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ORIGINAL RESEARCH

CD147 is required for matrix metalloproteinases-2 production and germ cell migration during spermatogenesis

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ABSTRACT: Spermatogenesis is a highly programmed process that requires the degradation of the extracellular matrix and the remodeling of tight junctions (TJ) to facilitate differentiating germ cell migration. Matrix metalloproteinases (MMPs) are essential in regulating Sertoli cell TJ in the testis. CD147 is known to stimulate the production of MMPs in tumor metastasis and its knockout mice are infertile. However, the functional relationship between CD147 and MMPs in spermatogenesis has not been investigated. In the present study, we examined the expression profile of CD147 and MMPs during mouse testicular development by RT–PCR, western blot and immunofluorescence staining. We also examined CD147 involvement in the production of MMP-2 and the migration of germ cells (GC-1 and GC-2 cells) using CD147 antibody or synthetic microRNA mimics-mediated knockdown. The results showed that CD147 was present at all stages of testicular development from 7 to 56 days post-partum (dpp). CD147 expression was found to increase after 21 days from moderate levels in 7 and 14 days. Of the eight MMPs studied, MMP-2, MMP-7, MMP-9 and MMP-23 were detected to have changes in expression during testicular development, with MMP-2 showing the largest change. CD147 and MMP-2 were co-localized in spermatogonia, spermatocytes and round spermatids in mouse testis, while in human testis, they were co-localized in spermatocytes and round spermatids. MMP-2 expression and migration of GC-1 and GC-2 cells were reduced by interfering with CD147 expression and function *in vitro*. These data suggest that CD147 regulates migration of spermatogonia and spermatocytes *via* induction of MMP-2 production during spermatogenesis.

Key words: spermatogenesis / CD147 / MMP-2

Introduction

CD147, also named as basigin (Bsg) or extracellular matrix (ECM) metalloproteinase inducer (EMMPRIN), is a highly glycosylated protein that mediates a number of physiological and pathological processes, including embryo implantation, embryo development, neural functions and tumor metastasis (Iacono *et al.*, 2007). While its functions in reproduction has not been well understood, previous studies in tumorigenesis have shown that CD147 is involved in regulating the migration of tumor cells by affecting the expression of MMPs. Notably, Hanata *et al.* (2007) have reported that soluble CD147 augmented the production of MMP-2 in human fibroblasts and induced the migration of HEp-2 (human laryngeal epidermoid carcinoma) cells. When overexpressed in squamous cell carcinoma cells CD147 was able to promote the motility of these cells while inducing the productions of MMP-2, -3 and -9 (Dang *et al.*, 2008). Therefore, a functional link between CD147 and metalloproteinase activity has been established in tumor metastasis.

CD147 expression has also been observed in the reproductive system. It has been reported that CD147 is expressed in male germ cells of different differentiation stages in the testis (Maekawa *et al.*, 1998). Interestingly, CD147 knockout mice are infertile and azoospermic, with unusual ectoplasmic specialization (ES), a testis-specific cell–cell actin-based adherens junction observed in these mice (Toyama *et al.*, 1999). These observations suggest that CD147 is required for the development of germ cells and its function in the communication between germ cells and Sertoli cells is obviously important. During spermatogenesis, developing germ cells, including spermatocytes,

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round, elongating and elongated spermatids, which are differentiated from spermatogonia migrate across the seminiferous epithelium from basal compartment to the luminal edge. This progress is associated with spermatogonial dissociation from basement membrane composed of ECM and dynamic alterations of the blood-testis barrier and Sertoli-germ cell adhesion junctions. Numerous studies have demonstrated that ECM-related molecules and proteases are involved in the regulation of dynamics of these junctions and spermatogenesis in the testis, including collegen IV (Dobashi *et al.*, 2003), focal adhesion kinase (FAK) (Parsons, 2003), integrins (Cheng and Mruk, 2002; Juliano, 2002) and matrix metalloproteinases (MMPs) (Siu and Cheng, 2004b). While the role of CD147 in activating MMPs production is quite clear in tumorigenesis, it is not known if and how CD147 can affect germ cell development and migration.

The functions of MMPs in spermatogenesis cannot be overlooked. In total, 28 MMPs have been identified in mammals and 18 have been found to be expressed in the testis (Nuttall *et al.*, 2004). MMP-2 is the most comprehensively studied MMP family member in testis. It is found to be secreted by Sertoli cells in culture (Hoeben *et al.*, 1996). Furthermore, MMP-2 and TIMP-2 expression and/or activation could be stimulated by FSH in Sertoli cells (Longin *et al.*, 2001; Slongo *et al.*, 2002). However, while MMP-2 activity is important for spermatogenesis, its activity must be properly regulated as partial activation of MMP-2 by tumor necrosis factor-α leads to Sertoli cell injury (Yao *et al.*, 2009). In germ cells, MMP-2 was shown to localize in the acrosome region of sperms (Buchman-Shaked *et al.*, 2002). In addition, MMP-2, TIMP-2 and MMP-14 (membrane-type (MT1)-MMP) physically interact with laminin γ3 and are co-localized at apical ES in rat testis, suggesting the pivotal role of these MMPs in ES dynamics (Siu and Cheng, 2004a). These reports have demonstrated the possible functions of MMP-2 in various reproductive tissues.

As the stimulatory function of CD147 on MMPs has been well established in tumorigenesis, one would likely speculate that CD147 may also exert its effect on the migration of germ cells, particularly spermatogonia and early spermatocytes, during spermatogenesis by activating MMPs. In this study, we tested the functional relationship between CD147 and MMPs during spermatognenesis by monitoring the expression patterns of CD147 and MMPs during the development of mouse testis. We demonstrated that CD147 and MMP-2 were co-localized in spermatocytes and round spermatids in mouse and human testis. Furthermore, knockdown of CD147 expression by a synthetic microRNA mimic caused decrease in MMP-2 production in GC-1 cells (immortalized spermatogonial cells). Anti-CD147 neutralizing antibody could inhibit the ability of GC-1 and GC-2 cells (immortalized spermatocytes) to migrate in culture condition and reduce the activity of MMP-2 with zymography. These results suggest that CD147 regulates migration of both spermatogonia and spermatocytes *via* induction of MMP production during spermatogenesis, defect of which may lead to azoospermia and infertility as seen in CD147 knockout mice.

Materials and Methods

Animals

Male C57/BL6 mice used in this study were purchased from Laboratory Animal Services Centre (LASEC) of the Chinese University of Hong Kong. All experiments were performed under the license from the government of the Hong Kong SAR and were endorsed by the animal experimentation ethics committee (AEEC) of the Chinese University of Hong Kong.

Human sample collection

Fertile human testis specimens were obtained from post-mortem studies and orchidectomies. The ethics committee of Shenzhen Hospital of Peking University granted research approval for the human sample collection before this study.

Cells

GC-1 and GC-2 cells were purchased from the American Type Culture Collection (ATCC) (ATCC number: CRL-2053 and CRL-2196) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% Penicillin–Streptomycin in 5% CO_2 incubators at 37°C. Adherent cells were passaged every 2 days.

RNA isolation and RT–PCR analysis

Total RNA were extracted from the different ages of mice testis by Trizol reagent (Invitrogen, California, USA) and 5 μg of RNA were reversed transcribed by SuperScript II reverse transcriptase kit (Invitrogen) using

Table I Sequences of the primers used for RT–PCR.

Gene	Forward (5′–3′)	Reverse (5′–3′)	Fragment size (bp)	Annealing temperature (°C)
CD147	GTCCGATGCATCCTACCCTCCTAT	CCCGCCTGCCCCACCACTCA	549	59
MMP-2	CTCTGCGTCCTGTGCTGCCTGTTG	AAAGTGAGAATCTCCCCCAACACC	573	62
MMP-3	GCTTTGAAGGTCTGGGAGGAGGTG	CAGCTATCTTCCTGGGAAATCCTG	850	58
MMP-7	GCGGAGATGCTCACTTTGAC	GCATCTATCACAGCGTGTTC	243	58
MMP-8	TCCATTACTGATCTTCCTCCACACAC	TTGTTGATGTCTGCTTCTCCCTGTAA	431	58
MMP-9	CTCAGAGATTCTCCGTGTCCTGTA	GACTGCCAGGAAGACACTTGGTTA	241	52
MMP-13	CATCCATCCCGTGACCTTAT	GCATGACTCTCACAATGCCA	383	55
MMP-23	CGGGTTGTGCCTTCTCTC	CGACATGGTCAACAGTCTGG	145	60
MMP-24	GTGCATGCACTGGGCCATG	TAGCCTTC CTGCACCCG	400	58

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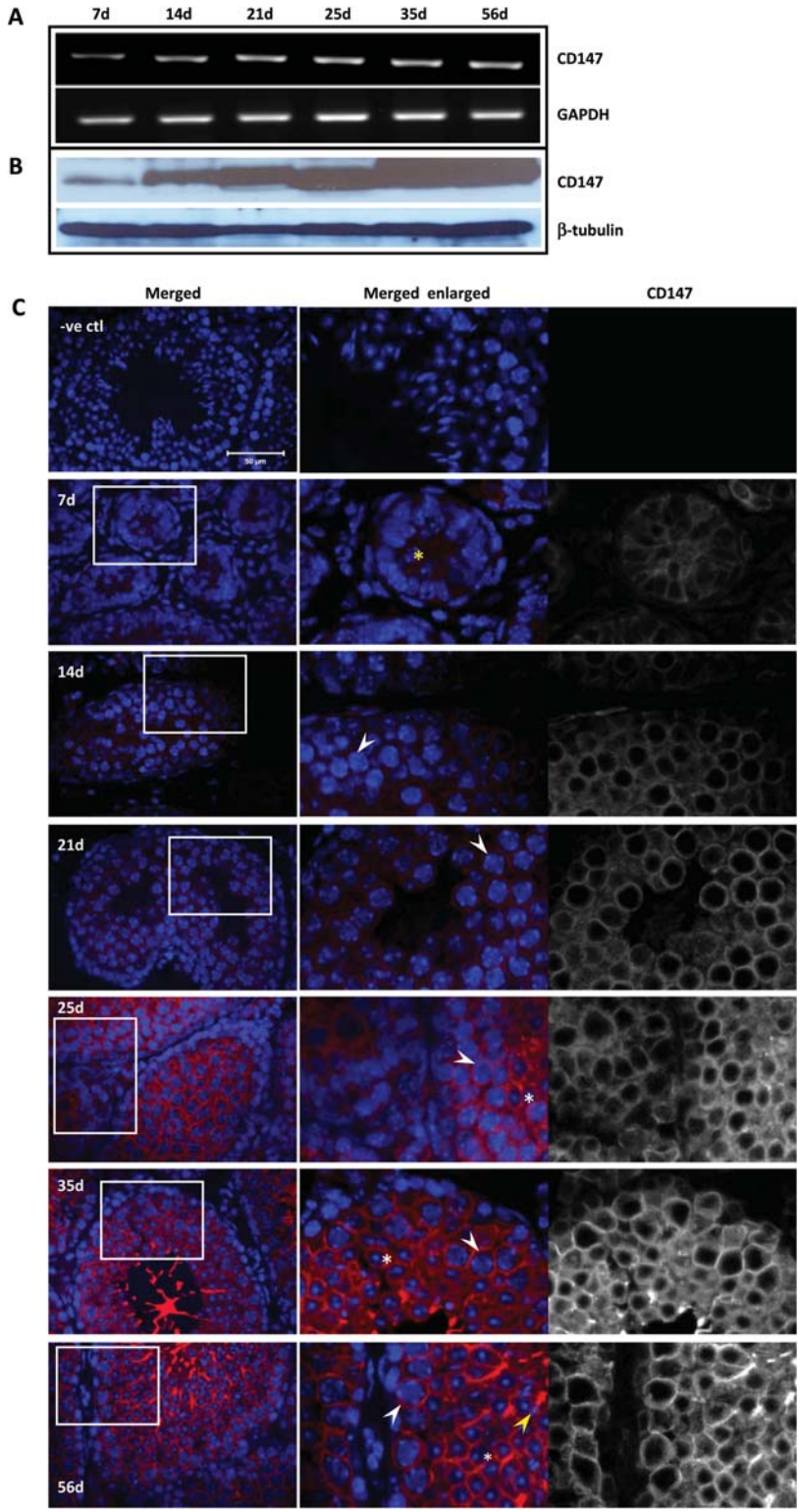

Figure 1 Expression profiles of CD147 (basigin; extracellular metrix metalloproteinase inducer, EMMPRIN) during mouse testicular development. (**A**) Expression of CD147 mRNA in developing mouse testis. (**B**) Western blot analysis of CD147 proteins in developing mouse testis. (**C**) Representative pictures of CD147 location in mouse testis development. Different stages of mouse testis were immunostained with goat anti-mouse CD147 antibody. CD147 immunoreactivity signal was found on the membrane of spermatogonia in 7 dpp testis (yellow asterisk) and spermatocytes (white arrow head) in 14 dpp testis. Intensive immunoreactivity is detected on the membrane of spermatocytes, round spermatids (asterisk) and spermatozoa (yellow arrow head) in 35 and 56 dpp testis.

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PolyA-Oligo(dT)$_{25}$ priming method according to manufacturer's instructions. RT–PCR primers for CD147 were designed by Primer 5 and primers of MMPs were described as previous studies (Pei, 1999; Chen et al., 2007; Kitamura et al., 2009). The sequences, annealing temperature and the expected sizes of these RT–PCR products are provided in Table I.

RNAi and western blot

The sequence of CD147 microRNA mimics were artificially designed with Invitrogen software (BLOCK-iT^{TM} RNAi Designer online). The designs are named with the start position of the targeted CD147 sequence: Knockdown (Kd)-323 Top strand, 5′-TGC TGA TTC CCT TCA AAC CAC CAC TGG TTT TGG CCA CTG ACT GAC CAG TGG TGT TGA AGG GAA T-3′; Bottom strand, 5′-CCT GAT TCC CTT CAA CAC CAC TGG TCA GTC AGT GGC CAA AAC CAG TGG TGG TTT GAA GGG AAT C-3′. Kd-825: Top strand, 5′-TGC TGA TTT CTT TCC GAC CTT GAT CCG TTT TGG CCA CTG ACT GAC GGA TCA AGC GGA AAG AAA T-3′; Bottom strand, 5′- CCT GAT TTC TTT CCG CTT GAT CCG TCA GTC AGT GGC CAA AAC GGA TCA AGG TCG GAA AGA AAT C-3′ (Invitrogen). The microRNA mimics were then cloned into pcDNA 6.2-GW/EmGFP-miR vector according to the manufacturer's protocol (Invitrogen). GC-1 cells were transfected with microRNA mimics using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Kd-negative control (Invitrogen) was used as a negative control under similar conditions. The silencing effects of CD147 were examined by western blot. Seventy-two hours after transfection, GC-1 cells were harvested in a lysis buffer (RAPI buffer: 50 mM Tris–HCl, pH8.0, 150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS). Western blot analysis was performed as described in our previous study (Chen et al., 2010). Total lysates of testis (40 μg per lane) at different stages were subjected to SDS–polyacrylamide gel electrophoresis and were transferred onto nitrocellulose membranes (Schleicher & Schuel, Dasse, Germany). The transferred membrane was blocked by incubating in tris-buffered saline tween 20 (TBST) plus 5% fat-free milk. The membrane was then washed three times with TBST and incubated with primary antibody (goat anti-mouse CD147, 1:500) polyclonal antibody, rabbit anti-β-tubulin (1:1000) polyclonal antibody (Santa cruz, California, USA) in TBST plus 1.5% fat-free milk at 4°C for overnight. The membrane was subsequently washed with TBST and incubated for 1 h with peroxidase-conjugated secondary antibody. The membrane was washed three times with TBST and then detected by enhanced chemiluminescence (Amersham, Piscataway, NJ, USA).

Immunohistochemistry

Normal testis of C57/ BL6 mice was fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 h and embedded in paraffin after tissue processing. Thin sections (4 μm thick) were cut from each testis and performed for double-labeling immunofluorescence staining. Sections were incubated for 1 h in blocking solution (10% normal donkey serum diluted in PBS) to avoid non-specific sites of antibody absorption. The specimen was covered with diluted rabbit-anti-MMP2 (1:100) (Santa cruz) or goat anti-mouse CD147 (1:200) (Santa cruz) overnight at 4°C. Normal IgG from homologous species of primary antibody was used as negative control. Mouse anti-human CD147 antibody (1:200, kindly from Dr Jianli Jiang) was used in the paraffin sections of human testis. The primary antibody solutions were removed from the slides and washed three times (5 min each) with 0.05% Tween-20 in 1 × PBS. Secondary antibodies (donkey anti-rabbit IgG NL 493 and donkey anti-goat IgG NL557, R&D system, Abingdon, USA) were added for 1 h at room temperature. The nuclei were stained with 300 nM 4′,6-diamidino-2-phenylindole (DAPI). In all cases, the slides were mounted with ProLong Gold Antifade Reagent (Invitrogen) and visualized on NIKON microscope (NIKON Corp, Tokyo, Japan) using 40× objectives.

Migration assay

Migration assay was used to assess the impact of immunodepleting CD147 on migration of GC-1 and GC-2 cells. This protocol was modified from a previously described method (Jiang et al., 1999). Briefly, 4 × 10^5 cells were cultured in a 6-well plate until they reached a near confluent monolayer. This monolayer was subsequently scratched with a 1 ml culture tip and the medium was replaced with fresh medium containing a 10 μg/ml goat anti-CD147 polyclonal antibody (Santa cruz). Normal goat IgG of 10 μg/ml (Santa cruz) was used as negative control. Cellular migration from the two wound fronts was tracked and recorded over a 24-h period using a live imaging system (Carl Zeiss, Jena, Germany). Migration rates were those calculated at appropriate hours intervals within the 24-h period. At least five imaging views were investigated on each plate to quantify the migration rates.

Protease expression by gelatin zymography

The expression and activation of MMP-2 after treatment with anti-CD147 antibody for 24 h in GC-1 or GC-2 cells were analyzed by gelatin zymography in cell lysates. Samples were applied onto a 7.5% SDS–polyacrylamide gel copolymerized with 0.5% gelatin. Gels were incubated for 30 min at room temperature in 2.5% Triton X-100 after electrophoresis. The Triton X-100 solution was decanted, replaced with developing buffer (50 mM Tris pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$ and 0.02% Brij35) and incubated overnight at 37°C. Gels were then stained with 0.5% Coomassie blur G in 30% methanol and 10% acetic acid for 30 min at room temperature and thereafter destained with destain solution (50% methanol and 10% acetic acid in water). Gelatinase activity was visualized as white bands on a blue background, during which the protein substrate (gelatin) has been degraded. The experiments were repeated two times.

Statistical analysis

All morphometric data were collected blindly. Statistical significance for comparison between two measurements was determined using the unpaired two-tailed Student *t*-test. One way or two way analysis of variance was used for evaluation of the three measurements. Values of $P < 0.05$ were considered significant.

Results

Development-dependent expression of CD147 mRNA and protein

RT–PCR results showed that CD147 mRNA was expressed at all stages of mouse testicular development and increased from the 7 to 56 days post-partum (dpp) (Fig. 1A). The 459 bp PCR amplification product of CD147 was validated by sequencing to ensure our RT–PCR system devoid any non-specific PCR amplification. Consistent with the RT–PCR results, protein expression of CD147 also increased in a developmental-dependent manner (Fig. 1B). In immunofluorescent study, CD147 immunoreactive signal was found in spermatogonia before Day 7 testes. CD147 signal was detected in spermatogonia and primary spermatocytes and enriched on the membrane with stronger signal on spermatocytes in 14 and 21 dpp. After Day 25, expression of CD147 was increased and mainly localized on the surface of spermatocytes and round spermatids. In the Day-56-testes, intense

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immunoreactivity for CD147 was observed on the surface of round spermatids and spermatozoa (Fig. 1C).

Development-dependent expression of mRNAs for MMPs

To determine the expression profile of different MMPs at various stages of testicular development, we perform RT–PCR to amplify the mRNAs of the MMPs using post-natal testis from 7 to 56 dpp and GC-1 cells. All amplicons were validated by sequencing. Of eight MMPs studied, the expressions of MMP-2, MMP-7, MMP-9 and MMP-23 changed in different stages of testicular development. The mRNA expression of MMP-2 and MMP-23 was also detected in GC-1 cells (Fig. 2). Interestingly, we observed a gradual decrease of expressions in MMP-2 and MMP-9, and to a slight extent, in MMP-7 and MMP-23. It is noteworthy that MMP-7 expression dropped off abruptly from 35 to 56 dpp. Together, these results show dynamic gene expression levels of MMPs, reflecting a possible role in spermatogenesis.

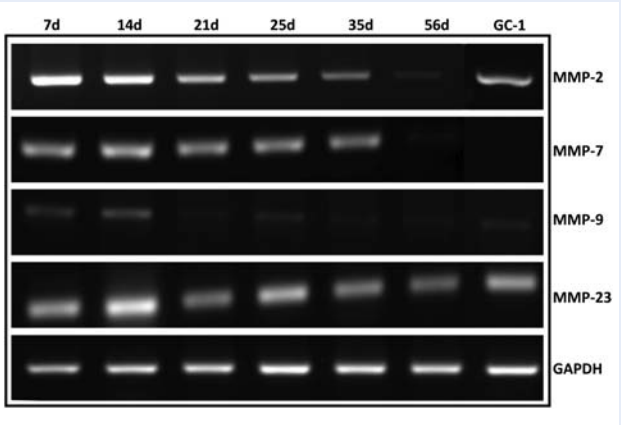

Figure 2 Transcription of MMP mRNAs in developing mouse testis. Total RNA from mouse testis at different stages and GC-1 cells was subjected to RT–PCR for MMP-2, MMP-7, MMP-9, MMP-23 and GAPDH.

Co-localization of CD147 and MMP-2

To study the functional relationship between CD147 and MMP-2, we tried to determine the localization of CD147 and MMP-2 signals in mouse testis by immunofluorescence. The results showed that CD147 and MMP-2 signals were detected in spermatogonia, spermatocytes and round spermatids (Fig. 3). Additionally, co-localization of CD147 and MMP-2 signals was found beside the elongated spermatids as a punctuate distribution. These results suggest that a functional relationship may exist between CD147 and MMP-2.

CD147 knockdown reduces the production of MMP-2 in GC-1 cells

With the observation that CD147 and MMP-2 are co-localized in spermatogonia and spermatocytes, together with the fact that CD147 regulates the production of MMP-2 in cancer cells (Chandru *et al.*, 2007; Xu *et al.*, 2007; Huang *et al.*, 2008), we hypothesize that CD147 may stimulate the production of MMP-2 in spermatogonia

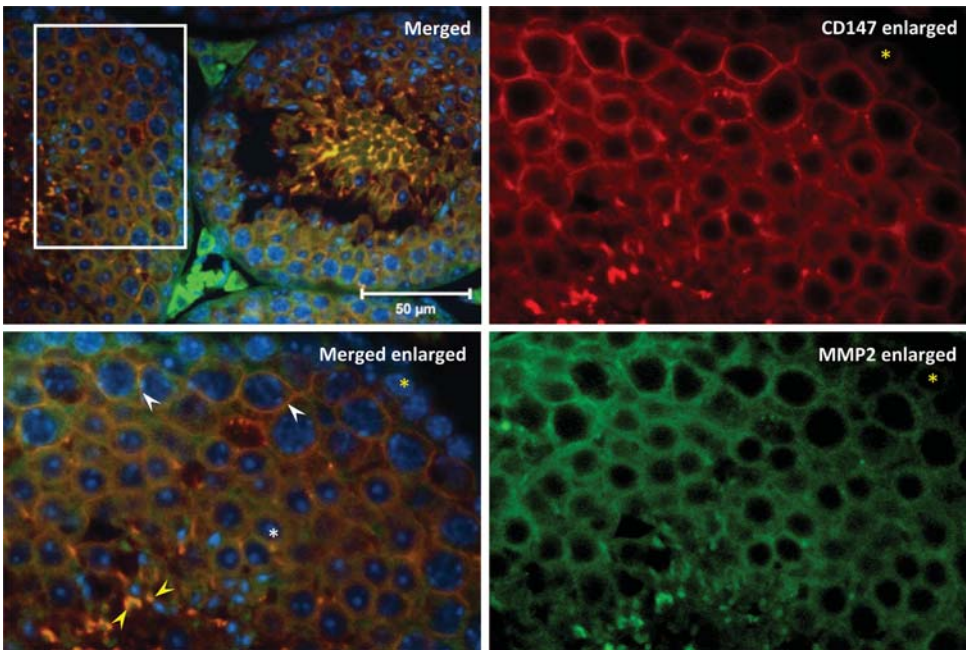

Figure 3 Co-localization of CD147 and MMP-2 in mouse testis. Thirty-five days mouse testis was double labeled with goat anti-CD147 (red) and rabbit-anti-MMP2 (green). Nuclei were counterstained with DAPI (blue). Co-localization of CD147 and MMP-2 was detected in spermatogonia (yellow asterisk) and spermatocytes (white arrow head), round spermatids (asterisk) and spermatozoa (yellow arrow head).

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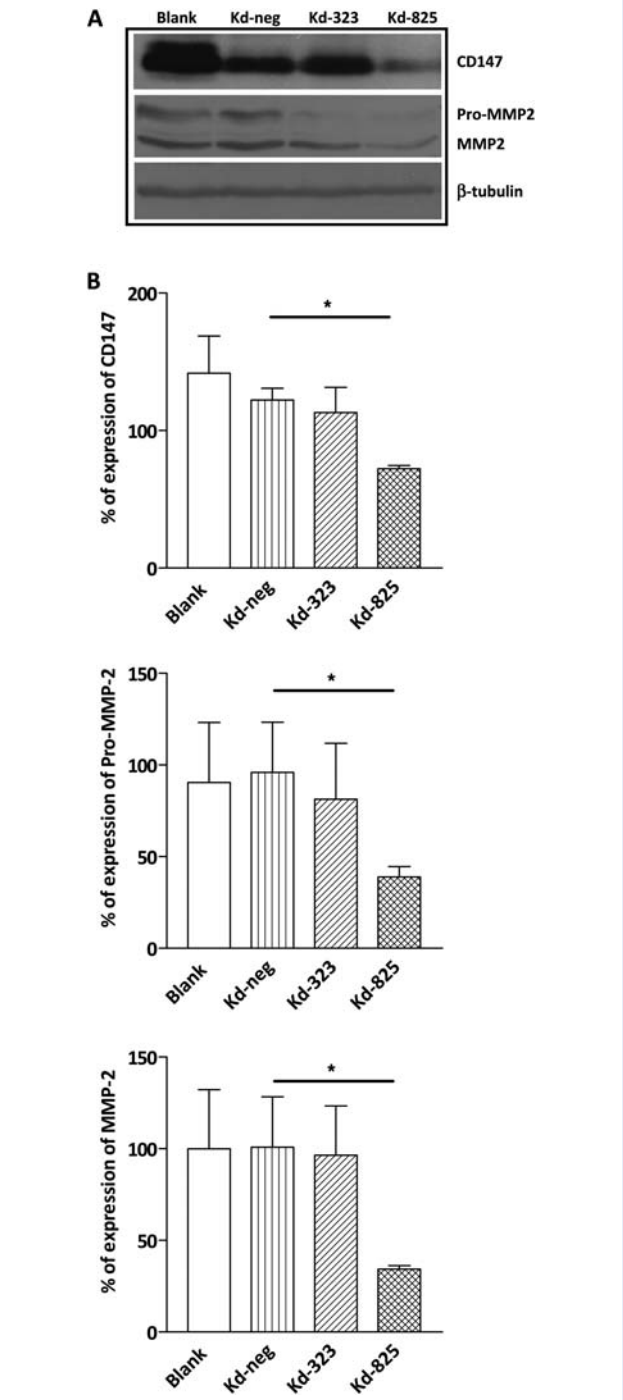

Figure 4 MicroRNA mimic-mediated CD147 knockdown reduces the production of MMP-2. (**A**) Western blot of two individual design Kd-325 and Kd-825. CD147 expression was knocked down by expression of Kd-825. MMP-2 production was significantly decreased in CD147 knockdown GC-1 cells, with beta-tubulin used as loading control. (**B**) The corresponding statistical analysis (*$P < 0.05$), the experiments were repeated three times. Values represent the mean ± SEM.

and spermatocytes for their movement during spermatogenesis. To investigate the role of CD147 on MMP-2 production, we employed a well-established microRNA-mediated knockdown strategy (McLaughlin *et al.*, 2007; Mhyre *et al.*, 2009) to knockdown the expression of CD147 in cultured mouse spermatogonia cell line GC-1. The artificially designed CD147-specific microRNA mimics (Kd-323 and Kd-825) and negative control were tested for their abilities to specifically suppress CD147 expression. The protein expression of CD147 was obviously reduced in Kd-825-transfected cells, but not in Kd-323-transfected cells 72 h after transfection. Importantly, MMP-2 expression was decreased in Kd-825-transfected cells (Fig. 4), suggesting that CD147 is required for the production of MMP-2.

Role of CD147 in MMP-2 activation and germ cell migration in culture

To determine whether the observation that CD147 regulates MMP-2 production is of functional significance, we performed a migration assay in GC-1 and GC-2 cells treated with a neutralizing antibody specific to CD147 targeting the extracellular domain (Fig. 5). We used a dose of antibody (10 μg/ml) that we have been previously determined to have no detrimental effect on cells. As shown in Fig. 5A and B, anti-CD147 antibody resulted in slower cell migration after 7 h incubation and shown the time-dependent manner, as the distance of migration at 9 h is significantly smaller in cells treated with anti-CD147 (Mean ± SEM: 64.4 ± 1.72), compared with IgG-treated control (Mean ± SEM: 72.0 ± 2.64). No significant difference was observed between blank control (Mean ± SEM: 73.3 ± 1.315) and IgG control (Mean ± SEM: 72.0 ± 2.64). Similar phenomenon was observed on GC-2 cells with time-dependent manner. The distance of migration was significantly slower after 5 h treatment with anti-CD147 antibody comparing with IgG control (Fig. 5C and D). Furthermore, blocking CD147 function with anti-CD147 antibody was accompanied by a significant decrease in MMP-2 activity both in GC-1 and GC-2 cells (Fig. 5E), represented by gelatin zymography. These results demonstrate that inhibiting the function of CD147 could reduce the production of MMP-2 and thus, the degradation of ECM is reduced. Ultimately cell migration, which requires the degradation of ECM and dynamic of cell–matrix adhesion junctions, is impaired.

Distribution of CD147 and MMP2 in normal human testis

So far, the present study has demonstrated that CD147 is required for the MMP2 activation and subsequent migration of GC-1 and GC-2 cells. As the basic mechanisms of testis development and spermatogenesis are similar between mouse and human, we believe that CD147 may contribute the spermatogenesis in human beings. One of the key findings obtained from our study in mice that can also be tested in human testis sample is whether CD147 and MMP2 are co-localized in normal human testis. To detect expressions of CD147 and MMP-2 in human testis, we performed immunofluorescence staining of these two proteins and found that they were localized in spermogonia, spermatocytes and round spermatids, much like their expressions found in mouse testis (Fig. 6). As CD147 and MMP-2 are co-localized in human testis, it suggests that CD147 is also required for MMP-2 production in human testis and has function on the germ cell migration during spermatogenesis in humans.

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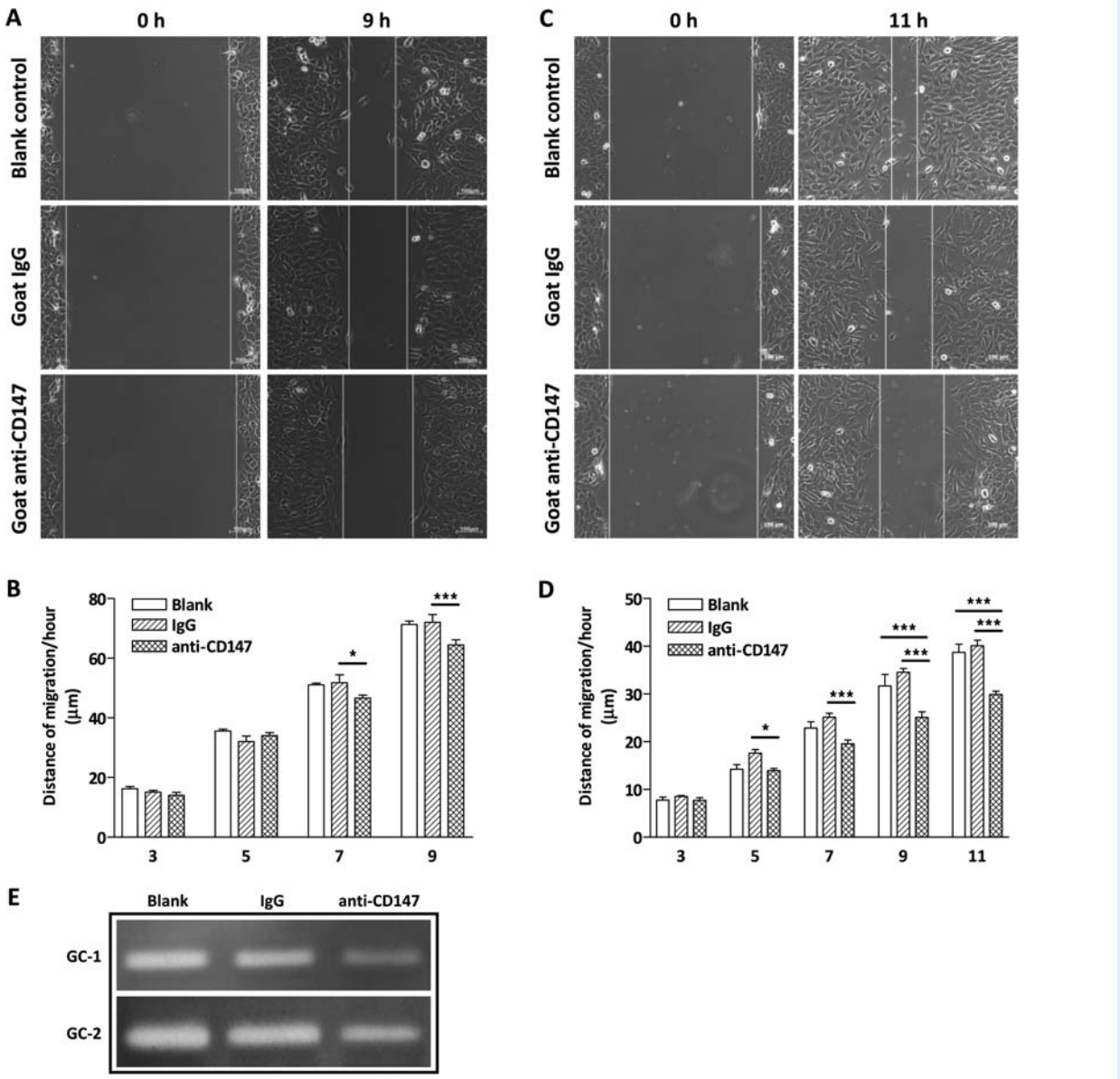

Figure 5 Ability of germ cells migration and MMP-2 activity was inhibited by blocking CD147 functions. (**A** and **B**) Representative photographs of treated and untreated GC-1 cells are presented (×10 magnification). Cells were treated with 10 μg/ml of goat anti-CD147 antibody or normal goat IgG for 9 h. Statistical analysis of migration ability of GC-1 cells. Values represent the mean ± SEM (*$P < 0.05$; ***$P < 0.001$). (**C** and **D**) Representative photographs of treated and untreated GC-2 cells are presented (×10 magnification). Statistical analysis of migration ability of GC-2 cells. Values represent the mean ± SEM (*$P < 0.05$; ***$P < 0.001$). (**E**) MMP-2 activity by zymography in GC-1 (or) GC-2 cell lysate.

Discussion

The present study has clearly demonstrated the expression of CD147 and MMPs in mouse testicular development ranging from Day 7 to adult stage and the co-localization of CD147 and MMP-2 both in mouse and human testis. Developmental expression of CD147 mRNA and protein were assessed in all stages of mouse testis. An obvious increase of CD147 signal was observed after Day-21-mouse testis. We observed a developmental dependent expression pattern of CD147 in mouse testis and inhibition of migration of immortalized spermatogonia and spermatocytes, GC-1 and GC-2 cells, respectively, by CD147 knockdown with a reduction in MMP-2. These observations, together with the previously observed azoospermia and infertility in CD147 knockout mice (Toyama *et al.*, 1999), suggest its important role in spermatogenesis.

CD147 has been characterized as an inducer of MMP expression in tumor metastasis (Biswas *et al.*, 1995; Zucker *et al.*, 2001). It also stimulates the expressions of MMPs in a number of cell culture systems (Guo *et al.*, 1997; Li *et al.*, 2001). This prompted us to evaluate the relationship between CD147 expression and MMPs expression during mice testicular development. The distribution of MMP-2 appears to be around the spermatogonia, spermatocytes, round and

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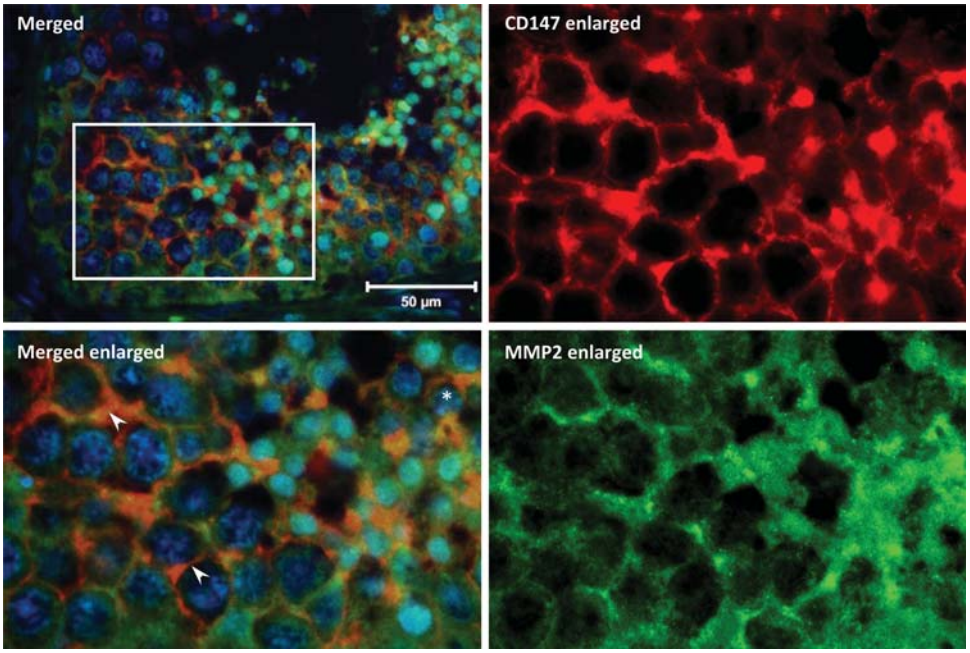

Figure 6 Co-localization of CD147 and MMP-2 in normal human testis. Samples of human testis were double labeled with mouse-anti-human CD147 (red) and rabbit-anti-MMP2 (green). Nuclei were counterstained with DAPI (blue). Co-localization of CD147 and MMP-2 was mainly detected on the surfaces of spermotocytes (white arrow head) and round spermatids (asterisk).

elongated spermatid in the adult testis. It seems to indicate that MMP-2 may be involved in the migration of germ cells and the release of differentiating germ cells from the basal lamina in the seminiferous tubules. Intriguingly, CD147 and MMP-2 are co-localized on the surface of spermatogonia, spermatocytes and round spermatids in adult mouse seminiferous tube. These data indicate that CD147 might function as a MMP-2 inducer in developing germ cells, particularly spermatogonia and spermatocytes, for their migration. However, the expression patterns of CD147 and total MMP-2 do not seem to have the same trends during testicular development, which might suggest that CD147 could have other function than to induce MMP-2 expression.

In the present study, we have demonstrated a similar expression profile for MMP-2 and MMP-9 mRNA in mouse testis. Interestingly, MMP-2 mRNA in mouse testis decreased during testicular development in our experiments. While MMP-2 mRNA was also detected during rat testicular development in a previous study, it was only observed in somatic cells (Longin *et al.*, 2001). However, Siu and Cheng (2004c) has reported that MMP-2 was found in the seminiferous epithelium-associated elongating/elongated spermatids in rat testis. In the present study, CD147 and MMP-2 were both detected in a spermatogonial cell line (GC-1 spg), suggesting that the source of MMP-2 was not only restricted to somatic cells in mouse testis. Interestingly, Baumgart *et al.* have evaluated MMP-2 and MMP-9 contents in human seminal plasma and their association with sperm count. Their results showed that the concentrations of MMP-2 in normozoospermic plasma were significantly higher than that of the azoospermic plasma (Baumgart *et al.*, 2002). Consistent with this observation, the production of activated MMP-2 was decreased by the knockdown of CD147 in our study. Interfering with CD147 function using its antibody also resulted in significant reduction in GC-1 and GC-2 cell migration in culture and decrease of MMP-2 activity. However, it should be noted that anti-CD147 only caused a 10% decrease in cell migration in GC-1 cells. The most likely explanation is that the antibody used may not fully neutralize CD147 function. Another possibility is that the migration of spermatogonia does not entirely depend on MMP production by the germ cells. In fact, it has reported that somatic cells in the testis also contribute to the activation of MMP-2 (Hoeben *et al.*, 1996), while what we measured in GC-1 cells was only the contribution from the germ cells in our experiments. Interestingly, the inhibition of migration in GC-2 cells was more prominent compared with GC-1 cells, suggesting that CD147 may be more important for spermatocytes development and migration. Collectively, the results we presented here and those from previous studies suggest that MMP-2, regulated by CD147, is involved in germ cell maturation during spermatogenesis. It should be noted, however, the increase in CD147 expression during development is not accompanied with an increase in MMP-2 expression, indicating possible role(s) of CD147 in spermatogenesis other than regulating MMPs. Further investigation of this is currently undertaken in the authors' laboratory.

We have also examined the distribution of CD147 and MMP-2 in normal human testis and found that they co-localized the same way as their mouse counterparts. This result supports a functional role of CD147 in spermatogenesis in humans, probably in regulating MMP production. Interestingly, a recent study showed that the concentration of MMP-2 in human seminal plasma was correlated with sperm count in azoospermic patients, confirming an important role of MMP-2 in sperm production. Given the importance of MMP-2 in

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spermatogenesis, the presently demonstrated effect of CD147 on MMP-2 expression, spermatogonia and spermatocytes migration, together with their co-localization in both mouse and human testes, has indicated a potentially important role of CD147 in spermatogenesis. This study thus provides the first explanation at the molecular level to why CD147 knockout mice are azoospermic and infertile. Further study examining the expression of CD147 in testes of azoospermic patients may elucidate the molecular pathogenesis of azoospermia and help evaluate its potential as a target for contraceptives.

Authors' roles

H.C.C. and Z.C. conceived and designed the experiments and final approval of the version to be published. H.C. and K.L.F. performed the experiments. H.C. analyzed the data. H.C., S.Y. and H.C.C. drafted the article. Y.G., J.J. and Z.C. revised the article critically.

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