Published in final edited form as:

Gene Expr Patterns. 2008 July ; 8(6): 389–396. doi:10.1016/j.gep.2008.05.001.

EXPRESSION OF EGFL7 IN PRIMORDIAL GERM CELLS AND IN ADULT OVARIES AND TESTES

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Abstract

We have previously reported the isolation and characterization of a novel endothelial-restricted gene, *Egfl7*, that encodes a secreted protein of about 30-kDa. We and others demonstrated that *Egfl7* is highly expressed by endothelial cells during embryonic development and becomes down-regulated in the adult vasculature. In the present paper we show that during mouse embryonic development, *Egfl7* is also expressed by primordial germ cells (PGC). Expression is down-regulated when PGCs differentiate into pro-spermatogonia and oogonia, and by 15.5 dpc *Egfl7* can no longer be detected in the germ line of both sexes. Notably, *Egfl7* is again transiently up-regulated in germ cells of the adult testis. In contrast, expression in the ovary remains limited to the vascular endothelium. Our results provide the first evidence of a non-endothelial expression of EGFL7 and suggest distinctive roles for *Egfl7* in vascular development and germ cell differentiation.

1. Results and Discussion

In a genetic screen for genes expressed in the cardiovascular system using a gene-trap approach in ES cells, we have identified a novel gene, EGF-like domain 7 (*Egfl7*) (Kuhnert and Stuhlmann, 2004; Fitch et al., 2004). The *Egfl7* gene spans 11.5-kb of chromosome 2 and encodes a 278 amino acids secreted protein that is highly conserved in vertebrates. *Egfl7* was independently identified by two other laboratories (Parker et al., 2004; Soncin et al., 2003). The protein contains an N-terminal signal peptide required for secretion, a cysteine-rich EMI domain, and two centrally located EGF domains. A region with similarity to an extracellular domain that is conserved in ligands of Notch receptors (Delta-Serrate-LAG-2) and is required for ligand-receptor binding is located within the first EGF domain (Fitch et al., 2004). EGFL7 acts as a chemoattractant for endothelial cells and embryonic fibroblasts. EGFL7 alone has no effect on the migration of vascular smooth muscle cells (VSMC) (Soncin et al., 2003; Campagnolo et al., 2005), but blocks PDGF-induced VSMC migration (Soncin et al., 2003). In addition, a fraction of secreted EGFL7 binds efficiently to the extracellular matrix of endothelial cells (Schmidt et al., 2007; M. Fitch and H. Stuhlmann, unpublished).

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We previously showed that embryonic expression of *Egfl7* is restricted to the vascular endothelium of the embryo proper and the yolk sac (Fitch et al., 2004). In the adult, expression is overall down-regulated in the quiescent vasculature, but remains detectable in highly vascularized adult organs, such as lung, heart, uterus, and ovary (Campagnolo et al., 2005). Moreover, *Egfl7* is transiently up-regulated during physiological angiogenesis in the pregnant uterus and upon vascular injury in the regenerating endothelium (Campagnolo et al., 2005). Together, these results show a correlation between *Egfl7* expression and an active, proliferative state of the endothelium. Studies in zebrafish embryos demonstrated that knock-down of *Egfl7* results in disruption of vascular tube formation, suggesting that *Egfl7* plays a crucial role during vascular development (Parker et al., 2004).

Here we report that a second site of *Egfl7* expression is detected in a temporal window during embryogenesis in primordial germ cells, the precursors of the germ cells in both sexes. In addition, we demonstrate that in the adult, *Egfl7* expression is **de novo** up regulated in the male germ line, while in the ovaries its expression is restricted to endothelial cells. Importantly, analysis of the avascular cloche mutant indicated that *Egfl7* in zebrafish is not expressed outside the cardiovascular system (Parker et al., 2004). This suggests an important difference regarding a potential involvement of *Egfl7* in germ cell and gonad development between mammals and fish.

1.1. *Egfl7* expression in pre-implantation embryos

In a previous study we showed that *Egfl7* mRNA is already detectable at the blastocyst stage and in embryonic stem (ES) cells that are derived from the inner cell mass of the blastocyst. In early post-implantation embryos its expression is localized to the extra-embryonic mesoderm and in the most anterior and posterior regions of the embryo proper (Fitch et al., 2004). To determine the developmental stage at which *Egfl7* is first expressed, RT-PCR analysis was performed on unfertilized oocytes and staged early pre-implantation embryos. Using primers that discriminate against genomic DNA, a 348-bp *Egfl7*-specific PCR product was amplified from 8-cell stage embryos, while no band was detected from 1-cell or 2-cell stage embryos or unfertilized ovulated oocytes (metaphase II oocytes) (Figure 1). Undifferentiated embryonic stem cell (ES) mRNA was used as a positive control and yielded a strong RT-PCR band. By the 8-cell stage, mRNA of maternal origin is already degraded and synthesis of embryonic RNA has begun (Schultz 1993). We therefore conclude that the *Egfl7*-specific amplification product obtained from 8-cell morulas reflects the onset of *Egfl7* transcription during mouse embryonic development.

1.2. *Egfl7* expression in primordial germ cells

During post-implantation development, EGFL7 expression was found to be restricted to the developing vascular system and its progenitors in the yolk sac (Fitch, et al, 2004; Soncin, et al, 2004; Parker, et al., 2004). RNA *in situ* hybridization studies also showed that in 7.5 dpc embryos, *Egfl7* mRNA can be detected in the extra-embryonic mesoderm close to the posterior end of the proximal epiblast (Fitch et al., 2004), at sites where primordial germ cells (PGCs) are located. This observation stimulated a thorough analysis of EGFL7 expression in PGCs using affinity-purified polyclonal anti-EGFL7 antibodies (Fitch et al., 2004, Campagnolo et al., 2005).

Between 8.5 and 10.5 dpc, PGCs are defined as migratory, since they move from the base of the allantois, migrate along the hindgut endoderm, and finally through the dorsal mesentery they reach the urogenital ridge. By 11 dpc, PGCs have entered the gonads and are defined as post-migratory cells. However, they are not yet organized in their definitive structures, cords and follicles, thus rendering male and female gonads morphologically indistinguishable until 12.5 dpc. Double immunostaining using specific antibodies against EGFL7 and the germ cell

marker SSEA1, respectively, shows that isolated PGCs from 8.5 dpc embryos (Fig. 2A, panels a,d), as well as 10.5 dpc migrating PGCs (Fig. 2A, panels b,e) do not express EGFL7 at significant levels. In contrast, PGCs immuno-magnetically sorted from 11.5 dpc gonads (DeFelici, 1998) displayed co-immunolocalization of EGFL7 and SSEA1 (Fig. 2A, panels c,f). After each PGC purification, we routinely performed staining for alkaline phosphatase, another marker for PGCs, to assure purity of the PGC population (not shown). Expression of EGFL7 in 11.5 dpc PGCs was confirmed by Western blot analysis (Fig 2D). A 30–32-kDa EGFL7 immuno-reactive band was detected in PGC extracts, and its specificity was verified by comparing with total cell extracts from 293 cells transiently transfected with an EGFL7 expression vector (Fitch et al., 2004), the empty vector, and an unrelated control vector, respectively (Fig. 2D). However, the intensity of the band was low in 11.5 dpc PGCs when compared to cell extracts from EGFL7-transfected HEK293 cells. A band of low intensity was also detected in the somatic cell fraction of 11.5 dpc gonads, presumably due to the presence of endothelial cells lining the primitive vasculature of the gonads. Next we investigated EGFL7 expression on cryostat sections of male and female gonads starting at 12.5 dpc, when sex specification becomes morphologically evident, and up to 15.5 dpc, when proliferation of germ cells has ceased, pro-spermatogonia have entered mitotic arrest, and oocytes are arrested at the prophase of the first meiotic division. Germ cells were identified by co-staining with anti-GCNA1 antibodies, which stains the nuclei of pro-spermatogonia and oocytes (Enders and May 1994). Starting around 13.5 dpc, we observed a progressive down-regulation of EGFL7 protein in the germ cells in both sexes (Figure 2B and 2C), and by 15.5 dpc, EGFL7 expression was undetectable (Fig. 2B, panels c,g,m, and 2C, panels c,g,m).

We also investigated if expression of EGFL7 is altered in the gonads of White-spotting (W) mice. Mice homozygous for the W mutation contain very few PGCs that reach the gonads, and they die soon after birth as a result of severe anemia (Mintz and Russell, 1957). We performed double immuno-localization of GCNA1 and EGFL7 on sections of gonads from 13.5 dpc W^{-/-} female embryos and wild type littermates. Clearly, EGFL7-positive germ cells were absent in W^{-/-} gonads (compare Fig. 3D,F with Fig. 3C,E), while EGFL7 persisted in a vascular restricted expression that was comparable between mutant and control wild type mice (Fig 3B with Fig. 3A).

Together, these results show that EGFL7 expression in PGCs is regulated in a temporal manner and is only detectable in proliferating, post-migratory PGCs. Since EGFL7 interacts with extracellular matrix components (Schmidt et al., 2007; M. Fitch and H. Stuhlmann, unpublished), it is possible that EGFL7 is involved in homing of PGCs once they reach their new environment in the gonads, or in the organization of testis cords and follicles. The generation of mice with a conditional knock-out in PGCs will help to address this possibility. Recently knock-out mice for *Egfl7* have been generated (Schmidt et al., 2007), 50% of which die in utero between 13.5 and 15.5 dpc. Interestingly, although those mice that developed to term showed a delay in vascular development, fertility appeared not to be impaired; however, the development of PGCs and embryonic gonads was not examined in these mutants.

1.3. *Egfl7* expression in adult male germ cells

The temporal expression of EGFL7 in PGCs prompted us to investigate if EGFL7 could be detected at later stages during germ cell differentiation and gonadal development. Using RNA *in situ* hybridization on postnatal testis, we found that *Egfl7* transcripts in the pre-pubertal testes (P19) were localized exclusively in the capillary network that surrounds seminiferous tubules (Fig. 4 A and D, arrows). In contrast, in the testis of adult mice, *Egfl7* expression is also detected in germ cells. In the seminiferous tubules, germ cells are spatially organized in a pluristratified epithelium, and they are distributed, following their maturational stages, from the periphery to the center of the tubules. Spermatogonia, the stem cells of the testis, reside in the outer ring of

the tubules adjacent to the basal membrane. Moving toward the lumen reside spermatocytes which enter meiosis to give rise to haploid spermatids that are located closer to the lumen of the tubules. During spermiogenesis, spermatids undergo profound morphological and functional modifications leading ultimately to the differentiation of spermatozoa, which become released into the lumen. *Egfl7* transcripts were specifically identified in a ring of cells surrounding the lumen of the seminiferous tubules (Fig. 4 B and E, arrowhead), consistent with being expressed by post-meiotic spermatids, but not spermatocytes. Of interest, signal was detected only on some of the tubules, reflecting that they are at different stages of maturation, and consistent with *Egfl7* expression being restricted to specific stages of spermatid maturation (steps 1–8).

To confirm the expression of EGFL7 on post-meiotic cells, we performed immunostaining of testes sections using anti-EGFL7 antibodies. EGFL7 protein was detected in capillaries of the interstitial compartment (not shown), and in a subpopulation of cells within the tubules (Fig. 5A and B, arrowheads). This population can be identified as post-meiotic germ cells by the smaller size of their nuclei and the presence of a single nucleolus. In contrast, cells with larger, heterochromatic nuclei, located closer to the tubular basal membrane were negative for EGFL7 (Fig. 5A and B, arrows). Immunofluorescence analysis was also performed on stage-specific, enriched populations of adult male germ cells, obtained by cell elutriation (Sette et al, 1999; Meistrich 1977). These experiments revealed the presence of a strong signal associated with spermatids and spermatozoa (Fig. 5C and D, respectively). No staining was detected on spermatocytes (not shown), confirming the results from RNA *in situ* hybridization. Western blot analysis using cell extracts from germ cells populations confirmed the results obtained from immunostaining (not shown). Together, these data demonstrate restricted expression of EGFL7 during male germ cell differentiation, and they suggest a possible role in spermatid maturation and sperm cell release. In order to test this hypothesis, we are currently generating a conditional knock-out of *Egfl7*.

We also analyzed *Egfl7* expression in pre-pubertal (not shown) and mature ovaries. In contrast to the male gonads, *Egfl7* transcripts are exclusively located on the endothelium of the capillaries surrounding the follicles and in the corpus luteum, while no signal was detected on maturing oocytes (Fig. 4C and F, arrows; compare also Fig. 1). Thus, our results demonstrate that post-natal expression of *Egfl7* is restricted to the endothelium and the male germ line.

2. Experimental procedures

2.1. Isolation of pre-implantation embryos and primordial germ cells

Outbred CD1 mice (Charles River, Calco, Italy) were housed and mated under standard laboratory conditions. Six weeks-old females were induced to superovulate by administration of pregnant mare's serum gonadotropin (PMSG, 5U.I./female) and human chorionic gonadotropin (hCG, 5U.I./female) with a 48 hours interval, and then mated with CD-1 males. Pre-implantation embryos at various developmental stages were collected (Nagy et al, 2003). Pregnant females were sacrificed at staged post-coital times (midday of the day of the vaginal plug was considered 0.5 day post-coitum (dpc)). Metaphase II oocytes and embryos were collected in RLT buffer (Qiagen) and stored at -80°C . To isolate primordial germ cells (PGCs) at 8.5 dpc, embryos were collected in enriched M2 medium (M2 supplemented with 1mg/ml BSA, 0.2 mg/ml N-Acetyl-Cystein and 0.036 mg/ml sodium pyruvate). The posterior half of the embryo, including the allantois, was dissected using fine needles, and digested for 5 min at RT in 0.5% trypsin. Digestion was blocked with FBS, and the suspension pipetted several times in order to completely dissociate the cells. After a short centrifugation step at 1000 rpm, cells were resuspended in M2 without BSA and spotted on poly-L-lysine coated slides for 10 min, followed by fixation in 2% paraformaldehyde. PGCs from 11.5 dpc embryos were isolated according to DeFelici (1998a and 2000). Gonads were dissociated in 0.5% trypsin -EDTA and

the resulting cell suspension incubated with anti-SSEA1 antibody, or EMA-1 (Hybridoma Bank), respectively, in enriched M2 at 4°C. After 45 min of rotation, 10 µl magnetic microbead-conjugated anti-mouse IgM (Miltenyi Biotec GmbH) were added to the suspension and incubated for a further 20 min at 4°C. PGCs were sorted by using the MiniMACS System according to the manufacturer's specifications (Miltenyi Biotec). 10 µl of the sorted cell suspension was spotted on a slide and stained for alkaline phosphatase (DeFelici 2000) to evaluate the efficiency of purification. Sorted PGCs with at least 90–95% purity were spotted on slides for immunofluorescence analysis or pelleted and stored at –80°C for Western blot analysis. Whole 10.5 dpc embryos were embedded in OCT and frozen on N2 vapor. Sections containing PGCs were identified by alkaline phosphatase staining, and the adjacent sections were stained for SSEA1 and EGFL7. Male and female urogenital ridges from 12.5, 13.5 and 15.5 dpc CD1 embryos, and 13.5 dpc White-spotting (W) embryos, were collected in M2, embedded in OCT and frozen on N2 vapor. Eight micron sections were fixed in methanol for 5 min at –20°C, air dried and processed for immunofluorescence.

2.2. Isolation of adult male germ cells

After removal of their albuginea membrane, testes from adult CD1 mice (Charles River Italia) were digested for 15 min in minimum essential medium (MEM, Invitrogen.), 2 mM lactate, 1 mM sodium pyruvate (Sigma) and 0.25% (w/v) collagenase (type IX, Sigma), at 30°C under constant shaking. Tubules were sedimented and incubated in 0.5% trypsin-EDTA for 30 min at 30°C. Undigested tissue debris were sedimented at unit gravity for 10 min and the supernatant containing the released germ cells was collected, pelleted at 1200 g and resuspended in elutriation medium (120.1 mM NaCl, 4.8 mM KCl, 25.2 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄·7H₂O, 1.3 mM CaCl₂, 11 mM glucose, 0.2% BSA). Spermatocytes, round spermatids, and elongated spermatids were enriched by elutriation of the single cell suspension as described previously (Meistrich, 1977; Sette et al., 1999). Homogeneity of cell populations, determined by morphological analysis, ranged between 80 and 85% (pachytene spermatocytes) and 95% (round spermatids). Spermatozoa from cauda were obtained from mature mice as described by Sette et al (1997).

2.3. RNA in situ hybridization

Procedures for RNA *in situ* hybridization were essentially as previously described (Campagnolo et al, 2005; Sassoon, 1993). Paraffin embedded pre-pubertal and pubertal mouse testis and mature ovaries were sectioned, de-paraffinized and digested with Proteinase K, followed by 16 hr. hybridization with a ³⁵S-[UTP]radiolabeled full-length *Egfl7* riboprobes (final probe concentration was 35 dpm/ml in hybridization buffer). Sense control probes were synthesized for all experiments. After a 5-day exposure, slides were counter-stained with hematoxylin and eosin. Dark field and bright field photographs were taken using a Zeiss Axioplan2 microscope with an attached digital camera (AxioCam, Zeiss). For immunofluorescence analysis of testes sections, adult mouse testes were dissected, cut in half and OCT embedded.

2.4. Western blot analysis

PGCs and somatic cells collected and purified from one hundred 11.5 dpc gonads were homogenized in 2x Laemmli buffer. Denatured protein samples were resolved on 10% SDS-polyacrylamide gels and electro blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore Corporation, Bedford, MA) using standard procedures. Adult germ cells were homogenized in lysis buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mg/ml SDS, 15 mM MgCl₂, 1 mM EGTA, 2 mM PMSF, 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin, 0.2 U/ml aprotinin, and 50 mM benzamidine), followed by centrifugation at 13000g for 30 min to remove the insoluble material. Protein concentrations were evaluated by

Bradford assay. Pre-stained molecular weight standards (Bench Mark Pre-stained, Invitrogen) and 15 µg of denatured proteins were resolved on 10% SDS gels and transferred to PVDF membranes. Filters were incubated with an affinity purified rabbit polyclonal anti-EGFL7 antibody (1:250 dilution) (Fitch et al., 2004). As a loading control, blots were stripped and re-probed with mouse anti-β-tubulin antibody (1:1000 dilution). Horseradish peroxidase conjugated anti-rabbit IgG and anti-mouse IgG (Amersham Biosciences, Piscataway, NJ) were used at a 1:10000 and 1:5000 dilution, respectively. Specific bands were detected with SuperSignal West Pico Chemiluminescent Substrate (Pierce).

2.5. Immunofluorescence and histochemical analysis

For immunofluorescence analysis, anti-EGFL7 and anti-SSEA1 antibodies were used at 1:200 and 1:100 dilutions, respectively. Germ cells from 12.5, 13.5 and 15.5 dpc embryos were identified by anti-GCNA1 antibody (a kind gift from Dr. G.C. Enders, University of Kansas Medical Center, Kansas City, KS). Phalloidin FITC-labeled (Sigma) was used to stain spermatozoa tails. Secondary antibodies were Cy3-labelled goat anti-rabbit IgG, AlexaFluor 488-labeled anti-mouse IgM, and AlexaFluor 488-labeled anti-rat IgM, all used at a 1:500 dilution. Control slides were incubated with rabbit IgG and rat or mouse IgM in the same conditions as the primary antibodies. Fluorescent images were taken using a Zeiss Axioplan2 microscope. After immunofluorescence analysis, adult mouse testis sections were stained with hematoxylin and eosin (H&E) according to standard protocols. The procedure of H&E staining caused the specimen to shrink slightly in size..

2.6. RT-PCR Analysis

RNA from pre-implantation embryos was isolated using the RNeasy Micro Kit (Qiagen). Total RNA was reverse transcribed using oligo dT with the Superscript First Strand Synthesis System (Invitrogen) according to the manufacturer's protocol. PCR was performed using 2 µl of the reverse transcriptase reaction in a volume of 25 µl with Platinum Taq polymerase (Invitrogen). PCR conditions were as follows: initial denaturation at 95°C (5 min) followed by 38 cycles of denaturation at 95°C (1 min), annealing at a temperature indicated below (1 min), and extension at 72°C (1 min). Amplified PCR products were analyzed by electrophoresis on a 1% agarose gel. PCR primer pairs were designed to span introns in order to avoid amplification of genomic DNA. Sequences were as follows:

<i>Egfl7</i> (T _A 59°C):	5'-GAGCATGGGCTACAAGATCC -3', 5'-TGGAGACAATCCCCACCTTA-3';
<i>Hprt</i> (T _A 58°C):	5'-CTGCTGGATTACATTAAAGCACTG-3', 5'-CTGAAGTACTCATTATAGTCAAGG -3';

Acknowledgements

We would like to thank Professor Massimo De Felici and Professor Susanna Dolci for precious advises and critical reading of the manuscript, and Graziano Bonelli for graphic assistance. The monoclonal antibody SSEA1 (developed by B.B. Knowles and D. Solter) was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, USA. Support for this study was provided by Public Health Service Grant NIH R21 HL072270 and NIH R01 HL082098 to H.S.

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Figure 1.

RT-PCR analysis of *Egfl7* expression in pre-implantation mouse embryos. Using intron spanning primers for *Egfl7*, a band of 348 bp was amplified from RNA isolated from ES cells and 8-cell stage embryos, but not from 1- and 2-cell stage embryos, and ovulated oocytes. Hprt primers were used as an amplification control.

Figure 2. Expression of EGFL7 in primordial germ cells (PGCs), gonocytes and oogonia

A: immunofluorescence analysis of EGFL7 and SSEA1 expression in isolated PGCs at 8.5 dpc (a, d, g) and 11.5 dpc (c, f, i) and in sagittal sections of 10.5 dpc embryos (b, e, h). Alkaline phosphatase staining was used to identify sections containing PGCs; adjacent sections were then incubated with antibodies. Arrows point to a migrating germ cell identified by the SSEA-1 antibody (e) that appears negative for EGFL7 (b); an EGFL7-positive and SSEA1-negative blood vessel is shown in b and e, respectively (arrowheads). The inserts show migrating PGCs and the arrows identify the cell shown at a higher magnification in b, e and h. a, b, c: EGFL7; d, e, f: SSEA1; g, h, i: Hoechst. Magnification 100x;

B: Immunofluorescence analysis of EGFL7 and GCNA1 on cryostat sections of embryonic mouse testis from 12.5 dpc, 13.5 dpc (longitudinal sections) and 15.5 dpc (cross section). Arrows indicate location of testicular cords. a, b, c: EGFL7; e, f, g: GCNA1; i,l,m,: merge of EGFL7 and GCNA1; d and h: section of 15.5 dpc testis incubated with non-specific rabbit IgG and rat IgM, respectively. Magnification 40x.

C: Immunofluorescence analysis of EGFL7 and GCNA1 on cryostat sections of embryonic mouse ovaries from 12.5dpc up to 15.5 dpc. a, b, c: EGFL7; e ,f ,g: GCNA1; i,l,m: merge of EGFL7 and GCNA1; d and h: section of 15.5 dpc ovaries incubated with non-specific rabbit IgG and rat IgM, respectively. Magnification 40x.

D: Western blot analysis of EGFL7 expression in PGCs isolated from 11.5 dpc embryos. Somatic cells represent the flow through fraction of the immuno-magnetic purification and presumably contain endothelial cells. Cell extract from 293 cells transiently transfected with an Egfl7 expression vector, the empty vector and an unrelated vector (pEGFP-C1), were used as positive and negative controls, respectively. Anti-tubulin antibody was used as a loading control.

Figure 3.

Immunofluorescence analysis of EGFL7 in gonads from 13.5 dpc White-spotting (W) females. Germ cells, identified by using the anti-GCNA1 antibody, were present in the wild type but not in the W^{-/-} homozygous mutants (C and D). Wild type germ cells were also positive for EGFL7 (compare A and B: some of the germ cells were indicated by arrows). No evident differences in the vascular EGFL7 were observed (arrowheads depict some of the capillaries). A–B: EGFL7; C–D: GCNA1; E–F: Hoechst. Magnification 40X.

Figure 4.

In situ hybridization analysis of *Eglf7* in mouse testes and ovaries. A: dark-field image of a pre-puberal mouse testis section hybridized with an *Eglf7* antisense probe showing expression of *Eglf7* in the vasculature surrounding the tubules (arrows); B: adult mouse testis section hybridized with an *Eglf7* antisense probe. The grains form a ring (arrowhead) surrounding the lumen of seminiferous tubules (*), a presumptive localization of post-meiotic germ cells; in situ signal is also present at low levels on the interstitial vasculature (arrows). C: *Eglf7* in situ hybridization analysis of adult mouse ovaries showing *Eglf7* expression limited to the vasculature surrounding the follicles (arrows). D, E, F: bright-field images of A, B and C, respectively. G and H sections of adult testis hybridized with an *Eglf7* sense control probe. Magnification 20X.

Figure 5.

Immuno-fluorescence analysis of EGFL7 expression in adult mouse testis and isolated adult germ cells. A-D: cross sections of adult mouse testis showing two seminiferous tubules (A,B and C,D, respectively); arrows in A and B indicate few of the EGFL7 positive spermatids. The tubule in C shows EGFL7 expression on spermatozoa; A, C: anti-EGFL7 antibody; B: Hoechst staining; D: hematoxylin and eosin counterstaining of section shown in C. Due to the H&E staining procedure, the tubule in D appears slightly shrunk compared to the corresponding image in C. Asterisks indicate the lumen of the tubules. E: spermatids isolated from adult testes by elutriation show strong immunoreactivity to the EGFL7 antibody. F: EGFL7 was also detected on the head of caudal sperm cells, possibly associated with the acrosomal vesicle;

sperm tails were stained using FITC-phalloidin. G: control slide of sperm cells incubated with non-specific rabbit IgG and stained with FITC-phalloidin. H: control slides of spermatids incubated with non-specific rabbit IgG. I: Hoechst staining of H. Magnification in A–D: 40X. Magnification in E–I: 100X.