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CD147 deficiency is associated with impaired sperm motility/acrosome reaction and offers a therapeutic target for asthenozoospermia

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Patients with asthenozoospermia often present multiple defects in sperm functions apart from a decrease in sperm motility. However, the etiological factors underlying these multifaceted defects remain mostly unexplored, which may lead to unnecessary treatment and unsatisfactory assisted reproductive technologies (ART) outcome. Here, we show that the protein levels of CD147 were lowered in sperm obtained from asthenozoospermic infertile patients exhibiting defects in both sperm motility and the acrosome reaction. Whereas CD147 maintained sperm motility before capacitation, female tract-derived soluble CD147 interacted with sperm-bound CD147 to induce an acrosome reaction in capacitated sperm. Soluble CD147 treatment restored the acrosome reaction and improved the fertility of sperm from patients with asthenozoospermia. Mechanistically, CD147 promotes sperm motility and acrosome reaction (AR) by eliciting Ca^{2+} influx through soluble CD147 binding to sperm-bound CD147. Notably, the level of soluble CD147 in seminal plasma was positively correlated with the fertilization rate and pregnancy outcome in infertile couples undergoing *in vitro* fertilization. Our study has identified a marker for the diagnosis and a therapeutic target for the defective AR capability in asthenozoospermia and a candidate for the prediction of *in vitro* fertilization outcomes for male infertile patients that facilitates the development of precision medicine in ART.

INTRODUCTION

Sperm undergo a series of processes to acquire their fertilizing capacity. These processes include the initiation and maintenance of motility, the induction of hyperactivation and capacitation during transit in the uterus and oviduct, and the acrosome reaction (AR).[1–3] Defects in any of these processes lead to subfertility or infertility.[4] A classic example is asthenozoospermia, a condition defined as sperm with motility lower than the normal threshold,[5] which accounts for approximately 18% of all male cases of subfertility and infertility.[6] A previous study has shown that deficiencies in human β-defensin 1, a small antimicrobial peptide released in the epididymis and female reproductive tract, underlie the poor sperm motility observed in patients with asthenozoospermia.[7] Besides, structural proteins required for flagella formation and proteins involved in the energy metabolism are known to be associated with asthenozoospermia.[8–10] A more recent study has also suggested the involvement of non-coding RNAs and circular RNA in asthenozoospermia.[11] Despite these efforts, the etiology of asthenozoospermia remains far to be illustrated. Intriguingly, sperm from patients with asthenozoospermia also showed a significantly lowered rate of ionophore-induced AR,[12] suggesting that the infertility outcomes of these patients could be attributed to multifaceted factors. However, AR defects in asthenozoospermia have received limited attention and, thus, the molecular mechanisms underlying defects in asthenozoospermia leading to infertility remain incompletely understood.

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Table 1. The baseline information of sperm parameters

	Men with normozoospermia (n = 162)	Men with asthenozoospermia (n = 86)
Age (years)	33.5 ± 6.9	33.1 ± 6.4
Sperm concentration (million/mL)	85 ± 29.1	69.1 ± 34.6
Total motility (%)	67 ± 10	25 ± 8.7
Progressive motility (%)	55 ± 10.2	14.8 ± 8.2

Data are the mean ± SD.

The acrosome is a Golgi-derived organelle on the anterior sperm head that stores hydrolytic enzymes capable of digesting the egg coat. AR, the exocytosis of these hydrolytic enzymes, occurs when the outer acrosomal membrane fuses with the inner leaflet of the sperm membrane.[13] During the voyage of sperm in the female reproductive tract, AR is known to be triggered in at least two locations: the cumulus oophorus and zona pellucida (ZP) outside the egg coat.[3,14] Sperm are known to release protein factors that initiate crosstalk with cumulus cells, which in turn release AR-inducing factors, such as progesterone, to trigger AR.[14,15] AR is also elicited when sperm bind to the ZP3 protein that constitutes the ZP.[16–18] Regardless of the location of induction, AR is often associated with the influx of extracellular Ca^{2+},[19] although the Ca^{2+}-independent protein kinase C pathway has also been identified.[20]

CD147, also known as EMMPRIN and Basigin, is a member of the immunoglobulin (Ig) superfamily. While CD147 is normally detected in the reproductive tract, brain, eye, muscle, kidney, colon, and other glandular epithelial cells,[21–24] CD147 is highly expressed in several cancers and has been associated with tumor progression and invasion.[21] CD147 is expressed in both membrane-bound and soluble forms.[25] The soluble form is considered to act in a paracrine fashion by binding to the membrane-bound form (i.e., dimerization).[26] CD147 dimerization is essential for promoting tumor invasion through its involvement in the production/activation of matrix metalloproteinases (MMPs).[27]

In a physiological context, CD147 has been shown to play essential roles in reproduction. Knockout of CD147 in mice resulted in both male and female infertility.[28] In the female reproductive tract, CD147 is expressed in cumulus cells and the uterine endometrium. CD147-null cumulus-oocyte complexes resulted in a significant decrease in the fertilization rate with wild-type sperm in *in vitro* fertilization (IVF),[29] suggesting an essential role of cumulus cell-expressed CD147 in fertilization. Blocking CD147 with a neutralizing antibody also inhibited fertilization of cumulus-invested zona pellucida-intact and zona pellucida-free oocytes but not sperm-egg binding in rats.[30] However, the exact role of CD147 in fertilization is not clear. CD147-null mutant male mice are sterile and exhibit impaired interactions between germ cells and Sertoli cells.[28] CD147 regulates the migration of meiotic male germ cells and the apoptosis of mitotic male germ cells by modulating the NF-κB pathway.[21,31–33] CD147 is also expressed in sperm. During maturation in the epididymis, the localization of CD147 shuttles from the principal piece to the midpiece of mouse sperm.[34] However, the function of CD147 in sperm remains unexplored.

Here, we show that the protein levels of CD147 are lowered in sperm and seminal fluid of infertile patients with asthenozoospermia who exhibit defects in both sperm motility and AR. CD147 promotes sperm motility and AR by eliciting Ca^{2+} influx. More importantly, recombinant CD147 (rCD147) treatment significantly restored sperm motility and AR capability of sperm from patients with asthenozoospermia. The protein level of CD147 in seminal plasma was positively correlated with the fertilization rate and pregnancy outcome after IVF. Our results suggest that CD147 is a diagnostic marker for defective AR and that recombinant CD147 represents a feasible approach for enhancing the fertility outcome of infertile patients with asthenozoospermia. Our results also suggest that the level of CD147 in seminal plasma may be used to predict the fertilization rate and pregnancy outcome, which will guide personalized ART regimens.

RESULTS

We obtained sperm samples from 162 individuals with normal spermiograms (normozoospermia, normal) and 86 patients with asthenozoospermia (Astheno) to carry out the experiments in this study. The baseline information of these patients is listed in Table 1. For the IVF outcome analysis, sperm samples from 63 male donors were collected through routine IVF treatment (Table S2).

Deficiency of CD147 in the sperm of patients with asthenozoospermia

To study the role of CD147 in sperm functions, we first examined the levels of CD147 in sperm obtained from normal men and a cohort of infertile patients with asthenozoospermia by immunofluorescence staining. The results showed that CD147 was expressed in the head and midpiece regions of sperm from normal and asthenozoospermic individuals (Figures 1A and S1A). Consistent with previous findings in rat sperm,[35] a stronger signal of CD147 was observed in the head region upon capacitation, suggesting a shift in the distribution of CD147 to the head region upon capacitation (Figures 1A, 1B, and S1A). Similar results were obtained with a separate antibody against CD147 (Figure S1B). This finding also suggests the possible differential role of CD147 before and after capacitation. Next, we compared the protein level of CD147 in sperm obtained from normal men (n = 26) and patients with asthenozoospermia (n = 21) by western blots. The results showed a significantly lower level of CD147 in sperm from the patients with asthenozoospermia (Figures 1C, 1D, and S2, p = 0.00557). It should be noted that a decrease in the expression of CD147 in sperm was also observed in a small group of normal individuals. Consistently, the RNA level of CD147 in sperm from the patients with asthenozoospermia was significantly decreased compared with that in sperm from the normal individuals (Figure 1E). Of

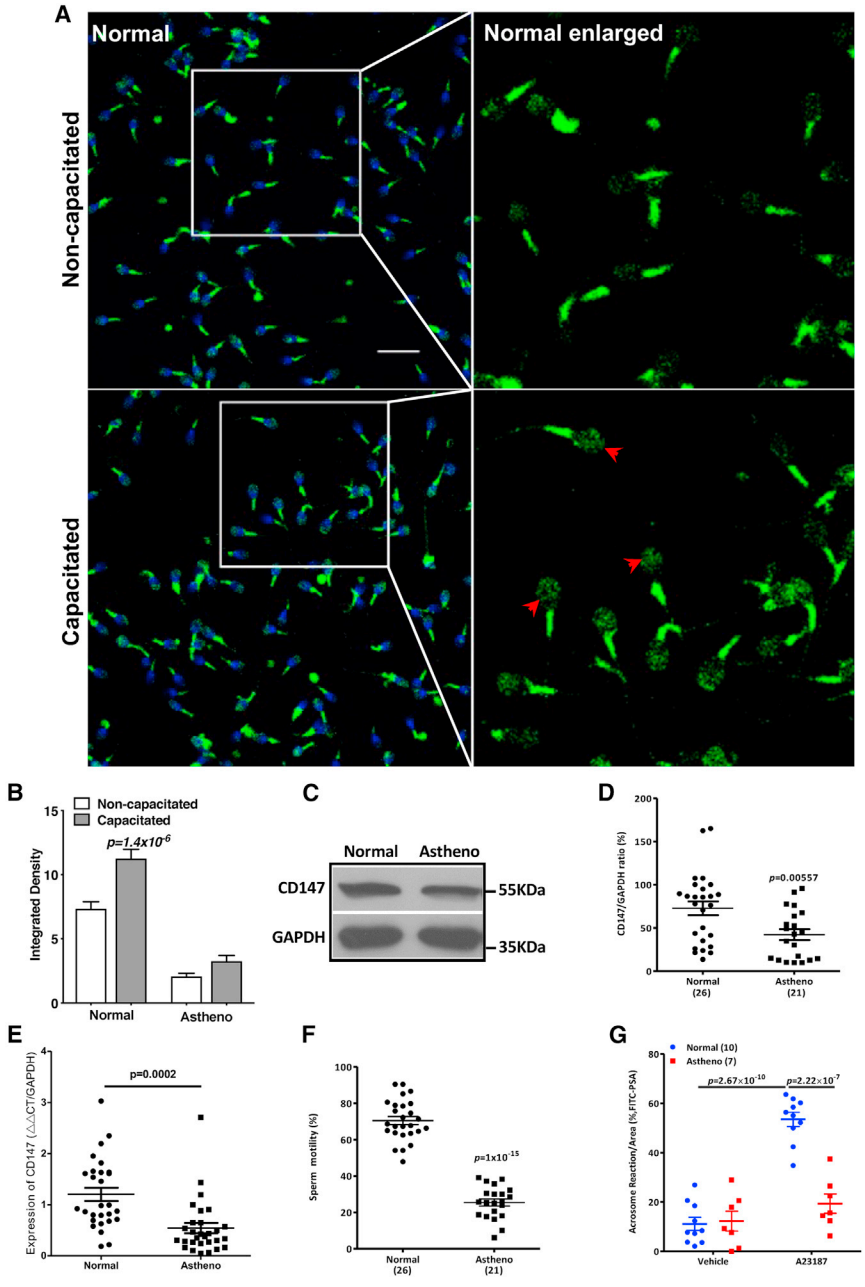

Figure 1. CD147 deficiency and impairment of the acrosome reaction in the sperm of patients with asthenozoospermia

(A) Representative confocal images showing the expression and localization of CD147 (green) in the head and midpiece before capacitation in normal sperm. The intensity of CD147 on the sperm head was increased after capacitation (red arrowheads). Nuclei were counterstained with DAPI (blue). Scale bar, 10 μm. (B) Quantification of fluorescent signals showing a significant increase in the integrated density of CD147 in the normal sperm head region after capacitation. (C) Representative western blot of normozoospermic (normal; n = 26) and asthenozoospermic patient samples (Astheno; n = 21) showing decreased CD147 expression in asthenozoospermic patients compared with normal men. GAPDH was used as a loading control. (D) Quantification of CD147 expression in sperm from the normal and asthenozoospermia cohorts. (E) qPCR assay of CD147 mRNA levels in sperm from the normal (n = 28) and asthenozoospermia groups (n = 29). (F) Computer-assisted sperm analysis of the motility of sperm from normozoospermic men (normal, n = 26) and asthenozoospermic patients (Astheno, n = 21). (G) Quantification of the ionophore-induced (A23187) acrosome reaction measured by FITC-PSA staining. The acrosome reaction of sperm from patients with asthenozoospermia (Astheno, n = 7) was significantly lower than that from normozoospermic men (normal, n = 10). Data are presented as the mean ± SEM.

note, the RNA levels of MMP-2 and MMP-9, two downstream proteases of CD147 in male germ cells,[31] in asthenozoospermic sperm were also lower than those in normal sperm (Figure S3). As expected, sperm motility was lower in the patients with asthenozoospermia than the controls and was correlated with the expression of CD147, despite a comparable sperm concentration (Figures 1F and S4). Intriguingly, the ionophore-induced AR was also significantly lower in asthenozoospermic sperm than in normal sperm (Figure 1G). Taken together, these results suggest that the sperm of the patients with asthenozoospermia exhibited not only reduced motility but also decreased levels of the AR, which may be associated with CD147 deficiency.

Soluble CD147 interacts with sperm-bound CD147

Next, we investigated the involvement of CD147 in sperm functions. CD147 is known to be expressed in membrane-bound and soluble forms, and its dimerization triggers the signaling required for CD147 function in cancer cells.[25,26] We observed the protein level of CD147 in sperm (herein referred to as sperm-bound CD147). Since CD147 is also known to be expressed in cumulus cells,[29] we reasoned that cumulus cells might secrete soluble CD147 physiologically, which might regulate sperm functions. We confirmed the mRNA expression of CD147 in human cumulus cells (Figure S5) and further assessed the protein level of soluble CD147 in human follicular fluid (FF) by ELISAs. Indeed, soluble CD147 was detected in human FF (Figure 2A). The release of soluble CD147 from the female reproductive tract was also confirmed in the KGN cell line, a human granulosa-like tumor cell line.[36] We chose the KGN cell line because a human cumulus cell line was not available, and granulosa cells are considered the precursor of cumulus cells.[37] As expected, ELISA results confirmed the presence of soluble CD147 in

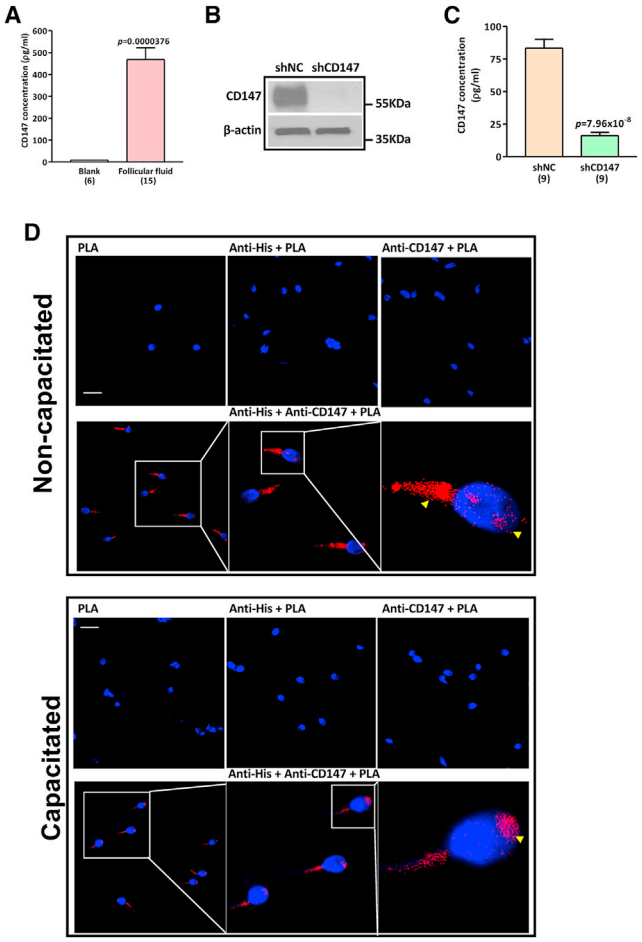

Figure 2. Soluble CD147 interacts with sperm-bound CD147

(A) ELISA results showing the presence of soluble CD147 in human follicular fluid (n = 15). Sample dilution buffer in the ELISA kit was used as a blank control. (B) Western blot results showing the expression of CD147 in vector control-transfected (shNC) or CD147 shRNA-transfected (shCD147) KGN cells. β-actin was used as a loading control. (C) ELISA results showing the presence of soluble CD147 in serum-free conditioned medium obtained from CD147 knockdown (shCD147) or control (shNC) KGN cells. (D) PLA results showing the interactions between His-tagged CD147 and membrane-bound CD147 on human sperm with or without capacitation. Human sperm that endogenously expressed membrane-bound CD147 were treated with His-tagged rCD147, which lacks the C-terminal cytoplasmic tail. The potential interaction was detected by PLA using anti-His and anti-CD147 (C terminus) antibodies (red signals in bottom panel). An enlarged image is shown on the bottom right panel. Sperm samples labeled with either PLA only (top left), anti-His antibody with PLA (top middle), or anti-CD147 antibody with PLA (C terminus) (top right) were used as controls. Scale bar, 10 μm. Data are presented as the mean ± SEM.

conditioned media collected from KGN cells (Figures 2B and 2C). Stable knockdown of CD147 in KGN cells resulted in a lowered level of soluble CD147, validating the ELISA results.

To investigate the binding of soluble CD147 to sperm-bound CD147, we treated human sperm from normal men with His-tagged C terminus truncated CD147 (His-ΔC-CD147) that mimicked soluble CD147. Thus, sperm-bound CD147 can be specifically recognized by an anti-CD147 antibody (C-19) that targets the C terminus of CD147, while soluble His-ΔC-CD147 can be recognized by an anti-His tag antibody only. Using the proximity ligation assay (PLA),[38,39] we revealed that soluble His-ΔC-CD147 came close to the proximity with sperm-bound CD147 at the midpiece and head of human sperm (Figure 2D), suggesting the interaction between these two forms. Intriguingly, more soluble CD147 was bound to the sperm head after capacitation (Figure 2D). Together, our results suggest that soluble CD147 in seminal fluid or those released by the female reproductive tract interact with sperm-bound CD147.

Involvement of sperm-bound CD147 in maintaining sperm motility

To investigate the role of CD147 in sperm functions, we established a loss-of-function model by blocking sperm-bound CD147 with a well-characterized neutralizing antibody (MEM-M6/6) that targeted the extracellular region of CD147.[40] We also tested the effect of rCD147 protein (22–205 amino acids [aa] of CD147) that resembled soluble CD147. In this set of experiments, we treated sperm from normal men with either the CD147-neutralizing antibody or rCD147 and examined sperm motility by computer-assisted sperm analysis (CASA). As shown in Figure 3A, the addition of rCD147 (10 μg/mL) did not further increase sperm motility. However, the anti-CD147 antibody treatment significantly reduced sperm motility (n = 30) compared with that after treatment with normal IgG (n = 30). Of note, the CD147 antibody did not lead to agglutination of sperm (<5 sperm per agglutinate; Figure S6A), suggesting that the effect of the CD147-neutralizing antibody on sperm motility was primarily due to the immunodepletion of CD147 but not agglutination of sperm. In addition, blocking CD147 with a separate source of monoclonal CD147 antibody (MEM-M6/1), which has also been used as a CD147-neutralizing antibody,[32,40] or by the CD147 antagonist AC-73[41] also resulted in a similar reduction of sperm motility in sperm obtained from normal men (Figures S6B and S6C). These results suggest that sperm-bound CD147 contributes to the maintenance of sperm motility.

Soluble CD147 induces the AR

The dynamic localization of CD147 upon capacitation together with the decreased expression in sperm from patients with asthenozoospermia who also showed defects in AR led us to investigate the involvement of CD147 in these processes. In this set of experiments, we treated capacitated sperm from normal men with a CD147-neutralizing antibody or rCD147 and examined hyperactivated motility and AR, which are associated with capacitation, by CASA and *Pisum sativum* agglutinin (PSA) staining, respectively. The results showed that while progesterone (P4), a well-known physiological inducer of hyperactivation and AR,[42] induced a 2-fold increase in the percentage of sperm that showed hyperactivated motility, rCD147 treatment did not affect this process (Figure 3B), suggesting that soluble CD147 is dispensable in sperm hyperactivation. Interestingly, rCD147 treatment induced a 3-fold increase in AR, which was

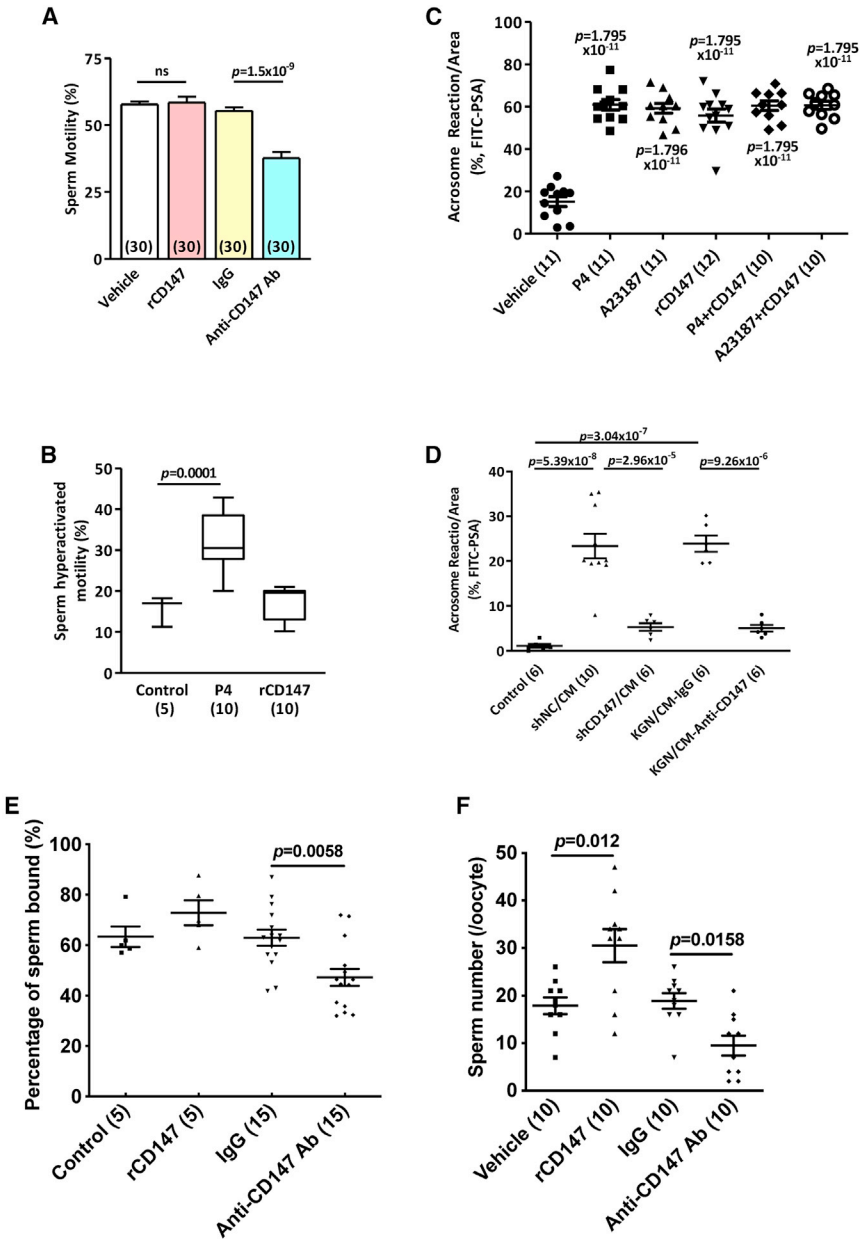

Figure 3. CD147 promotes sperm motility and induces the acrosome reaction

(A) CASA results showing the effect of recombinant CD147 (rCD147; 10 μg/mL) and CD147-neutralizing antibody (anti-CD147 Ab, 10 μg/mL) on sperm motility. The same amount of vehicle or normal IgG was used as a control. (B) CASA results showing the effect of rCD147 (10 μg/mL) and progesterone (P4, 20 μM) on the hyperactivated motility of capacitated sperm. (C) FITC-PSA staining results showing the effect of rCD147 (10 μg/mL), P4 (20 μM), the ionophore A23187 (5 μM), or the indicated combined treatments on the sperm acrosome reaction. (D) FITC-PSA staining results showing the effect of conditioned medium of CD147 knockdown (shCD147/CM) or control (shNC/CM) KGN cells on the acrosome reaction in the presence or absence of a CD147-neutralizing antibody (10 μg/mL). (E and F) Hyaluronan binding assay (E) and human sperm-hamster oocyte penetration assay (F) results showing the effect of rCD147 (10 μg/mL) and CD147-neutralizing antibody (anti-CD147 Ab, 10 μg/mL) on the hyaluronan binding ability and oocyte-penetrating ability of treated sperm. The same amount of vehicle or normal IgG was used as a control. Data are presented as the mean ± SEM.

comparable with those triggered by P4 and ionophore A23187 (Figure 3C). Simultaneous treatment of rCD147 with P4 or A23187 showed no additive effect on the induction of AR (Figure 3C). As the non-relevant protein control, the 27 kDa GST tag protein did not induce AR (Figure S7A). These results suggest that rCD147 is a potent AR inducer. To test the effect of soluble CD147 on AR in a more physiological context, we treated capacitated sperm with conditioned medium obtained from KGN cells that contained soluble CD147 (Figure 2A). As expected, KGN-conditioned medium induced a significant increase in AR (Figure 3D). Moreover, the AR-inducing ability was blunted by knockdown of CD147 in KGN cells or by CD147-neutralizing antibody treatment in conditioned medium (Figure 3D). These results suggest a physiological role of soluble CD147 in inducing AR but not hyperactivation.

Effect of CD147 on sperm fertilizing ability

As CD147 was involved in maintaining sperm motility and inducing AR, we further examined the impact of rCD147 and CD147-neutralizing antibody on fertilization outcome by the hyaluronan binding assay (HBA) and the human sperm-hamster oocyte penetration assay, both of which are recognized analyses for fecundity rate.[43,44] The results showed that anti-CD147 antibody treatment significantly decreased the percentage of sperm bound to hyaluronan and the number of oocyte-penetrating sperm (Figures 3E and 3F). Intriguingly, rCD147 significantly increased the number of oocyte-penetrating sperm but showed a modest increase in the percentage of sperm bound to hyaluronan (Figures 3E and 3F). Together, these results suggest an important role of CD147 in fertilization by regulating sperm motility and AR.

CD147 induces Ca^{2+} influx in human sperm

Ca^{2+} mobilization in sperm, which is mainly attributed to extracellular Ca^{2+} influx due to their lack of endoplasmic reticulum,[45] plays central roles in sperm functions, such as sperm motility and AR.[46,47] CD147 has been shown to induce Ca^{2+} mobilization required for the invasion of liver cancer cells.[48,49] Since CD147 was involved in these sperm functions, we speculated that CD147 might elicit Ca^{2+}

mobilization in human sperm. To test this hypothesis, we examined the effect of rCD147 and CD147-neutralizing antibody on Ca^{2+} mobilization in sperm using a Ca^{2+}-sensitive fluorescent probe, with P4 as a positive control for Ca^{2+} mobilization in sperm. As shown in Figures 4A–4C, despite the weaker induction effect compared with that induced by P4, rCD147 triggered a 2-fold increase in the intracellular Ca^{2+} level ($[Ca^{2+}]_i$) in the presence of extracellular Ca^{2+}, whereas the induction effect was blunted in Ca^{2+}-free condition. A similar increase in the Ca^{2+} signal was observed by another source of rCD147 (same protein sequence of 22–205 aa) (Figure S7B), confirming the effect of rCD147 in triggering Ca^{2+} influx. Ca^{2+} mobilization was not observed in the GST tag protein-treated group (Figure S7C). Moreover, the rCD147-evoked Ca^{2+} influx was blocked by the CD147-neutralizing antibody (Figures 4A–4C).

Since the localization of CD147 shifted to the head region upon capacitation, we speculated that the Ca^{2+} response induced by soluble CD147 might be different in capacitated sperm. Indeed, we observed an ~2-fold increase in the rCD147-induced $[Ca^{2+}]_i$ elevation in capacitated sperm compared with that in noncapacitated sperm (Figure 4D). Together, these results suggest that rCD147 induces differential Ca^{2+} responses in human sperm under noncapacitated and capacitated conditions that may underlie disparate sperm functions in the female reproductive tract.

rCD147 improves sperm functions in asthenozoospermia

We next tested whether augmenting the binding of soluble CD147 to sperm-bound CD147 by increasing the amount of soluble CD147 might restore sperm functions. As shown in Figure 5A, rCD147 significantly enhanced sperm motility in sperm from patients with asthenozoospermia. Sperm motility was not affected by interfering with CD147 with the neutralizing antibody in sperm from patients with asthenozoospermia. However, the CD147-neutralizing antibody abolished the rCD147-mediated rescue of sperm motility (Figure 5A). Of note, despite a lowered level of AR, rCD147 was able to induce a 3.7-fold increase in AR of sperm from the patients with asthenozoospermia (Figure 5B). Similarly, the rCD147-induced AR in sperm from the patients with asthenozoospermia was inhibited by the anti-CD147 antibody (Figure 5B). More importantly, rCD147 treatment significantly increased the percentage of bound sperm in samples obtained from the patients with asthenozoospermia, as revealed by HBA (Figure 5C), suggesting an improvement in fertilizing capacity. Taken together, our results demonstrated that rCD147 treatment could improve sperm functions and fertilization outcomes in sperm obtained from the patients with asthenozoospermia.

The seminal CD147 level is associated with IVF outcomes

The capability to undergo the acrosome reaction is associated with fertility status and ART outcome.[50–52] Our results demonstrated the involvement of CD147 in the acrosome reaction. Therefore, we reasoned that the level of CD147 may be a marker to predict the fertilization rate and ART outcome. To test this hypothesis, we extended our patient cohort to include infertile couples undergoing IVF treatment and examined the level of CD147 in seminal plasma. To control the potential bias caused by oocyte quality in the IVF setting, we included only patients with female partners younger than 40 and with retrieved oocytes >5 in the analysis. Male infertility patients with oligozoospermia (number of sperm <15 million/mL) were also excluded, as the level of CD147 may be affected by the number of sperm, i.e., sperm-bound CD147. Our results showed that the level of CD147 in seminal plasma was positively correlated with the fertilization rate, which was defined as the percentage of fertilized eggs in mature eggs (Figure 5D). Interestingly, the level of CD147 in seminal plasma was significantly lowered in sperm from men whose partners did not become pregnant after IVF treatment (Figure 5E), suggesting an association between seminal CD147 levels and IVF outcomes. Together, our results suggest that the soluble CD147 levels in seminal plasma may be a marker for predicting the fertilization rate and IVF outcome.

DISCUSSION

This study identified a novel role of CD147 in sperm functions and provided new insights into the molecular basis of multifaceted defects in asthenozoospermia contributing to infertility outcomes. Here, we show that sperm-bound CD147 was involved in sperm motility, AR, and sperm-egg interactions in sperm from normal men. Therefore, the deficiency of sperm-bound CD147 may underlie poor motility and impaired AR in patients with asthenozoospermia. Interestingly, a small group of normal/fertile patients with normal sperm motility demonstrated a decreased level of sperm-bound CD147, suggesting that CD147 may not be the sole determinant of sperm motility. Nonetheless, the significant decrease in sperm-bound CD147 in patients with asthenozoospermia with dual defects in sperm motility and AR suggests that CD147 might be a diagnostic marker for male infertility associated with defects in these sperm functions.

Although blockade of CD147 significantly decreased sperm motility, a considerable percentage of sperm still retained motility. This finding suggests the involvement of other mechanisms in regulating sperm motility. Our previous study demonstrated that the expression of DEFB1 is correlated with sperm motility.[7] Sperm from patients with asthenozoospermia also showed a lowered level of DEFB1 compared with that in normal men. Intriguingly, while DEFB1 triggered hyperactivation but had no effect on AR, CD147 induced AR but not hyperactivation. In corroboration, the Ca^{2+} response induced by CD147 was markedly stronger than that induced by DEFB1.[7] These results suggest that DEFB1 and CD147 have different roles in sperm functions and that the multifaceted defects in sperm functions of patients with asthenozoospermia may stem from deficiency in either DEFB1 or CD147 or both in sperm.

Our results suggest that soluble CD147 interacts with sperm-bound CD147 to elicit sperm functions. Since soluble CD147 is present in both seminal fluid and FF, sperm must possess a mechanism to distinguish signals induced by different sources of soluble CD147. Capacitation appears to play a role in this process. During capacitation, the

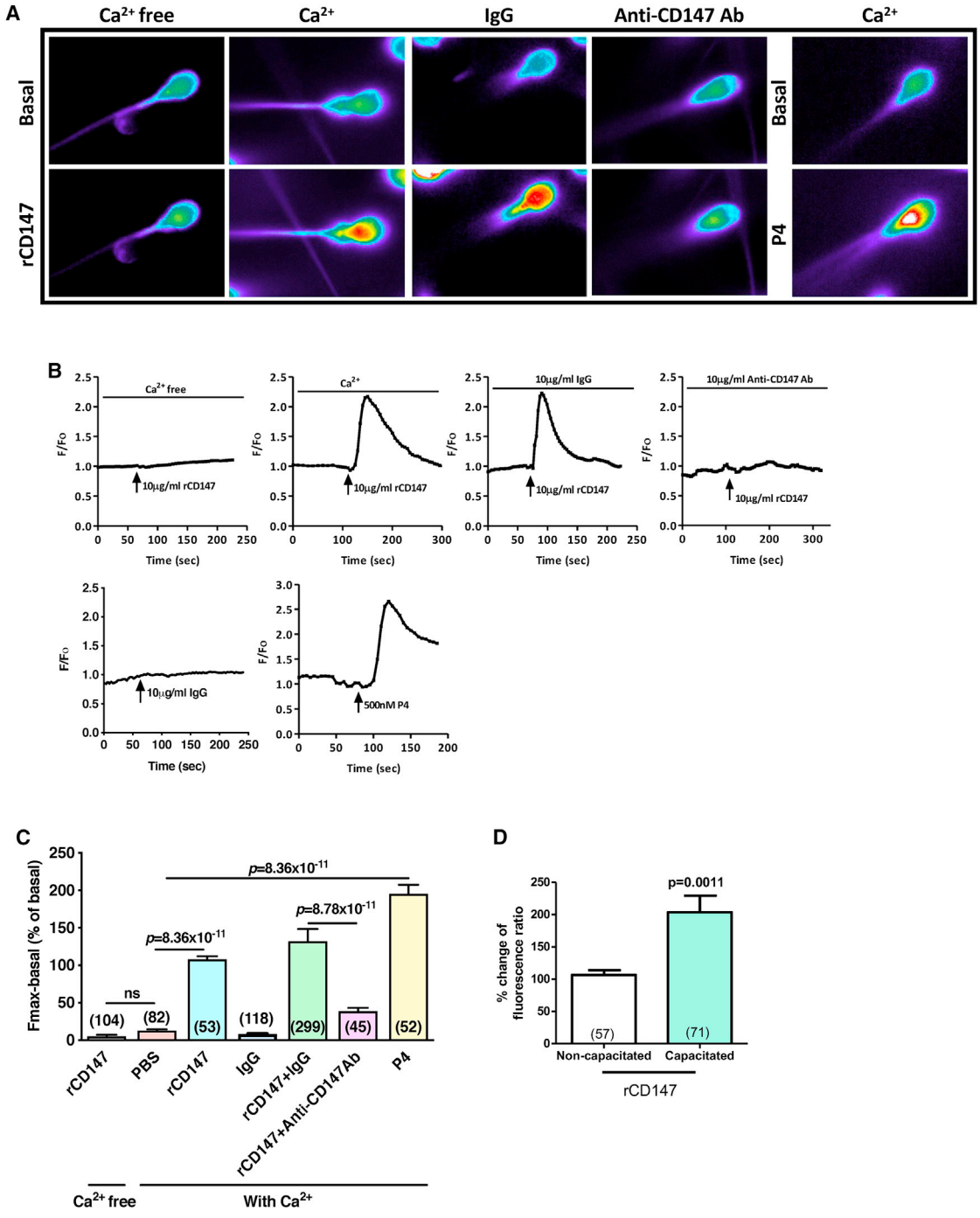

Figure 4. CD147 induces Ca^{2+} influx in human sperm

(A) Representative fluorescence images of human sperm loaded with Fluo-4, a Ca^{2+}-sensitive dye, before (basal) and after (rCD147) treatment with rCD147 (10 μg/mL) in Ca^{2+}-free or Ca^{2+}-containing media with or without CD147-neutralizing antibody (10 μg/mL). Sperm samples treated with P4 (500 nM) were used as a positive control. (B) Representative time course changes in Fluo-4 intensity (F) normalized to the initial intensity (F_0) in the human sperm treated with rCD147 or P4 as in (A). (C) Quantification of changes in intracellular Ca^{2+} in sperm samples treated as in (A). The calcium values of responsive sperm were calculated. Numbers in brackets represent the number of sperm recorded in at least three independent experiments. (D) Quantification of rCD147-induced (10 μg/mL) changes in intracellular Ca^{2+} in sperm samples of normal men under noncapacitated and capacitated conditions. Data are presented as the mean ± SEM.

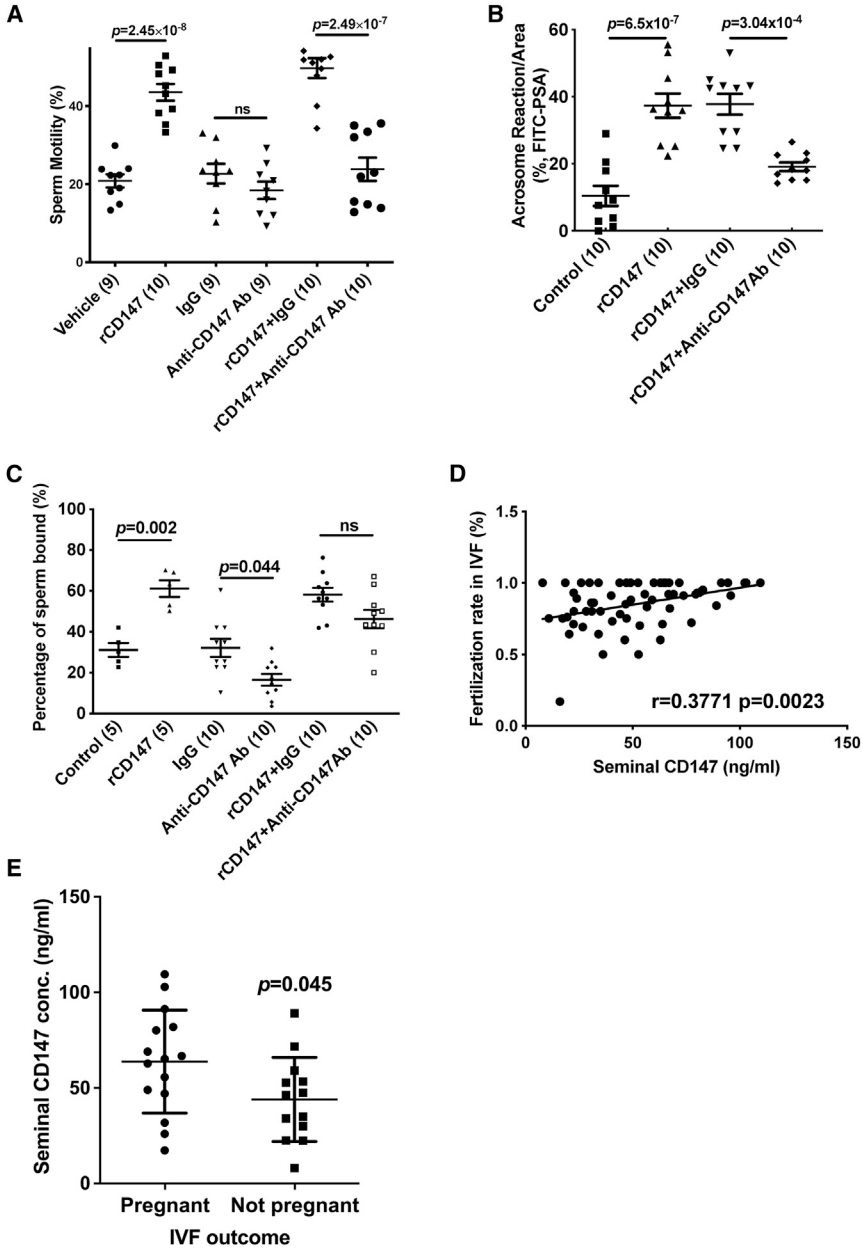

Figure 5. Recombinant CD147 treatment improves functions in infertile sperm

(A) CASA results showing the effect of a CD147-neutralizing antibody (10 μg/mL) and/or rCD147 treatment (10 μg/mL) in sperm obtained from patients with asthenozoospermia. (B) FITC-PSA staining results showing the effect of rCD147 (10 μg/mL) on the acrosome reaction of sperm obtained from patients with asthenozoospermia in the presence or absence of the CD147-neutralizing antibody (10 μg/mL). (C) Hyaluronan binding assay results showing the effect of the CD147-neutralizing antibody (anti-CD147 Ab, 10 μg/mL) and/or rCD147 (10 μg/mL) on the hyaluronan binding ability of sperm obtained from patients with asthenozoospermia. (D) Correlation analysis of seminal plasma CD147 levels as determined by ELISAs with the fertilization rate in IVF procedures. (E) ELISA results showing the level of CD147 in the seminal plasma of infertile men with the indicated IVF outcome. Data are presented as the mean ± SEM.

sperm head membrane undergoes biochemical modification that poises sperm for the induction of AR.[53] We observed a shift in the localization of CD147 from the midpiece toward the head region upon capacitation. Moreover, soluble CD147 induced a markedly stronger Ca^{2+} response in capacitated sperm than in noncapacitated sperm. Therefore, we propose that the shift in localization of CD147 underlies the differential effects elicited by different sources of soluble CD147. In noncapacitated ejaculates, soluble CD147 in the seminal fluid binds to sperm-bound CD147 at the midpiece and maintains sperm motility. Upon capacitation, female tract-derived soluble CD147 binds to sperm-bound CD147 at the head region and induces AR.

The presently demonstrated ability of rCD147 to induce AR and augment CD147 signaling to improve sperm motility and fertilizing capacity has high translational value. Asthenozoospermia is a common cause of infertility and accounts for approximately 18% of male cases of subfertility and infertility.[6] Apart from empiric regimens, such as vitamins and minerals, the therapeutic effects of which are often debatable,[54,55] IVF and intrauterine insemination (IUI) are two common options for patients with asthenozoospermia who desire to father a child. Of note, the success of IVF and IUI relies on the motility of sperm.[56] The use of rCD147, which mimics the physiological source of soluble CD147 in semen and the female reproductive tract, represents a feasible approach to improve the success rate of IVF and IUI by restoring sperm motility and enhancing fertilizing capacity.

Intracytoplasmic sperm injection (ICSI) is usually applied when patients do not benefit from IVF procedures due to the lack of motile or morphologically normal sperm.[57,58] Since AR is essential for fertilization, more recent studies have proposed the assessment of acrosome reaction capability to guide the use of IVF versus ICSI.[59] Our results showed that the level of soluble CD147 in seminal plasma was correlated with the fertilization rate and pregnancy rate, suggesting that CD147 may be used as an indicator to guide the ART regimen in this regard. In addition, the introduction of large amounts of acrosomal enzymes into the cytoplasm of oocytes that disrupt the cytoskeleton and eventually damage oocytes remains one of the limiting factors for the success of ICSI.[60,61] The removal of acrosomes before ICSI has been shown to improve the survival and embryonic development of oocytes.[62,63] Our finding that

AR can be triggered by rCD147 treatment provides an efficient and physiological way to remove acrosomal enzymes, which in turn increases the success rate of ICSI.

Taken together, the present findings reveal a pivotal role of CD147 in sperm functions. CD147 deficiency results in poor sperm motility and impaired AR, which contribute to infertile outcomes associated with asthenozoospermia. Finally, rCD147 represents a new treatment regimen to improve the success rate of assisted reproductive technologies, and the level of soluble CD147 in seminal plasma may be used as an indicator for guiding a personalized ART regimen.

MATERIALS AND METHODS

Clinical samples

The use of human semen samples in this study was approved by the Medical Ethics Committee of Nanfang Hospital, Southern Medical University (NEFC-2019-026) and the Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee (CREC ref. no. 2016.499). Written informed consent was obtained from all patients who participated in this study. Semen samples were obtained from 21- to 45-year-old donors undergoing routine semen analysis for fertility assessment in the andrology laboratory (Table S1). Subjects with a history of genetic, endocrine, or anatomical disorders were excluded. Semen analysis was performed manually according to World Health Organization criteria (version V) (WHO 2010). The measurement process conformed to the checklist recommended by Bjorndahl et al.[64] Sperm concentration and motility were assessed by a CASA system (SCA version 4.0.0.5, Microptic S.L., Barcelona, Spain). Duplicate assessments were performed, and at least 200 spermatozoa were assessed for each sample. To evaluate sperm morphology, we used Tygerberg Strict Criteria after staining the slides with a Diff-Quik staining kit (Dade Behring AG, Düdingen, Switzerland),[65] and assessments were performed under a microscope with an oil immersion ×100 objective (Olympus BX43, Tokyo, Japan). Normal sperm were selected according to the following criteria: a sperm concentration of $\geq 15 \times 10^6$ spermatozoa/mL, sperm motility >40%, progressive motility $\geq$32% and normal morphology $\geq$4. Samples with progressive motility <32% were classified into the asthenozoospermia group.

Human semen samples used to analyze the relationship between seminal and sperm CD147 concentrations and IVF outcomes were obtained from male donors who underwent routine IVF treatment (Table S2). For control of the bias caused by oocyte quality, only male partners of normal ovarian responder women (retrieved oocytes more than 5, age under 40 years old) after ovarian stimulation by standard GnRH antagonist and long GnRH agonist were included in the study. Semen samples were collected for research purposes when sufficient semen samples remained after the routine IVF procedure.

Human sperm preparation by density gradient

Sperm samples were isolated from liquefied semen using 40% and 80% phase gradient centrifugation (SupraSperm no. 10970060, Origio) before experiments. In brief, 2 mL of the 80% phase gradient was transferred into a sterile conical-bottom tube. A second 2-mL layer of 40% phase gradient was gently placed on top of the 80% phase using a transfer pipette. A distinct line was observed between two layers. Then, approximately 2 mL of liquefied semen was gently placed onto the upper phase. The sample was centrifuged for 30 min at 400 × *g*. The supernatant was discarded, and the pellet was washed with 2 mL of human tubal fluid (HTF). After that, the sample was centrifuged for 10 min at 1,500 rpm. After removal of the supernatant, the final pellet was resuspended in 1 mL of HTF.

Cell line

KGN cells were cultured in DMEM/F12 with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in 5% CO_2 incubators at 37°C. The lentivirus-mediated CD147 shRNA knockdown experiment was performed as described previously.[66] In brief, the lentivirus was purchased from a commercially available source (5′-GCACAGTCTT CACTACCGTAG-3′, GenePharma, China). For transduction, KGN cells were seeded into a 24-well plate at 1×10^4 cells/well with 200 μL of medium. After cell attachment, the medium were replaced with fresh medium, and cells were transduced with 1×10^7 TU lentiviruses plus 0.1% polybrene. Stably transduced cells were selected by adding 4 μg/mL puromycin for 5 days. For soluble CD147 detection, confluent KGN-shCD147 cells and control cells (KGN-shNC) were washed three times with PBS, and then fresh DMEM/F12 without FBS was added to the cells. The conditioned media were collected after 48 h, and the concentration of soluble CD147 was measured using a human BSG (CD147) ELISA Kit (Abcam, USA) according to the manufacturer's instructions.

Sperm motility assay and hyperactivation examination

Isolated human sperm from normozoospermic men and men with asthenozoospermia were adjusted to 1×10^6 cells/mL in HTF and then incubated with rCD147 (10 μg/mL; obtained from Abcam, USA, and Sino Biotech, China; both with residues 22–205 aa) or anti-CD147 antibody (10 μg/mL, clone MEM-M6/6, Abcam, USA) for 1 h at 37°C under 5% CO_2 as indicated. PBS or normal mouse IgG was included as vehicle controls. Sperm motility was determined by CASA as described.[7] Spermatozoa were defined as hyperactive if they had a curvilinear velocity $\geq$150 μm/s, amplitude of lateral head displacement $\geq$7.0 μm/s, and linearity $\leq$50%.[67]

HBA

The hyaluronan binding assay was carried out using the Origio HBA Assay according to the manufacturer's instructions (Origio, Denmark). In general, the HBA assay reflects the maturity of sperm through the percentage of motile sperm binding to hyaluronan.[68] In brief, isolated sperm from normozoospermic men and asthenozoospermic patients were centrifuged and capacitated in capacitation medium (HTF plus 5% bovine serum albumin and 25 mM HCO_3^-) for 3 h at 37°C under 5% CO_2. After adjustment of the sperm concentration, aliquots were incubated with rCD147 (10 μg/mL) or anti-CD147 antibody (10 μg/mL) for 30 min. PBS or normal mouse IgG was used as a control. Ten microliters of diluted sperm was placed near the

center of the chamber, which was covered with a coverslip and incubated for 20 min at 37°C. Motile sperm bound to hyaluronan were counted. Hyaluronan binding is expressed as the percentage of bound sperm/total motile sperm.

Sperm penetration assay

Sperm penetration assays were performed as described previously.[7] In brief, hamster oocytes were collected from oviducts after superovulation. The ZP was removed, and then oocytes were transferred to 100 μL drops of pre-equilibrated IVF medium under mineral oil. The capacitated sperm were incubated with either rCD147 (10 μg/mL) or anti-CD147 antibody (10 μg/mL) for 30 min at 37°C under 5% CO_2. Treated sperm (50 μL) were deposited in IVF medium containing 15–20 oocytes and incubated for 2 h. The oocyte-spermatozoon complexes were washed thoroughly and captured using a phase contrast microscope. The penetration index was calculated as the total number of sperm penetrations per ovum.

AR assessment

The acrosome reaction was analyzed using a SpermFunc ARIC kit (BRED Life Science Technology, China) according to the manufacturer's protocol. In brief, sperm were centrifuged at 1,500 rpm for 10 min, resuspended in 500 μL of sperm-capacitation medium (from SpermFunc ARIC kit), and incubated in 5% CO_2 incubators for 3 h at 37°C. After capacitation, sperm were treated as indicated for 30 min. Sperm were washed twice with sperm washing medium and overlaid onto slides and air dried. Samples were stained with FITC-PSA solution overnight at 4°C in darkness. After staining, samples were washed in PBS and mounted in antifade mounting medium (Invitrogen). The presence of green fluorescence of the sperm head was considered an intact acrosome, while a partial or total absence of fluorescence was considered an acrosome-reacted sperm. A23187-induced AR was performed in each AR experiment as a positive control to ensure the vitality of the examination. The calculation for AR is acrosome reaction/area means (%FITC-PSA) = the number of acrosomal reacted sperm/total number of sperm in each captured area × 100%.

In situ PLA

A proximity ligation assay was performed using a Duolink PLA kit (Sigma, USA) according to the manufacturer's instructions. Sperm were incubated with rCD147 (His-tagged) for 1 h at 37°C. Then, slides with sperm were subjected to protein block and incubated overnight at 4°C with goat anti-C-terminal CD147 (C-19) and rabbit primary anti-His tag antibodies (H-15). PLA probes were added to slides. After incubation in Duolink ligation stock, Duolink polymerase was used for hybridization and rolling circle DNA amplification. PLA signals were visualized by confocal microscopy (Zeiss, Germany). Negative controls for PLA included the omission of both or either one of the primary antibodies.

Western blot

Isolated human sperm were resuspended in lysis buffer (RIPA buffer: 50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) and lysed with ultrasonication. Sperm lysates (30 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4%–15%, v/w), and resolved proteins were transferred to nitrocellulose membranes. After the membranes were blocked with 5% nonfat milk for 1 h, they were immunoblotted with anti-CD147 (1:500, Santa Cruz sc-9725) or anti-GAPDH (1:2,000, Santa Cruz sc-47724) overnight at 4°C. The membranes were washed three times with TBST and incubated with anti-mouse IgG-peroxidase (Bio-Rad) for 1 h at room temperature. Signals were detected with enhanced chemiluminescence (Amersham, Piscataway, NJ, USA). The intensities of the bands were quantified by densitometry with an Alpha Imager HP.

Immunofluorescence staining and confocal microscopy

Isolated human spermatozoa were fixed in 4% paraformaldehyde for 30 min at room temperature and spread on coverslips. Samples were blocked with 5% BSA in PBS for 1 h and then incubated with mouse anti-CD147 antibody (1:200) (Santa Cruz, USA) or rabbit-anti-CD147 monoclonal antibody (1:100, 10186-R125, Sino Biological) overnight at 4°C. Normal mouse IgG was used as a negative control. After that, the coverslips were washed three times with 0.05% Tween-20 in 1× PBS. Then, Alexa 488-conjugated donkey-anti-mouse antibody (1:500) (Invitrogen, Camarillo, CA, USA) was added for 1 h at room temperature. Nuclei were stained with 1 μg/mL Hoechst 33258 (Invitrogen). In all cases, coverslips were mounted in ProLong Gold Antifade Reagent (Invitrogen) and visualized using a confocal microscope (Zeiss, Germany).

Intracellular Ca^{2+} measurement

Isolated sperm were loaded with 5 μM Fluo-4 in modified sperm washing medium (97.8 mM NaCl, 4.69 mM KCl, 0.2 mM $MgSO_4$, 0.37 mM KH_2PO_4, 2.04 mM $CaCl_2$, 4 mM $NaHCO_3$, 21 mM HEPES, 2.78 mM glucose, 0.33 mM Na-pyruvate, 21.4 mM Na lactate, and 5 mg/mL BSA) in darkness for 30 min at 32°C. After loading, sperm were washed with fresh modified sperm washing medium. Then, sperm suspensions were deposited on coverslips precoated with poly-L-lysine (0.01%, w/v) for 2 min. Unattached sperm were removed by gentle washing, and the chamber was filled with modified washing medium. The measurement was made on an epifluorescence microscope (Nikon Eclipse Ti, Japan) with a 60× oil objective lens (1.40 NA) (Nikon, Japan) and a CCD camera (Spot Xplorer, USA) controlled by MetaFluor software (Universal Imaging). Excitation at 488 nm was used, and emission at 510 nm was collected.

RT-PCR analysis

RT-PCR analysis was performed as described in a previous study.[69] In brief, total RNA of human cumulus cells was extracted by TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. After reverse transcription using a first-strand cDNA synthesis kit, PCR assay was performed with CD147 primers (forward, 5′-CCCTT CCTGGGCATCGT-3′; reverse, 5′-CGGCGTCGTCATCATCC-3′). The glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was used as a loading control.

Measurement of CD147 concentration by ELISA

The sperm and seminal plasma were collected after treatment procedures in the clinical laboratory following the criteria described in the previous section. Sperm samples were lysed with cell lysis buffer (10×, 9803s, Cell Signaling Technology, USA) at 4°C according to the manufacturer's instructions. The concentrations of all extracted samples were determined. Forty micrograms of total protein from each sample was used for ELISAs with a human CD147 ELISA kit (Abcam, ab119592, USA) according to the manufacturer's protocols. This kit is designed for the quantitative measurement of human CD147 in cell culture supernatants and multiple body fluids through enzyme-linked immunosorbent assays. In brief, 100 μL of standards and diluted samples was added to wells in a 96-well precoated plate and incubated for 90 min at 37°C. After the wells were rinsed, 100 μL of 1× biotinylated anti-human CD147 antibody was added to each well and incubated for 60 min. The wells were washed three times with 300 μL of 0.01 M TBS and then incubated with 1× avidin-biotin-peroxidase complex working solution. The OD_{450} values were determined using a microplate reader (Molecular Devices, USA) when the TMB color development was finished. The minimum detection sensitivity was 2 pg/mL. The concentration of CD147 in the samples was interpolated from the standard curve. The amount of CD147 in the seminal plasma and FF was also examined by the same ELISA kit.

Statistical analysis

All morphometric data were collected blind. Comparisons between two measurements were performed by unpaired or paired two-tailed Student's t test. One-way or two-way analysis of variance was used for analysis involving more than two groups. Values of $p < 0.05$ were considered statistically significant. GraphPad Prism 7.0 (GraphPad Software, CA, USA) was used for all statistical analyses.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2021.11.009.

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AUTHOR CONTRIBUTIONS

H.C. and K.L.F. conceived and designed the experiments. H.C., X.S., Xiaofeng Li, R.D., J.J., M.K.Y., C.W., and C.L. performed the experiments and analyzed the data. Z.Q., C.L., Q.M., Xianxin Li, and D.Y.L.C. provided clinical samples. Z.C., F.L., A.Z.Z., and F.S. provided guidance. H.C. and K.L.F. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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