Int. J. Dev. Biol. 63: 623-629 (2019)
https://doi.org/10.1387/ijdb.190053mm

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Identification and expression analysis of zebrafish *testis-specific gene 10 (tsga10)*

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ABSTRACT Several clinical studies suggest that testis-specific gene antigen 10 (TSGA10) is a cancer-testis antigen with a discernible expression pattern in the testis. Recent studies have highlighted that TSGA10 overexpression in HeLa cells impairs the transcriptional activity of hypoxia-inducible factor alpha (HIF-1α) and inhibits angiogenesis. In this study, we used the zebrafish as a powerful model organism to identify and characterize the orthologue of TSGA10. We analyzed the gene expression pattern by RT-PCR and whole mount *in situ* hybridization and overexpressed the tsga10 protein by mRNA microinjection. Our results revealed that during early development, *tsga10* expression is enriched, but gradually subsides between 0 and 72 hours post fertilization (hpf). There was no detectable transcript at the larval stages. In adult fish, we found high expression levels of *tsga10* in the testis and unfertilized egg and low levels of gene expression in the brain, eyes and muscle. Overexpression of *tsga10,* using *tsga10* mRNA microinjection into one-cell stage embryos, resulted in angiogenic and morphological defects at 24 and 48 hpf. This study clarified the expression pattern of *tsga10* in different developmental stages and adult tissues, suggesting that t*sga10* may have a related biological role in different cell types and tissues. Our results indicate that *tsga10* mRNA at embryonic stages is maternally deposited, indicating a transient functional role during embryogenesis. Our findings suggest that *tsga10* is a human orthologous gene relevant for future studies to elucidate its mechanism of action in angiogenesis.

KEY WORDS: *zebrafish, tsga10, angiogenesis, development*

The TSGA10 gene, predominantly expressed in testis, has an important role in spermatogenesis. It is mapped to chromosome 2q11.2, consists of 19 exons and is transcribed into a 3 kb RNA that is subsequently translated into a 698-amino acid protein (Behnam *et al*., 2006, Modarressi *et al*., 2004, Modarressi *et al*., 2001).

TSGA10 is overexpressed in various types of cancers, including solid tumors and blood malignancies (Behnam *et al*., 2009, Mobasheri *et al*., 2007, Mobasheri *et al*., 2006, Reimand *et al*., 2008, Smith *et al*., 2011).

It has been shown that the C-terminus fragment of TSGA10 protein, which co-localizes with hypoxia-inducible factor (HIF-1α) in the midpiece of spermatozoa, impairs the nuclear localization of HIF-1α (Hagele *et al*., 2006). HIF-1α activates the transcription of genes involved in critical aspects of cancer development, such as angiogenesis and cell survival (Semenza, 2003). A recent study has shown that TSGA10 overexpression decreases HIF-1α transcriptional activity and inhibits angiogenesis, cell growth, and invasion of HeLa cells (Mansouri *et al*., 2016). Although these studies support the concept that TSGA10 has a repressive role in angiogenesis, these are still insufficient to explain the precise role of TSGA10 particularly in a living organism. In another study using whole-genome chromosomal microarray analysis, TSGA10 deletions at 2q11.2 have been found in patients with developmental disabilities and congenital anomalies (Kaminsky *et al*., 2011).

Zebrafish is an attractive model for the study of gene function and organogenesis (Lieschke and Currie, 2007). As a model organism, zebrafish has unique advantages for studying vasculogenesis,

Abbreviations used in this paper: HIF, hypoxia-inducible factor; hpf, hours post fertilization; TSGA, testis-specific gene antigen.

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Supplementary Material (two figures) for this paper is available at: https://doi.org/10.1387/ijdb.190053mm

Submitted: 6 April, 2019; *Accepted:* 24 June, 2019

ISSN: Online 1696-3547, Print 0214-6282

Fig. 1. Analysis of the *tsga10* transcript and protein. *Number of exons, transcript length in basepairs, and translation length (number of amino acids in the protein)* **(A)**. *Multiple sequence alignment of Human, zebrafish, and mouse Tsga10 protein using the T-Coffee algorithm* **(B)**. *Schematic representation of the position of COG1196 (SMC_N superfamily member), the conserved domain of zebrafish Tsga10* **(C)** *and human TSGA10* **(D)** *proteins.*

as well as angiogenesis, *in vivo* (McKinney and Weinstein, 2008). Seventy percent of human genes, including TSGA10, have at least one orthologue in zebrafish (Howe *et al.*, 2013). The small size, optical transparency, high number of offspring, and external fast embryonic development make zebrafish an excellent model system for forward-genetic analysis (Gore *et al.*, 2012). Moreover, the early development of the cardiovascular system in zebrafish embryos provides a unique opportunity for direct blood circulation observation. Due to the availability of zebrafish transgenic lines with endothelial expression of fluorescent proteins mediated by a vascular gene promoter, such as Tg(flk:EGFP) and Tg(fli1:EGFP), vascular and blood development can be visualized and analyzed in great detail. Finally, genetic studies have uncovered the preservation of molecular pathways between mammals and fish, making vasculogenesis research in zebrafish doubtlessly applicable for human health (Chavez *et al.*, 2016).

Given the emerging evidence of *tsga10* overexpression role in cell growth and angiogenesis, we employed a zebrafish-based *in vivo* functional study to characterize the zebrafish *tsga10* and to analyze the mRNA overexpression of *tsga10* in developmental and aberrant angiogenesis.

Results

Identification and in silico *analysis of zebrafish* tsga10

NCBI database analysis indicates the presence of one orthologue of the human TSGA10 in zebrafish, namely, *tsga10*, which is mapped to chromosome 1. The Danio rerio *tsga10* gene (NC_007112.6, Ensembl Id: ENSDARG00000052512) has 18 exons from which, similar to its human counterpart, 16 exons are protein-coding exons (Fig. 1A). It is expressed mainly as a 2.5 kb transcript isoform encoding a 710-amino acid protein (NP_001070757.2) with 43% identity to the human TSGA10 protein (Fig. 1B). A single *tsga10* gene is present in other species (data not shown). UniGene analysis of zebrafish *tsga10*, represented by 47 Expressed Sequence Tags (ESTs) from cDNA libraries, shows a restricted expression pattern of the gene in the reproductive system from hatching to adult stages; in addition to this expression pattern, our study finds *tsga10* expression in other developmental stages and tissues.

The comparison of zebrafish Tsga10 protein with its orthologues in human, mouse, chimpanzee, clawed frog, dog, and brown rat indicates that the COG1196 domain (Fig. 1C, D) has been highly conserved across different species. This domain, with a coiled-coiled structure, is a member of the SMC (structural maintenance of chromosomes) superfamily of proteins, involved in the control of cell cycle, cell division, chromosome segregation, regulation and repair of the gene (Hirano, 2006).

Analysis of the EPD database reveals that zebrafish *tsga10* has one promoter, (Chromosome [NC_007112.5]; Strand [+];

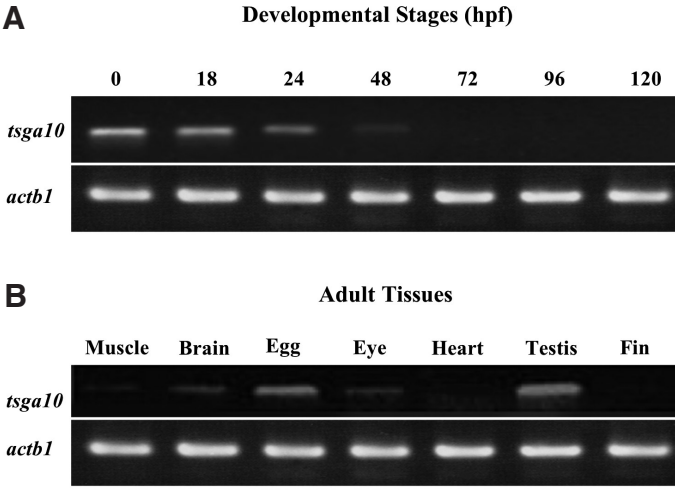

Fig. 2. *tsga10* expression profiles. *RT-PCR analysis of* tsga10 *expression using embryos of the stages indicated* **(A)** *and dissected embryonic tissues* **(B)** *demonstrating strong expression at one-cell, 18 hpf, 24 hpf, and also in adult testis and unfertilized egg. Weak expression was detected at 48 hpf and in the adult zebrafish eye, muscle and brain. Actin beta1 (*actb1*) was used as a reference gene.*

Position [50157442]), compared to its human counterpart, which has two promoters. Data obtained from the TRANSFAC database shows that nuclear transcription factor Y (NF-Y) binds to *tsga10* promoter at the CCAAT box region and activates gene expression. NF-Y also has different target genes in human, mouse, and rat.

Temporal and spatial expression pattern of tsga10 *during zebrafish development*

The mRNA levels of *tsga10* were investigated in zebrafish embryos at different developmental stages, and in adult tissues using RT-PCR. Our results showed that *tsga10* expression is enriched during early development, detectable as early as the one-cell stage and 18 hpf, but gradually subsides between 24 and 72 hpf with no expression at 72 hpf. There was no detectable transcript at the larval stages (Fig. 2A). In the adult stage, *tsga10* was highly expressed in testis and unfertilized egg and at low levels in brain, eye, and muscle. The gene was not expressed in adult caudal fin and heart. (Fig. 2B).

To study the spatial and tissue-specific expression pattern of *tsga10*, whole mount *in situ* hybridization was conducted on zebrafish embryos from 0.2 (one-cell) to 48 hpf. A sense probe was used in parallel control experiments with embryos of the same stages, which gave no detectable signal (Supplementary Fig. S1 in supplementary materials). We detected a ubiquitous signal in zygote (one-cell stage) and in the early stages of development (Fig. 3). During early segmentation period (10 hpf-24 hpf), a more defined expression pattern appeared in somite regions and at the level of the developing central nervous system (Fig. 3 B,C,E). At 18 hpf, *tsga10* was expressed at the midbrain and hindbrain level, in spinal cord neurons and in somites (Fig. 3C). *tsga10* expression persisted at 24 hpf in somites and different structures in brain including telencephalon (Fig. 3 E,H), and expression appeared also in lens and retina in the eye (Fig. 3D). Later in development, at 30 hpf, while *tsga10* expression pattern continued in head (brain and eye), the expression was found in intersomitic boundaries, which are likely indicative of growing intersegmental vessels (ISVs) (Fig. 3F, I). At 48 hpf, *tsga10* expression appeared to be very low and almost undetectable by whole mount *in situ* hybridization (Fig. 3G, J). These results were supported by RT-PCR where we found the expression of *tsga10* transcripts gradually decreased from their highest level at one-cell stage to the very low level at 48 hpf. Together, these expression-based data suggest that *tsga10* mRNA present at embryonic stages is maternally deposited. The strong expression in the central nervous system and in the trunk region suggest that *tsga10* may have a role in brain and axis development.

Overexpression of tsga10 *causes zebrafish angiogenic development and morphological defects*

To investigate the role of *tsga10* in zebrafish angiogenesis, we overexpressed the protein by injecting *tsga10* mRNA into one-cell stage flk-GFP zebrafish embryos (n=50), which resulted in embryos with angiogenic and morphological phenotypes (56% n=28), normal embryos (24% n=12), and dead embryos (20% n=10). The presence of the injected mRNA was confirmed by RT-PCR using the T1 and T2 primers (Supp. Fig. S2). The embryos injected with *tsga10* mRNA showed a decrease in the number and length of ISVs compared to control embryos injected with nuclease-free water (Fig. 4A, B, and C). At 24 hpf, mRNA-injected embryos had an average number of 7 angiogenic sprouts in tail, compared to 14 angiogenic sprouts in control embryos (Fig. 4B and E, $p<0.001$, n=28). In addition, the caudal vein plexus was not completely formed in mRNA-injected embryos (Fig. 4B).

At 48 hpf, the analysis of complete ISVs showed that mRNA-injected embryos had an average number of 6 complete ISVs and 5 angiogenic sprouts in tail, compared to 20 fully extended ISVs in control embryos (Fig. 4C and F $p<0.001$, n=28). Injected embryos also had less developed blood vessels in the brain compared to control embryos (Fig. 4H). Neither angiogenic defects nor morphological phenotypes were detected at 72 hpf (Fig. 4D and G). Our data suggest that decreasing the concentration and activity of *tsga10* mRNA, between 48 and 72 hpf, may probably lead to the ISV recovery and the rescue of flk-GFP mRNA-injected embryos. In addition to angiogenic defects, the overexpression of *tsga10* mRNA resulted in a range of morphological defects in 48 hpf embryos, including a small head, heart edema, and an abnormally bent fin (Fig. 4H, $p<0.001$, n=28). All control injected zebrafish embryos developed normally (Fig. 4H).

Discussion

TSGA10 gene in human localizes to region q11.2 of chromosome 2 and is mainly expressed in testis and some tumors (Behnam *et al.*, 2009, Behnam *et al.*, 2006, Mobasheri *et al.*, 2007, Modarressi *et al.*, 2004, Modarressi *et al.*, 2001). In recent years, intense efforts to identify the function of TSGA10 led to the discovery of the inhibitory effect of TSGA10 on tumor angiogenesis (Mansouri *et al.*, 2016). Previous studies have shown that TSGA10 is expressed during spermatogenesis (in testis), embryogenesis (undifferentiated embryonic stem cells), brain development, and in different types of cancer (Behnam *et al.*, 2009, Behnam *et al.*, 2006, Miryounesi *et al.*, 2014, Mobasheri *et al.*, 2007, Mobasheri *et al.*, 2006, Reimand *et al.*, 2008, Smith *et al.*, 2011). In this study, we characterize the zebrafish homologue of TSGA10 and analyze its expression pattern and the effect of its overexpression in zebrafish angiogenic

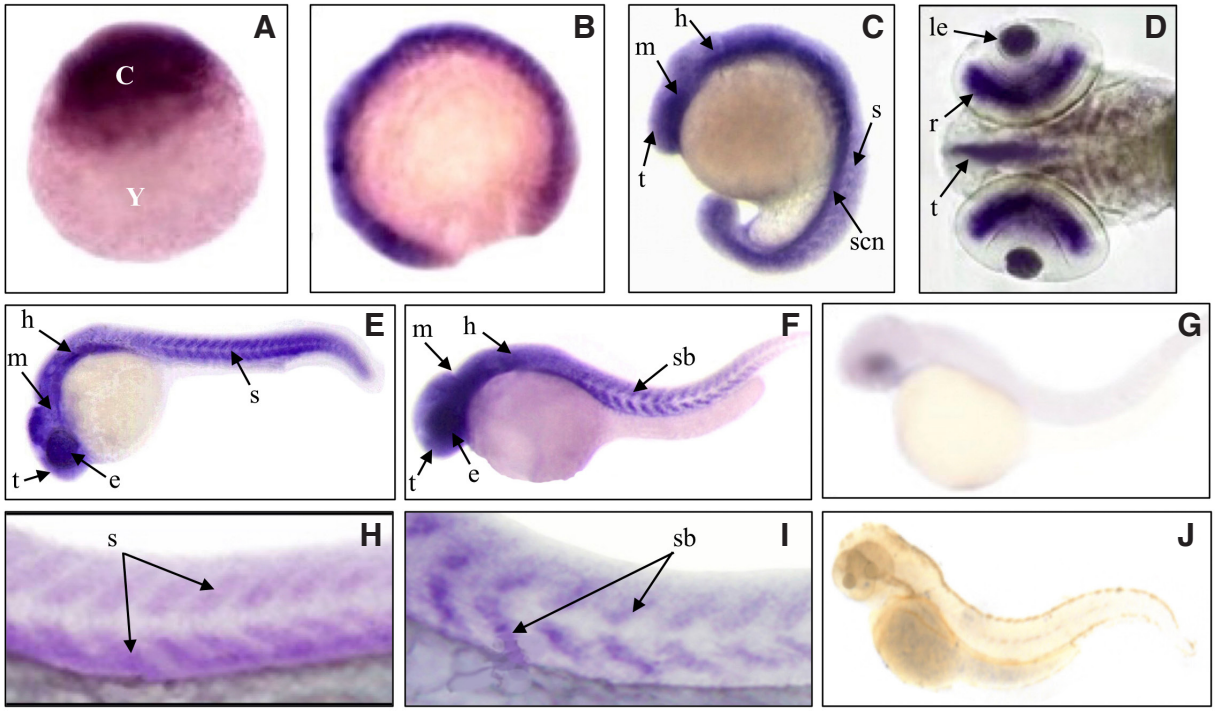

Fig. 3. Spatial expression pattern of zebrafish *tsga10*. In situ *hybridization of embryos at one-cell stage to 48 hpf using an antisense probe against* tsga10. tsga10 *was expressed ubiquitously in the rostral part of the embryo during the early stages of development at one-cell stage and at 12 hpf* **(A,B)**. *At 18 hpf the signal was present at somites and central nervous system* **(C)**. *A lateral view of embryos shows the* tsga10 *expression in somites, defined brain regions, and in the eye at 24 hpf* **(D,E)**. *Later on, at 30 hpf* **(F)** tsga10 *expression is maintained in brain regions and eye, but appears in somatic boundaries. Magnification of somites at 24 hpf* **(H)** *and somitic boundaries at 30 hpf* **(I)**. *No detectable signal, except a very weak expression around eye, was found at 48 hpf* **(G,J)**. *c, cell; y, yolk; m, midbrain; h, hindbrain; scn, spinal cord neurons; e, eye; le, lens; r, retina; s, somite; sb, somitic boundaries; t, telencephalon.*

development. We show that zebrafish *tsga10* mRNA is present as a maternal transcript during early developmental stages, from the newly fertilized egg until 48 hpf, indicating a transient functional role during embryogenesis. Furthermore, *tsga10* in the adult stage is expressed in the reproductive organs, brain, eye, and muscle, which is similar to the TSGA10 expression pattern in human. These data suggest that *tsga10* might have a related biological role in different cell types and tissues. We also report the *tsga10* expression pattern in the head, intersomitic regions, and different parts of the brain in zebrafish embryos. These results are in line with data obtained from previous studies in which TSGA10 is expressed in a broad range of tissues (Behnam *et al.*, 2006, Dianatpour *et al.*, 2012, Fratta *et al.*, 2011, Mobasheri *et al.*, 2007, Mobasheri *et al.*, 2006) and suggest that *tsga10* may have a role in head and axis development. There is a TSGA10 orthologue in mouse, chimpanzee, clawed frog, dog, and brown rat (Church *et al.*, 2011, Gibbs *et al.*, 2004, Hellsten *et al.*, 2010, Lindblad-Toh *et al.*, 2005, McConkey, 2004). By comparing Tsga10 protein domains, we found that the COG1196 domain (a member of the superfamily SMC: structural maintenance of chromosomes) is conserved in human, zebrafish, and all the animals mentioned above. Our bioinformatics analysis also shows that the nuclear transcription factor Y (NF-Y) binds to the CCAAT box region of *tsga10* promoter and acts as a transcriptional activator. Given the central role of SMC proteins as ATPases involved in cell cycle control, chromosome segregation in mitosis and meiosis, gene regulation, and recombinational DNA repair (Hirano, 2006), and the fact that NF-Y target genes are mostly cell-cycle-related and related to human disease such as cancer (Ly *et al.*, 2013), *tsga10* could have an important role in cell survival in different diseases and cancer. Further investigation is necessary to better understand and to confirm these findings.

A previous study of TSGA10 found that tumor cell characteristics such as migration, proliferation, invasion, and angiogenic activity are remarkably affected by TSGA10 overexpression. According to this study, which was done on HeLa cells, TSGA10 overexpression has an inhibitory role in angiogenesis for tumor cells (Mansouri *et al.*, 2016). In another study, TSGA10 has been identified as a tumor suppressor gene (Jakhesara *et al.*, 2013, Yuan *et al.*, 2013). Interestingly, our results support the published data showing the inhibitory role of *tsga10* on angiogenesis, as evidenced by deficiencies in ISV, brain vasculature, and caudal vein plexus formation at 24 and 48 hpf after *tsga10* overexpression by *tsga10* mRNA injection. The mechanisms that regulate angiogenic sprouting and extension of zebrafish ISVs are different than the *de novo* vasculogenic formation of the posterior cardinal vein and the dorsal aorta (Herbert *et al.*, 2009). This could explain the effect of *tsga10* overexpression on zebrafish ISV sprouting and extension but not on the main vasculature. The effect of *tsga10* on ISV angiogenesis is probably indirect as we found that the expression of *tsga10* is mainly located in the head, trunk, and intersomitic boundaries; however, more experimental evidence is needed to support these conclusions.

The obtention of different morphological phenotypes after *tsga10* overexpression such as heart edema, small head, and abnormal

fin, is similar to the results of a human study in which TSGA10 deletions at 2q11.2, were found by whole-genome chromosomal microarray analysis in patients with developmental and congenital anomalies (Kaminsky *et al.*, 2011). These findings suggest additional roles for *tsga10* in zebrafish development, which need to be further investigated.

Zebrafish specific biology allows easy access to all developmental stages, and the optical transparency of embryos and larvae make real-time imaging of developing pathologies possible (Lieschke and Currie, 2007). Zebrafish has also become a valuable vertebrate model to study the process of vasculogenesis and angiogenesis among various *in vitro* and *in vivo* models (Chavez *et al.*, 2016, Thisse and Zon, 2002). Zebrafish have a closed circulatory system, and the anatomy of the vasculature, the vessel congregation process, and the molecular mechanisms of vasculogenesis are highly similar to those in humans (Isogai *et al.*, 2001). The external and fast embryonic development and the straightforward access to the developing vasculature due to available fluorophore-tagged strains, made zebrafish an ideal *in vivo* model to study the effects of gene modification on angiogenesis and tumor neovascularization (Gore *et al.*, 2012, Santoro, 2012).

Our study is the first to identify zebrafish *tsga10* and to generate *in vivo* data that demonstrate the role of *tsga10* overexpression in zebrafish angiogenesis at the early stages of development. There is no evidence yet that our results regarding the gene angiogenic effects could be the same in the human pathology; therefore, investigating the role of *tsga10* in mammalian experimental models of angiogenesis could be of interest. We are not aware of any mechanism linking *tsga10* overexpression and reduced angiogenesis, so our findings may open a new perspective to investigate

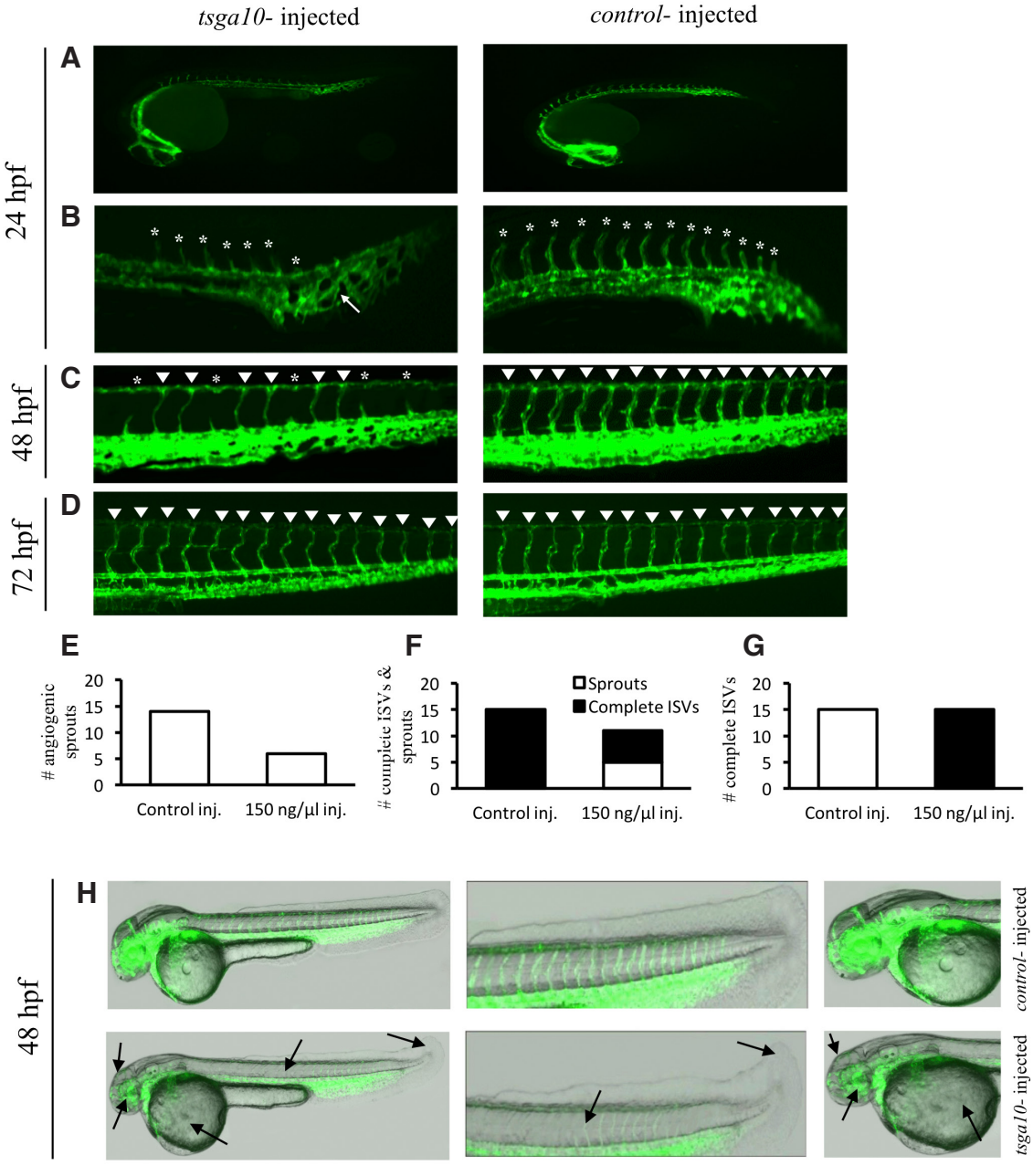

Fig. 4. *tsga10* overexpression causes angiogenic defects and morphological phenotypes. *Tg(flk1-EGFP) embryos were injected at one-cell stage with 150 ng/µl* tsga10 *mRNA (final concentration of 0.3 per embryo). Overexpression of* tsga10 *resulted in a decrease in the number of angiogenic sprouts in tail; see asterisks in* **(A,B)** *and incomplete formation of the caudal vein plexus; see arrow in* **(B)** *at 24 hpf* **(A,B,E)**, *a decrease in the number of complete ISVs in tail; see arrowheads in* **(C)** *at 48 hpf* **(C,F)**, *fewer brain vessels, a small head, heart edema, and an abnormally bent fin at 48 hpf; see arrows in* **(H)**. *Defective angiogenesis was recovered at 72 hpf* **(D,G)**.

those mechanisms. Knowing that zebrafish is a valuable model organism to study the *tsga10* function, and given the emerging evidence that TSGA10 has an important role in tumor angiogenesis and metastasis (Mansouri *et al.*, 2016), further studies using tumor cell xenograft in zebrafish are required to confirm the angiogenic inhibitory role of *tsga10* in cancer.

Materials and Methods

Zebrafish husbandry

All zebrafish experiments were performed under St. Michael's Hospital Animal Care Committee approved protocol ACC403. Zebrafish were maintained on a 14 h light/ 10 h dark cycle at 28°C in the Li Ka Shing Knowledge Institute Research vivarium. Embryos were collected and raised in E3 embryo medium (5mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, and 0.33 mM MgSO4), and treated with 0.2 mM 1-phenyl-2-thiourea (PTU) at 24 hpf to minimize pigmentation. The following genetic strains were used: wild-type Tupfel Long fin (TL) (Zebrafish International Resource Center (ZIRC)) and transgenic Tg(flk1:EGFP) (Jin *et al.*, 2005).

Bioinformatics analysis

Zebrafish genomic sequences were analyzed using the NCBI (National Center for Biotechnology Information) and the Ensembl zebrafish genome database. The Basic Local Alignment Search Tool (BLAST) was used to compare nucleotide and amino acid sequences to sequences from the NCBI database. *tsga10* promoter and transcription factor analyses were achieved using EPD and TRANSFAC databases, respectively. Multiple protein sequence alignment was obtained using the T-Coffee algorithm.

RT-PCR

Total RNA of wild-type TL zebrafish embryos was isolated from different developmental stages (1-cell stage to 120 hpf, 30 embryos/stage) and from adult tissues, including testis, fin, heart, eyes, brain, muscle, and unfertilized egg (14 RNA groups), using an RNeasy mini kit (Qiagen) and DNase treatment. For tissue dissection, adult fishes were anesthetized with 0.2% Tricaine and then euthanized by submersion in ice water for 15 minutes (Gupta and Mullins, 2010). Four dissected samples were used for each tissue RNA extraction. RNA was quantified using a NanoDrop Spectrophotometer and verified by gel electrophoresis on a 1% TAE-agarose gel. Total cDNA was synthesized from 1μg of total RNA using Superscript II reverse transcriptase kit (Invitrogen) and random hexamer primers following the manufacturer's protocol. Oligonucleotides TR1, 5'-AGGATCTTGAGACAACTCTAATAGC-3' (forward), and TR2, 5'-TGAGTCGAAGTATCTCCGAATTAGC-3' (reverse), were used to isolate a 450 bp *tsga10* DNA fragment from each total cDNA group. PCR was performed in a 25 μl volume using a Taq DNA polymerase (Qiagen), and consisted of an initial denaturation step (94°C for 2 min); 40 cycles of denaturation (94°C for 30 s), annealing (57°C for 30 s), and extension (72°C for 1 min); followed by a final extension step (72°C for 10 min). RT-PCR products were gel-purified using QIAquick Gel Extraction Kit (Qiagen) and cloned into the pCRII-TOPO vector (Invitrogen). We used zebrafish actin, beta 1 (actb1) as a reference gene. Oligonucleotides 5'- GAAATTGTCCGTGACATCAA -3' (Forward), and 5′- CACTGTGTTGGCATACAGGT -3' (Reverse), were used to amplify zebrafish actb1. PCR was performed in a 25 μl volume using a Taq DNA polymerase (Qiagen), and consisted of an initial denaturation step (94°C for 2 min); 36 cycles of denaturation (94°C for 30 s), annealing (57°C for 30 s), and extension (72°C for 1 min); followed by a final extension step (72°C for 10 min).

Riboprobe synthesis and whole-mount in situ *hybridization*

Whole-mount *in situ* hybridization was performed as previously described (Thisse and Thisse, 2008). To make the *tsga10* riboprobe, a 450 bp fragment (the same fragment used in the RT-PCR) was amplified from a mixture of testis and one-cell stage cDNA of WT TL zebrafish with the TR1 and TR2 primers. The PCR product was gel-purified using QIAquick Gel Extraction Kit (Qiagen), subcloned into the pCRII-TOPO vector (Invitrogen) and verified for the orientation and sequence of the insert. The resulting plasmid was used as a template for *in vitro* transcription to make a 450 bp *tsga10* RNA probe. Antisense and sense riboprobes were synthesized by *in vitro* transcription using 10x Dig RNA labeling mix (Roche) and SP6 and T7 RNA polymerases, respectively (mMESSAGE mMACHINE *in vitro* transcription kit, Invitrogen). WISH images were taken with a fluorescent dissection microscope (Leica M205 FA).

Generation of tsga10 *expression construct and capped* tsga10 *mRNA*

Total testis RNA was extracted from adult zebrafish using an RNeasy mini kit (Qiagen) and treated with DNase (tissue dissection explained above). RNA was quantified using a NanoDrop Spectrophotometer and gel electrophoresis on a 1% TAE-agarose gel. Total cDNA was synthesized from 1μg of total RNA using Superscript II reverse transcriptase kit (Invitrogen) and random hexamer primers following the manufacturer's protocol. The full-length protein coding sequence of zebrafish *tsga10* was PCR amplified using T1 primer, 5'-ATGTTGAGGCCTCGTCGC-3' (forward), and T2 primer, 5'-TCAGTCGTGAAGGTCCCTAA-3' (reverse), starting at the start and stop codons of the *tsga10* coding sequence, respectively. PCR was performed in a 25 μl volume using a Taq DNA polymerase (Qiagen), and consisted of an initial denaturation step (94°C for 2 min); 40 cycles of denaturation (94°C for 30 s), annealing (57°C for 30 s), and extension (72°C for 1 min); followed by a final extension step (72°C for 10 min). The PCR product was gel-purified using the QIAquick Gel Extraction Kit (Qiagen) and cloned into the pCRII-TOPO vector (Invitrogen). Clones with sense orientation were confirmed by restriction digestion (SpeI and EcoRV restriction enzymes, FastDigest) and sequencing. Capped mRNA was transcribed *in vitro* using the mMESSAGE mMACHINE T7 kit (Invitrogen) and the Poly A sequence added to the product of mMESSAGE mMACHINE T7 kit using Poly(A) tailing kit (Invitrogen), according to the manufacturer's instruction. The mRNA was ethanol-precipitated, resuspended in nuclease-free water, and stored in aliquots at -80°C.

tsga10 *mRNA microinjection and imaging*

We injected flk-GFP embryos with 150 ng/μl of *tsga10* mRNA and final concentration of 0.3 per embryo (n=80, injection volume=2 nl/embryo) using an N2 driven microinjector 5171 (Eppendorf, USA) through the chorion into the cell at the one-cell stage. The dye tracer phenol red was added to the mRNA before injection (0.1% final concentration) to track the successful microinjection. We also injected control flk-GFP embryos with nuclease-free water (n=80). After microinjection, embryos were raised at 28°C and imaged periodically using fluorescent dissection microscopy (Leica M205 FA) equipped with GFP fluorescence filter. To obtain the desired orientation, we anesthetized the embryos/larvae with 0.16 mg/ml of Tricaine and placed them in 2.5% methyl cellulose (Sigma). The mRNA microinjection was performed with three technical replicates.

Acknowledgements

We are grateful to Dr. Xiao-Yan Wen (Keenan Research Center for Biomedical Science, Li Ka Shing Knowledge Institute, St. Michael's Hospital) for providing the lab facility, the Animal Models Shared Resource (AMSR) from Georgetown University for the discussion and consultation, and Koroboshka Brand-Arzamendi for the technical assistance in whole-mount in-situ *hybridization.*

Competing interests

The authors declare that the research was conducted with no competing interests.

Funding

This research has been supported by the International Campus, Tehran University of Medical Sciences, and the Keenan Research Center for Biomedical Science, Li Ka Shing Knowledge Institute, St. Michael's Hospital (Xiao-Yan Wen).

References

BEHNAM, B., CHAHLAVI, A., PATTISAPU, J. and WOLFE, J. (2009). TSGA10 is Specifically Expressed in Astrocyte and Over-expressed in Brain Tumors. *Avicenna J Med Biotechnol* 1: 161-166.

BEHNAM, B., MODARRESSI, M.H., CONTI, V., TAYLOR, K.E., PULITI, A. and WOLFE, J. (2006). Expression of Tsga10 sperm tail protein in embryogenesis and neural development: from cilium to cell division. *Biochem Biophys Res Commun* 344: 1102-1110.

CHAVEZ, M.N., AEDO, G., FIERRO, F.A., ALLENDE, M.L. and EGANA, J.T. (2016). Zebrafish as an Emerging Model Organism to Study Angiogenesis in Development and Regeneration. *Front Physiol* 7: 56.

CHURCH, D.M., SCHNEIDER, V.A., GRAVES, T., AUGER, K., CUNNINGHAM, F., BOUK, N., CHEN, H.C., AGARWALA, R., MCLAREN, W.M., RITCHIE, G.R. *et al.,* (2011). Modernizing reference genome assemblies. *PLoS Biol* 9: e1001091.

DIANATPOUR, M., MEHDIPOUR, P., NAYERNIA, K., MOBASHERI, M.B., GHAFOURI-FARD, S., SAVAD, S. and MODARRESSI, M.H. (2012). Expression of Testis Specific Genes TSGA10, TEX101 and ODF3 in Breast Cancer. *Iran Red Crescent Med J* 14: 722-726.

FRATTA, E., CORAL, S., COVRE, A., PARISI, G., COLIZZI, F., DANIELLI, R., NICOLAY, H.J., SIGALOTTI, L. and MAIO, M. (2011). The biology of cancer testis antigens: putative function, regulation and therapeutic potential. *Mol Oncol* 5: 164-182.

GIBBS, R.A.WEINSTOCK, G.M.METZKER, M.L.MUZNY, D.M.SODERGREN, E.J.SCHERER, S.SCOTT, G.STEFFEN, D.WORLEY, K.C.BURCH, P.E. *et al.,* (2004). Genome sequence of the Brown Norway rat yields insights into mammalian evolution. *Nature* 428: 493-521.

GORE, A.V., MONZO, K., CHA, Y.R., PAN, W. and WEINSTEIN, B.M. (2012). Vascular development in the zebrafish. *Cold Spring Harb Perspect Med* 2: a006684.

GUPTA, T. and MULLINS, M.C. (2010). Dissection of organs from the adult zebrafish. *J Vis Exp*. 37. pii: 1717. doi: 10.3791/1717.

HAGELE, S., BEHNAM, B., BORTER, E., WOLFE, J., PAASCH, U., LUKASHEV, D., SITKOVSKY, M., WENGER, R.H. and KATSCHINSKI, D.M. (2006). TSGA10 prevents nuclear localization of the hypoxia-inducible factor (HIF)-1alpha. *FEBS Lett* 580: 3731-3738.

HELLSTEN, U., HARLAND, R.M., GILCHRIST, M.J., HENDRIX, D., JURKA, J., KAPITONOV, V., OVCHARENKO, I., PUTNAM, N.H., SHU, S., TAHER, L. *et al.,* (2010). The genome of the Western clawed frog *Xenopus* tropicalis. *Science* 328: 633-636.

HERBERT, S.P., HUISKEN, J., KIM, T.N., FELDMAN, M.E., HOUSEMAN, B.T., WANG, R.A., SHOKAT, K.M. and STAINIER, D.Y. (2009). Arterial-venous segregation by selective cell sprouting: an alternative mode of blood vessel formation. *Science* 326: 294-298.

HIRANO, T. (2006). At the heart of the chromosome: SMC proteins in action. *Nat Rev Mol Cell Biol* 7: 311-322.

HOWE, K. CLARK, M.D. TORROJA, C.F. TORRANCE, J. BERTHELOT, C. MUFFATO, M. COLLINS, J.E. HUMPHRAY, S. MCLAREN, K. MATTHEWS, L. *et al.,* (2013). The zebrafish reference genome sequence and its relationship to the human genome. *Nature* 496: 498-503.

ISOGAI, S., HORIGUCHI, M. and WEINSTEIN, B.M. (2001). The vascular anatomy of the developing zebrafish: an atlas of embryonic and early larval development. *Dev Biol* 230: 278-301.

JAKHESARA, S.J., KORINGA, P.G. and JOSHI, C.G. (2013). Identification of novel exons and transcripts by comprehensive RNA-Seq of horn cancer transcriptome in Bos indicus. *J Biotechnol* 165: 37-44.

JIN, S.W., BEIS, D., MITCHELL, T., CHEN, J.N. and STAINIER, D.Y. (2005). Cellular and molecular analyses of vascular tube and lumen formation in zebrafish. *Development* 132: 5199-209.

KAMINSKY, E.B., KAUL, V., PASCHALL, J., CHURCH, D.M., BUNKE, B., KUNIG, D., MORENO-DE-LUCA, D., MORENO-DE-LUCA, A., MULLE, J.G., WARREN, S.T. *et al.,* (2011). An evidence-based approach to establish the functional and clinical significance of copy number variants in intellectual and developmental disabilities. *Genet Med* 13: 777-784.

LIESCHKE, G.J. and CURRIE, P.D. (2007). Animal models of human disease: zebrafish swim into view. *Nat Rev Genet* 8: 353-367.

LINDBLAD-TOH, K., WADE, C.M., MIKKELSEN, T.S., KARLSSON, E.K., JAFFE, D.B., KAMAL, M., CLAMP, M., CHANG, J.L., KULBOKAS 3rd, E.J., ZODY, M.C. *et al.,* (2005). Genome sequence, comparative analysis and haplotype structure of the domestic dog. *Nature* 438: 803-819.

LY, L.L., YOSHIDA, H. and YAMAGUCHI, M. (2013). Nuclear transcription factor Y and its roles in cellular processes related to human disease. *Am J Cancer Res* 3: 339-346.

MANSOURI, K., MOSTAFIE, A., REZAZADEH, D., SHAHLAEI, M. and MODARRESSI, M.H. (2016). New function of TSGA10 gene in angiogenesis and tumor metastasis: a response to a challengeable paradox. *Hum Mol Genet* 25: 233-244.

MCCONKEY, E.H. (2004). Orthologous numbering of great ape and human chromosomes is essential for comparative genomics. *Cytogenet Genome Res* 105: 157-158.

MCKINNEY, M.C. and WEINSTEIN, B.M. (2008). Chapter 4. Using the zebrafish to study vessel formation. *Methods Enzymol* 444: 65-97.

MIRYOUNESI, M., NAYERNIA, K., MOBASHERI, M.B., DIANATPOUR, M., OKO, R., SAVAD, S. and MODARRESSI, M.H. (2014). Evaluation of *in vitro* spermatogenesis system effectiveness to study genes behavior: monitoring the expression of the testis specific 10 (Tsga10) gene as a model. *Arch Iran Med* 17: 692-697.

MOBASHERI, M.B., JAHANZAD, I., MOHAGHEGHI, M.A., AARABI, M., FARZAN, S. and MODARRESSI, M.H. (2007). Expression of two testis-specific genes, TSGA10 and SYCP3, in different cancers regarding to their pathological features. *Cancer Detect Prev* 31: 296-302.

MOBASHERI, M.B., MODARRESSI, M.H., SHABANI, M., ASGARIAN, H., SHARIFIAN, R.A., VOSSOUGH, P. and SHOKRI, F. (2006). Expression of the testis-specific gene, TSGA10, in Iranian patients with acute lymphoblastic leukemia (ALL). *Leuk Res* 30: 883-889.

MODARRESSI, M.H., BEHNAM, B., CHENG, M., TAYLOR, K.E., WOLFE, J. and VAN DER HOORN, F.A. (2004). Tsga10 encodes a 65-kilodalton protein that is processed to the 27-kilodalton fibrous sheath protein. *Biol Reprod* 70: 608-615.

MODARRESSI, M.H., CAMERON, J., TAYLOR, K.E. and WOLFE, J. (2001). Identification and characterisation of a novel gene, TSGA10, expressed in testis. *Gene* 262: 249-255.

REIMAND, K., PERHEENTUPA, J., LINK, M., KROHN, K., PETERSON, P. and UIBO, R. (2008). Testis-expressed protein TSGA10 an auto-antigen in autoimmune polyendocrine syndrome type I. *Int Immunol* 20: 39-44.

SANTORO, M.M. (2012). Modeling Tumor Angiogenesis in Zebrafish. In *Tumor Angiogenesis*, (Sophia Ran, Ed.) IntechOpen, DOI: 10.5772/26568.

SEMENZA, G.L. (2003). Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* 3: 721-732.

SMITH, C.J., OSCARSON, M., RONNBLOM, L., ALIMOHAMMADI, M., PERHEENTUPA, J., HUSEBYE, E.S., GUSTAFSSON, J., NORDMARK, G., MELONI, A., CROCK, P.A. *et al.,* (2011). TSGA10 - A target for autoantibodies in autoimmune polyendocrine syndrome type 1 and systemic lupus erythematosus. *Scand J Immunol* 73: 147-153.

THISSE, C. and THISSE, B. (2008). High-resolution *in situ* hybridization to whole-mount zebrafish embryos. *Nat Protoc* 3: 59-69.

THISSE, C. and ZON, L.I. (2002). Organogenesis--heart and blood formation from the zebrafish point of view. *Science* 295: 457-462.

YUAN, X., HE, J., SUN, F. and GU, J. (2013). Effects and interactions of MiR-577 and TSGA10 in regulating esophageal squamous cell carcinoma. *Int J Clin Exp Pathol* 6: 2651-2667.

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