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Disruption of ADAM3 Impairs the Migration of Sperm into Oviduct in Mouse[1]

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ABSTRACT

Sperm from four different gene-disrupted mouse lines (*calmegin* [*Clgn*], *Adam1a*, *Adam2*, and *Ace*) are known to have defective zona-binding ability. Moreover, it is also reported that the sperm from all of these mouse lines exhibit another common phenotype of impaired migration into oviduct despite the large number of sperm found in uterus after coitus. On the other hand, the sperm from the *Adam3*-disrupted mouse line was reported to have defects in binding ability to zona, but were able to move into the oviduct. In order to clarify the difference, we investigated the migration of ADAM3-null sperm into oviduct precisely by visualizing the sperm by using acrosin-green fluorescent protein as a tag. As a result, in contrast to previous observations, it was demonstrated that the *Adam3*-disrupted sperm were unable to migrate into the oviduct after coitus. It was ultimately shown that, in five out of five different gene-disrupted mouse lines, the phenotype of impaired sperm binding to zona pellucida was accompanied by the loss of ability of sperm to migrate into the oviduct. This indicates a close relationship between the two phenomena, and also that sperm migration into the oviduct is a crucial step for fertilization.

female reproductive tract, fertilization, gamete biology, male sexual function, sperm motility and transport

INTRODUCTION

Fertilin is one of the most intensively investigated proteins in the study of the mechanism of fertilization [1]. Fertilin was initially reported as a heterodimer of fertilin α and β, which are now officially called *Adam1* (a disintegrin and metallopeptidase domain 1) and *Adam2* (a disintegrin and metallopeptidase domain 2), respectively. In mouse, two *Adam1* genes have been found, and are named *Adam1a* and *Adam1b* [2]. Both ADAM1A and ADAM1B are reported to form a heterodimer with ADAM2, and are expressed in germ cells in separate differentiation steps [3]. ADAM1A/ADAM2 is found only in testis, and is referred to as t-fertilin, while, on sperm, only ADAM1B/ADAM2 is found, and is referred to here as s-fertilin (T. Baba, personal communication). The disruption of *Adam2* by homologous recombination led to the disappearance of s-fertilin from sperm, and resulted in the impairment of zona-binding ability of sperm, in agreement with previous reports indicating the involvement of s-fertilin in fertilization [4]. However, surprisingly, when s-fertilin was eliminated by disrupting *Adam1b* instead of *Adam2*, the fertilizing ability of sperm remained intact [5]. This enigma can be explained by an experiment on *Adam1a* disruption [6] in which, when *Adam1a* gene was eliminated, t-fertilin disappeared from the testis, while s-fertilin on sperm remained unaffected. It was found that spermatogenesis seemed to be normal without t-fertilin in the testis. However, the sperm produced without t-fertilin could not bind to zona pellucida, and males became infertile despite having s-fertilin on sperm [6]. These data indicate that t-fertilin is the crucial factor for sperm to acquire the zona-binding ability, but s-fertilin is not. Further investigation revealed the disappearance of ADAM3 from t-fertilin-disrupted mouse sperm, while ADAM3 remained the same on s-fertilin-disrupted sperm [6]. Since the disruption of *Adam3* caused a loss of zona-binding ability without affecting the existence of t-fertilin and s-fertilin [6], it was assumed that ADAM3 is the most closely related factor involved in sperm zona binding.

In our previous paper, we reported that testis-specific molecular chaperone, calmegin (CLGN), specifically interacts with ADAM1A, ADAM1B, and ADAM2, and the disruption of *Clgn* leads to the disappearance of t-fertilin in testis, resulting in the loss of ADAM3 from sperm [7]. We also reported that the disruption of ACE caused an aberrant distribution of ADAM3 on sperm membrane [8]. These findings also reinforce the assumption that ADAM3 is the key molecule in sperm zona binding.

However, a question remains to be addressed. In *Clgn*-, *Adam1a*-, *Adam2*-, and *Ace*-disrupted mouse lines, another important phenotype relating to male infertility is reported: the failure of sperm migration into the oviduct. However, in *Adam3*-disrupted mouse line, which seems to be the most important gene-enabling sperm to bind to zona, sperm migration into the oviduct was reported not to be impaired [9]. Where does the difference arise? In the present experiment, we sought to re-examine the oviduct-migrating ability of *Adam3*-disrupted mouse sperm precisely by tagging sperm with transgenically expressed green fluorescent protein (GFP) in sperm acrosome [10], which enabled us to visualize sperm in the oviduct by fluorescence dissection microscopy.

MATERIALS AND METHODS

Animals

The *Adam3*$^{-/-}$ mice were originally produced by Shamsadin et al. (*Adam3tm1Ihgg*/*Adam3tm1Ihgg* [involves: 129S1/Sv* 129X1/SvJ*CD-1]) [9]. Transgenic rescue mouse lines were produced by injecting the purified fragment, indicated below, into *Adam3*$^{-/-}$ × *Adam3*$^{+/-}$ fertilized eggs. The cDNA encoding mouse *Adam3* was amplified by PCR with primers, 5′-TCTCGAGGGATCCGCCACCATGCTGCCCTTATTCCTAGTCC-3′ and 5′-ACTCGAGGGATCCTTACTGCTGGCTGTCGTTACC-3′ using *Adam3* full-length clone (MGC150317; Invitrogen) as a template. His-tag was added in front of the transmembrane region by PCR amplification with primers, 5′-CCTCGAGCTTAAGTACCACCACCACCACCACCACAAAAAA

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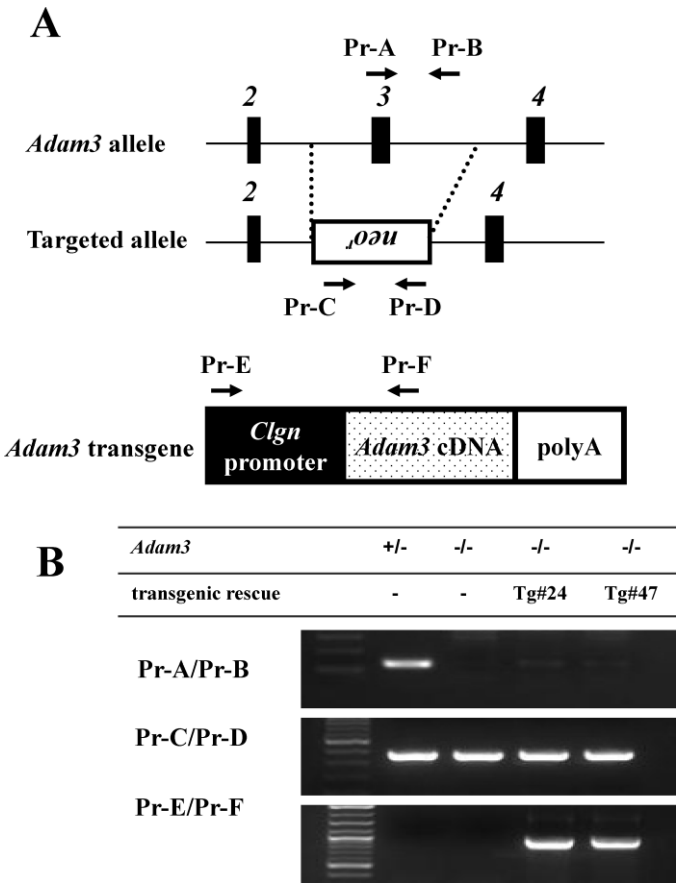


FIG. 1. **A**) The upper panel shows the target disrupted mouse *Adam3* allele. The lower panel shows the transgene to express mouse *Adam3* under the control of *Clgn* promoter. The arrows indicate the positions of primer sets to detect the targeted allele. **B**) Genotyping of tail tip DNA from various groups of mice by PCR amplification with indicated primers.

GTGCTTTTGATCAG-3′ and 5′-ACTCGAGGGATCCTTACTGCT GGCTGTCGTTACC-3′. The *Xho*I sites included in the PCR primers were used to introduce the amplified *Adam3* cDNA into a SKII^{+} expression vector containing the *Clgn* promoter and bovine globin polyadenylation signal [7]. The transgene was excised with *Kpn*I and *Sac*I and gel purified.

In order to investigate sperm migrating ability, females from a transgenic mouse line with enhanced GFP (EGFP) in sperm acrosome *Tg(Acr-EGFP)1Osb* (also called *Acr-EGFP*) [10] were crossed with *Adam3*$^{+/-}$ males to obtain double-transgenic F1 offspring (*Adam3*$^{+/-}$ and *Acr-EGFP*$^{+/-}$).

All experiments were performed with the approval of the Animal Care and Use Committee of Osaka University.

Antibodies

Monoclonal antibodies against mouse ADAM2 (fertilin β; 9D2.2) and ADAM3 (cyritestin; 7C1.2) were purchased from Chemicon International Inc. (Temecula, CA).

Immunoblot

Immunoblot analysis was performed as described previously [8]. Briefly, sperm from the epididymis and vas deferens were collected and incubated in lysis buffer containing 1% TritonX-100 for 20 min on ice. The testes were excised and homogenized in lysis buffer, and then placed on ice for 1 h. The sperm and testis extracts were centrifuged, and the supernatants were collected. Proteins were separated by SDS-PAGE under reducing conditions and transferred to polyvinylidene fluoride membranes. After blocking, blots were incubated with primary antibodies overnight at 4°C, and then incubated with horseradish peroxidase-conjugated secondary antibodies. The detection was performed using an enhanced chemiluminescence Western blotting detection kit (GE Healthcare).

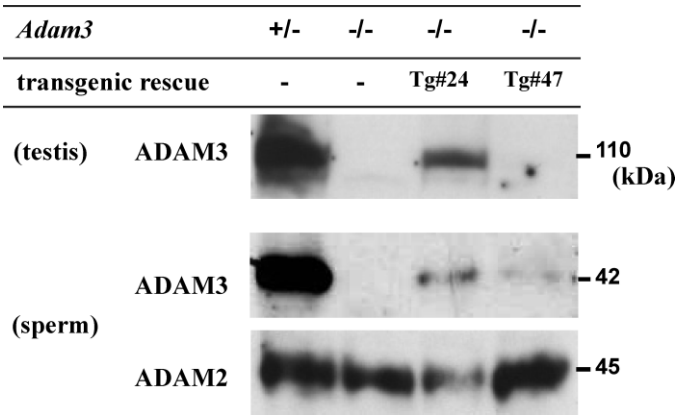
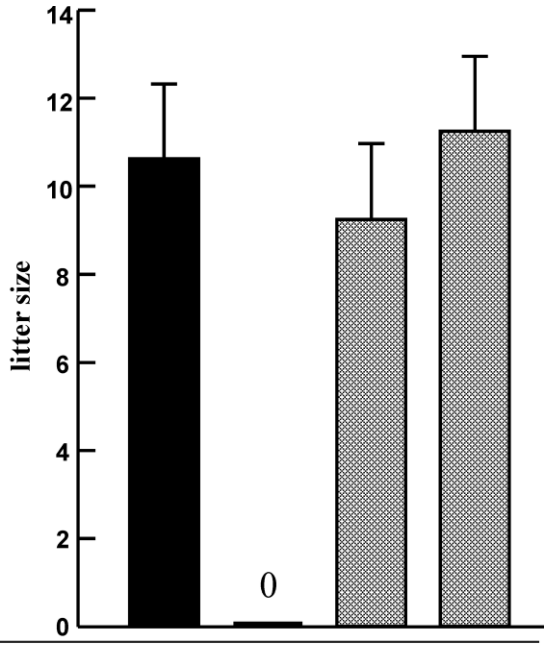


FIG. 2. Protein extracts from *Adam3*$^{+/-}$, *Adam3*$^{-/-}$, and transgenically rescued *Adam3*$^{-/-}$ testis (top panel) and sperm (middle and bottom panels) were separated by electrophoresis and subjected to immunoblot analysis using antibodies against ADAM3. ADAM2 was indicated as a reference marker for successful extraction of proteins.

Sperm Migration Analysis

(C57BL/6J × DBA/2N)F1 (also known as B6D2F1) females were superovulated by intraperitoneal injection of 5 U equine chorionic gonadotropin (CG) followed 48 h later by 5 U human CG (hCG). Superovulated females were caged together with test males 12 h after hCG injection, and the formation of vaginal plug was observed every 30 min. Once plug formation was confirmed, the male was pulled away from the female. About 2 and 6 h after copulation, oviducts were excised, together with the connective part of the uterus. Oviducts were carefully separated from the uterine horns and straightened out by cutting the mesosalpinx. They were transferred to slides as whole mounts, covered with coverslips, and examined by fluorescence



Adam3	+/-	-/-	-/-	-/-
Transgenic rescue	-	-	Tg#24	Tg#47
No. of mating	8	7	4	4

FIG. 3. Average litter sizes obtained by mating *Adam3*$^{+/-}$, *Adam3*$^{-/-}$, and transgenically rescued *Adam3*$^{-/-}$ males with B6D2F1 wild-type female mice. The sterile phenotype of *Adam3*$^{-/-}$ mice was restored by the addition of *Adam3* transgene. Error bars represent 1 SD.

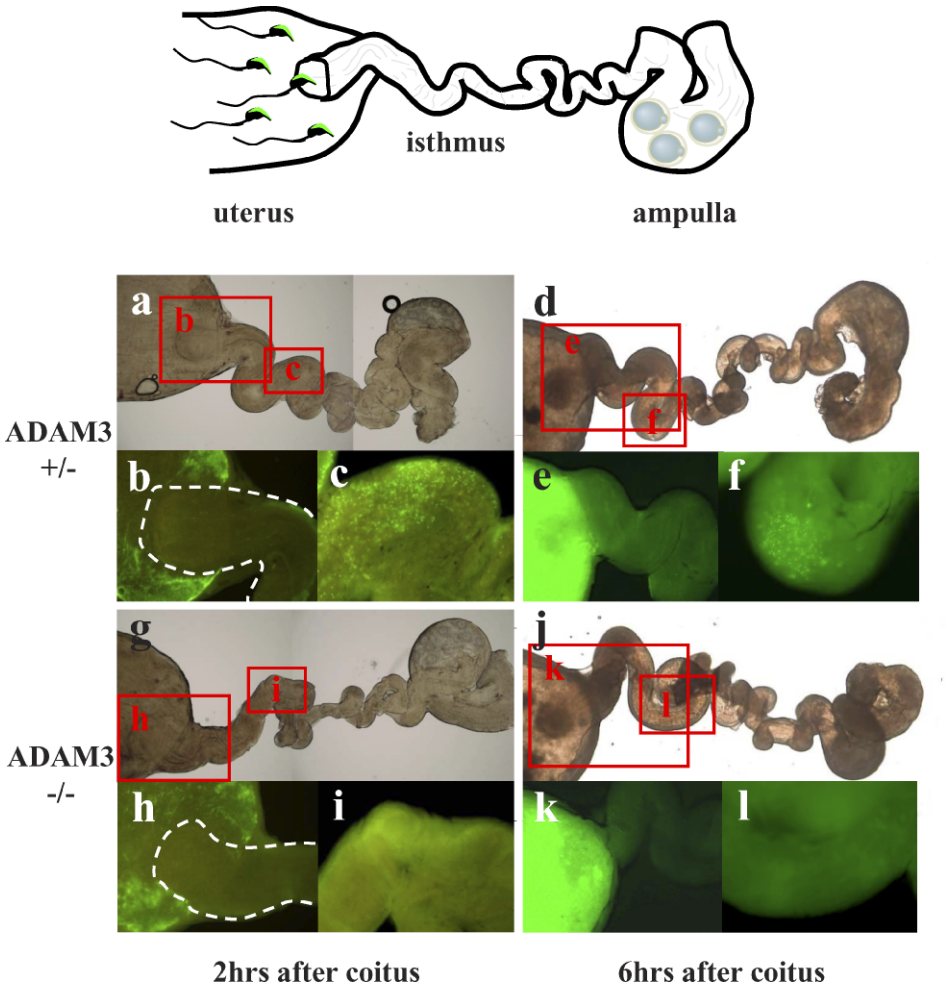


FIG. 4. Observation of UTJ and oviduct from a female mated with an *Adam3*$^{+/-}$ male 2 and 6 h after coitus (**a**, **d**) and an *Adam3*$^{-/-}$ male (**g**, **j**) with *Acr-EGFP*-tagged sperm. The boxes indicate the UTJ (**b**, **e**, **h**, **k**) and isthmic regions (**c**, **f**, **i**, **l**). Fluorescent illumination of the boxed areas in **a** and **d** indicate the existence of wild-type sperm within the uterus (**b**, **e**) and the oviductal isthmus (**c**, **f**). However, when *Adam3*$^{-/-}$ males were mated, a similar number of sperm were seen in the uterus lumen (**h**, **k**), but there was no indication of migrated sperm into the oviductal isthmus (**i**, **l**). The filter set used for fluorescence microscopy was the Olympus XF52 (excitation, 490–550 nm; emission, 520–580 nm; dichroic mirror, 490–550 nm) and the Olympus U-MNIBA2 (excitation, 470–490 nm; emission, 510–550 nm; dichroic mirror, 505 nm) at 2 and 6 h, respectively.

microscopy with fluorescence microscopes (Olympus BX50 and Keyence Corporation BZ-8000) to determine the presence of sperm containing the acrosomal EGFP marker. Six females were examined in each group.

RESULTS

Rescue of Infertility of Adam3 *Disruption by* Adam3 *Transgene*

In some cases, the phenotypes exhibited by the gene disruption varied depending on the research groups when different targeting vectors were used. It has been reported that this discrepancy is caused by an unexpected influence of the targeting vector to neighboring genes of the target site [11, 12]. One method to avoid misinterpretation is to examine whether the observed phenotype could be rescued by transgenically reintroducing a targeted gene into the knockout mouse line [7, 13]. *Adam3* disruption was known to cause male infertility, but the rescue experiment had not been performed before. Based on the concept of the importance of a rescue experiment, we transgenically introduced *Adam3* into *Adam3*-disrupted mouse lines to determine whether the phonotype could be rescued. In order to generate transgenic mouse lines, *Adam3* coding sequence with (or without) His-tag sequence on the C-terminal end was connected downstream of the testis-specific *Clgn* promoter and injected into pronuclei of fertilized eggs. The *Adam3*-transgenic mouse lines were then introduced into *Adam3*-null genetic background by crossing (Fig. 1).

Western blot analysis was performed to examine the expression of the transgene in various lines. As indicated in Figure 2, no ADAM3 protein was detected in the *Adam3*$^{-/-}$ mouse line, while in the transgenic mouse line no. 24 (tg#24 [His^{-}]), ADAM3 was detected in testis and also in sperm. In *Adam3* tg#47 (His^{+}) line, the amount of ADAM3 in testis was less than that of tg#24, but the existence of ADAM3 was evident in sperm. These mice were mated with wild-type females, and their fertilizing ability examined. As shown in Figure 3, the infertile phenotype was rescued by transgenically produced ADAM3 in both of the transgenic mouse lines.

Oviduct-Migrating Ability of Adam3$^{-/-}$ *Sperm*

In order to examine the oviduct-migrating ability in *Adam3*$^{-/-}$ mice, we introduced transgenic mice with GFP-tagged acrosome to the *Adam3*$^{-/-}$ genetic background to visualize the sperm inside the uterus and oviduct. The GFP-tagged sperm in the uterus and oviduct were easily detected by observation through the uterine and oviductal wall under a dissection-type fluorescence microscope. When females were mated with *Adam3*$^{+/-}$ males, and the oviducts were gently sliced out, many sperm were seen in the uterus, and in the isthmus region of the oviduct. On the other hand, when females were mated with *Adam3*$^{-/-}$ males, no sperm were found in the isthmus of the oviducts, but a large number of sperm with green acrosome were seen in the uterus about 2 h and also 6 h after coitus (Fig. 4). This clearly indicates that, when *Adam3* is disrupted, the sperm lose their oviduct-migrating ability.

DISCUSSION

In our previous paper, we produced chimeric mice using embryos from wild-type and transgenic GFP-tagged "green sperm" *Clgn*-knockout mice. When we observed the sperm migrations from these chimeric mice, we found wild-type sperm exclusively within the oviducts [14]. Thus, it was indicated that the presence of wild-type sperm cannot compensate for the disabled oviduct-migrating ability of sperm from *Clgn*-knockout mice. The results led us to assume that sperm migration into the oviduct depends on the interaction of individual sperm with the uterotubal junction (UTJ).

In the present paper, utilizing the "green sperm" technique [10], the sperm migrating ability into oviducts from *Adam3*-disrupted mice was examined. In contrast to the previous observation [9], *Adam3*$^{-/-}$ sperm were not migrating into oviduct when observed about 2 and 6 h after coitus, while many sperm were seen inside the uterus; the reason for this discrepancy unclear. However, at least by preparing oviductal sections as described in previous papers [6, 15], we confirmed that the absence of "green sperm" in oviduct was not caused by the acrosomal loss in the oviduct, but by the actual absence of sperm in the oviduct (Supplemental Figure S1 available at www.biolreprod.org). The "live imaging" observation method was also applied to the *Clgn* (which leads to the loss of ADAM2 and ADAM3 from sperm)-knockout mouse line, and our new method was confirmed to show the same results as those previously reported for the sectioning method (data not shown). Thus, we postulate that all five gene-disrupted mouse lines (*Clgn*, *Adam1a*, *Adam2*, *Adam3*, and *Ace*) reported to have defects in sperm zona binding shared the defects in sperm migration into oviduct around the time suitable for fertilization [4, 6, 9, 15–17]. Although sperm seem to pass through the UTJ not only using swimming ability but also by sperm selection [14], the mechanism of how the selection is achieved remains unclear. One could reasonably speculate that sperm are using a different recognition system in UTJ penetration and zona binding. However, in five out of five different gene disruptions, it was found that ADAM3 completely disappeared from sperm or changed its localization in sperm membrane [6, 8]. Moreover, after learning that five different gene disruptions resulted in a defect in the zona-binding and UTJ penetration steps, it is tempting to imagine that a common recognition system is utilized in zona-sperm and UTJ-sperm interactions.

It was reported that, when mouse *Zp3* gene was replaced by human *ZP3*, the resulting chimeric zona showed affinity only to mouse, but not to human sperm [18]. This indicates that, if ZP3 is the responsive element on zona pellucida for sperm to bind, it may not be the peptide sequence that sperm are recognizing. On the other hand, there are many papers indicating the importance of glycans in sperm-egg recognition. If this is the case, the zona pellucida and the surface of the UTJ might share similar glycans on their surface to interact with sperm. Although the importance of O-glycans in zona pellucida is reported [19, 20], the assumption was challenged by recent gene disruption experiments. For example, four kinds of basic core structures are known in O-glycans, and among the four, only core 1 and core 2 were found on zona pellucida; however, the disruption of GCNT1, which is the key enzyme in the formation of core 2 glycans, revealed that the oocytes without core 2 glycan are fertile [21]. Moreover, the disruption of C1GALT1 (previously termed T-syn), which is also a key enzyme in the formation of core 1 glycans, revealed that core 1 in zona pellucida is not essential for fertilization [22], diminishing the role of O-glycans on zona pellucida. Although these findings do not indicate alternative candidate molecules for sperm to bind, they are not supportive of the hypothesis considering a common glycan on zona pellucida and epithelium of the UTJ.

Thus, the results described here present a new enigma in terms of the mechanism of fertilization; however, if there is a factor that exists on both zona pellucida and UTJ, it could be a potential key molecule to solve this mystery. We can also imagine an alternative scenario, an epididymis- or seminal plasma-derived sperm-binding protein (BSP: see review [23]) reported to function in sperm migration inside oviduct, where a gradual release/degradation of BSP would break the bridge and free the sperm. If this binding was applicable to the UTJ region, the absence of *Clgn*, *Adam1a*, *Adam2*, *Adam3*, or *Ace* genes might affect the binding of BSP to sperm more or less persistently, possibly leading to trapping of (or, conversely, failure to trap) the sperm in the UTJ region.

In summary, the ability of *Adam3*$^{-/-}$ sperm to migrate into the oviduct was found to be impaired, as were other gene-disrupted mouse sperm. This phenotype was compensated for by transgenically expressed ADAM3. Although the reason is unclear, the amount of ADAM3 required for sperm to migrate into the oviduct was much less than the amount of ADAM3 present on wild-type sperm. After all, sperm penetration through the UTJ must be as important as the intensively studied zona-binding ability. However, the molecular mechanism of sperm migration through the UTJ, and the relationship between sperm-UTJ and sperm-zona binding, are questions yet to be conclusively addressed.

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