins. Further, it was demonstrated that the *sparc* gene expression is dynamically regulated during post-embryonic turbot development with a significant level of transcript abundance during stage-specific post-embryonic remodeling.

INTRODUCTION

Osteonectin, also named Sparc (secreted protein, acidic, rich in cysteine) or BM-40, is an extracellular matrix protein that takes part in multiple processes further than maintain the cellular structural integrity. Sparc was firstly described as the major non collagenous protein in extracellular bone matrix (Termine *et al*., 1981) but was also found in other tissues like skin, reproductive organs, alimentary tract, central nervous system and hematopoietic system (Sage *et al*., 1989; Vincent *et al*., 2008; Ceinos *et al*., 2012). It is well-known its high affinity to calcium, hydroxyapatite and collagen (Holland *et al*., 1987). SPARC is highly expressed during the first stages of life, playing an important role in morphogenesis (Damjanovski *et al*., 1997; Rotllant *et al*., 2008; Kang *et al*., 2008). However, in adults the expression of SPARC seems to be restricted to tissues undergoing repair or remodeling (Schelling *et al*., 2004; Padhi *et al*., 2004). Recently, the use of deficient Sparc animal models, provides new insights about the Sparc-associated effects such as cataract formation (Gilmour *et al*., 1998), enhanced growth of tumors (Brekken *et al*., 2003), adiposity increment (Bradshaw *et al*., 2003), osteopenia and decreased bone formation (Delany *et al*., 2000; Rotllant *et al*., 2008) or haematopoiesis alteration (Ceinos *et al*., 2012).

Sparc of teleost species consists of three distinct domains: an acidic domain in the N-terminus of the polypeptide; a follistatin like domain, which is a cysteine-rich region that contains a 1 putative site of *N*-linked glycosylation (Laizé *et al*., 2005), and an extracellular Calcium binding (EC) domain that contains two EF-hand motifs located in the C-terminus of Sparc (Sage *et al*., 1989). Phylogenetic analyses indicate that Sparc is a highly conserved protein among species being the domain I the most variable part (Kawasaki *et al*., 2004). Several studies confirmed not only the reduction of the N-terminal in triploblastic invertebrates as *Drosophila melanogaster*, *Artemia maritima* or *Caenorhabditis elegans* but also the absence of this domain in the diploblastic cnidarian *Nematostella vectensis* (Koehler *et al*., 2009).

Turbot (*Scophthalmus maximus*), is an important reared species with a high economic value. Several descriptive studies were made to provide basic knowledge of the embryonic and post-embryonic development under reared conditions (Weltzien *et al*., 1999; Sadiq *et al*., 1984; Tong *et al*., 2012). Most of these studies have focused on the metamorphosis stage since it is the most relevant event produced in flatfish larvae. Therefore, flatfish, including the turbot (*Scophtalmus maximus*), change from a symmetrical pelagic larva to an asymmetric benthic juvenile. This metamorphic remodeling is characterized by several drastic physiological changes that involve a high active remodeling in different organs such as skin (Campinho *et al*., 2007), musculo-skeletal system (Saele *et al*., 2006), nervous system (Graf and Baker, 1990) and intestinal system (Tanaka *et al*., 1996). Although, turbot metamorphosis is now well described and the central role of thyroid hormone in this process is well established (Power *et al.,* 2008; Infante *et al*., 2008; Roberto *et al*., 2009), few data are available about the regulation of specific possible target genes in the frame of the gene network responsible for metamorphic remodeling of larval tissues. During metamorphosis a significant number of transcription factors and development genes have altered expression, specifically it has been shown that in the late stages of remodeling, genes necessary for cell proliferation, signal transduction, and cell–cell signaling are significantly upregulated (Kulkarni and Buchholz, 2013). However, there is not currently an evidence of the possible specific regulation of any extracellular matrix protein.

Therefore, because the well-documented implication of SPARC to modulate cell–matrix interactions, particularly during tissue remodeling and at sites of high cellular turnover during development (Metsäranta *et al*., 1989; Rotllant *et al*., 2008; Kang *et al*., 2008; Renn *et al*., 2006), the key objective of the present study was to molecular characterize SPARC in turbot and its transcriptional regulation during turbot metamorphic remodeling. Additionally, the effect of rearing water temperature on turbot development and on Sparc transcriptional regulation was also evaluated.

MATERIAL AND METHODS

Experimental design and sampling

Turbot gametes were obtained from turbot broodstock reared at the facilities of the Centro Oceanógrafico de Vigo (NW Spain). Eggs from one ovulated female and milt from one male were obtained by abdominal massage. Egg quality was assessed according to the criteria of McEvoy (1984). First, eggs were coated with milt in the proportion (~200 µl of milt for each 10 ml of eggs). Then, each volume of eggs plus milt was mixed with 2 volumes of seawater. This moment was considered time zero. Thirty seconds after fertilization, eggs were gently rinsed with excess sea water. Viability was assessed by placing them in a graduate cylinder, letting them sit for about 5 min and measuring the proportion that floated. Subsequently, fertilized eggs were placed in 450l tank under a constant stream of water maintained at 18ºC. Larvae were sampled at 6h, 14h, 24h post fertilization and 2d, 4d, 6d,8d, 11d, 16d, 19d, 22d, 25d, 28d, 31d, 34d and 40d after hatching (h, hours; d, days).

For temperature experiment, 1dph embryos were randomly separated into 4 groups each group was introduced in one of four 450 l tanks with two different water temperatures (14 and 18°C). The experimental temperatures (14 and 18°C) were chosen in order to include the range of the suggested developmental temperatures that turbot encounters in natural environment and be at the same time inside the range of the accomplishable successful rearing in aquaculture facilities. The regulation of temperature was achieved with the use of heater-coolant devise (Pasquarium®, Vigo, Spain).

All fish were fed ad libitum initially with rotifers (from 2 to 7 dpf), rotifers plus newlyhatched Artemia sp. Nauplii (from 7 to 10 dpf), newlyhatched Artemia sp. Nauplii (from 10 to 17 dpf), Mutigain enriched-artemia (17-30dpf), and from 30 dpf onward with GEMMA-Micron ® (Skreeting, Norway). Larvae maintained either at 14 or 18 °C were sampled at 4, 15, 30, 40, 50 and 80 dph. All fish sampled were previously

euthanized using MS-222(Sigma). Fish were staged according to Al-Maghazachi and Gibson (1984).

Ethical approval (N011011) for all animal studies was obtained from the Institutional Animal Care and Use Committee of the IIM-CSIC Institute in accordance with the National Advisory Committee for Laboratory Animal Research Guidelines licensed by the Spanish Authority (1201/2005).

Molecular cloning of turbot *sparc* gene

A multiple alignment using MAFFT VERSION 7 (http://mafft.cbrc.jp/alignment/server/) was performed with the coding sequences of *sparc* in zebrafish (NM_001001942.1), seabream (AY239014.1) and fugu (NM_001032550.1). A degenerate primer pair (F: 5'-ACTGCAAGAAGGGMAAAGTG-3' and R: 5'-GGTGGTGCAYTGCTCCAT-3') was designed in highly conserved regions.

Total RNA was extracted from turbot larvae using TrizolReagent (Ambion) and first strand cDNA was synthesized according to the Maxima First Strand cDNA Synthesis Kit (Fermentas) protocol with 1µg RNA.

PCR was carried out using the PfuUltra® II Fusion HS DNA Polymerase (Agilent). The reaction volumes were: 5µl of 10X PfuUltra® II Reaction Buffer, 1.25µl dNTP mix (25mM each), 2µl cDNA template, 1 µl of each primer, 1 µl PfuUltra® II Fusion HS DNA Polymerase and 38.75 µl of sterile water. The PCR standard conditions were performed for 35 cycles and a 55ºC of annealing temperature. A 575bp fragment was amplified by RT-PCR. PCR product was extracted and gel-purified with QIAquick® Gel Extraction Kit. 3µl of the purified band were ligated into 1 µl of Pgem T-easy (Promega). For DNA sequencing, SP6 and T7 primers were used.

For rapid amplification of cDNA ends, 5' RACE (5'-CACTTTGCCCTTCTTGCAGT-3') and 3' RACE (5'-ATGGAGCACTGCACCACC-3')-PCRs reaction mixtures were performed by using Smart-RACE PCR cDNA amplification system (Clontech) following

the manufacturer's manual and specific primers designed according to the previously obtained sequence. Purified fragments were treated as above.

To corroborate that 5' and 3' ends correspond to the same transcript, full *sparc* sequence was amplified by using specific primers (F: 5'-ATGAGGGTGTGGATCATCTTCGT-3'; R: 5'-TCAGATGACGAGGTCTTTGTCCA-3') targeting the cDNA extremes. Full cDNAs were cloned and sequenced as before. The nucleotide sequence has been deposited with EMBL Nucleotide Sequence Database under accession number KF192603.

Larvae RNA isolation and RT-PCR

Turbot samples were collected at 6, 14, 24 hpf (hours post fertilization) and at 2, 4, 6, 8, 11, 16, 19, 22, 25, 28, 31, 34, and 40dpf (days post hatching) at 18ºC (control temperature) and conserved in Trizol Reagent (Ambion) for RNA extraction. First strand cDNA was synthesized as before.

Temporal expression profiles of *sparc* were determined by RT-PCR using the primers F: 5'-ATGAGGGTGTGGATCATCTTCGT-3' and R: 5'-TCAGATGACGAGGTCTTTGTCCA-3'.

PCR was also carried out to amplify *β-actin* cDNA as a positive control. Turbot *β-actin* primers were F: 5'-TGAACCCCAAAGCCAACAGG-3' and R: 5′-CAGAGGCATACAGGGACAGCAC-3′.

Absolute-quantitative real time PCR (qRT-PCR)

Sparc mRNA absolute quantification was used as a method to particularly characterize the transcriptional regulation of *sparc* gene during turbot post-embryonic development and the effect of water temperature.

Turbot larvae were collected at 4, 15, 30 and 50dph at two different temperatures and also at 80dph at low temperature and conserved in Trizol Reagent for RNA extraction. 10 individuals for each age and temperature group were sampled for qRT-PCR. cDNA was synthesized as before. Dilutions 1:10 of cDNAs were made for quantifying the number of *sparc* transcripts.

Turbot *sparc* cDNA cloned into pGEM-T easy was used as standard. 10-fold serial dilution of pGEM-Teasy-*sparc* construct, ranging from 10^{-4} to 10^{-9} copies/µL were used to make a standard curve. The plasmid copy number was calculated following the formula: DNA (copy) = [6.02×10^{23} (copy/mol) x DNA amount (g)] / [DNA length (bp) x 660 (g/mol/bp)] (Lee *et al*., 2006). PCR quantification was performed in 96-well optical plates in triplicate on an Applied Biosystems 7500 analyzer with Maxima® SYBR Green/ROX qPCR Master Mix (2X) (Fermentas). The following primer sequences were used for qRT-PCR: (5'primer/3'primer) 5'-TGAACCACCACTGCAAGAAG-3' and 5' -TCAGATGACGAGGTCTTTGTCCA-3'. The total reaction volume was 25 µl with 12.5µl of SYBR green, 0.5 µl of each primer, 9.5µl of nuclease free water and 1 µl of cDNA template. Two-step cycling conditions were: an initial denaturation for 10 min. at 95ºC, 40 cycles of 15 sec at 95ºC and 60 sec. at 60ºC. Finally, a melting curve analysis was carried out at 95ºC 15 seconds, 60ºC 30 sec and 95ºC 15sec for testing the primers specificity. A standard curve was drawn by plotting the natural logarithms of the threshold cycle (C_T) against the number of molecules, respectively. C_T was calculated under default settings for the real-time sequence detection software (Applied Biosystems). The equation drawn from the graph was used to calculate the precise number of specific *sparc* cDNA molecules present per microgram of total primed cDNA, tested in the same reaction plate as the standard.

Whole mount in situ hybridization

For whole mount *in situ* hybridization, samples at 1d, 4d and 15dph were fixed in parafolmaldehide 4% in 1X PBS overnight at 4ºC and stored in 100% methanol at -

20ºC. Bleaching was necessary with 3% H_2O_2 and 1% KOH in all larval stages. Whole-mount in situ hybridization was performed using digoxigenin-labeled antisense probes as previously described (Rotllant *et al*., 2008). Antisense riboprobes were made from linearized full length *Scophthalmus maximus sparc* cDNA (GenBank Accession number: KF192603).

Cartilage-bone staining

10 fish from each temperature were collected at 4, 15, 30, 50 and 80dph. Fish were anesthetized with MS-222 (500 mg/L, Sigma–Aldrich, Madrid, Spain) and they were fixed in 4% paraformaldehyde. Fish were stained with Alcian-Blue/Alizarin Red S protocol adapted to turbot for cartilage-bone observation (Walker and Kimmel, 2007). Stained embryos were preserved and observed in 50% glycerol, 0.1% KOH solution.

Data analysis and Statistics

Specimens were observed and photographed under a Leica M165FC stereoscope (Leica Microsystems, Germany) equipped Leica DFC 500 digital camera. Adobe Photoshop™ software was used to adjust contrast levels in all images.

Translation was carried out using the EMBOSS Transeq (http://www.ebi.ac.uk/Tools/services/web_emboss_transeq/toolform.ebi). Signal peptide was predicted using SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). Multiple alignments were performed with some of the available Sparc proteins from NCBI using the MAFFT software version 7 (http://mafft.cbrc.jp/alignment/server/). The accession numbers of those proteins selected were ABI85389.1 (*Hippoglossus hippoglossus),* CAD91895.1 (*Sparus aurata*), NP_001027722.1 (*Takifugu rubripes*), NP_001001942.1 (*Danio rerio*), NP_989741.1 (*Gallus gallus*), AAA60993.1 (*Homo sapiens*) and NP_001079590.1 (*Xenopus laevis*).

Pairwise alignments were made to calculate the overall and domains percentage of sequence identity between turbot and other Sparc sequences (http://www.ebi.ac.uk/Tools/psa/emboss_matcher/). Phylogenetic analysis was carried out with domains II and III using the Mega 4/ClustalX software following the Neighbor Joining Method and displayed by FigTree v1.4.0. Phylogenetic tree was bootstrapped 1000 times. The accession numbers of the sequences were: AAA60993.1 (*Homo sapiens*), ABQ12988.1 (*Bos taurus*), AAH61777.1 (*Rattus norvegicus*), AAA40125.1 (*Mus musculus*), AAD12179.1 (*Coturnix coturnix*), NP_989741.1 (*Gallus gallus*), CAA44350.1 (*Xenopus laevis*), AAC99813.1 (*Oncorhynchus mykiss*), AAT01213.1 (*Danio rerio*), AAT01217.1 (*Oryzias latipes*), AAT01214.1 (*Takifugu rubripes*), AAP04488.1 (*Sparus aurata*), ABI85389.1 (*Hippoglossus hippoglossus*), ABM21523.1 (*Ginglymostoma cirratum*), ABM21522.1 (*Petromyzon marinus* A), ABM21524.1 (*Petromyzon marinus* B), AAT01212.1 (*Ciona intestinalis*), CCJ09602.1 (*Patella vulgata*), BAB20042.1 (*Artemia franciscana*), AAA16827.1 (*Caenorhabditis elegans*), XP_001640958.1 (*Nematostella vectensis* 1), XP_001626442.1 (*Nematostella vectensis* 2), XP_001641541.1 (*Nematostella vectensis* 3), XP_001629356.1 (*Nematostella vectensis* 4). Differences in gene expression were assayed by the non-parametric test Kruskal-Wallis for each temperature group followed by the Bonferroni test. U-Mann Whitney test was used to detect differences between temperatures at the same days of development. Statistical significance was considered at $p < 0.05$. Results are given as mean ± SEM.

RESULTS

Cloning and phylogenetic analysis of turbot *sparc*

The full-length cDNA of zebrafish *sparc* was obtained through a combination of reverse transcription (RT) and RACE-PCR amplifications. RT-PCR using degenerated primers designed by alignments of available fish *sparc* sequences produced a partial cDNA fragment of 575 bp. To obtain the complete sequence of turbot peptide precursor RACE-PCR was performed in the 3' and 5'direction with specific primers. 5' RACE generated unique band of 358bp and 3' RACE generated also an unique band of 259bp. The complete cDNA is 1154 bp long and consists of an open-reading frame (ORF) of 930bp, encoding a predicted polypeptide of 310 amino acid residues with a putative signal peptide of 17 amino acids, a 94 bp 5'-untranslated region (UTR) and a 130 bp 3'-UTR (Fig.1).

Like other vertebrate SPARCs, the mature turbot Sparc protein has three highly conserved domains. Domain I (acidic rich domain) comprises 58 amino acids, domain II (Follistatin like domain) includes 80 amino acids with the typical pattern of cysteines and domain III (extracellular calcium binding domain) includes the last 155 amino acids (Figure 1).

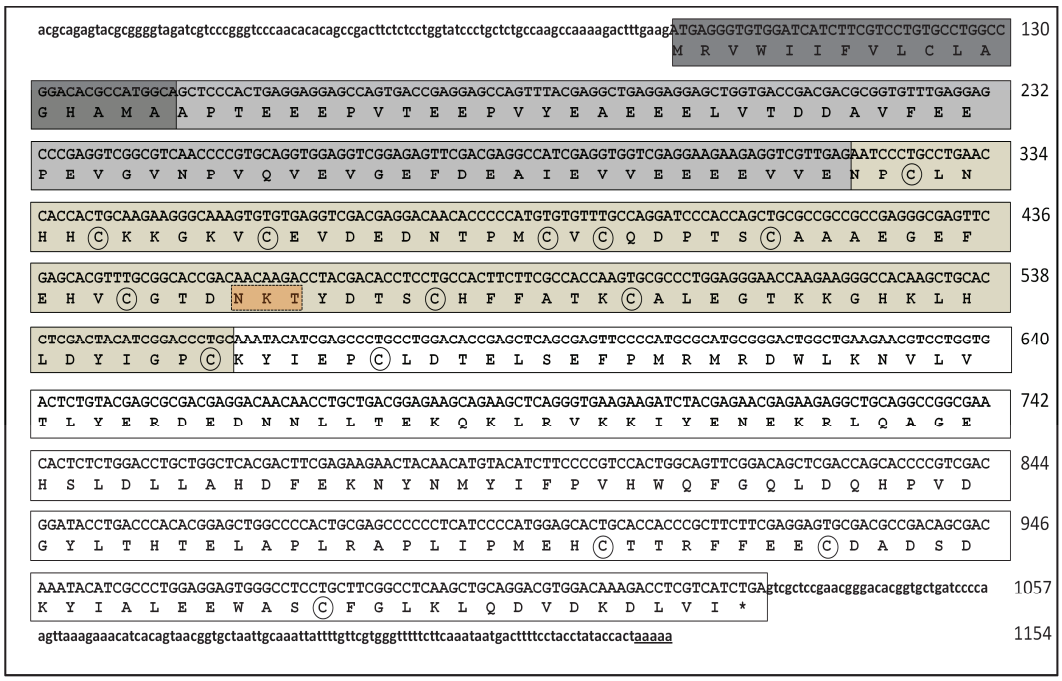

Figure 1. Nucleotide and deduced amino acid sequence of turbot (*S. maximus*) Sparc cDNA. Nucleotides are numbered on the right of the sequence, and the amino acids are represented below. The coding region is shown in upper-case letters with the 5' and 3' untranslated regions in lowercase letters. The putative polyadenylation signal is underlined, and the stop codon is shown at position 1022bp. Conserved cysteine residues are circled. Dark grey box indicates signal peptide; Light grey box indicates Acidic rich domain; brown box indicates Follistatin-like domain; White box indicates Extracellular domain. The nucleotide sequence have been submitted to the GenBank nucleotide sequence databases and have been assigned the accession no KF192603.

A multiple sequence alignment performed with most of the available Sparc protein sequences shows high sequence conservation among vertebrates (Fig.2).

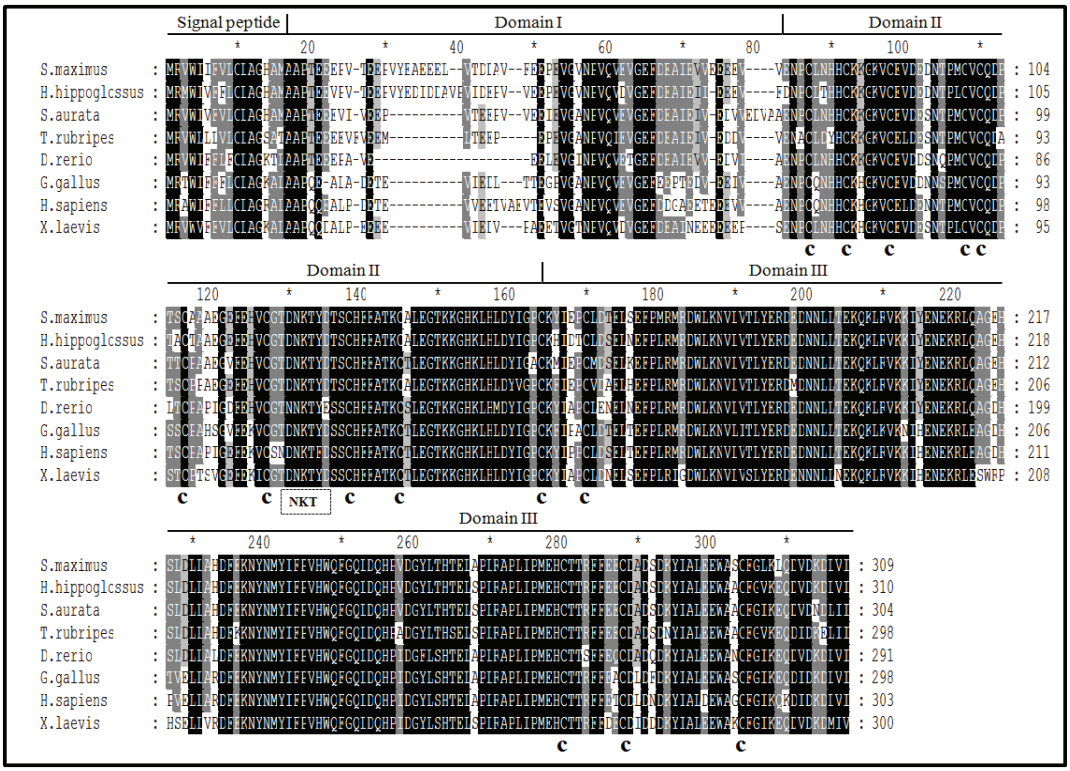

Figure 2. Functional domains of other vertebrate Sparc's are conserved in turbot. Sequence alignment of Sparc proteins. Identical amino acids are represented by dark boxes. The conservative amino acids are indicated by grey boxes and semi-conservative substitution amino acids are depicted by light grey boxes. Conserved cysteine residues, signal peptide and functional domains are highlighted. Domain I (Acidic domain), Domain II (Follistatin-like domain) and Domain III (Extracellular domain).

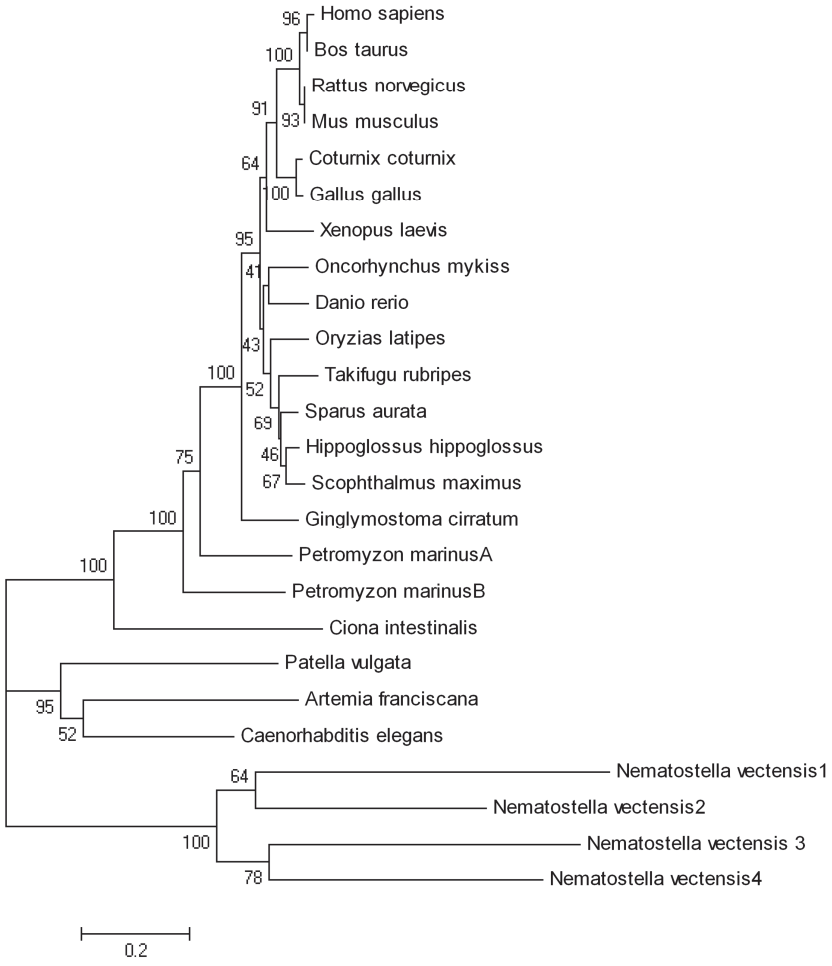

Figure 3. Phylogenetic tree showing the relationships among metazoan Sparc FS–EC domains. The numbers on the branches are bootstrap values. The scale for branch length (0.2 substitutions/site) is shown below the tree. See GenBank accession codes of the sequence used at the material and methods section.

The amino acid sequence of turbot Sparc is 75–89% identical to those of other vertebrate species (Fig.2; Table 1).

Species	Overall	Domain I	Domain II	Domain III
H. sapiens	77	44	84	84
B. taurus	78	44	84	86
R. norvegicus	77	56	84	86
M. musculus	77	56	83	86
C. coturnix	78	63	84	86
G. gallus	77	61	84	85
X. laevis	75	63	84	80
O. mykiss	80	64	86	89
D. rerio	80	71	83	90
O. latipes	84	65	89	90
T. rubripes	82	59	89	89
S. aurata	86	73	93	93
H. hippoglossus	89	71	94	94
G. cirratum	69	47	75	79
P. marinus A	56	52	70	64
P. marinus B	54	35	55	63
C. intestinalis	41	31	44	48
P. vulgata	31	33	37	39
A. franciscana	30	27	26	39
C. elegans	34	28	32	39
N. vectensis 1	32	-	40	32
N. vectensis 2	31	-	28	31
N. vectensis 3	29	-	27	32
N. vectensis 4	33	-	32	30

Table 1. Values represent overall and structural identity percentages between turbot and the species selected based on pairwise alignments.

Additionally, the extracellular calcium binding domain is 89-94 % similar to other teleost orthologs and 84–86% similar to mammalian Sparc (Fig.2; Table 1), being the most highly conserved domain, while the two other structural domains, acidic rich domain and follistatin like domain are also conserved in the turbot Sparc protein and share,

respectively 44–73% and 83-94% amino acids sequence homology with vertebrate Sparc orthologs (Table 1).

Similar to other Vertebrate Sparc orthologs, turbot sparc contains 14 cysteine residues, 10 of which are conserved in domain II, the follistatin-like domain of SPARC. Furthermore, a putative N-linked glycosylation site (N-X-S/T) in the middle of domain II conserved across all phyla is also found in turbot Sparc ortholog. In order to study the evolutionary relationship of genes coding for Sparc orthologs, we constructed a phylogenetic tree by aligning 24 collected amino acid sequences (Fig.3). Genes coding for Sparc orthologs have been identified in organisms ranging in complexity from basal metazoans to mammals (Fig.3). Since domain I is absent from cnidarian Sparc orthologs (Koehler *et al*., 2009), an amino acid alignment of the follistatin-like (II) and the extracellular calcium (III) domains of Sparc sequences was used to construct a phylogenetic tree using likelihood and Bayesian methods. *sparc* gene family phylogentic tree is consistent with accepted taxonomic relationships, with reasonably well-supported clades representing Cnidaria, Protostomia, and Deuterostomia (Fig.3). Even within each of these groups, the *sparc* gene structure is essentially fitting with organisma relationships. Within deuterostomes, vertebrates form a well-supported monophyletic group. Therefore, the phylogenetic tree revealed the relationship between the turbot SPARC amino acid sequence with other known SPARC orthologues teleost Sparc family members (Fig.3).

Temporal and spatial *sparc* expression during embryonic and larval development

The temporal expression of *sparc* mRNA during embryonic and larval development was analyzed by RT-PCR and absolute real-time PCR (Fig. 4A,B). By RT-PCR turbot *sparc* mRNA was first identified at 14 hours post fertilization, approximately at the midblastula transition period and expression subsequently increased and persisted throughout embryonic stages and early larval development (Fig. 4A). Because the RT-PCR has

inherent limitations, particularly those that result in biases in the template to product ratios of target sequences, to specifically characterize the transcriptional regulation of Sparc during the critical period of postembryonic remodeling in turbot we used absolute real-time PCR. Absolute Real-time PCR showed that a high level *sparc* mRNA expression was associated with metamorphosis (developmental stages 3b-4d; 15-30dph) (Fig. 4B). Expression was also evident at initial post-metamorphic stage 5a (from 30dph) and then began to decrease until the end of post-metamotphic phase (Stage 5c; 50dph). Expression was also evident at the end of pre-metamorphic Phase (Stage 3a; until 15dph) then began to increase.

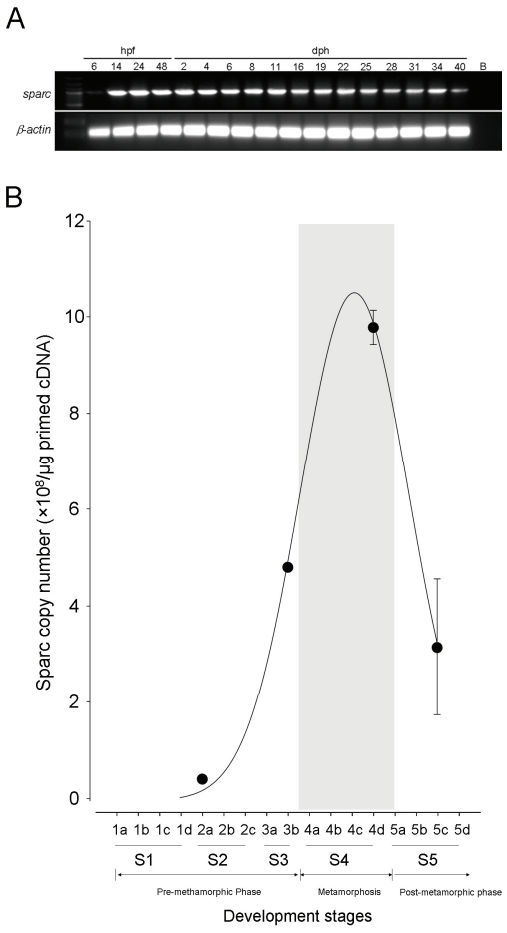

Figure 4. Osteonectin gene expression during turbot embryonic and larval development. (**A**) RT-PCR analyses of osteonectin and β-actin expression. hpf, hours after fertilization; dph, days after hatching. (**B**) Represents osteonectin absolute gene expression. *sparc* gene copy number was quantified by absolute qRT-PCR. The average sparc gene copy number per µg of primed cDNA was calculated from10 individuals analyzed each time in triplicate. Curve is Gaussian Peak Least-Squares Regression line. Data are expressed as mean± SEM.

The spatial expression of *sparc* was determined by whole-mount in situ hybridization. A strong expression was clearly detected in the pectoral fin and lower jaw in 1dph turbot larvae (Fig.5A). At 4 dph (pre-methamorphic phase, developmental stage 2b), *sparc* was detected in cranioencephalic region mainly in upper and low jaws (Fig.5B) but also was detectable in the caudal pterigiophores (Fig.5C). At 15dph (beginning of metamorphosis phase, developmental stage 3b) , *sparc* transcripts show the same location as at 4dph in branchial arches, jaws (5D) and caudal fin (5E) but additionally in pterigiophores of dorsal and anal fins (5F). All these areas are cartilaginous elements that afterward undergo mineralization processes.

Figure 5. Spatial expression of *sparc* by whole amount in situ hybridization in (**A**) 1 day , (**B**) 4 days and (**D**) 15 days turbot larvae. (**C**) caudal fin at 4 d, (**E**) caudal fin at 15 days and (**F**) pterigiophores at 15 days (dph). Scale bar: 250µm

Effect of temperature on growth, post-embryonic remodeling and osteonectin expression

Eggs of turbot were capable of complete development at constant temperatures of 14 and 18 ºC. Percentage mortality was similar (35%) at both temperatures. Thus, over the range of temperatures proposed in this work, mortality differences were not significant and egg mortality occurred primarily during the early developmental stages (2-8 dpf).

During the experimental period, the animals were sampled and staged at 4, 15, 30, 40, 50 and 80 dph. No significant differences in dry weight between groups were found until day 15 dph (Fig. 6A). Thereafter, significant differences in mean wet weight were recorded between both groups. 18 ºC reared larvae grew faster than 14 ºC larvae. The developmental rate (time necessary to reach a certain development stage) at the two temperatures tested was significantly different. Thus, it decreases from almost 50d at 18ºC to 30d at 14 ºC (Figure 6B). 50 dph 18 ºC reared larvae were at the same developmental stage than 80 dph 14 ºC reared larvae (Figure 6C). To further shown the effects of temperature in the post-embryonic remodeling events during turbot development, differences in the sequential steps of ossification are shown in Figure 6c. The time necessary to reach a certain stage of skeletal development was significantly different between both temperature groups (Fig. 6C).

Thus, Cranioencephalic region (CR) and jaws start to ossify at 15dph in both groups but with higher grade in 18ºC specimens. At this time point, Neural (NS) and haemal spines (HS) are also undergoing mineralization in 18ºC group but this process will appear later (between 15-30dph) in 14ºC samples. Pterigiophores (PT) still do not appear in 14ºC but they are visible as cartilaginous structures in 18ºC specimens. Relating to caudal fin hyoid1 (HY1) appear in both groups but hyoid2 (HY2) appears only at 18ºC. At 30 dph, an anterior-posterior ossification of the vertebral column is observed in fish raised at 14°C water temperature when only a few first abdominal vertebrae were ossified meanwhile in 18°C water temperature all the vertebrae are completely ossified and only the two last caudal vertebrae (preural vertebrae) are in the ossification process. Dorsal,

anal and caudal fin remains cartilaginous at this point in both groups. In 50dph samples raised at 14°C water temperature, the vertebral ossification is finished. However, radial fins are still in ossification process but not in 18ºC group where all the structures appear already calcified. Finally, the mineralization is ended at 80 dph in 14ºC water temperature fish samples when all the vertebrae and radial fins are totally calcified.

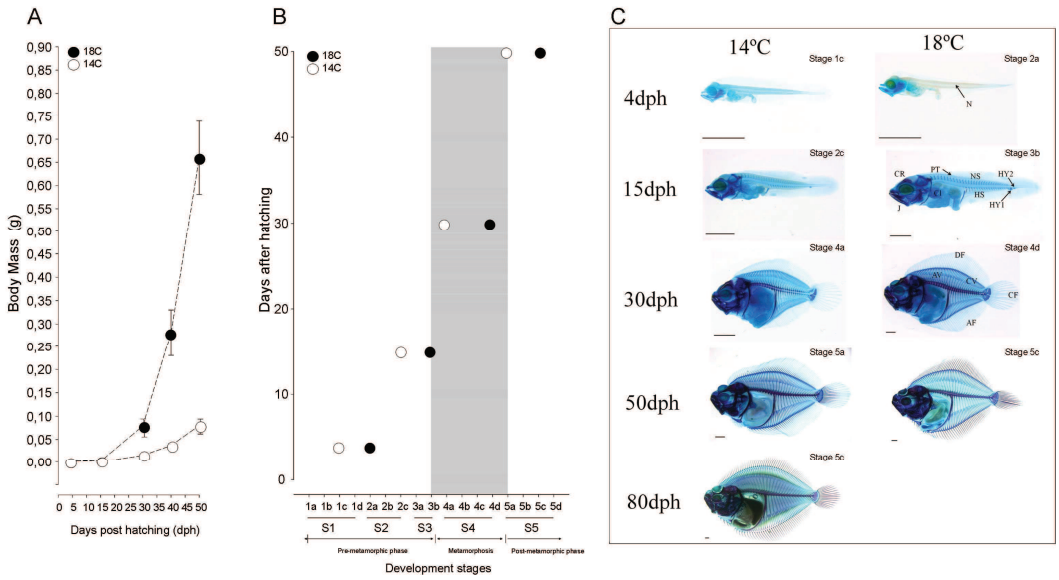

Figure 6. Effect of rearing water temperature on growth and post-embryonic remodeling in turbot. (**A**) Wet body mass of developing turbot larvae reared and fed ad libitum at 14 ºC (white dots) and 18ºC (dark dots) in relation to dph (**B**) Age (dph) in turbot reared at 14ºC (white dots) and 18 ºC in relation to stage in development (**C**) Time-course comparison of skeletal development from 4dph to 80dph in turbot raised at 14ºC and 18ºC using Alcian blue-Alizarin red double staining. AF (anal fin), AV (abdominal vertebrae), CF (caudal fin), CV (caudal vertebrae), CR (cranium), Cl (cleitrum), CR (cranium), DF (dorsal fin), HS (haemal spines), HY (hypuralia), J (jaw), NS (neural spines), N (notochord); PT (pterygiophores). Scale bar= 1mm

In order to determine the effect of rearing water temperature on *sparc* expression, *sparc* gene copy number were measured in 4, 15, 30, 50 and 80dph samples reared at 14ºC and 18ºC by absolute real time PCR (Fig.7). Transcripts of *sparc* keep in a quite low level at 4 dph in both temperature reared larvae. In larvae reared at 18ºC the levels of *sparc* mRNA reached the peak at 30dph and then began to decrease until 50dph in 18ºC. In larvae reared at 14ºC *sparc* mRNA had a sharply increase in 15 to 45dph, and gradually decreased and got to a nearly identical level with 15dph at 80dph, and it got to a higher level at 45-50 dph (Fig.7) when the larvae were just at metamorphic climax (Fig. 6B).

Therefore, there were not significant differences in the *sparc* gene copy number dynamics, with both groups having the highest *sparc* gene copy number when the larvae were just at metamorphic climax but such specific expression pattern is time delayed in low temperatures reared larvae (Fig.7). Thus, comparing *sparc* levels at the same developmental stage in both temperatures, no significant differences were obtained in all the collection points from 15 days post hatching (data not shown).

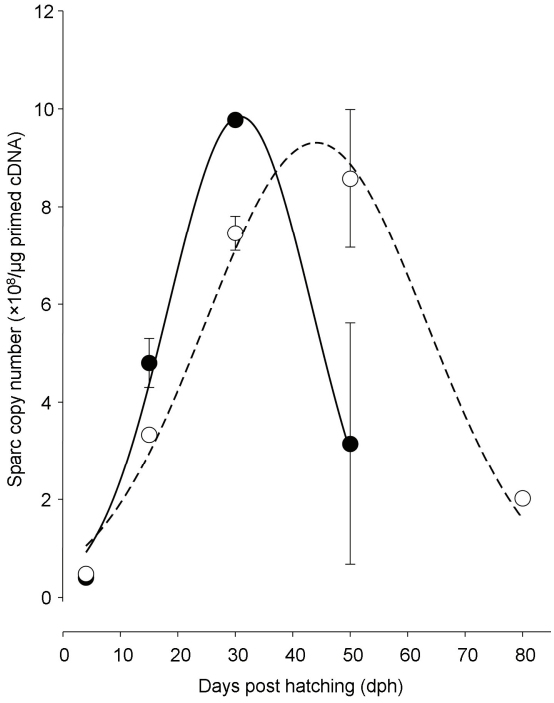

Figure 7. Effect of rearing water temperature on *sparc* gene expression during turbot post-embryonic development. *sparc* gene copy number was quantified by absolute qRT-PCR. The average *sparc* gene copy number per µg of primed cDNA was calculated from 10 individuals analyzed each time in triplicate. Data are expressed as mean SEM. The sample points were 4, 15, 30, 50 in 18 ºC reared larvae and 4, 15, 30, 50 and 80 days in 14 ºC reared larvae. 14ºC (white dots) and 18ºC (black dots). Curves are Gaussian Peak Least-Squares Regression line.

DISCUSSION

Metamorphosis is irreversible developmental and physiological change that affects multiple traits during postembryonic development. These changes include major remodeling of existing features as well as the formation of entirely new tissues and organs; thus, metamorphosis requires extensive differentiation as well as the morphogenetic processes of cellular migration, proliferation, growth, and death. However, for most of the metamorphic events, the underlying cellular mechanics remain unexplored. In order to provide new insights into the molecular and cellular mechanisms underlying the morphogenetic processes required during post-embryonic remodeling, we report the cloning, protein organization and post-embryonic remodeling stage–specific expression of turbot osteonectin, a extracellular matrix protein that has been shown to affects cellular differentiation by promoting the withdrawal of cells from the cell cycle and contributes to modulate cell–cell and cell–matrix adhesion (Koehler *et al*., 2009).

We found that, turbot *sparc* cDNA is 1154 bp long and consists of an open-reading frame (ORF) of 930bp, encoding a predicted polypeptide of 310 amino acid residues with a putative signal peptide of 17 amino acids, a 94 bp 5'-untranslated region (UTR), and a 130 bp 3'-UTR. Turbot Sparc protein keep the same protein structure exhibited by all vertebrate Sparc proteins (Laizé *et al*., 2005; Koehler *et al*., 2009; Kos and Wilding 2010). The putative turbot Sparc precursor have the characteristics of a secreted protein, displaying a putative signal peptide. Processing of the potential signal peptide produces 293-amino acid mature protein, including a glutamic acid-rich N-terminal domain (I), a follistatin-like (FS) central domain (II) with a high proportion of Cysteine residues as well as a N-linked glycosylation site that precedes the C-terminal domain or also called extracellular calcium domain (III) which is an alpha helix-rich region containing two high-affinity Ca^{2+}-binding EF-hands (EF-hand1 and EF-hand2). The glutamic acid-rich N-terminal domain (I) is an acidic region that binds Ca^{2+} with low affinity (Lane *et al*., 1994; Brekken and Sage, 2001), interacts with hydroxyapatite (Brekken and Sage, 2001), it is involved in the mineralization of cartilage and bone (Brekken and Sage, 2001) and

contains the major immunological epitopes of the protein (Stenner *et al*., 1984). In turbot, this domain contains 21 glutamic acid residues and most likely has functions comparable with those in mammalian vertebrates. This domain is highly variable among invertebrates and it is absent in cnidarians (Koehler *et al*., 2009). Therefore, it has been proposed that Ca^{2+}-dependent activities emerged with the acquisition of the acidic N-terminal domain in triplobastic organisms (Koehler *et al*., 2009). The second domain is a cysteine-rich follistatin-like domain, which includes a Kazal -like domain and an EGF-like motif (Hohenester *et al*., 1997). It has been shown to binds activin, inhibin, heparin and proteoglycans and may regulate proliferation of endothelial cells and angiogenesis (Funk and Sage, 1993; Yan and Sage, 1999). The tertiary structure of Sparc is maintained by seven disulphide bridges provided by the 10 conserved cysteines located at this region. Additionally, this domain contains a highly conserved N-linked glycosylation site that seems to be an important feature for collagen affinity and for protein functionality (Kaufmann *et al*., 2004).

The third and last domain is a calcium-binding extracellular domain, which includes two EF-hand motifs with high affinity for extracellular Ca^{2+}. It has been shown to binds collagen types I, III, and IV in a Ca^{2+}-dependent (Sasaki *et al*., 1997; Sasaki *et al*., 1998).

Our multiple sequence alignment indicated that the deduced amino acid sequence of turbot Sparc revealed strong overall conservation with its vertebrate counterparts. Furthermore, our phylogenetic analysis also indicated that turbot Sparc clusters together with its vertebrate orthologues and teleost Sparcs were arranged into a single clade. Additionally, we found that the expression pattern of turbot *sparc* is comparable to zebrafish, medaka and seabream (Rotllant *et al*., 2008; Renn *et al*., 2006a,b; Redruello *et al*., 2005, Estêvão *et al*., 2005). Therefore, the comparative analysis of turbot Sparc primary sequence with other Sparc proteins from diverse vertebrate species and specifically with its teleost orthologs suggest a strong evolutionary pressure to conserve this protein and indicate that there should be an evident conservation of function.

Numerous studies indicate that Sparc, has complex multiple functions during development. Sparc is dynamically expressed in skeletal and non-skeletal tissues from

early development to adulthood, suggesting also a potentially wide range of action (Rotllant *et al*., 2008; Estêvão *et al*., 2005; Renn *et al*., 2006a,b). However, its functions are not limited to embryonic development as Sparc has been shown to remain associated with adult tissues undergoing turnover, remodeling, secretion and repair in several species (Lane and Sage 1994). However, despite the knowledge gained from recent *in vivo* and *in vitro* studies (Bradshaw *et al.,* 2003; Brekken *et al*., 2003; Redruello *et al.,* 2005; Rotllant *et al*., 2008; Ceinos *et al*., 2013), the precise morphogenetic functions of SPARC during development are poorly understood. It is well-known that flatfish undergo a spectacular morphological metamorphosis, comparable to the metamorphosis of anuran amphibians. Thus, flatfish including the turbot (*Scophtalmus maximus*) change from a bilaterally symmetrical pelagic larva to an asymmetric benthic juvenile (Sadiq *et al*., 1984). Thus, post-embryonic development in turbot encompasses a broad spectrum of complex morphogenetic processes with an active cellular migration, proliferation, growth and death events.

In this study, we demonstrated that turbot *sparc* RNA was first identified at 14 hours post fertilization, approximately at the midblastula transition period (Tong *et al*., 2012), when zygotic transcriptional activity starts, thus appearing not to be maternally inherited, and then remained highly expressed throughout embryonic stages and early larval development. Similar results were found in zebrafish and sea bream (Estêvão *et al*., 2005; Rotllant *et al*., 2008). Furthermore, analysis of turbot *sparc* mRNA expression showed a dynamic stage-specific expression during post-embryonic turbot development with high *sparc* mRNA levels when the larvae were just at metamorphic climax, indicating that it might be necessary for turbot metamorphosis. Although, turbot metamorphosis is now well described and the central role of thyroid hormone in this process is well established (Power *et al.,* 2008; Infante *et al*., 2008; Roberto *et al*.,2009), there is not evidences of the possible regulatory role of TH on *sparc* expression. However, a number of different factors, such as parathyroid hormone (PTH; Nakajima *et al.,* 2002) and dexamethasone (Sawhney, 2002), have been shown to regulate *sparc* and indicate it may be an important intermediate in hormone action. Thus, further showing its

possible role in turbot metamorphic development, which could help us further explain the complex genetic network that controls processes of turbot metamorphosis and provide insight into metamorphic changes.

To further validate the Sparc dynamic stage-specific expression during post-embryonic turbot development, we determined the *sparc* mRNA expression in turbot development at two different rearing temperatures. It is well known that the relative timing of turbot embryonic and post-embryonic development varied with temperature (Gibson and Johnston, 1994). We found that turbot embryonic development time doubled for a 4°C decrease in water rearing temperature. Turbot embryos reared at 14°C showed a reduction in the rate of ossification and growth. Such observations are in agreement with previous studies on other teleosts species in which growth and skeletal development are compromised at temperatures lower than the optimum thermal environment (Anken *et al.,* 1993; Campinho *et al*., 2004).

The effect of temperature on *sparc* mRNA expression measured in chronological time units (Days post hatching) appears to coincide with that found for development time, skeletal development and growth rate. Thus the relative timing of *sparc* mRNA expression seems to be delayed at 14°C having high expression levels at higher specific fish age but concomitant with a the specific stage of development. Therefore, *sparc* mRNA expression appears to be dependent on the development time rate. Thus, further supporting the dynamic stage-specific *sparc* expression during post-embryonic turbot development.

In conclusion, we report, for the first time, the cloning of turbot *sparc* and the analysis of its protein structure and temporal distribution during embryonic/larval development. Given its evolutionary conservation in terms of protein organization when compared to mammalian genes and the data presented here, it is likely that, as in mammals, Sparc plays an important role in tissue remodeling in fish, its function seems to be maintained through evolution. Furthermore, the dynamic stage-specific expression during turbot post-embryonic development described in this paper suggest a useful framework for

future studies, which should address the succession of morphological and molecular events that take place during flatfish metamorphosis.

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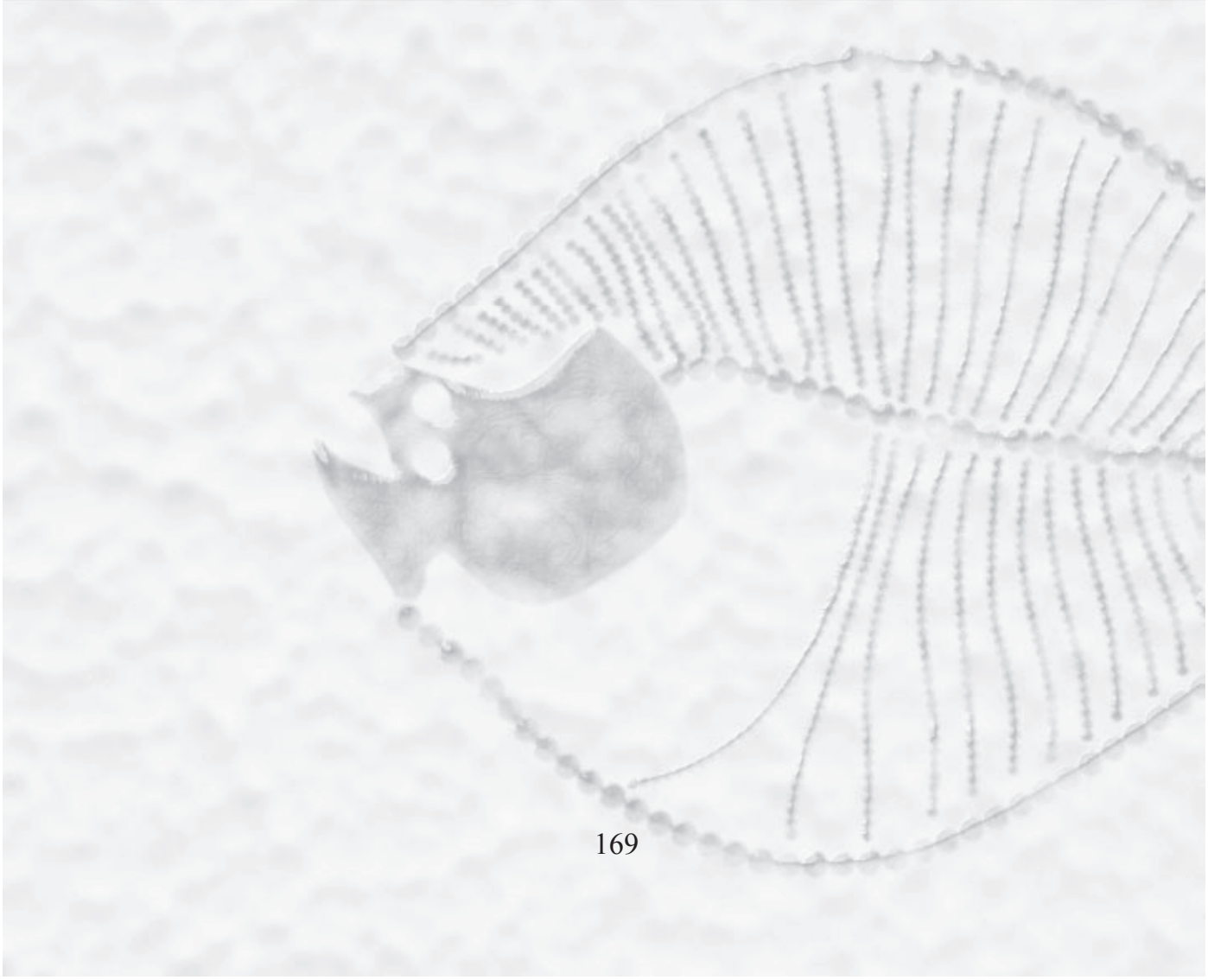
Discussion



The main objective of this thesis was to contribute to the understanding of Sparc due to the existence of gaps in our knowledge regarding its regulation and the contradictory roles found in different tissues. Since teleosts apparently have less Sparc functional homologs than mammals, the observations in non-mammalian vertebrates can likely uncover key functions of Sparc. Therefore, the studies presented in this thesis project were carried out in zebrafish and turbot, two species of teleosts, which is the largest and most diverse group of vertebrates.

For that purpose, first, we investigated the functional role of Sparc in haematopoiesis; second we determined the potential role of Sparc on the developmental abnormalities produced by solar UV radiation exposure in fish embryos and we carried out functional analysis to characterize the transcriptional regulation of the *sparc* gene in zebrafish embryos using reporter gene expression. Finally, we have cloned and characterized the turbot (*Scophthalmus maximus*) *sparc* gene to unravel the spatio-temporal expression pattern of *sparc* in flatfish metamorphic remodeling.

Our data provide strong evidences that *sparc* play a critical role in haematopoiesis during zebrafish development and the placement of *sparc* in the fgf signaling pathway. Regarding its regulation, we demonstrated a possible role for Sparc in underlying molecular mechanisms responsible for developmental abnormalities produced by UVR exposure in fish embryos and we identified an intron necessary for the transcriptional regulation of *sparc*. Finally, sequence similarities, domain organization of the deduced peptide and expression analysis allow us to conclude that Sparc in turbot is highly similar to all vertebrate Sparc proteins and it is stage-specific expressed during post-embryonic turbot development. Therefore, our studies add new pieces to the puzzling functional roles of Sparc in teleosts and reveal significant differences but also striking similarities that help to improve our understanding of the Sparc function in vertebrate in general.

The discussion follows to a large extent the chronology of the chapters but it has been structured to specifically address the most important findings:

1. *Sparc* is as an important regulator of embryonic haematopoiesis.

A recent study on morpholino antisense oligonucleotide (MO)-based functional screening in zebrafish showed a potential hematopoietic function of 14 genes (Eckfeldt *et al*., 2005), and *sparc* was among them. Therefore, we investigated the function of Sparc in zebrafish haematopoiesis in more detail.

The present study demonstrated that *sparc* knockdown significantly reduced embryonic haematopoiesis at the lineage-committed cellular level. In particular, genes associated with primitive and transient erythroid progenitor cell development (*gata 1* and *βe3globin)* were down-regulated in the *sparc* morphants. By contrast, genes associated with primitive and transient myeloid progenitor cell development and genes associated with definitive haematopoiesis were not deregulated. This suggests a critical role of *sparc* by modulating the lineage-specific transcription factor *gata 1* expression levels or activity. However, this assumption raises a puzzling question of how a matricellular protein can regulate expression of transcription factor genes. The role of *sparc* in cell-matrix interactions may hold the answer; *sparc* may mediate or trigger signal transduction pathways required for activation or maintenance of target genes transcription. This concept could be explored by identifying extracellular signalling molecules that act upstream of these genes encoding for *gata1* and *sparc*.

2. *Sparc* acts downstream of *fgf21* signaling.

Members of the Fgf family are known to regulate *sparc* gene expression in different species (Brekken and Sage, 2001; Whitehead *et al*., 2005). In mammals, although it is well known that *sparc* gene expression is regulated by members of the *fgf* family and in turn the *fgf* pathway regulates primitive haematopoiesis by modulating *gata1* expression level and activity, its function in haematopoiesis is not clear. Furthermore, the expression of *gata1* transcription factor gene is regulated by Fgf signalling pathways in chick and zebrafish (Nakazawa *et al*., 2006; Songhet *et al*., 2007), as altered *fgf* expression leads to perturbation of expression of this gene (Yamauchi *et al*., 2006). The disruption of expression of *gata1* mRNA

in *sparc* morphants raises the possibility that the effects of *sparc* on haematopoiesis may at least in part be due to perturbed *fgf* signalling. This hypothesis is supported by the fact that the *sparc* morphant blood phenotype is very similar to the *fgf21* morphant blood phenotype, which is characterized by a severe disruption of erythroid/myeloid progenitor cell development in zebrafish (Yamauchi *et al*., 2006).

To corroborate this theory, we examined whether *sparc* gene expression was perturbed in *fgf21* morphants and if exogenous *sparc* could rescue *gata1* deficiency in zebrafish embryos. We found that *sparc* expression was substantially reduced or missing in *fgf21* morphant embryos. In addition, we also tested the *fgf21* gene expression in *sparc* morphants and found that *fgf21* mRNA expression was not altered.

Our findings therefore suggest that *sparc*, at least in part, acting downstream of the *fgf21* signalling pathway, is critically required in mediating erythroid progenitor cell development in zebrafish. Surprisingly, mice deficient in *sparc* have no severe hematopoietic defects (Siva *et al*., 2012). It has been hypothesized that the presence of more *sparc* functional homologues in mammals functionally compensates for the lack of *sparc* expression, possibly leading to mild defects in *sparc*-null mice. However, studies carried out in other organisms such as *Caenorhabditis elegans* and zebrafish, where there is less redundancy, reduction in *sparc* produces much more significant defects. Consequently, our observations in zebrafish likely uncover the significant roles of *sparc*.

3. UV exposure induces an increase in the *p53* and *sparc* expression

The effects caused by UV exposure are known to produce irreparable alterations at different levels from organism survival and reproduction (Tietge *et al*., 2001; Häder *et al*., 2007; Marquis *et al.,* 2008; Charron *et al*., 2000) to cellular metabolism and viability (Dahms and Lee, 2010; Rastogi *et al*., 2010). However, the molecular responses triggered in an animal organism after a UV exposure are not yet understood. Under natural conditions the direct effects of UV radiation are difficult to study due to the interaction with other environmental factors and changes in irradiance caused by the variability in cloud cover, atmospheric

composition and/or the amount of the colored dissolved organic matter, among others. In this study an incubator which emits PAR, UVA and UVB in similar proportions as those observed under natural conditions was used to evaluate the consequences of exposure (Neale and Fritz, 2001). We demonstrated that UV exposure can induce an exposure-dependent increase in the gene *p53* expression. Therefore zebrafish embryos do show an increase in *p53* gene expression in response to UVR like in mammals. In analogy with these other organisms, *p53* expression in zebrafish is expected to be beneficial by increasing DNA repair, but other functions of *p53*, like apoptosis may have contributed to abnormal development. It has been shown that keeping *p53* at low levels during embyogenesis is critical to protect normal development (Zeng *et al.*, 2009).

In parallel with *p53* induction, we also demonstrated a UV exposure-dependent increase in the expression of the matricellular protein, Sparc. To date, there is little information on the direct effect of UV on *sparc* gene expression regulation. Aycock *et al.*, 2004 showed that *sparc* was present in relatively high quantities in UV-induced squamous cell carcinoma, however, was undetectable in skin from the nonirradiated control group. In addition, *sparc* null mice were tumor resistant, developing no squamous cell carcinoma in response to UV radiation. Therefore, they suggested that *sparc* had a critical role in mediating skin tumor formation in response to UV irradiation.

Regarding the spectral dependence of gene expression, exposure to a combination of UVB and UVA radiation produced a greater *sparc* and *p53* expression increase than UVA alone, thus probably indicating a higher capability of UVB to produce cellular damage in zebrafish. However, significant differences in the expression of both genes were observed in the shorter wavelengths of the UVA, in which *p53* was activated by less damaging spectral treatments than *sparc*. Longer wavelengths of UVA did not produce a significant expression increase of both genes compared with embryos exposed to nondamaging PAR. Previous work in zebrafish has demonstrated the capacity of UVA to activate a mechanism, the photoenzymatic repair (PER), which repairs the DNA damage caused by UVB exposure (Dong *et al*., 2007). The evidence of the mentioned

repair system is the initial detection of photolyase enzyme in 3 hpf zebrafish embryos (Dong *et al.,* 2008). The induction of PER partially compensates for a considerable decrease in tolerance of UVB exposure at this developmental stage (Dong *et al*., 2008). It is suggested that the higher UVB tolerance at the egg stage may be related to other (dark) repair mechanisms as well as possible shielding by the chorion and other maternally derived photoprotective compounds. In conclusion, it has to be considered that sensitivity to UV radiation may vary between developmental stages.

4. *Sparc* expression could be a possible underlying molecular mechanism of UV-radiation induced phenotypic anomalies

A decrease in survival percent and an increase in developmental abnormalities were observed in UV-exposed embryos. The increase of *sparc* expression detected by qRT-PCR and *in situ* hybridization could be an additional cause for these mentioned effects in UV-exposed embryos. The phenotypic abnormalities revealed by previous overexpression and loss-of-function studies (Damjanovski *et al*., 1997) also support this possibility. It has been shown that injection of *sparc* RNA into early blastomeres is associated with head and axis defects in *Xenopus*. Histological analysis revealed somite malformations that corresponded with the kinked axis (Damjanovski *et al*., 1997). In this study we also show that ectopic expression of *sparc* affects zebrafish development. Microinjection of capped and poly(a)-tailed full-length zebrafish *Sparc* mRNA into 1–2 cell zebrafish embryos generated phenotypic malformations, with caudal (posterior) notochord bending/torsion as the most frequent deformity. The fact that similar phenotypic malformations linked to an increase in *sparc* gene expression were found in UV-exposed and in ectopic *sparc* expression experiments therefore suggests *sparc* expression as one of the possible molecular mechanisms of UV-radiation induced phenotypic anomalies.

5. 5'UTR-intron is a key control region for the transcriptional regulation of *sparc*.

A common characteristic of the *sparc* gene organization in all vertebrates species studied is the presence of an intronic sequence between first non coding and the second coding exon. However, the size of the first intron seems to be species-specific being of 7kb in zebrafish, of 10kb in humans and 2kb in *Xenopus* (Damjanovski *et al.,* 1998). We found that the 0.2-kb *sparc* promoter and its 5'-flanking sequence 7 kb upstream of the translated exon drove GFP expression in the notochord, otic vesicle, fin fold, somites, intermediate cell mass, skeletal and cardiac muscles, which mimicked the already well described expression pattern of the endogenous *sparc* mRNAs (Rotllant *et al*., 2008; Ceinos *et al*., 2013). Similar results were also found in mouse, where *sparc* transcripts were detected in developing tissues, such as the otic vesicle (Mothe and Brown, 2001), notochord, somites and the embryonic skeleton (Holland *et al*., 1987; Mason *et al*., 1986).

In addition, the 0.2-kb *sparc* promoter and its 5'-flanking sequence 7 kb upstream of the translated exon drove the expression of the GFP reporter gene in the olfactory epithelium. Although, specific expression of *sparc* in the olfactory epithelium of mice it has already been reported (Mendis and Brown, 1994), this is the first report to demonstrate the possible expression on *sparc* in the olfactory epithelium in non-mammalian vertebrate. In this study, we were not able to detect *sparc* expression in olfactory epithelium by whole-mount in situ hybridization. One possible explanation for this discrepancy may relate to the limited sensitivity of our whole-mount in situ-hybridization assay to detect faint expression of *sparc* in some regions.

It also should be noted that, although the conclusion was based on transient and transgenic expression analysis, it is unlikely that the tissue-specific spatial expression pattern of the e*gfp* reporter gene expression was due to position effect of the integration site, because the pattern of *egfp* expression, in many ways, mimicked the endogenous *sparc* expression. However, we cannot exclude the possibility that there might be position effect on the activity of the promoter, which might explain the specific *egfp* expression in the olfactory epithelium in transgenic and mosaic fish.

Transient expression analyses in zebrafish embryos demonstrated that promoter activity resides in the 5′-UTR unique intronic region (nt+126/+7168). The specific deletion of this region resulted in a complete reduction of promoter activity. Transcriptional regulation of other genes (ej. ubiquitine C) has also been shown to be exclusively regulated by the 5'UTR intron sequence (Bianchi *et al.,* 2009). Therefore, the 5′-UTR unique intronic region (nt+126/+7168) provides the proper regulatory elements required for the expression of a reporter gene in a subset of the tissues that normally express the endogenous *sparc* gene in zebrafish embryos.

6. *Sparc* is transcriptionally regulated by DNA methylation.

Sequence analyses of the zebrafish *sparc* 5′-UTR intron 1 region revealed a number of transcription factors binding sites. Additionally, a CpG-rich sequence (CpG island) was also identified in the zebrafish 5′-UTR intron 1 region. It has been shown that *sparc* is transcriptionally regulated by DNA methylation and CpG-rich sequence were also identified in mammalian *sparc* promoter sequences (Rodríguez-Jiménez *et al*., 2007; Gao *et al.,* 2010; Tajerian *et al*., 2011). Therefore, in order to obtain insights into the transcriptional regulation of *sparc* expression, we investigated the role of DNA methylation in the expression of *sparc* in zebrafish embryos. 5'-Azacytidine exposure approach was use to artificially induce DNA hypomethylation. This method has already been used to induce aberrant DNA hypomethylation in zebrafish embryos (Martin *et al*., 1999; Christman, 2002). Our results show that (i) 5'-Aza exposure produce distinctive phenotypic abnormalities in zebrafish larvae, including shortened tail, torsion of spinal cord, head malformations and depigmentation, (ii) 5'-Aza exposure produced significant global DNA demethylation in zebrafish larvae, (iii) 5'-Azacytidine exposure specifically reduced CpG-rich sequence (CpG island) methylation in the zebrafish Sparc 5′-UTR intron 1 region and (iv) SPARC is highly expressed in 5'-Azacytidine treated zebrafish larvae. Therefore, these results suggest that *sparc* is transcriptionally regulated by DNA methylation.

7. Sparc has been highly conserved among species

We found that, turbot *sparc* cDNA is 1154 bp long and consists of an open-reading frame (ORF) of 930bp, encoding a predicted polypeptide of 310 amino acid residues with a putative signal peptide of 17 amino acids, a 94 bp 5'-untranslated region (UTR), and a 130 bp 3'-UTR. Turbot Sparc protein keep the same protein structure exhibited by all vertebrate Sparc proteins (Laizé *et al*., 2005; Koehler *et al*., 2009; Kos and Wilding 2010). The putative turbot Sparc precursor have the characteristics of a secreted protein, displaying a putative signal peptide. Processing of the potential signal peptide produces 293-amino acid mature protein, including a glutamic acid-rich N-terminal domain (I), a follistatin-like (FS) central domain (II) with a high proportion of Cysteine residues as well as a N-linked glycosylation site that precedes the C-terminal domain or also called extracellular calcium domain (III) which is an alpha helix-rich region containing two high-affinity Ca^{2+}-binding EF-hands (EF-hand1 and EF-hand2). The glutamic acid-rich N-terminal domain (I) is an acidic region that binds Ca^{2+} with low affinity (Lane *et al*., 1994; Brekken and Sage, 2001), interacts with hydroxyapatite (Brekken and Sage, 2001), it is involved in the mineralization of cartilage and bone (Brekken and Sage, 2001) and contains the major immunological epitopes of the protein (Stenner *et al*., 1984). In turbot, this domain contains 21 glutamic acid residues and most likely has functions comparable with those in mammalian vertebrates. This domain is highly variable among invertebrates and it is absent in cnidarians (Koehler *et al*., 2009). Therefore, it has been proposed that Ca^{2+}-dependent activities emerged with the acquisition of the acidic N-terminal domain in triplobastic organisms (Koehler *et al*., 2009). The second domain is a cysteine-rich follistatin-like domain, which includes a Kazal -like domain and an EGF-like motif (Hohenester *et al*., 1997). It has been shown to binds activin, inhibin, heparin and proteoglycans and may regulate proliferation of endothelial cells and angiogenesis (Funk and Sage, 1993; Yan and Sage, 1999). It has been shown that the tertiary structure of Sparc is maintained by seven disulphide bridges provided by the 10 conserved cysteines located at this region. Additionally, this domain contains a highly conserved N-linked glycosylation site, which it has been shown to be an important feature for collagen affinity and therefore for protein functionality (Kaufmann *et al*., 2004).

The third and last domain is a calcium-binding extracellular domain, which includes two EF-hand motifs with high affinity for extracellular Ca^{2+}. It has been shown to binds collagen types I, III, and IV in a Ca^{2+}-dependent (Sasaki *et al*., 1997; Sasaki *et al.,* 1998).

Our multiple sequence alignment indicated that the deduced amino acid sequence of turbot Sparc revealed strong overall conservation with its vertebrate counterparts. Furthermore, our phylogenetic analysis also indicated that turbot Sparc clusters together with its vertebrate orthologues and teleost Sparcs were arranged into a single clade. Additionally, we found that the expression pattern of turbot *sparc* is comparable to zebrafish, medaka and seabream (Rotllant *et al*., 2008; Renn *et al*., 2006a,b; Redruello *et al*., 2005, Estêvão *et al*., 2005). Therefore, the comparative analysis of turbot Sparc primary sequence with other Sparc proteins from diverse vertebrate species and specifically with its teleost orthologs suggest a strong evolutionary pressure to conserve this protein and indicate that there should be an evident conservation of function.

8. *Sparc* is stage-specific expressed during flatfish metamorphic remodeling

Numerous studies indicate that Sparc has complex multiple functions during development. *Sparc* is dynamically expressed in skeletal and non-skeletal tissues from early development to adulthood, suggesting also a potentially wide range of action (Rotllant *et al*., 2008; Estêvão *et al*., 2005; Renn *et al*., 2006a,b). However, its functions are not limited to embryonic development as Sparc has been shown in several species to remain associated with adult tissues undergoing turnover, remodeling, secretion and repair (Lane and Sage 1994). However, despite the knowledge gained from recent in vivo and in vitro studies, the precise morphogenetic functions of Sparc during development are poorly understood.

It is well-known that flatfish undergo a spectacular morphological metamorphosis, comparable to the metamorphosis of anuran amphibians. Thus, flatfish including the turbot (*Scophtalmus maximus*) change from a bilaterally symmetrical pelagic larva to an asymmetric benthic juvenile (Sadiq *et al*., 1984). Thus, post-embryonic development in turbot

encompasses a broad spectrum of complex morphogenetic processes with an active cellular migration, proliferation, growth and apoptotic events.

In this study, we demonstrated that turbot *sparc* RNA was first identified at 14 hours post fertilization, approximately at the midblastula transition period (Tong *et al*., 2012), when zygotic transcriptional activity starts, thus appearing not to be maternally inherited, and then remained highly expressed throughout embryonic stages and early larval development. Similar results were found in zebrafish and sea bream (Estêvão *et al*., 2005; Rotllant *et al*., 2008). Furthermore, analysis of turbot *sparc* mRNA expression showed a dynamic stage-specific expression during post-embryonic turbot development with high *sparc* mRNA levels when the larvae were just at metamorphic climax, indicating that it might be necessary for turbot metamorphosis. Although, turbot metamorphosis is now well described and the central role of thyroid hormone in this process is well established (Power *et al.,* 2008; Infante *et al*., 2008; Roberto *et al*.,2009), there is not evidences of the possible regulatory role of TH on *sparc* expression. However, a number of different factors, such as parathyroid hormone (PTH; Nakajima *et al*., 2002) and dexamethasone (Sawhney, 2002), have been shown to regulate Sparc and indicate it may be an important intermediate in hormone action. Thus, further indicating its possible role in turbot metamorphic development, which could help us further explain the complex genetic network that controls processes of flounder metamorphosis and provide insight into metamorphic changes.

To further validate the *sparc* dynamic stage-specific expression during post-embryonic turbot development, we determined the *sparc* mRNA expression in turbot development at two different rearing temperatures. It is well known that the relative timing of turbot embryonic and post-embryonic development varied with temperature (Gibson and Johnston, 1994). We found that turbot embryonic development time doubled for a 4°C decrease in water rearing temperature. Turbot embryos reared at 14°C showed a reduction in the rate of ossification and growth. Such observations are in agreement with previous studies on other teleosts species in which growth and skeletal development are compromised at temperatures lower than the optimum thermal environment (Anken *et al*., 1993; Campinho *et al.,* 2004).

The effect of temperature on *sparc* mRNA expression measured in chronological time units (Days post hatching) appears to coincide with that found for development time, skeletal development and growth rate. Thus the relative timing of *sparc* mRNA expression seems to be delayed at 14°C having high expression levels at higher specific fish age but concomitant with a the specific stage of development. Therefore, *sparc* mRNA expression appears to be dependent on the development time rate. Thus, further supporting the dynamic stage-specific *sparc* expression during post-embryonic turbot development.

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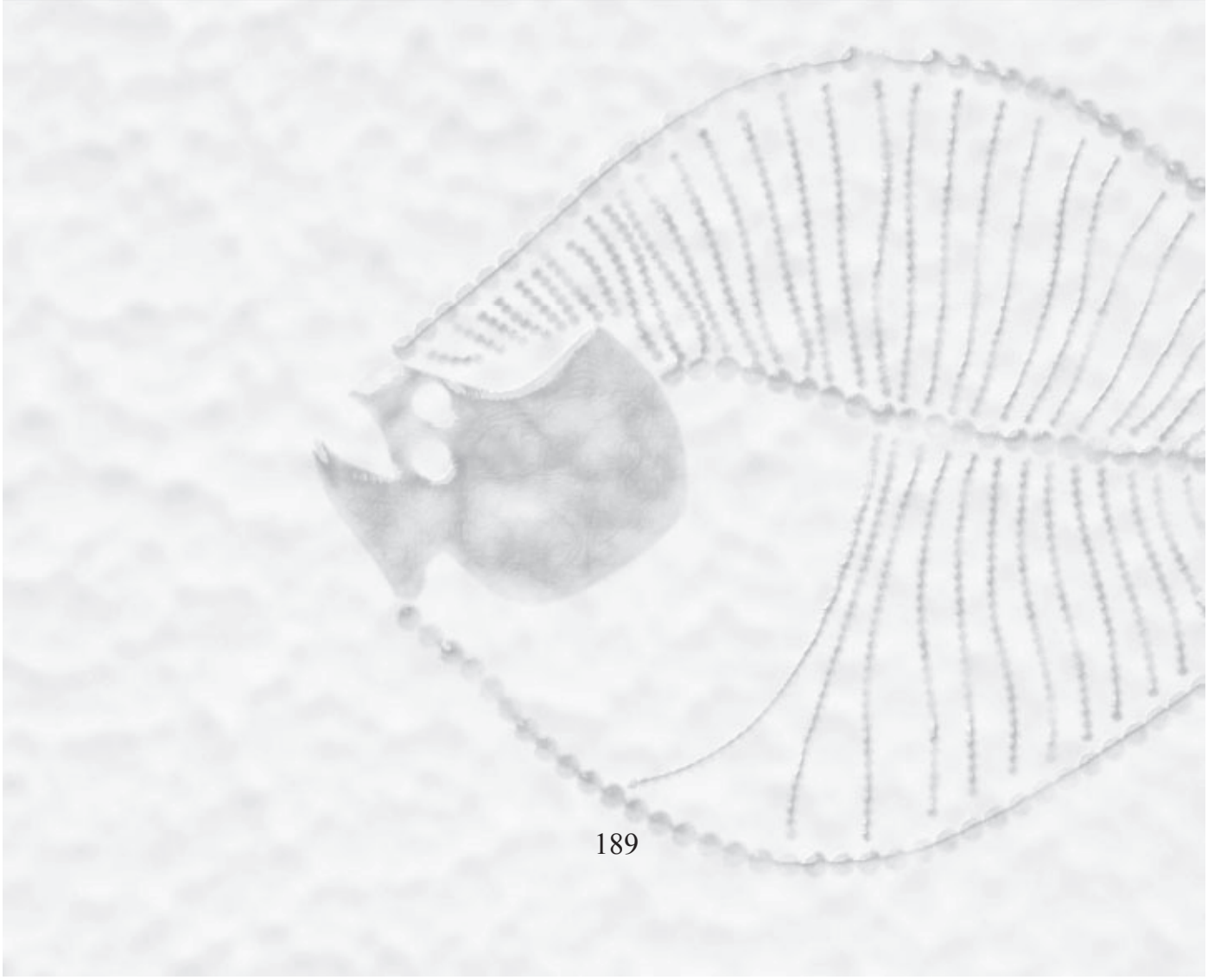
Conclusions



Analysis of the results obtained during this PhD Thesis leads to the following conclusions:

- Sparc is as an important regulator of embryonic haematopoiesis during early development in zebrafish. Specifically, it mediates erythroid progenitor cell development regulating *gata1* and *βe3globin* expression.

- Similar defects in blood phenotypes of *sparc* and *fgfs* knockdowns and the capacity to partially rescue the *fgf21* blood phenotype places *sparc* downstream of fgf21 signaling genetic network.

- UV exposure induces an increase in the *p53* and *sparc* expression

- According with the conclusion 3, a possible molecular mechanism induced by *sparc* after UV-radiation is suggested to be the responsible in part of the increment in developmental abnormalities.

- 5'UTR-intron is key transcriptional regulatory region of *sparc* gene since gene-construct containing simply this region predominantly displayed *egfp* expression in notochord, intermediate cell mass, otic vesicle, olfactory epithelium and muscle fibers in injected zebrafish embryos.

- *Sparc* is transcriptionally regulated by DNA methylation through the CpG island detected immediately upstream the 5' translation start site which is located within the intron sequence.

- Turbot Sparc protein keeps the same protein structure exhibited by all vertebrate Sparc proteins. The predicted turbot Sparc protein sequence shares high similarity to the Sparc proteins of other vertebrates. Phylogenetic analysis also indicated that turbot Sparc clusters together with its vertebrate orthologs. Additionally, we found that the expression pattern of Turbot *sparc* is comparable to other teleost species.

Therefore, these results suggest a strong evolutionary pressure to conserve this protein and indicate that there should be an evident conservation of function.

- *Sparc* mRNA expression showed a dynamic stage-specific expression during post-embryonic turbot development with high levels at metamorphic climax, indicating that it might be necessary for turbot metamorphosis.

Summary

INTRODUCCIÓN

La matriz extracelular es una red compleja secretada por las células que sirve como un elemento estructural de los tejidos y que incluso interviene en su desarrollo y fisiología (Alberts *et al*., 2002). Concretamente ayuda a las células a unirse entre sí y también regula diversas funciones celulares tales como migración, proliferación y diferenciación (Teti, 1992). Está compuesta por factores de crecimiento, proteoglicanos, proteínas estructurales y proteínas matricelulares.

Osteonectina, también llamada Sparc o BM-40, es una glicoproteína multifuncional que pertenece a la familia de las proteínas matricelulares. Este grupo de proteínas modula las interacciones entre la matriz y las células e interviene en múltiples funciones más que limitarse a jugar un papel meramente estructural (Brekken and Sage, 2000). Sparc tiene una alta afinidad por los iones calcio y fue descubierta por primera vez como el componente mayoritario de la matriz extracelular de tejidos mineralizados. Más tarde, se localizó en muchos otros tejidos. La expresión de Sparc es elevada durante el desarrollo temprano y disminuye durante la edad adulta. Sin embargo, su expresión aumenta en tejidos que requieren cierto grado de renovación, reparación o en tumorigénesis (Yan y Sage, 1999). Debido a que Sparc es capaz de interactuar con múltiples moléculas, se le han atribuido importantes funciones como antiadhesión, regulación del ciclo celular y actividad angiogénica (Yan y Sage, 1999).

Estructura

Por lo general la estructura génica de *sparc* está conservada en las diferentes especies donde se ha identificado con algunas excepciones. En mamíferos, el gen *sparc* está compuesto por 10 exones (Lane y Sage, 1994). Los dos primeros exones contienen la 5'UTR y el péptido señal, mientras que el exón 10 codifica para los últimos ocho aminoácidos de la proteína así como la 3'UTR. Esta estructura la comparten también *Xenopus* y medaka (Damjanovski *et al*., 1998; Renn *et al*., 2006) a diferencia del

nematodo *C.elegans* que no posee los exones 1, 3 ni 10 y los exones 6 y 7 están fusionados (Schwarzbauer y Spencer, 1993).

La Tabla 1 resume las variaciones de tamaño del transcrito en diferentes especies.

Species	cDNA size	5'UTR (bp)	cds (bp)	3'UTR (bp)
H.sapiens	2133	57	912	1164
B.taurus	2141	54	915	1172
R.norvegicus	2019	68	906	1045
G.gallus	1009	43	897	69
X.laevis	1615	58	903	654
D.rerio	1359	138	876	345
O.mykiss	1431	85	903	443
D.melanogaster	1170	109	915	146
A.franciscana	1083	51	876	156
C.elegans	1109	18	795	296

Tabla 1. Comparación del transcrito de *sparc* entre diferentes especies (adaptada de Redruello *et al.,* 2005)

Además, el promotor de *sparc* en mamíferos no cuenta con la clásica caja TATA pero contiene cajas GCA como también cAMP, elementos de choque térmico y elementos de respuesta a glucocorticoides. Sin embargo, se ha visto que *Xenopus* sí que contiene una caja TATA (Damjanoski *et al.,* 1998).

Sparc es por lo general un gen de copia única en la mayoría de especies pero se encontraron cuatro ortólogos en la especie diploblástica *Nematostella vectensis* (nvSparc 1, 2, 3 y 4) (Koehler *et al*., 2009). A esta excepción también hay que sumarle el único organismo triploblástico que contiene más de una copia, *Petromyzon marinus* (-A y –B) (Kawasaki *et al*., 2007).

Sparc está localizada en el cromosoma 5 humano (Hsa5) y en el LG14 en pez cebra. *sparc* y dos de los tres genes vecinos están localizados en el mismo locus en ambas especies, por tanto esto demuestra la conservación de este segmento cromosómico desde el antecesor común de pez cebra y humanos (Fig.1). El gen humano *atox1* no parece

tener su ortólogo en el genoma de pez cebra (Zv7). Este descubrimiento demuestra la utilidad de estudiar Sparc en el pez cebra debido a la conservación entre ambas especies.

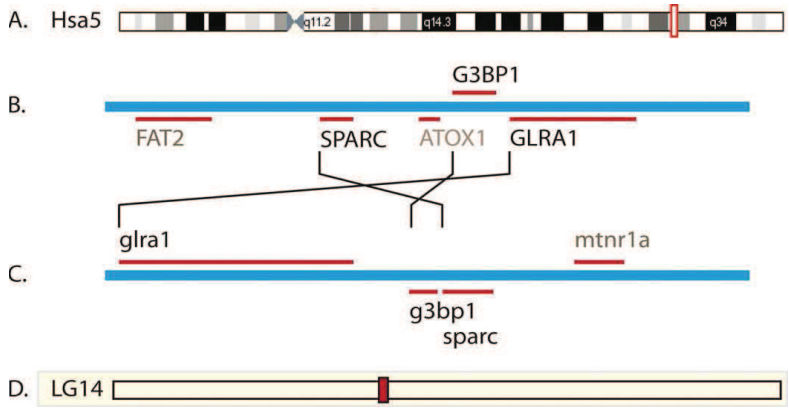


Figura 1. Sintenia que confirma la ortología de *sparc* en pez cebra y humanos. A. Cromosoma 5 humano (Hsa5) localizando *sparc* mediante una recuadro rojo y expandido en B. C. región donde se localiza *sparc* en el genoma del pez cebra, la cual pertenece a LG14, en el recuadro rojo en D (Rotllant *et al*., 2008)

La proteína Sparc de humanos de 32K-Da contiene un péptido señal de 17 aminoácidos, un dominio N-terminal (I) de 50 aminoácidos, de los cuales 18 están cargados negativamente, seguido de un dominio Folistatina (II) con 10 cisteínas en un patrón conservado y por último un dominio extracelular de unión al calcio (III) con dos motivos EF, cada uno de los cuáles tiene la capacidad de unirse al calcio (Fig.2).

Más detalladamente, los 286 residuos que componen la proteína se dividen en 3 regiones:

- Dominio I/módulo I aa 3-51: está compuesto por los exones 3 y 4. Es altamente ácido y sensible a los cambios de concentración en calcio. Este NH_2-terminal se une al calcio con baja afinidad e interactúa con hidroxiapatita por lo que regula los procesos de mineralización.

- Dominio II/módulo II aa 52-132: está compuesto por los exones 5 y 6. Es una secuencia rica en cisteína (contiene 10 cisteínas) que da lugar a una estructura homóloga al dominio folistatina (dominio FS). La proteólisis de Sparc genera

distintos péptidos activos con diferentes propiedades a la proteína completa. En particular, el péptido 2.1 inhibe la proliferación de células endoteliales mientras que el péptido (K)GHK estimula la proliferación endotelial y la angiogénesis.

– Dominio III/módulo III aa 133-285: codificado por los exones del 7 al 9. Constituye la parte de unión extracelular de unión al calcio y contiene dos motivos EF. El colágeno también se une a este dominio. Además, contiene el péptido 4.2 el cual tiene la capacidad de unirse a las células endoteliales e inhibir su proliferación.

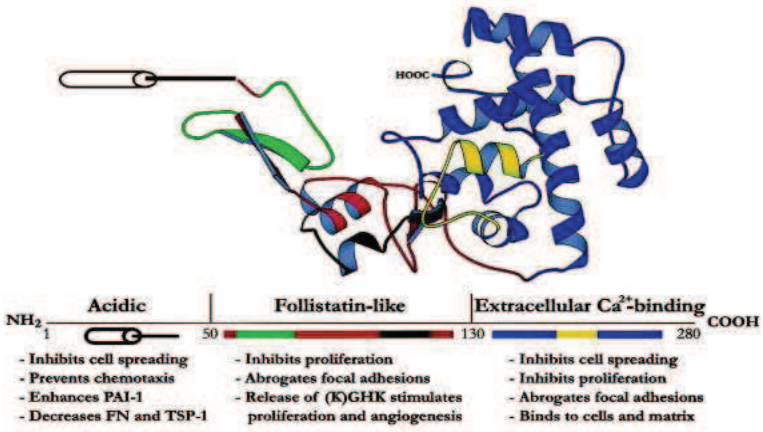


Figura 2. Estructura de la proteína Sparc en humanos. El dominio folistatina se muestra en rojo excepto para el péptido 2.1 y el péptido (K)GHK, los cuales se muestran en verde y negro, respectivamente. El dominio EC está señalado en azul excepto el péptido 4.2 en amarillo. PAI-1: inhibidor del activador del plasminógeno-1; FN: fibronectina; TSP-1: trombospondina (Brekken y Sage, 2000)

La estructura de Sparc está altamente conservada cuando se compara con otros vertebrados, pero el porcentaje de identidad disminuye en invertebrados (Tabla 2). La razón de esta diferencia se encuentra en el domino I que es altamente variable. En especies más primitivas como *C. elegans* donde no existen tejidos mineralizados, el número de residuos ácidos se reduce aproximadamente al 35% comparado con mamíferos.

	Ci	Bm	Dm	Dy*	Ce	Af	Ag	Mm	Rn	Cf	Mmu	Hs	Ss	Bt	Oc	Gg	Cc	Ts*	Cn*	Es*	Xt	Xl	Rc	Ga	Tr	Tn	Ol	Ip	Dr	Om1	Om2	Ssa1	Ssa2	Sa*	Ca*
Ci																																			
Bm	20																																		
Dm	22	44																																	
Dy*	20	31	96																																
Ce	22	29	33	31																															
Af	20	49	39	30	34																														
Ag	20	45	46	42	31	42																													
Mm	32	21	21	20	28	22	21																												
Rn	32	21	21	20	28	22	21	98																											
Cf	33	21	21	21	28	22	21	92	92																										
Mmu	33	21	21	20	28	22	21	92	91	97																									
Hs	33	21	21	20	28	22	21	92	92	97	100																								
Ss	33	21	21	20	28	22	21	92	91	96	96	96																							
Bt	33	22	21	21	28	22	21	92	92	98	98	99	97																						
Oc	33	22	21	21	28	22	21	90	90	94	94	94	93	95																					
Gg	32	20	21	19	27	21	20	83	84	86	85	85	87	85	84																				
Cc	33	21	21	19	28	22	21	84	85	88	87	87	88	88	86	97																			
Ts*	46	28	31	33	32	29	30	90	90	91	91	91	90	91	91	93	94																		
Cn*	45	28	29	31	31	28	30	90	90	91	91	91	91	91	90	96	96	93																	
Es*	46	29	29	31	30	28	30	89	89	90	90	90	89	89	88	91	91	90	93																
Xt	33	22	20	18	26	21	21	79	79	81	81	81	82	81	79	80	81	89	89	89															
Xl	32	22	20	19	27	22	21	79	79	81	81	81	82	81	80	80	82	89	88	88	96														
Rc	32	22	21	20	26	22	21	77	77	81	81	81	81	80	79	79	79	87	87	86	86	86													
Ga	33	21	20	18	26	20	21	75	76	76	75	75	75	75	74	73	73	83	83	83	77	78	76												
Tr	33	20	20	19	25	20	20	72	73	73	73	73	73	73	71	72	72	82	81	81	73	73	70	81											
Tn	33	21	20	19	26	20	20	74	75	75	74	74	74	75	73	74	74	83	81	81	75	75	73	81	93										
Ol	34	20	20	19	26	22	22	77	78	78	78	78	78	78	77	78	78	85	84	83	77	77	74	82	82	86									
Ip	33	21	22	21	27	23	22	74	74	74	74	74	75	74	73	74	75	83	82	80	75	76	72	78	76	77	82								
Dr	33	21	21	19	27	22	21	76	77	76	75	75	76	75	74	76	76	84	83	81	77	77	72	78	76	79	83	89							
Om1	32	22	21	19	27	20	22	74	75	75	74	74	75	74	73	73	73	82	82	81	74	74	70	78	76	77	79	78	80						
Om2	32	22	21	21	27	21	22	74	75	76	75	75	76	75	74	73	74	84	83	81	73	75	73	80	75	77	78	79	79	94					
Ssa1	32	22	21	20	27	21	22	74	74	74	74	74	74	74	73	74	84	83	81	73	74	72	78	76	77	79	78	79	98	94					
Ssa2	32	22	21	21	27	21	22	75	75	77	76	76	76	76	75	74	74	84	83	81	74	76	73	80	76	78	79	80	80	95	99	95			
Sa*	42	22	27	32	23	20	23	84	84	83	84	84	83	83	83	82	83	84	83	83	83	84	81	90	90	91	87	83	86	88	89	88	89		
Ca*	41	24	26	25	27	22	24	80	80	77	77	77	77	77	76	81	80	80	78	77	82	85	77	76	76	81	78	87	92	78	77	78	77	80	

Tabla 2. Porcentaje de identidad entre secuencias proteicas de Sparc. En blanco, especies de invertebrados; en gris, vertebrados; * secuencias parciales. Ci, *C.intestinalis*; Bm, *B.mori*; Dm, *D.melanogaster*; Dy, *D.yakuba*; Ce, *C.elegans*; Af, *A.franciscana*; Ag, *A.gambiae*; Mm, *M.musculus*; Rn, *R.norvegicus*; Cf, *C.familiaris*; Mmu, *M.mulatta*; Hs, *H.sapiens*; Ss, *S.scrofa*; Bt, *B.taurus*; Oc, *O.cuniculus*; Gg, *G.gallus*; Cc, *C.coturnix*; Ts, *T.scripta*; Cn, *C.myloticus*; Es, *Elaphe sp.*; Xt, *X.tropicalis*; Xl, *X.laevis*; Rc, *R.catesbeiana*; Ga, *G.aculeatus*; Tr, *T.rubripes*; Tn, *T.nigroviridis*; Ol, *O.latipes*; Ip, *I.punctatus*; Dr, *D.rerio*; Om, *O.mykiss*; Ssa, *S.salar*; Sa, *S.aurata* ; Ca, *C.auratus* (Laizé *et al*., 2005)

Recientemente se ha descubierto una diferencia estructural de Sparc entre los grupos radiata y bilateria. Mientras que la estructura trimodular se mantiene en todos los organismos bilaterales con algunas diferencias de tamaño (vertebrados *vs* invertebrados), el alineamiento de Sparc incluyendo el cnidario *Nemastotella vectensis* mostró que el dominio I está ausente en nvSparc1-4 (Koehler, *et al*., 2009) sugiriendo que el dominio I puede ser una incorporación más tardía en la evolución, después de la aparición de los organismos bilaterales. Debido a este dominio tan variable entre especies, se construyó un árbol filogenético basado en los dominios FS y EC (Fig.3). La filogenia de Sparc es

consistente con los grupos taxonómicos, mostrando 3 divisiones, cada uno de los cuales corresponde a uno de los 3 clados: cnidaria, protostomia y deuterostomia.

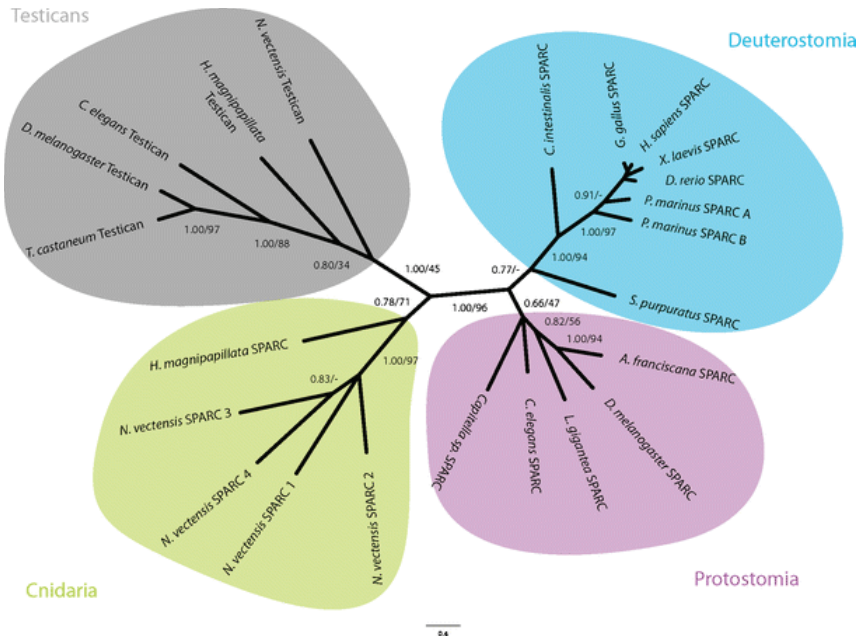


Figura 3. Filogenia bayesiana de los dominios FS-EC de Sparc en metazoos, con los dominios FS-EC de testicanos, incluidos como secuencias fuera del grupo (Koehler *et al*., 2009)

Atendiendo a la estructura de dominios, Sparc ha sido incluida en la familia Sparc Family-related Proteins la cual incluye 5 proteínas que se ha agrupado juntas debido a que comparten los dominios FS y EC (Fig.4). Estas proteínas son:

– Hevin/Sparc-like protein (SLP) comparte la estructura trimodular con Sparc pero el dominio N-terminal es de mayor tamaño. El dominio III está altamente conservado y hevina es junto con Sparc, la única proteína de este grupo capaz de unirse al colágeno. Está localizada principalmente en el sistema nervioso y se ha propuesto como supresora de tumores y reguladora de angiogénesis.

– El proteoglicano testicular humano testican/SPOCKs contiene un dominio folistatina, un tiropina y un EF. Fue originalmente encontrado en testículos pero la

expresión más abundante es en el cerebro. Está asociado con la regulación de la actividad proteasa.

– SMOC-1contiene un dominio EC común a Sparc y un dominio adicional folistatina, dos dominios de tiroglobulina y un dominio nuevo. Está localizada en membranas basales y también se encontró en gónadas y tracto reproductivo. Actúa como regulador de la señal BMP.

– SMOC-2 actúa como un estimulador de la angiogénesis a través de la unión a VEGF y bFGF y tiene la misma estructura que SMOC-1. Sin embargo, se encuentra predominantemente en corazón, músculo, hígado y ovario.

– Fstl-1 (Follistatin like protein-1)/TSC-36**.** El dominio EC no es funcional. Actúa como una proteína proinflamatoria, como regulador de la señal BMP y como regulador de la homeostasis en la sensación somática.

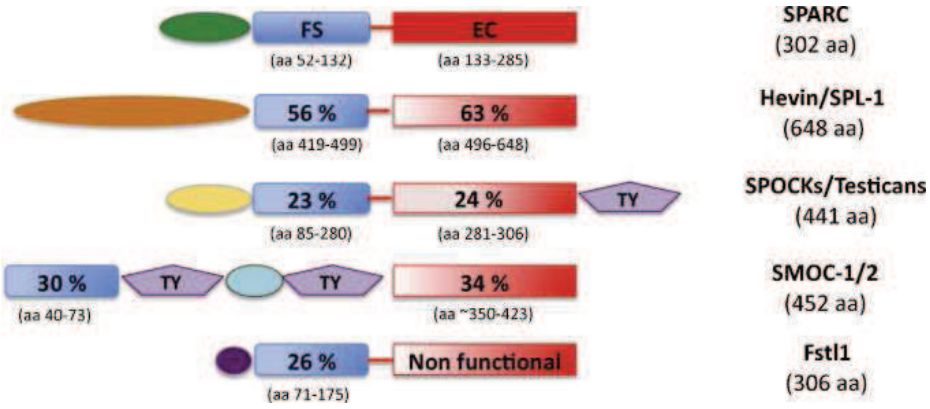


Figura 4. Representación esquemática de la estructura de dominios de las proteínas pertenecientes a la familia de Sparc (Bradshaw, 2012)

Expresión

Sparc es uno de los componentes mayoritarios localizados en la matriz extracelular. Fue descubierta en la matriz de tejidos mineralizados pero se expresa en una gran variedad de tejidos. Además, tiene una expresión alta durante la embriogénesis y está restringida en adultos a tejidos que sufren remodelación, tumorigénesis, curación de heridas o angiogénesis.

En humanos, *sparc* se encuentra en hueso, cartílago, dientes, riñón, glándula adrenal, pulmones, ojos, vasos sanguíneos, hígado, meninges y plexo coroideos durante el desarrollo embriogénico y fetal (Mundlos *et al*., 1992). En adultos, *sparc* se expresa también en el intestino (Lussier *et al*., 2001), piel (Hunzelmann *et al*., 1998) y aorta (Hao *et al*., 2004).
En embriones de ratón, se detectó expresión de *sparc* en el tracto alimentario (lengua, epitelio oral, esófago e intestino), timo, músculo esquelético, somitas, cartílago, hueso, corazón, pulmón y piel (Sage *et al*., 1989). En adultos, se identificó en el tracto alimentario (lengua, esófago, estómago e intestino), tejido glandular (glándula submaxilar, glándula parótida y glándula mamaria), sistema reproductivo y piel.

Sparc está presente en la notocorda, somitas floor plate en embriones de *Xenopus* (Damjanovski *et al*., 1994).

En el pez cebra se encontró durante la formación de la faringe y oído interno, notocorda, placa ventral mesencefálica, aletas y vesícula ótica (Rotllant *et al.,* 2008). Además durante la regeneración de la aleta caudal *sparc* se expresa diferencialmente en esta área (Padhi *et al*., 2004). En dorada es abundante en escamas, discos intervertebrales, vértebras, radios caudales, arcos branquiales y opérculo mientras que el neurocranio, cerebro, gónada e hígado contienen niveles bajos de *sparc* (Redruello *et al.,* 2005; Estêvão *et al*., 2005). Durante la embriogénesis en medaka, *sparc* se expresa en el esclerotoma, notocorda y placa ventral mesencefálica, sin embargo en el adulto está presente en órganos como el riñón, corazón, branquias, hígado, cerebro y ojo (Renn *et al*., 2006).

Se identificó *sparc* predominantemente en el manto y a más bajos niveles en branquias e intestino del bivalvo *Pinctada fucata* (Miyamoto y Asada, 2011). Esta localización sugiere que Sparc puede tener un papel importante en la formación de la concha.

En *C. elegans* se expresa en la faringe y gónadas (Fitzgerald y Schwarzbauer, 1998).

Por último, la expresión de *sparc* se restringe al endodermo desde el desarrollo post-gástrula de *Nematostella vectensis* (Koehler *et al*., 2009).

En resumen, estos resultados indican que la expresión de *sparc* se encuentra principalmente en tejidos esqueléticos pero también en muchos otros tejidos adultos que sufren procesos de remodelación.

Función y regulación

Sparc es una proteína multifunctional con una alta afinidad por cationes e hidroxiapatita que dan soporte a la matriz extracelular e interviene en las actividades de un amplio grupo de factores de crecimiento (Brekken y Sage, 2001). Malformaciones fenotípicas reveladas por estudios de pérdida de función también confirman la idea de que Sparc actúa principalmente en las interacciones célula-matriz (Gilmour *et al*., 1998; Delany *et al*., 2003; Bradshaw *et al*., 2002; Bradshaw *et al*., 2003; Brekken *et al*., 2003; Eckfeldt *et al*., 2005).

A Sparc se le atribuyen diferentes funciones biológicas ya que se une a un gran número de componentes de la matriz extracelular, factores de crecimiento y otras moléculas. Desde un punto de vista celular, Sparc tiene un amplio rango de acción en la organización de la matriz extracelular, migración, proliferación, antiadhesión, diferenciación y supervivencia (Bradshaw y Sage, 2001; Delany *et al.,* 2003).

A continuación se exponen las funciones más importantes de Sparc según el tipo de interacción.

a) Interacción con moléculas de la matriz extracelular

La unión de Sparc con el colágeno es la interacción mejor caracterizada de todas. Esta unión está modulada por iones Ca^{+2} e implica una alteración en la conformación que lleva a una reducción en la susceptibilidad a las proteasas y una alteración de su afinidad por el colágeno (Fig.5) (Maurer *et al*., 1995; Bradshaw 2009; McCurdy *et al*., 2010). Además, Martinek *et al*., 2007 sugiere un posible papel intracelular de Sparc como una chaperona conservada esencial para el plegamiento del colágeno en el retículo endoplasmático.

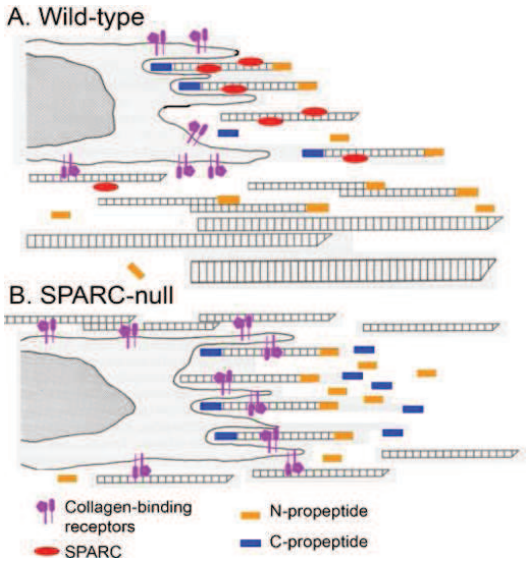


Figura 5. Actividad de Sparc modulando interacción celular con el colágeno y el procesado del procolágeno**.** En **A**, fibras de procolágeno se unen entre sí mediante Sparc, la cual disminuye la unión del colágeno a los receptores celulares. En ausencia de Sparc **B**, el procolágeno se une a receptores con mayor afinidad y es retenido en las superficies celulares. Las fibras que no poseen Sparc se agregan entre sí menos eficientemente que fibras de células con fenotipo salvaje. (Rentz *et al.,* 2007; Bradshaw, 2009)

Diferentes estudios mostraron la afinidad de Sparc por los colágenos I, II, III, IV, V y VIII. La interacción entre estas dos moléculas protege al colágeno de la degradación como por ejemplo pasa en el ligamento periodontal después de un tratamiento con LPS (Trombetta y Bradshaw, 2010) pero incluso es necesario para un proceso de remodelación de la matriz extracelular dando lugar a diferentes eventos como procesos morfogénicos. Por ejemplo, Vincent *et al*., 2008 detectó *sparc* en el cerebro de ratones durante la neurogénesis, sistema nervioso central, médula espinal, formación de vasos sanguíneos y células de la glia pero está retenida en el adulto en lugares que requieren un alto grado de plasticidad/remodelación. En el pez cebra, *sparc* es necesaria para la formación del cartílago faríngeo y oído interno (Rotllant *et al.,* 2008; Kang *et al.,* 2008). En medaka, *sparc* se expresa antes de la osificación de los somitas, notocorda, floorplate y vesícula ótica (Renn *et al*., 2006).

Usando ratones que no expresan Sparc se han detectado diferentes defectos como fibras de colágeno más pequeñas (Bradshaw *et al*., 2003), degeneración de discos intervertebrales (Gruber *et al.*, 2005), cataratas (Gilmour *et al*., 1998), aceleración en la curación de heridas (Bradshaw *et al*., 2002), aumento de crecimiento de tumores (Brekken, *et al*., 2003), osteopenia (Delany *et al*., 2003) o un incremento en tejido adiposo (Bradshaw *et al*., 2003; Nie y Sage, 2009). Estos defectos se asocian a cambios en la matriz extracelular principalmente por una disminución en la cantidad de colágeno o una incorrecta diferenciación celular (e.g. osteoblastos).
La sobreexpresión de Sparc en *Xenopus* interfiere con la morfogénesis de diferentes tejidos a través de la modificación de la forma celular, inhibición de la migración celular, proliferación y la incapacidad de formar adhesiones focales (Damjanovski *et al*., 1997; Huynh *et al*., 1999). Además, enfermedades tales como la fibrosis o esclerosis están causadas por una sobreexpresión de Sparc seguida por una anormal deposición de colágeno en la matriz extracelular (Trombetta y Bradshaw, 2012) que puede ser restaurada por una inhibición de Sparc (Zhou *et al*., 2006; Atorrasagasti *et al*., 2013).

La secuencia rica en glutamato en el dominio I se identificó como un posible sitio de unión para la hidroxiapatita y por tanto este lugar puede estar relacionado con procesos de mineralización en diferentes tejidos óseos. De hecho, Fujisawa *et al*., 1996 sugirió que Sparc incrementaba la mineralización en experimentos *in vitro*.

Sparc también regula la actividad de las metaloproteinasas (Bradshaw, 2012), que pertenecen a una familia que media la proteólisis de la matriz extracelular y su renovación. En algunos casos, Sparc induce la activación de las metaloproteinasas la cual desencadena en la invasión de tumores (Gilles *et al*., 1998; Shen *et al*., 2010). Sin embargo, Said *et al*., 2007 demostró que Sparc también puede disminuir la expresión de las metaloproteinasas por ejemplo en cáncer de ovarios. El papel funcional de Sparc en cáncer es dependiente del tejido y tipo de tumor, ya que se sabe que Sparc puede tanto promover como inhibir diferentes tipos de tumores.

Se sabe que el cobre se acumula en tejidos durante la respuesta inmune. Por lo que, proteínas de unión al cobre son necesarias para la reparación de tejidos y tienen un papel angiogénico. En experimentos in vitro con cerebros de ratón y dermis de adulto, se demostró que la degradación de Sparc libera el péptido activo (K)GHK que posee propiedades angiogénicas dependientes de cobre (Lane *et al.,* 1994).

El sitio de unión a la heparina de la proteína matricelular vitronectina es esencial para la interacción con sitio de unión al Ca^{+2} de Sparc en las paredes de los vasos sanguíneos en tejido de riñón **(**Rosenblatt *et al*., 1997). Debido a que ambas proteínas tienen efectos opuestos en la adhesión celular, la función de la interacción entre estas dos moléculas puede ser la de regular el papel de las células endoteliales durante la angiogénesis.

Finalmente, la trombospondina, otra proteína matricelular, es capaz de formar un complejo con Sparc. Dicha unión está implicada en procesos de agregación de plaquetas (Clezardin *et al*., 1991).

b) Interacción con factores de crecimiento

La unión a factores de crecimiento es otra de las características de Sparc. La actividad de los factores de crecimiento influye en la proliferación celular, migración y diferenciación (Taipale y Keski-Oja, 1997).
Se ha demostrado que Sparc es capaz de unirse al factor de crecimiento endotelial vascular (VEGF) en células endoteliales en humanos. Existen papeles contradictorios en el mecanismo de acción. Mientras que la proteína intacta Sparc no permite la unión entre VEGF y su receptor, inhibiendo así el efecto mitótico de VEGF, el péptido derivado de Sparc (K)GHK muestra un efecto angiogénico en las células endoteliales (Kato *et al*., 2001). Por tanto, Sparc parece ser un factor importante en la regulación del crecimiento vascular.

La expresión de Sparc y el factor de crecimiento de plaquetas (PDGF) es mínima en la mayoría de tejidos adultos pero incrementan después del daño. La interacción de Sparc

con la cadena B de evita la unión a su receptor en fibroblastos. Como consecuencia de esta unión, existe una inhibición en la progresión del ciclo celular endotelial, sugiriendo que Sparc puede ejercer cierto control en los procesos de reparación (Raines *et al*., 1992).

De manera similar, Sparc inhibe la migración de células endoteliales inducida por el factor de crecimiento de fibroblastos (bFGF) (Hasselaar y Sage, 1992). Sin embargo, bFGF recíprocamente disminuye la síntesis de Sparc en osteoblastos en cultivo (Delany y Canalis, 1998).

La capacidad de Sparc para inhibir VEGF, PDGF y bFGF, factores que se sabe que mejoran la curación, puede contribuir a la mejora de reparación del daño en ausencia de Sparc.

Sparc mantiene el balance entre la producción de proteínas de la matriz y la proliferación celular en el riñón. De hecho, Sparc modula la síntesis de colágeno I y la actividad de factores de crecimiento a través de una vía dependiente de TGF-β1 (Fig.6) en respuesta al daño (Francki y Sage, 2001).

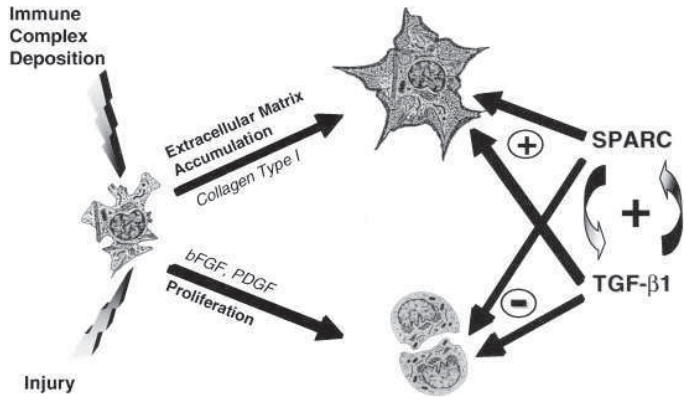


Figura 6. La proliferación y acumulación de la matriz extracelular en las células está regulada por Sparc y TGF-β1. Sparc modula la proliferación y la producción de la matriz, en particular la síntesis de colágeno I, a través de una vía dependiente TGF-β1. Sparc induce la síntesis de TGF-β1, un factor anti-mitótico de las células mesangiales. TGF-β1 disminuye la hiperproliferación de células activadas después de un daño glomerular y conduce a la acumulación de matriz extracelular mediante la síntesis de colágeno I y Sparc (Francki y Sage, 2001).

Como Sparc es un marcador de odontoblastos, se estudiaron diferentes factores de crecimiento para averiguar los mecanismos de su regulación génica. En humanos, la expresión de Sparc aumenta mediante TGF-β de una manera dosis-dependiente antes de la calcificación mientras que bFGF, TNF-α, PDGF y IL-1β disminuyen su expresión (Shiba *et al.,* 1998).

c) Interacción con sustancias químicas

El ácido retinoico estimula la maduración de condrocitos promoviendo la activación de ciertos genes relacionados con este evento tales como Sparc, colágeno X, fibronectina o osteopontina (Iwamoto *et al*., 1994).

La dexametasona es un miembro de la familia de los glucocorticoides que actúa como agente cataractogénico. Tratando las células bovinas de las lentes con este agente químico aumentan los niveles de Sparc. Debido a que Sparc se une a colágeno IV, uno de los componentes mayoritarios de de las membranas basales de las lentes, tiene una función en la deposición de las proteínas de la matriz extracelular (Sawhney, 2002).

Aparte de agentes químicos, también el choque térmico afecta a los niveles de Sparc en las células. La presencia de dos elementos de choque térmico en el gen *sparc* explica el incremento de la expresión de Sparc después de exponer condrocitos a altas temperaturas (Neri *et al*., 1992).

d) Interacción con otras moléculas

El dominio de interacción con el cobre media la supervivencia celular *in vitro* vía la interacción con la integrina β1 y la activación de la kinasa ligada a la integrina en las células epiteliales de las lentes después de condiciones de estrés (Weaver *et al*., 2008).

Arnold y Brekken, 2009 proponen a Sparc (SP) como una proteína extracelular de umbral (Fig.7) controlando las interacciones entre la matriz, integrinas (α, β) y receptores de factores de crecimiento (RTK).

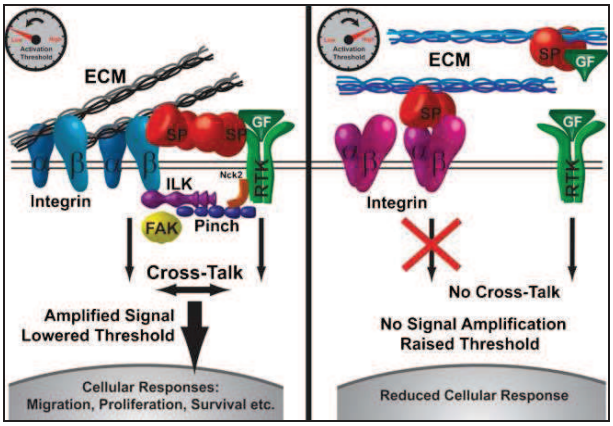


Figura 7. Sparc actúa como una proteína extracelular de umbral **(Izquierda)** Sparc puede disminuir el umbral de activación de ciertos factores de crecimiento (GF) formando el complejo y estableciendo señales entre integrinas y receptores de factores de crecimiento **(Derecha)** Sparc puede aumentar el umbral de activación de las integrinas y factores de crecimiento inhibiendo la unión de integrinas a la matriz extracelular (Arnold y Brekken, 2009)

En *Drosophila melanogaster*, Sparc inhibe la apoptosis aumenta la vida celular que están sufriendo apoptosis mediante la interacción con un factor secretado no identificado (KS=killer signal) e inmovilizándolo a través de la actividad kinasa ligada a integrinas (Fig. 8) (Bradshaw, 2012).

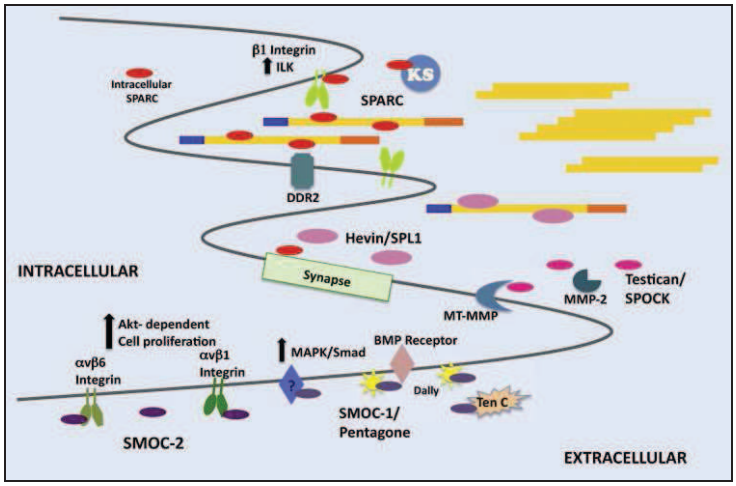


Figura 8. Sparc regula la formación de sinapsis mediante unión a factores no identificados en la matriz o en la superficie celular. (Bradshaw, 2012)

Por el contrario, Rahman, *et al*., 2011 encontraron que el extremo N-terminal de Sparc parece aumentar la apoptosis interactuando con la caspasa 8.

Entre las funciones de la albúmina está el transporte de hormonas y ácidos grasos. Se ha propuesto a Sparc como un receptor de esta molécula en tejidos epiteliales (Liddelow *et al*., 2011).

e) Sparc y metilación

Recientemente se están estudiando mecanismos epigenéticos como nuevos elementos de regulación de Sparc, y por tanto, se están explorando nuevas funciones. Los mecanismos epigenéticos se definen como cambios en el ADN heredables, que no afectan a la secuencia de bases, reversibles y que se manifiestan como patrones específicos de expresión génica La metilación del ADN y la modificación de histonas son ejemplos de tales modificaciones que sirven para regular la expresión de genes sin alterar la secuencia. Un aumento en metilación está asociado en muchos casos al silenciamiento de genes y una reducción en la metilación está asociación a su activación. Los cambios en el patrón de metilación en un organismo

pueden ser debidos a herencia pero también en respuesta a ciertos tipos de estrés de tipo ambiental, especialmente la temperatura (Varriale y Bernardi, 2006; Whittle *et al*., 2009), nutricional (Feil y Fraga, 2012), infecciones patógenas (Dowen *et al*., 2012) o por exposición a agentes que interfieren con la metilación del ADN.

Las islas CpG, regiones ricas en CG, son las dianas más frecuentes de metilación. Se ha demostrado que en Sparc la metilación es un potente regulador de su expresión, específicamente en la región del promotor donde se han identificado varias islas CpG.

La mayoría de los casos donde *sparc* está metilada, está descrita en un contexto canceroso ya que la matriz extracelular es la responsable de diferentes procesos que conducen al proceso tumoral como son la diferenciación, supervivencia, proliferación y migración (Larsen *et al*., 2006; Lu *et al.,* 2012).

La hipermetilación de *sparc* está asociada con la degeneración de discos intervertebrales (Tajerian *et al*., 2011) y con cánceres pancreáticos, colorectales u ováricos (Gao *et al*., 2010; Cheetham *et al.,* 2008; Socha *et al*., 2009) reduciendo la producción de Sparc. Por el contrario, demetilando *sparc*, se reactiva su expresión y se demostró que esto atenuaba el poder de invasión de cáncer de pulmón y colorectal reafirmando la idea de que Sparc puede actuar como un supresor de tumores (Pan *et al*., 2008; Chetham *et al*., 2008). Sin embargo, una sobreexpresión de *sparc* conduce a otros tipos de cáncer en cerebro, colon, riñón o páncreas (Arnold y Brekken, 2009).Por lo que el efecto tumorigénico de Sparc es específico del tipo celular y dependiente del ambiente que rodea a las células afectadas.

Debido al poco conocimiento respecto a la regulación de Sparc y los papeles contradictorios en diferentes tejidos, el objetivo principal de esta tesis es contribuir a un mayor entendimiento de este gen en peces teleósteos.

OBJETIVOS

Diversos estudios indican que Sparc tiene múltiples funciones durante el desarrollo. Sin embargo y a pesar de los conocimientos obtenidos de estudios in vivo e in vitro, las funciones morfogénicas que Sparc efectúa durante el desarrollo no están claras.

Esta tesis tiene como objetivo en general caracterizar la función y regulación de Sparc, particularmente durante el desarrollo temprano de dos teleósteos y se centra en los siguientes objetivos específicos:

1. Esclarecer la implicación de Sparc en el control de la hematopoyesis embriogénica en el pez cebra por medio de estudios de silenciamiento de genes (Capítulo I).

2. Establecer los posibles mecanismos en la regulación de Sparc en el pez cebra:

    i. Determinar el papel de Sparc en las malformaciones del desarrollo producidas por radiación solar ultravioleta en embriones (Capítulo II).

    ii. Establecer la regulación a nivel transcripcional y caracterización del promotor de *sparc* en embriones de pez cebra usando mecanismos de expresión diferencial génica (Capítulo III).

3. Clonar y caracterizar molecularmente Sparc en rodaballo para estudiar su posible papel en la remodelación durante la metamorfosis de peces planos (Capítulo IV).

RESÚMENES DE LOS CAPÍTULOS

1. Función de Sparc en el desarrollo de células progenitoras eritroides en pez cebra

Sparc (osteonectina) es una glicoproteína matricelular que desempeña multitud de funciones y que se expresa en gran variedad de células. Las proteínas matricelulares intervienen principalmente en interacciones célula-matriz más que limitarse a actuar como componentes estructurales de la matriz extracelular. Por lo tanto, este tipo de moléculas influyen en diversos eventos de remodelación, incluyendo la hematopoyesis.

La hematopoyesis es un proceso biológico que se refiere a la formación y desarrollo de componentes celulares sanguíneos. Este proceso ocurre en dos pasos sucesivos: el primero/primitivo que da lugar a eritrocitos y células mieloides en el mesodermo posterior lateral y en el mesodermo anterior lateral respectivamente, y el definitivo que produce no sólo eritrocitos y células mieloides sino también linfocitos y trombocitos.

Hemos investigado el papel de Sparc en la hematopoyesis generando "knowdown" de peces cebra a través de la inyección de morfolinos antisentido. La mayoría de los embriones inyectados con morfolino de *sparc* se desarrollan normalmente hasta las 30 horas post-fertilización (hpf), sin embargo, son más pequeños que aquellos inyectados con el morfolino control. Para describir más detenidamente los efectos de la pérdida de sparc en la hematopoyesis del pez cebra, se analizaron diferentes marcadores moleculares. Así, se ha visto que *gata1* y *βe3globin (hbbe3)*, importantes para la eritropoyesis, están muy reducidas en embriones knockdown para *sparc*; sin embargo la expresión de los factores de transcripción específicos para células mieloides *pu.1 y l-plastin (lcp1)* y *rag1,* específico para linfocitos, no se ve afectada. Estos resultados indican que sparc juega un papel importante en el desarrollo de células eritroides pero no en las mieloides ni linfoides.

Para comprobar la especificidad de los defectos obtenidos, hicimos un rescate inyectando ARNm de *sparc* en embriones inyectados con el morfolino de *sparc*. Así, se restableció la expresión de *gata1* aunque todavía se veía un desarrollo corporal anormal. Esto

demuestra que la *sparc* exógena es suficiente para corregir defectos en sangre causados por el morfolino de *sparc*.

La inyección del morfolino *fgf21* inducía una reducción significativa en la expresión de *sparc* del 80% en embriones de 24hpf. Mediante Q-PCR, se detectó también una reducción severa de *gata 1* y *hbbe3* pero no en *pu.1* y *l-plastin (lcp1)*. Esto demuestra la similitud en el fenotipo de embriones inyectados con morfolino de *fgf21* e inyectados con morfolino de *sparc* que se caracterizan por una disrupción severa de marcadores celulares eritroides y eritrocitos.

Inyectando ARNm de *sparc* en embriones knockdown de fgf21 conseguimos parcialmente rescatar la expresión de gata1 y *βe3globin* con lo que podemos sugerir que *sparc* actúa corriente abajo de *fgf21*.

En resumen, nuestro estudio muestra que Sparc tiene un papel importante en hematopoyesis embriogénica durante el desarrollo temprano del pez cebra actuando como modulador de factores de transcripción relacionados con la formación de eritropoyesis como gata1 y *βe3globin*. Sin embargo, el mecanismo preciso por el cual sparc afecta *gata1* (via fgf21) debe ser estudiado más detalladamente.

2. Respuesta molecular de embriones de pez cebra a la exposición de radiación ultravioleta: consecuencias para la supervivencia y desarrollo morfológico

Se ha demostrado que la radiación ultravioleta genera muchas alteraciones en los organismos tales como melanomas en mamíferos, malformaciones esqueléticas en anfibios o una reducción en la supervivencia y estrés oxidativo en especies acuícolas. Sin embargo, existe poca información sobre el mecanismo molecular que genera dichas alteraciones producidas por una exposición a radiación ultravioleta.

El daño a nivel de ADN causado por radiación UV provoca respuestas adaptativas que incluyen reparación de ADN, activación de cascadas de señalización y cambios en la transcripción. *p53* es un factor de transcripción muy importante en vertebrados que actúa como un mecanismo de protección frente algún tipo de estrés. Las funciones de *p53* son la de inhibidor del ciclo celular, actividad 3'-5' exonucleasa, reparación de nucleótidos o apoptosis. Entre las respuestas fotoprotectoras también se encuentran los cambios en la matriz extracelular sin embargo, el papel de proteínas matricelulares después de radiación todavía no está muy claro. Se ha demostrado que una de estas proteínas, Osteonectina/Sparc, incrementa su expresión en fibroblastos expuestos a UVB y que está asociada a ciertos tipos de cáncer como melanomas.

En este estudio caracterizamos los posibles mecanismos moleculares inducidos por radiación ultravioleta en embriones de pez cebra. Concretamente medimos la expresión de *p53* y *sparc* después de la exposición y caracterizamos la supervivencia y malformaciones producidas.

Sparc y *p53* aumentaban su expresión en embriones expuestos a radiación ultravioleta B más que cuando ésta era excluida del espectro. Osteonectina aumentaba con longitudes de onda más cortas de 335nm mientras que *p53* era inducido por longitudes más largas. Además, sólo el 53% de embriones expuestos a radiación ultravioleta sobrevivían hasta los 6 días mientras que el 94% de los controles sobrevivía. Asimismo, la incidencia de peces con malformaciones era más alta en embriones expuestos a ultravioleta siendo la torsión a nivel de notocorda el tipo más común de malformación.

Para relacionar el aumento de expresión de *sparc* con la alta incidencia de malformaciones, se inyectó ARNm de *sparc* en embriones y se observaron

malformaciones en la notocorda similares a las producidas por una exposición a ultravioleta.

En resumen, el presente estudio demuestra que la osteonectina de pez cebra juega un papel importante como posible inductor molecular de las malformaciones morfologicas detectadas en respuesta a la radiación UV. Sin embargo, el mecanismo de transducción en la que interviene osteonectina todavía está por determinar. Además, los resultados también demuestran un aumento de expresión de *p53* en respuesta a la radiación UV en peces.

3. 5'-UTR intrón es crucial para la regulación de la transcripción del gen *sparc* en el pez cebra

En diversas especies se ha visto que el extremo 5' de *sparc* contiene elementos reguladores que pueden ser responsables de la expresión diferencial característica de este gen durante el desarrollo.

Aunque se han detectado numerosos factores implicados en la regulación transcripcional de *sparc*, todavía existe poca información detallada de los mecanismos moleculares que lo regulan así como escasos estudios de promotor los cuales se restringen a mamíferos. El presente estudio intenta explorar los mecanismos moleculares que regulan la expresión de *sparc* mediante una caracterización funcional *in vivo* e identificar los posibles elementos reguladores que dirigen la expresión basal del promotor en el pez cebra.

El inicio de transcripción se ha localizado separado del inicio de traducción por un intrón de 7kb. Para estudiar la regulación de Sparc, se inyectó el promotor de 0.2kb más el intrón de 7kb asociado al gen EGFP en embriones de pez cebra. La expresión de EGFP se detectó inicialmente a las 24hpf y los lugares de expresión fueron la notocorda, masa celular intermedia, vesícula ótica, bulbo olfatorio, fibras musculares, corazón, mandíbulas y en la aleta caudal. Si eliminamos del vector de expresión el intrón de 7kb no detectamos fluorescencia en los embriones lo cual indica que ese intrón es una secuencia reguladora importante para la expresión de Sparc.

A continuación buscamos factores de transcripción relevantes en dicho intrón e identificamos elementos de choque térmico, elementos pertenecientes a la familia de gata, factores sox, elementos de respuesta a glucocorticoides… Además, se detectó una isla CpG compuesta por 9 dinucleótidos CG susceptibles demetilación por lo que hipotetizamos la regulación epigenética de sparc en peces. Por tanto, para investigar la relación entre regulación transcripcional y metilación del ADN en *sparc*, tratamos larvas de pez cebra con 5'azacitydina y observamos que aproximadamente el 40% de las larvas mostraban malformaciones como una torsión de la columna vertebral, cola más corta de lo normal, malformaciones craneales y despigmentación. Se hizo un genotipado de

dichos peces y se observó que los niveles de metilación totales en peces tratados eran significativamente más bajos, un 25%, que los peces controles. Específicamente detectamos que la metilación en la isla CpG de Sparc era de un 44% en controles y un 22% en peces expuestos. Esto fue corroborado con la medición de los niveles de expresión de *sparc* que era de hasta 3 veces más elevado en los peces tratados.

En resumen, nuestro estudio aporta la primera evidencia de que el intrón localizado en el extremo 5' de *sparc* contiene elementos reguladores requeridos para la expresión temporal y especial de este gen. Además, demostramos que *sparc* está regulado transcripcionalmente por metilación del ADN. Nuestros hallazgos por tanto establecen una base para que futuros estudios puedan caracterizar más detalladamente los elementos reguladores y esclarecer los mecanismos moleculares que llevan a la regulación transcripcional de *sparc*.

4. La expresión de *sparc* es específica del estado de desarrollo durante la remodelación post-embriónica de peces planos

Sparc está altamente expresado durante las primeras etapas de desarrollo, jugando un papel importante en morfogénesis (Damjanovski *et al*., 1997; Rotllant *et al*., 2008; Kang *et al*., 2008). Sin embargo, en adultos la expresión está restringida a tejidos en reparación o remodelación (Schelling *et al*., 2004; Padhi *et al*., 2004).

El rodaballo (*Scophthalmus maximus*), es una especie con alto valor económico. La mayoría de estudios se han centrado en la etapa de metamorfosis ya que implica una remodelación con cambios fisiológicos drásticos de diversos órganos como piel (Campinho *et al*., 2007), sistema músculo-esquelético (Saele *et al*., 2006), sistema nervioso (Graf y Baker, 1990) y sistema intestinal (Tanaka *et al*., 1996). El objetivo principal fue caracterizar molecularmente Sparc en rodaballo y su regulación transcripcional durante la remodelación metamórfica. Además, se evaluó el efecto de la temperatura del agua en el desarrollo y la regulación de Sparc. El ADNc *sparc* de rodaballo tiene 1154 bp y consiste en una pauta abierta de lectura de 930bp que codifica para 310 aminoácidos con un péptido señal de 17 aminoácidos, una región 5'UTR de 95 bp y una región 3'UTR de 130bp. Nuestro alineamiento múltiple indicó que la secuencia aminoacídica de rodaballo posee una alta conservación con la de otros vertebrados. Mediante una PCR en tiempo real absoluta se detectó un alto nivel de ARNm de *sparc* asociado a la metamorfosis (estadio de desarrollo 3b-4d; 15-30dph). Osteonectina se expresa diferencialmente en la región cranioencefálica, principalmente en mandíbulas, aleta pectoral, arcos branquiales y pterigióforos de las aletas caudal, dorsal y anal. Se midió el número de copias de *sparc* a los 4, 15, 30, 50 y 80 días en muestras cultivadas a 14ºC y a 18ºC para determinar el efecto de la temperatura de cultivo. Los transcritos se mantienen bajos a 4dph en ambas temperaturas. Larvas criadas a 18ºC alcanzan el pico máximo de niveles de ARNm a 30dph y a continuación empieza a bajar hasta los 50dph En las larvas que están a 14ºC los máximos niveles de ARNm se sitúan en 45-50dph (estado metamórfico), y gradualmente empieza a disminuir hasta que los de 80dph alcanzan los mismos niveles que los de 15dph. Por lo tanto, no hay diferencias significativas en la dinámica del número de copias, teniendo ambos grupos el más alto

nivel de expresión justo durante la metamorfosis pero el patrón de expresión es ralentizado en el grupo de 14ºC. Así, si se comparan los niveles de *sparc* en el mismo estadio de desarrollo en ambas temperaturas, no existen diferencias significativas en ninguno de los puntos de muestreo.

En resumen, por primera vez se clonó la osteonectina de rodaballo y se analizó la estructura de la proteína y su distribución tanto especial como temporal durante el desarrollo larvario. Además, su expresión estadio-específica durante el desarrollo post-embrionario descrito en este trabajo sugiere un marco útil para futuros estudios, los cuales servirán para caracterizar la sucesión de los diversos eventos morfológicos y moleculares que tienen lugar durante la metamorfosis de los peces planos.

DISCUSIÓN

El objetivo principal de esta tesis ha sido la caracterización tanto a nivel molecular como función del gen Sparc en vertebrados no mamíferos, concretamente los teleósteos. Por tanto los experimentos se realizaron con dos especies de teleósteos, pez cebra y rodaballo, ya que los peces teleósteos poseen aparentemente menos genes homólogos funcionales a Sparc y por tanto las observaciones en vertebrados no mamíferos podría descubrir funciones clave de Sparc.

Para ello, en primer lugar, se investigó el papel funcional de Sparc en la hematopoyesis, en segundo lugar se determinó el posible papel de Sparc en las anomalías del desarrollo producidas por exposición a la radiación solar UV en embriones de pez cebra y se realizó un análisis funcional para caracterizar la regulación transcripcional del gen *sparc* en embriones de pez cebra y por último, clonamos y caracterizamos el gen *sparc* en rodaballo (*Scophthalmus maximus*) con la finalidad de desentrañar el patrón de expresión espacio-temporal de este gen durante la remodelación metamórficas que sufren este tipo de teleósteos. Nuestros datos proporcionan evidencias concluyentes que SPARC juegan un papel crítico en la hematopoyesis durante el desarrollo de pez cebra y su colocación en la vía de señalización de FGF. En cuanto a su regulación, se demuestra un posible papel de Sparc en los mecanismos moleculares subyacentes responsables de las anomalías del desarrollo producido por la exposición a la radiación UVR en embriones de pez. Por último, las similitudes de secuencia, organización estructural y expresión génica permiten concluir que la Sparc de rodaballo es muy similar a todas las otras proteínas Sparc de vertebrados y su expresión es estadio-especifica durante el desarrollo post-embrionario en rodaballo. Por lo tanto, nuestros estudios añaden nuevas piezas en el complejo entramado funcional del gen Sparc en teleósteos y revelan así mismo diferencias significativas, pero también similitudes sorprendentes que ayudan a perfeccionar nuestra comprensión de la función de Sparc en vertebrados en general.

La discusión sigue en gran medida la cronología de los capítulos, pero se ha estructurado para abordar específicamente los hallazgos más importantes:

1. *Sparc* es un importante regulador de la hematopoyesis embriogénica.

Un estudio reciente usando morfolinos antisentido en pez cebra detectó una función potencial hematopoyética de 14 genes (Eckfeldt *et al*., 2005). Sparc, una proteína de la matriz extracelular también llamada Osteonectina, estaba entre ellas. En el presente trabajo investigamos la función de *sparc* más detalladamente en la hematopoyesis del pez cebra.

Demostramos que generando un knockdown de sparc se redujo significativamente la hematopoyesis durante el desarrollo embrionario. En concreto, disminuyó la expresión de genes asociados con el desarrollo primitivo de células progenitoras eritroideas (*gata 1* y *βe3globin)*. Por el contrario, genes asociados con el desarrollo de progenitores mieloides no estaban afectados. Esto sugiere que Sparc posee un papel importante en la modulación de la expresión de *gata 1*. Sin embargo, de esta propuesta surge la pregunta de cómo una proteína matricelular puede regular la expresión de un factor de transcripción. El papel de Sparc en la interacción célula-matriz puede tener la respuesta; Sparc puede mediar o desencadenar vías de transducción requeridas para la activación o mantenimiento de genes de transcripción. Este concepto podría comprobarse si se identifican las moléculas señalizadoras extracelulares que actúan corriente arriba de esos genes que codifican para *gata1* y *sparc*.

2. *Sparc* actúa en la vía de señalización de *fgf21*.

Se sabe que miembros de la familia Fgf regulan la expresión de *sparc* en diferentes especies (Brekken y Sage, 2001; Whitehead *et al*., 2005). En mamíferos, la función de Sparc no está clara en la hematopoyesis aunque se sabe que *sparc* está regulada por

miembros de la familia de fgf y que éstos a su vez regulan la hematopoyesis primitiva modulando la expresión del factor de transcripción *gata 1*. Además, la expresión de *gata 1* está regulada en por Fgf en otras especies como en aves y pez cebra (Nakazawa *et al*., 2006; Songhet *et al*., 2007), ya que una alteración en la expresión de fgf conduce a una perturbación de este gen (Yamauchi *et al*., 2006). La disrupción de la expresión de *gata1* mRNA hace pensar en la posibilidad de que los efectos de *sparc* en hematopoyesis puedan ser debidos en parte a una perturbación en la señalización fgf. Esta hipótesis está corroborada por el hecho de que el fenotipo de individuos inyectados con el morfolino de *sparc* es similar al de aquellos inyectados con el morfolino de *fgf21*, el cual se caracteriza por una disrupción severa del desarrollo de células eritroides en el pez cebra (Yamauchi *et al*., 2006).

Para corroborar esta teoría, examinamos si la expresión de *sparc* está alterada en individuos inyectados con el morfolino de *fgf21* y si inyectando *sparc* de manera exógena podría rescatar la deficiencia de *gata 1* en embriones. Encontramos que la expresión de *sparc* estaba drásticamente reducida en esos embriones. Además, también medidos la expresión de *fgf21* después de la inyección con morfolinos de *sparc* y se vio que la expresión de *fgf21* no estaba afectada.
Nuestros resultados por tanto sugieren que *sparc*, al menos en parte, actúa corriente abajo de *fgf21*, y que es importante en el desarrollo de células progenitoras eritroideas en el pez cebra.

Sorprendentemente ratones con deficiencia en los niveles de *sparc* no presentaban defectos hematopoyéticos severos (Siva *et al*., 2012). Una posible razón para este hecho es la hipótesis de la presencia de homólogos de *sparc* en mamíferos que compense la falta de la expresión de *sparc*. Sin embargo, estudios en *Caenorhabditis elegans* y zebrafish, donde hay menos redundancia, la reducción de *sparc* produce muchos más defectos significativos. Consecuentemente, nuestras observaciones en cebra probablemente definan mejor las funciones de Sparc.

3. Exposición a luz ultravioleta induce a un incremento en la expresión de *p53* y *sparc*

La exposición a luz ultravioleta produce alteraciones irreparable a diferentes niveles desde la supervivencia del organismo y reproducción (Tietge *et al*., 2001; Häder *et al*., 2007; Marquis *et al.,* 2008; Charron *et al*., 2000) hasta el metabolismo celular y viabilidad (Dahms y Lee, 2010; Rastogi *et al*., 2010). Sin embargo, las respuestas moleculares que se desencadenan en un organismo después de la exposición todavía no están caracterizadas. Bajo condiciones naturales los efectos directos de la radiación ultravioleta son difíciles de estudiar debido a su interacción con otros factores ambientales y cambios en la irradiancia causada por la variabilidad en nubes, composición atmosférica y/o la cantidad de materia orgánica disuelta, entre otros. En este estudio se usó un incubador que emite PAR, UVA y UVB en proporciones similares a las que se observaron en condiciones naturales para evaluar las consecuencias de dicha exposición (Neale y Fritz, 2001).

Demostramos que la exposición a ultravioleta puede inducir a un incremento en la expresión del gen *p53* dependiente de la exposición. Por lo tanto, embriones de pez cebra mostraban un incremento en la expresión de *p53* en respuesta a UV como en mamíferos. En analogía con otros organismos, se espera que la expresión de *p53* sea beneficiosa incrementando la reparación del ADN, pero otras funciones de *p53*, como apoptosis puede haber contribuido a un desarrollo anormal. En otro estudio se vio que mantener *p53* a niveles bajos durante la embriogénesis es crítico para proteger el desarrollo normal (Zeng *et al.*, 2009).

Paralelamente con la inducción de *p53*, demostramos que la expresión de la proteína matricelular Sparc era también dependiente de la exposición a UV. Hasta ahora, hay poca información de los efectos directos de UV en la regulación de *Sparc*. Aycock *et al.*, 2004 demostró que *sparc* estaba presente en altas cantidades en carcinomas inducidos por UV, sin embargo, no era detectable en la piel del grupo control no irradiado. Además, ratones que no expresaban *sparc* y resistentes a tumores, desarrollaban carcinomas en respuesta a la radiación. Por tanto, se sugiere que Sparc tiene un papel crítico en la formación de tumores en la piel en respuesta a radiación ultravioleta.

Con respecto a la dependencia espectral de la expresión génica, la exposición combinada de UVB y UVA produjo una expresión mayor de *sparc* y *p53* que UVA sola, por tanto esto parece indicar una capacidad más alta de UVB a producir daño celular en pez. Sin embargo, diferencias significativas en la expresión de ambos genes se observaron a longitud de onda más cortas de UVA, en las cuales *p53* se activaba por tratamientos espectrales menos dañinos que *sparc*. Longitudes de onda más largas de UVA no producen un aumento de expresión significativo en ninguno de los dos genes comparado con embriones expuestos al tratamiento PAR.

Trabajos previos en pez cebra demostraron la capacidad de la radiación ultravioleta A a activar un mecanismo, la reparación fotoenzimática (PER), la cual repara el daño del ADN causado por la exposición a ultravioleta B (Dong *et al*., 2007). La evidencia del sistema de reparación mencionado es la detección inicial de la fotoliasa a las 3 hpf en embriones de pez cebra (Dong *et al.,* 2008). La inducción de PER compensa parcialmente la disminución considerable de tolerancia a la radiación ultravioleta B en este particular estadio de desarrollo (Dong *et al*., 2008). Se sugiere que tolerancias más altas a UVB durante el estadio de huevo puede estar relacionado con otros mecanismos de reparación así como por la protección del corion y otros compuestos fotoprotectores heredados vía materna. En conclusión, la sensibilidad a la radiación ultravioleta puede variar entre estadios de desarrollo.

4. *Sparc* puede ser un posible mecanismo molecular inductor de las malformaciones fenotípicas producidas después de una exposición a luz ultravioleta.

Se observó una disminución del porcentaje de supervivencia y un incremento en las malformaciones en embriones expuestos a radiación ultravioleta. El incremento de la expresión de *sparc* detectada por qRT-PCR y por hibridación in situ puede ser una causa de estos efectos. Las malformaciones fenotípicas debidas a estudios de sobreexpresión y pérdida de función (Damjanovski *et al*., 1997) también afianzan dicha posibilidad. Se ha demostrado que la inyección de RNA de *sparc* en blastómeros está asociado con defectos

en cabeza y el eje de *Xenopus*. Análisis histológicos revelaron que malformaciones en somitas correspondían a un eje torcido (Damjanovski *et al*., 1997). En este estudio mostramos que la expresión ectópica de *sparc* afecta al desarrollo del pez cebra. La microinyección de mRNA de *sparc* en embriones de pez cebra de 1-2 células generó malformaciones fenotípicas, con torsión de la notocorda como la más frecuente.

El hecho de que se encontrasen malformaciones fenotípicas similares en embriones expuestos a UV y embriones inyectados, sugiere que la expresión de *sparc* es uno de los posibles mecanismos moleculares que inducen malformaciones fenotípicas después de una radiación ultravioleta.

5. El intrón situado en el extremo 5'UTR juega un papel importante en la regulación transcripcional de *sparc*.

Una característica común en la organización de *sparc* en todos los vertebrados es la presencia de una secuencia intrónica entre el primer no codificante y el segundo exón. Sin embargo, el tamaño del primer intrón parece ser especie específico siendo de 7kb en pez cebra, de 10kb en humanos y 2kb en *Xenopus* (Damjanovski *et al.,* 1998).

Encontramos que el promotor de 0.2-kb y la secuencia de 7kb situada anterior al primer exón codificante condujo la expresión de EGFP en notocorda, vesicular ótica, fin fold, somitas, músculos cardíaco y esquelético, lo cual reproduce el ya descrito patrón de expresión del ARNm endógeno de *sparc* (Rotllant *et al*., 2008; Ceinos *et al*., 2013). Se encontraron resultados parecidos en ratón, donde los transcritos de *sparc* se detectaron en tejidos en proceso de desarrollo, tales como vesícula ótica (Mothe y Brown, 2001), notocorda, somitas y esqueleto embriogénico (Holland *et al*., 1987; Mason *et al*., 1986). Además, la región de promotor de 0.2 kb y la secuencia situada inmediatamente después de 7kb condujeron la expresión de *egfp* en el epitelio olfatorio. Aunque se ha visto expresión de *sparc* en el epitelio olfatorio en ratón (Mendis y Brown, 1994), este es el primer trabajo donde se demuestra su posible expresión en un vertebrado no mamífero. En este estudio, no fuimos capaces de detectar *sparc* en el epitelio olfatorio mediante

hibridación *in situ*. Una de las posibles explicaciones puede estar relacionada con la limitada sensibilidad de la técnica para observar la expresión en determinadas regiones. Cabe mencionar que, aunque la conclusión está basada en expresión temporal y transgénica, es poco probable que el patrón de expresión de *egfp* sea debido al sitio de integración ya que *egfp* mimetiza la expresión de endógena de *sparc* en muchas regiones. Sin embargo, no podemos excluir la posibilidad de que sea debido a la integración en otra posición que puede explicar la expresión de *egfp* en el epitelio olfatorio en peces mosaicos y transgénicos.

La expresión dinámica demuestra que la actividad del promotor reside en el intrón 1 (nt+126/+7168). La eliminación específica de esta región resultó en una completa reducción de la actividad promotora. La regulación transcripcional de otros genes (ej. ubiquitina C) también se ha visto que es exclusiva de la secuencia intrónica (Bianchi *et al.,* 2009). Por tanto, el intrón (nt+126/+7168) aporta elementos regulatorios para la expresión en distintos en embriones de pez cebra.

6. *Sparc* está transcripcionalmente regulada mediante la metilación de regiones específicas de su promotor.

Análisis de la región intrónica situada en el extremo 5' de *sparc* reveló numerosos sitios de unión para factores de transcripción. Además, se encontró una secuencia rica en CGs (isla CpG) en dicho intrón. En mamíferos se ha visto que *sparc* está regulada transcripcionalmente por metilación del ADN y también se detectaron islas CpG en el promotor de *sparc* (Rodríguez-Jiménez *et al*., 2007; Gao *et al.,* 2010; Tajerian *et al*., 2011). Por tanto, investigamos el papel de la metilación del ADN en la expresión de *sparc* en embriones de pez cebra para adquirir nuevos conocimientos en su regulación transcripcional. Se hizo una exposición de los peces a 5'-Azacytidina para artificialmente inducir hipometilación en los individuos. Este método ya había sido usado anteriormente en embriones de pez cebra (Martin *et al*., 1999; Christman, 2002). Nuestros resultados mostraron que (i) la exposición a 5'-Aza produce distintas anomalías fenotípicas en

larvas, incluyendo una aleta caudal acortada, torsión de la columna vertebral, malformaciones craneales y despigmentación, (ii) 5'-Aza produjo una demetilación global significativa en larvas, (iii) 5'-Azacytidine redujo la metilación específicamente en la región CpG situada en el intrón 1 (iv) *sparc* está expresada de manera elevada en peces expuestos al tratamiento con 5'-Azacytidina. Por tanto, estos resultados sugieren que *sparc* está regulada mediante metilación del ADN a nivel transcripcional.

7. Sparc está altamente conservada en rodaballo (*Scophthalmus maximus)*

El ADNc de *sparc* en rodaballo tiene 1154 bp y consiste en una pauta abierta de lectura de 930bp que codifica para 310 aminoácidos con un péptido señal de 17 aminoácidos, una región 5'UTR de 95 bp y una región 3'UTR de 130bp. La proteína de rodaballo mantiene la misma estructura que poseen la de todos los vertebrados (Laizé *et al*., 2005; Koehler *et al*., 2009; Kos y Wilding 2010). El precursor de Sparc tiene las características de una proteína de secreción, con un péptido señal. Procesando este péptido señal se produce la proteína Madura de 293 aminoácidos, incluyendo un dominio N-terminal rico en ácido glutámico (I), un dominio central folistatina (II) con una alta proporción de residuos de cisteína así como también un sitio de glicosilación que precede a un dominio C-terminal o también llamado dominio extracelular de calcio (III) el cual es una región rica alfa hélice conteniendo dos sitios de unión para Ca^{2+}-binding EF-hands (EF-hand1 y EF-hand2). El ácido glutámico del dominio I se une al Ca^{2+} con baja afinidad (Lane *et al*., 1994; Brekken y Sage, 2000), interactúa con hidroxiapatita (Brekken y Sage, 2000), está envuelta en la mineralización de cartílago y hueso (Brekken y Sage, 2000) y contiene los epítopos de la proteína (Stenner *et al*., 1984). En rodaballo, este dominio contiene 21 residuos de ácido glutámico y lo más probable es que la mayoría de las funciones atribuidas a los mamíferos. Este dominio es altamente variable en los invertebrados y está ausente en cnidarios (Koehler *et al*., 2009). Por lo tanto, se ha propuesto que las actividades dependientes de Ca^{2+} surgieron con la adquisición del dominio N-terminal en los organismos triploblásticos (Koehler *et al*., 2009). El segundo dominio es rico en cisteína, e incluye un dominio Kazal y un motivo EGF (Hohenester *et*

al., 1997). Se ha demostrado que se une a activina, inhibina, heparina y proteoglicanos y puede regular la proliferación de células endoteliales y angiogénesis (Funk y Sage, 1993; Yan y Sage, 1999). La estructura terciaria de Sparc se mantiene mediante siete puentes disulfito. Además, este dominio contiene un sitio de glicosilación altamente conservado, el cual se ha demostrado que es importante para la afinidad por el colágeno y por tanto para la funcionalidad de la proteína (Kaufmann *et al*., 2004).

El tercer y último dominio es extracelular y de unión al calcio, el cual incluye dos motives EF. Se une a los colágenos tipo I, III, y IV en dependencia con el Ca^{+2} (Sasaki *et al*., 1997; Sasaki *et al.,* 1998).

Nuestro alineamiento múltiple indicó que la secuencia aminoacídica de rodaballo posee una alta conservación con la de otros vertebrados. También indicó que Sparc de rodaballo se agrupa con sus ortólogos de vertebrados y junto con las Sparcs de teleósteos forma un único clado. El patrón de expresión es comparable al pez cebra, medaka y dorada (Rotllant *et al*., 2008; Renn *et al*., 2006a,b; Redruello *et al*., 2005; Estêvão *et al*., 2005). Por lo tanto, el análisis comparativo de Sparc de rodaballo sugiere una fuerte presión evolutiva para conservar esta proteína.

8. *Sparc* se expresa de una forma estadio-específica en los procesos de remodelación metamórfica que tienen lugar durante el desarrollo post-embrionario de rodaballo

Sparc se expresa de forma dinámica en tejidos esqueléticos y no esqueléticos desde el desarrollo temprano hasta la vida adulta; esto sugiere un amplio rango de acción (Rotllant *et al*., 2008; Estêvão *et al*., 2005; Renn *et al*., 2006a,b). Sin embargo, sus funciones no están limitadas al desarrollo embriogénico ya que Sparc se ha demostrado que está asociado a tejidos adultos que requieren remodelación, y reparación (Lane y Sage 1994). A pesar del estudio en los últimos años, las funciones morfogénicas de Sparc durante el desarrollo están poco caracterizadas.

Se sabe que los peces planos están sometidos a un proceso de metamorfosis comparable a la que experimentan los anfibios anuros. Por tanto, los peces planos incluyendo el rodaballo (*Scophtalmus maximus*) cambian de una larva pelágica con simetría bilateral a

un juvenil bentónico asimétrico (Sadiq *et al*., 1984). Así, el desarrollo post-embriogénico en rodaballo implica una serie de procesos morfológicos complejos que activan la migración celular, proliferación, crecimiento y eventos apoptóticos.

El análisis de la expresión de ARNm de *sparc* mostró una expresión dinámica específica del estado de desarrollo con sus niveles más altos justo cuando la larva alcanzaba la metamorfosis, indicando que puede ser necesaria para la metamorfosis del rodaballo. Aunque la metamorfosis en rodaballo está bien descrita y el papel central de la hormona tiroidea está estudiado (Power *et al.,* 2008; Infante *et al*., 2008; Roberto *et al*., 2009), no hay evidencias de un posible papel regulatorio de esta hormona en la expresión de *sparc*. Sin embargo, factores como la hormona paratiroidea (PTH; Nakajima *et al*., 2002) y dexametasona (Sawhney, 2002) regulan *sparc*, esto indica que este gen puede ser importante un intermediario de la acción de diferentes hormonas.

Para comprobar la expresión dinámica dependiente del estadio de desarrollo durante el desarrollo post-embriogénico de rodaballo, determinamos la expresión del ARNm a dos temperaturas. Se sabe que el tiempo de desarrollo tanto embriogénico como post-embriogénico de rodaballo varía con la temperatura (Gibson y Johnston, 1994). Encontramos que el tiempo de desarrollo era el doble en las muestras cultivadas a 4ºC menos. Los rodaballos a esa temperatura mostraban una reducción en el grado de osificación y crecimiento. Tales observaciones apoyan los estudios previos en otros teleósteos en los que el crecimiento y desarrollo esquelético está alterado a bajas temperaturas comparando con un ambiente térmico óptimo (Anken *et al*., 1993; Campinho *et al.,* 2004).

El efecto de la temperatura en la expresión de ARNm de *sparc* parece coincidir con el encontrado en el tiempo de desarrollo., desarrollo esquelético y crecimiento. Por tanto, la expresión de *sparc* parece estar también retrasada a 14ºC teniendo su máxima expresión en un estadio específico de desarrollo. Concluimos que a expresión de *sparc* parece ser dependiente al grado de desarrollo del animal. Esto afirma la expresión dinámica de *sparc* específica del estadio de desarrollo durante el desarrollo post-embriogénico del rodaballo.

CONCLUSIONES

Los análisis de los resultados obtenidos en este proyecto de tesis llevan a las siguientes conclusiones:

- Sparc es un importante regulador de la hematopoyesis embrionaria durante el desarrollo temprano en el pez cebra. En concreto, interviene en el desarrollo de células progenitoras eritroides mediante la regulación diferencial de los genes GATA1 y βe3globin.

- Situamos *sparc* en la vía de señalización genética de *fgf21* debido a que se obtuvieron defectos similares en los knockdowns de *sparc* y *fgf21* y también por la capacidad de gen *sparc* de rescatar parcialmente el fenotipo derivado de la eliminación del gen *fgf21*.

- La exposición de embriones de pez cebra a luz ultravioleta induce a un aumento en la expresión de *p53* y *sparc*.

- En relación al punto anterior, sugerimos *sparc* como parte del mecanismo molecular responsable de las malformaciones morfológicas en el desarrollo embrionario. inducidas por la exposición a radiación UVR

- El intrón situado en el extremo 5' es un región clave para la regulación transcripcional del gen *sparc*, ya que un vector conteniendo únicamente esta región es capaz de expresa EGFP en las mismas regiones donde se expresa el transcrito endógeno.

- Asimismo, *sparc* está regulada a nivel transcripcional mediante metilación de una zona específica de su promotor. Concretamente, a través de una isla CpG detectada en la región del intrón.

- La proteína Sparc de rodaballo conserva la misma estructura proteica observada en otros vertebrados. Análisis filogenéticos engloban Sparc de rodaballo en el mismo clúster que los otros vertebrados. Asimismo, encontramos que la expresión en rodaballo es comparable a otras especies de teleósteos. Estos resultados sugieren por tanto una fuerte presión evolutiva para conservar esta proteína e indican que debe de haber una conservación en su función.

- La expresión de ARNm de *sparc* mostró una expresión dinámica es estadio-específica durante el desarrollo post-embrionario de rodaballo, con altos niveles en el momento de la metamorfosis, indicando así un posible papel clave de este gen en los procesos de remodelación metamórfica que tienen lugar durante el desarrollo post-embrionario de rodaballo

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