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Transgenic Mouse Sperm that Have Green Acrosome and Red Mitochondria Allow Visualization of Sperm and Their Acrosome Reaction *in Vivo*

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Abstract: In the present paper, we introduce a transgenic mouse line whose sperm express green fluorescent protein (GFP) in their acrosome and red fluorescent protein (RFP) in their mitochondria [*B6D2F1- Tg(CAG/su9-DsRed2, Acr3-EGFP)RBGS002Osb*]. The dual fluorescent sperm showed normal fertilizing ability in both *in vivo* and *in vitro* fertilization and the sperm could be observed through uterine and oviductal walls when female reproductive tracts were dissected out and placed under excitation light. This characteristic could facilitate examination of sperm migration inside the female reproductive tract as well as facilitating *in situ* live imaging of the acrosome reaction, the details of which have remained elusive.
Key words: live imaging, oviduct, sperm

Fertilization is a mysterious phenomenon that is carried out between an ovulated egg and one of hundreds of millions of sperm that have been ejaculated into the female reproductive tract in human and other mammalian species. In order to elucidate the mechanism of fertilization, researchers established an *in vitro* fertilization (IVF) system in which oocytes collected from oviducts are mixed with sperm incubated in a "capacitating" medium [8]. Although eggs can be fertilized by sperm in the IVF system and the fertilized eggs result in healthy pups when transferred into pseudopregnant females, the IVF system is very different from real fertilization *in vivo*. Under natural conditions, only a part of the sperm in the uterus is considered to migrate into the oviduct. In mice and rats, the entrance forms a conical projection into the uterus called colliculus tubarius. Within the lumen of the junction, there are large and small folds in the mucosa designed to entrap sperm and prevent further ascent [6]. Histological sections and direct observation of sperm through the oviductal wall have revealed that the sperm which migrate into the oviduct are bound to the oviductal wall at the isthmus area [5]. The sperm found in the isthmus are attached by their heads to the mucosal epithelium and are believed to be awaiting ovulation there. It was postulated that sensing ovulation, a proportion of the sperm in the isthmus detaches from the oviductal wall and migrates into the ampulla part of the oviduct where fertilization occurs [5]. As reported pre-

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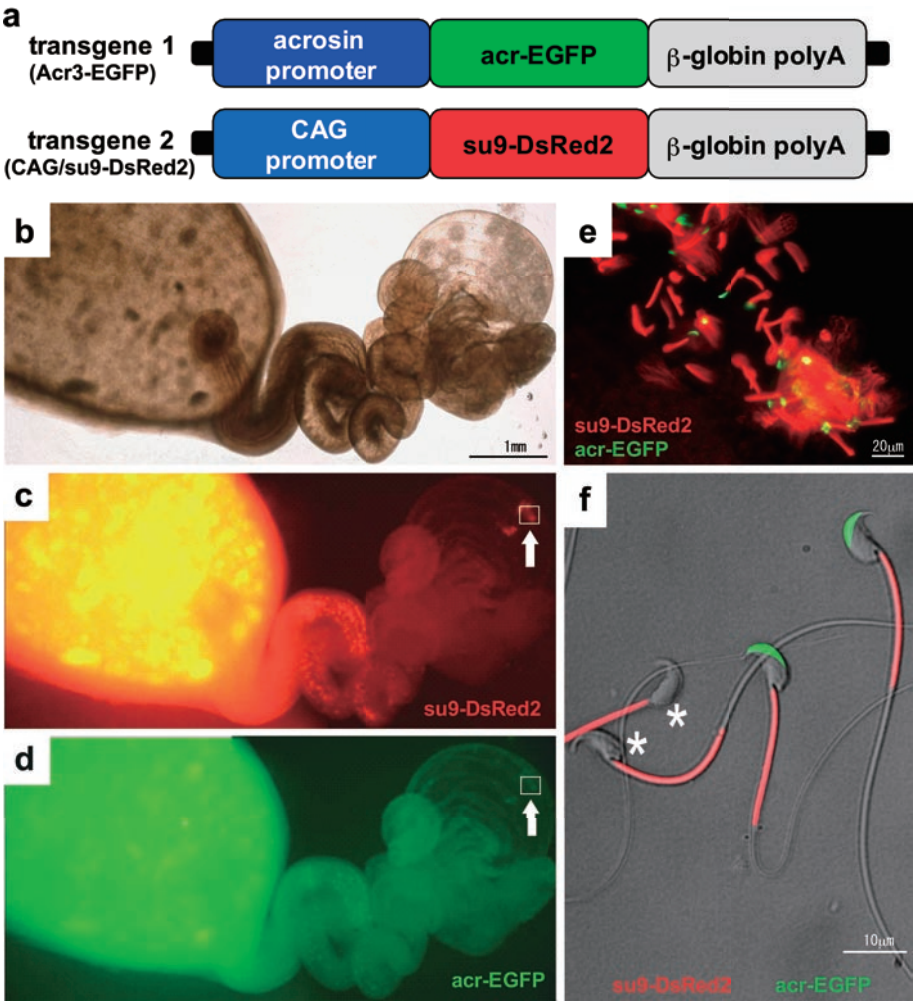

Fig. 1. Live imaging of sperm inside the female reproductive tract. a) RFP (DsRed2) was designed to be expressed ubiquitously under the CAG promoter with a mitochondrial import signal sequence of Atp5g1 (su9) and the GFP was designed to be expressed under the acrosin promoter with a proacrosin signal sequence [3]. One of the resultant transgenic mouse lines was designated as *B6D2F1- Tg(CAG/su9-DsRed2, Acr3-EGFP)RBGS002Osb* (red body and green sperm). The RBGS males were mated with wild-type females and the sperm were observed through the uterine and oviductal walls: b) normal lighting, c) observation of RFP, d) observation of GFP. Most of the ejaculated sperm resided in the uterus and showed strong fluorescence, while only a limited number of sperm were seen as individual dots in the oviduct. e) Magnified and double-exposed views of the ampulla portion of the oviduct surrounded by a square indicated by arrows in b) and c). Since the oviduct and sperm are alive and moving, the distribution of the sperm photographed in e) is somewhat different from those in c) and d). Note that acrosome reacted sperm and acrosome intact sperm are clearly distinguishable from each other. f) Transgenic sperm before and after the acrosome reaction. Acrosome-reacted sperm are shown with asterisks.

viously, wild-type sperm can be seen through the oviductal wall [5]. However, wild-type sperm are very difficult to observe, especially when their tails are not vigorously moving. Therefore, it is not clear what percentage of sperm this observation represents. The difficulty of observing the sperm has hindered the study of the mechanisms that regulate sperm residing in and leaving the isthmus for the ampulla.

We established "green sperm," which allowed us to observe the acrosome reaction [3, 4]. We were also able

to examine sperm localization inside the female reproductive tract and showed that *Adam3*-disrupted sperm were unable to migrate into the oviduct after coitus [7]. However, that transgenic mouse line has intrinsic drawbacks, as GFP is expressed inside the acrosome. The acrosome reaction is the prerequisite for sperm to acquire fertilizing ability and discharges the content of the acrosome by exocytosis. Thus, in that transgenic mouse line, the acrosome reacted sperm lost their fluorescence. To observe sperm through the oviductal wall irrespective of their acrosomal status, another marker that remains in sperm even after the acrosome reaction was required. To achieve this, we established transgenic mouse sperm which express GFP in the acrosome and RFP (DsRed2) in their mitochondria.

A fragment (222 bp) containing the mitochondrial import signal of the mouse *Atp5g1* [ATP synthase Fo complex subunit 9 (*su9*)] was amplified using mouse brain cDNA with two PCR primers, su9-forward: 5'-CCCAAGCTTGAGATTGAAAAATGCAGACCA-3' (carrying *Hin*dIII site at the 5' end) and su9-reverse: 5'-CGGGATCCGTGTCGATGTCCCGGGAAATGA-3' (carrying *Bam*HI site at the 5' end). The amplified DNA fragment was cleaved with *Hin*dIII and *Bam*HI and cloned into the multiple cloning site of pDsRed2-N1 (Clontech. Lab). A DNA fragment (*Hin*dIII/*Not*I) carrying *su9* and *DsRed2* sequences was excised out and both ends were ligated to *Eco*RI linkers. After cleaving with *Eco*RI, the DNA fragment was cloned into the *Eco*RI site of pUC-CAGGSneo.

The transgene Acr3-EGFP, in which EGFP with a proacrosin signal peptide is connected to the acrosin promoter, was prepared as described previously [3]. The *Xba*I/*Hin*dIII fragment of Acr3-EGFP and *Sal*I/*Hin*dIII fragment of CAG/su9-DsRed2 fragment (Fig. 1a) were co-injected into B6D2 F_1 × B6D2 F_1 fertilized eggs. Two transgenic mouse lines were obtained and in both of the lines, the males showed normal fertilizing ability when mated with wild-type females. All males also showed normal fertilizing ability in an IVF system (data not shown). After observing the vaginal plug, the female reproductive tracts were dissected out and examined under both normal and fluorescence excitation light. As shown in Fig. 1b, the colliculus tubarius can be seen through the uterine wall. The oocytes are also visible in the ampulla part of the oviduct. In this condition, the sperm are basically not visible unless the tail movement is detected under higher magnification. However, under a fluorescence microscope, the location of the sperm was clearly visible owing to their green (acrosome) and red (midpiece; mitochondria) fluorescence even though they were hardly moving (Figs. 1c–f).

Sperm localization was detectable by red fluorescence even after the acrosome reaction (Fig. 1e). Importantly, this characteristic enables us to observe acrosomal status and sperm localization inside the oviduct. Recently, it was reported that in five out of five gene-disrupted mouse lines, males became infertile by the loss of the ability of sperm to migrate into the oviduct [7]. Thus, sperm migration into the oviduct was indicated to be critical for successful fertilization. The transgenic mouse line reported in the present paper could be helpful in elucidating the sperm migration mechanism when used in combination with various infertile model mouse lines. It also provides a useful means of clarifying the timing of the acrosome reaction *in vivo*, which remains imprecisely understood. The transgenic mouse line reported here has been submitted to Riken BRC [1] and CARD [2] and is open to the scientific community, if used for academic research purposes.

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