Transcripts encoding the sperm surface protein tMDC II are non-functional in the human

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Five members of the MDC (**m**etalloproteinase-like,**d**isintegrin-like **c**ysteine-rich domain) family of proteins (fertilin α, fertilin β, tMDC I, tMDC II and tMDC III) are expressed on the surface of macaque (*Macaca fascicularis*) sperm, where they have been proposed to play a role in sperm–egg binding via an interaction between their disintegrin-like domain and one or more integrins on the egg plasma membrane. Of these, two (fertilin α and tMDC I) have recently been shown to be non-functional in the human. Here we report the existence of multiple isoforms of human tMDC II transcripts in the human, all of which are also non-functional owing to the presence of deletions and in-frame termination codons, when compared with the macaque orthologue, a finding which is further supported by the lack of immunoreactivity on Western blots of human testis and sperm extracts probed with a macaque anti-tMDC II polyclonal anti-serum. These results are discussed in the context of our proposed model for multiple proteins implicated in sperm–egg interactions.

Key words: aberrant splicing, disintegrin, fertility, sperm–egg recognition, testis.

INTRODUCTION

Ever since the pioneering work of Bronson, Fusi and others almost a decade ago, integrin interactions have been implicated in the process of mammalian sperm–egg binding prior to fertilization [1–3]. The discovery that the α and β subunits of the guinea-pig sperm surface fertilin complex each contain a disintegrin-like domain [4], reminiscent of the integrin-binding disintegrins associated with some snake venoms, subsequently led Blobel and co-workers to propose a model [4]. They suggested that the disintegrin-like domain of the fertilin β subunit played a role in oolemma binding, and a putative fusogenic peptide within the fertilin α subunit mediated subsequent membrane fusion. Experimental evidence to support a role for fertilin β in integrin-mediated oolemma interactions has stemmed largely from studies using peptide mimics of the disintegrin-like domain [5,6] or recombinant fertilin β [7], both of which inhibit sperm–egg interactions *in vitro*. However, fertilin α and fertilin β are not the only sperm surface proteins possessing a disintegrin-like domain. Indeed they represent members of a large and growing family of integral membrane proteins, commonly known as the MDC [8] or ADAM [9] family, which possess **m**etalloproteinase-like, **d**isintegrin-like and **c**ysteine-rich domains, with an organization characteristic of many snake-venom haemorrhagic proteins. A number of these MDC proteins are abundantly expressed in the male reproductive tract, and several have been shown to be present on the surface of rodent and macaque (*Macaca fascicularis*) spermatogenic cells [8–10,11], including fertilin α, fertilin β, tMDC I (also known as cyritestin), tMDC II and tMDC III. Each possesses a conserved XCD (Xaa-Cys-Asp) tripeptide within the disintegrin-like domain, which we believe represents the integrin-binding motif [8,10,11]. This common structural feature, together with their localization on spermatogenic cells, makes tMDC I, tMDC II and tMDC III equally attractive egg-binding candidates although, to date, most functional studies on reproductive MDC proteins have concentrated on the fertilin subunits. However, recent studies on a fertilin β knockout mouse [12] have demonstrated that, while fertilin β-null males have greatly decreased fertility, this could be largely attributed to causes other than impaired oolemma binding, supporting the view that other MDC proteins may be equally, if not more, important in sperm–egg interactions. Indeed, recent *in vitro* studies on mouse tMDC I have supported a role for this protein in rodent sperm–egg interactions [13,14].

For practical reasons, virtually all *in vitro* studies aimed at establishing a role for disintegrin–integrin interactions in sperm–egg recognition have utilized rodent model systems. Nevertheless, in view of the obvious attraction of such interactions as a target for human contraceptive intervention, we have embarked on a study of the corresponding MDC orthologues, expressed in the human testis.

Human fertilin β transcripts have been cloned ([15,16]; J. Frayne and L. Hall, unpublished work) and the corresponding mature protein demonstrated in human sperm membrane extracts [17] by Western blotting. However, in light of the results with the fertilin β knockout mouse, we have also cloned and characterized transcripts encoding additional, abundant human sperm surface MDC proteins as potential egg-recognition proteins. Human fertilin α transcripts, although expressed, proved to be non-functional [18], owing to the presence of insertions, deletions and in-frame termination codons within the single fertilin α gene, a property they share with gorilla (*Gorilla gorilla*) fertilin α [19]. More recently, we have found that human tMDC I transcripts are also non-functional, again due to the presence of deletions, insertions and in-frame termination codons [17]. Hence two of the major MDC proteins (fertilin α and tMDC I) implicated in sperm–egg interactions in rodents are not present on human sperm, raising important questions about their dispensability. We now present compelling evidence that tMDC II, one of the few remaining, abundantly expressed, sperm surface MDC

Abbreviations used: RT-PCR; reverse-transcription PCR; MDC proteins, a novel family of multidomain integral membrane proteins all of which possess a **m**etalloproteinase-like domain, a **d**isintegrin-like domain and a **c**ysteine-rich domain.

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The nucleotide sequence data discussed (but not shown) in this paper appear in the EMBL, GenBank® and DDBJ Nucleotide Sequence Databases under the accession numbers AJ132820–AJ132827 inclusive (human tMDC II mRNA isoforms [a]–[h] respectively).

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proteins identified in rodent and macaque systems, is also non-functional in the human. This finding is discussed in the light of our current understanding of integrin-mediated sperm–egg interactions and their proposed role in mammalian fertility.

MATERIALS AND METHODS

Reverse-transcriptase PCR (RT-PCR)

A pool of human testis total RNA, isolated from 29 sudden-death victims (aged 23–65), was obtained from Clontech Labs (Palo Alto, CA, U.S.A.). Human heart, skeletal-muscle and uterus total RNA was extracted as described previously [8]. Total RNA (5 μg) was used as a template for oligo$(dT)_{12-18}$-primed Expand™ (Boehringer Mannheim, Lewes, East Sussex, U.K.) reverse-transcriptase-directed cDNA synthesis using buffer and conditions supplied by the manufacturer. Subsequent PCR amplification of tMDC II sequences (1 min at 94 °C; 2 min at 58 °C; 1 min at 72 °C; 30 cycles) used 10 % of this cDNA and Expand™ (Boehringer Mannheim) High Fidelity PCR system. The resulting PCR products were resolved on low-melting-temperature agarose gels and appropriate bands excised, purified and cloned into pGEM®-T Easy plasmid vector (Promega U.K., Southampton, U.K.). Cloned cDNA inserts were completely sequenced on both DNA strands using an ABI 377 automated DNA sequencer and a custom primer walking strategy.

Western-blot analysis of human tMDC II

Macaque and human testis extracts were prepared by homogenization of tissues in PBS containing 1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride (ICN Biomedicals Ltd., Thame, Oxon, U.K.), followed by incubation on ice in the same buffer containing 1 % (v/v) Triton X-100 (Sigma Chemical Co., Poole, Dorset, U.K.). Homogenates were then centrifuged at 10000 ***g*** and supernatants collected.

Plasma-membrane-enriched fractions of macaque cauda-epididymidal sperm and human ejaculated sperm were prepared by detergent extraction with 1 % (v/v) Triton X-100 in PBS and vortex-mixing [20] in the presence of 1 mM 4-(2-aminoethyl)-benzenesulphonyl fluoride.

Proteins (100 μg/lane) were separated by electrophoresis under reducing conditions on 12 % (w/v) polyacrylamide gels containing SDS, then electroblotted on to PVDF membranes (PolyScreen™; NEN Life Science Products, Brussels, Belgium). Blots were probed as described previously [10] with affinity-purified polyclonal antisera raised against recombinant proteins containing the region of macaque tMDC II (antiserum J105) or macaque fertilin β (antiserum J73) from the end of the disintegrin-like domain through to the natural C-terminus [10]. A polyclonal antiserum (H104), raised against a synthetic peptide (RKKLRELCYRGETESESSVS) from the C-terminal cytoplasmic domain of macaque tMDC II was also used to probe Western blots of macaque testis protein extracts and human sperm plasma-membrane-enriched protein preparations.

RESULTS

Sequence analysis of human tMDC II transcripts.

The entire coding region of human tMDC II cDNA was obtained as a series of three overlapping PCR fragments as follows.

Initially, a 650 bp fragment encoding the C-terminal half of the cysteine-rich domain, the transmembrane domain and the C-terminal cytoplasmic domain of human tMDC II was amplified by PCR using primers based on the macaque orthologue [21] with human testis cDNA as the template. DNA sequence analysis of this cloned PCR product confirmed a high degree of sequence identity (90 %) with macaque tMDC II.

The next set of PCR reactions utilized reverse primers based on this cloned human tMDC II 3′ cDNA fragment and macaque-based forward primers derived from the C-terminal end of the pro-domain. Several PCR products were obtained (ranging from about 0.7 to 1.0 kbp), all significantly shorter than the predicted size (1.52 kbp) based on the macaque tMDC II cDNA sequence (see Figure 1B). The four major PCR products were excised from an agarose gel and directly sequenced. In each case, comparison with the corresponding macaque tMDC II region revealed the presence of one or more large deletion(s) within the human sequence (Figure 1A, isoforms [a]–[d]); however, within common regions the level of sequence identity was high (greater than 80 %). Sequence analysis of some of the minor PCR products observed in Figure 1(B) revealed the presence of intronic sequences, indicative of incomplete or aberrant RNA splicing.

Several additional PCR primer pairs, based on the human tMDC II sequence obtained above, were used to amplify across the deleted regions in the human tMDC II transcripts, in an attempt to establish whether any transcripts contained the regions absent in isoforms [a]–[d].

The remaining protein coding region (signal peptide and N-terminal end of pro-domain) was obtained by PCR using a reverse primer based on the above human tMDC II sequence and a forward primer based on the macaque sequence. Three PCR products were obtained (430, 375 and 290 bp), and all were sequenced directly. The largest product showed a very high degree of sequence identity (96 %) with the corresponding macaque tMDC II region, but possessed a 2 bp deletion at position 158–159, resulting in disruption of the expected reading frame (Figure 1A, isoforms [a]–[d]). The two smaller products (Figure 1A, isoforms [e]–[f]) contained internal deletions of the larger product, both of which would also interrupt the expected reading frame.

Compilation of sequence data from all PCR products described above demonstrated the presence of many human tMDC II transcript isoforms in the testis. However, the relative abundance of these different isoforms varied significantly, as judged by the relative intensities of the corresponding PCR bands, with four more prominent isoforms. These four major isoforms (Figure 1A, isoforms [a]–[d]) all possessed either one or two large deletions when compared with macaque tMDC II cDNA, as well as a two-base deletion at position 158–159. Although the positions of introns with the tMDC II gene are unknown, we have previously observed that many of the exon–intron boundaries are conserved throughout the MDC genes abundantly expressed in the male reproductive tract [17], with the notable exception of the fertilin α gene, which is intronless [18,19]. Hence, when the exon–intron boundaries of the mouse fertilin β gene [22] are mapped on to the human tMDC II transcripts (see Figure 1A), the large deletions are all found to correspond precisely to one or more predicted exons; transcript [a] lacks fertilin β exons 8, 9, 10, 11 and 13; transcript [b] lacks exons 7, 8, 9, 10, 11 and 13; transcript [c] lacks exons 8–13 inclusive; and transcript [d] lacks exons 7–13 inclusive. No transcripts containing putative tMDC II exons 8, 9, 10 or 12 were detected using a variety of flanking PCR primers. It would therefore appear that all human tMDC II transcripts are incorrectly spliced.

In addition to the four major tMDC II transcripts, a number of more minor species were also observed. Direct sequence analysis of these identified a number of additional deletions, two near the 5′ end of the transcript (Fig. 1A, [e] and [f]) and two near to the 3′ end (Fig. 1A, [g] and [h]). Each of these deletions

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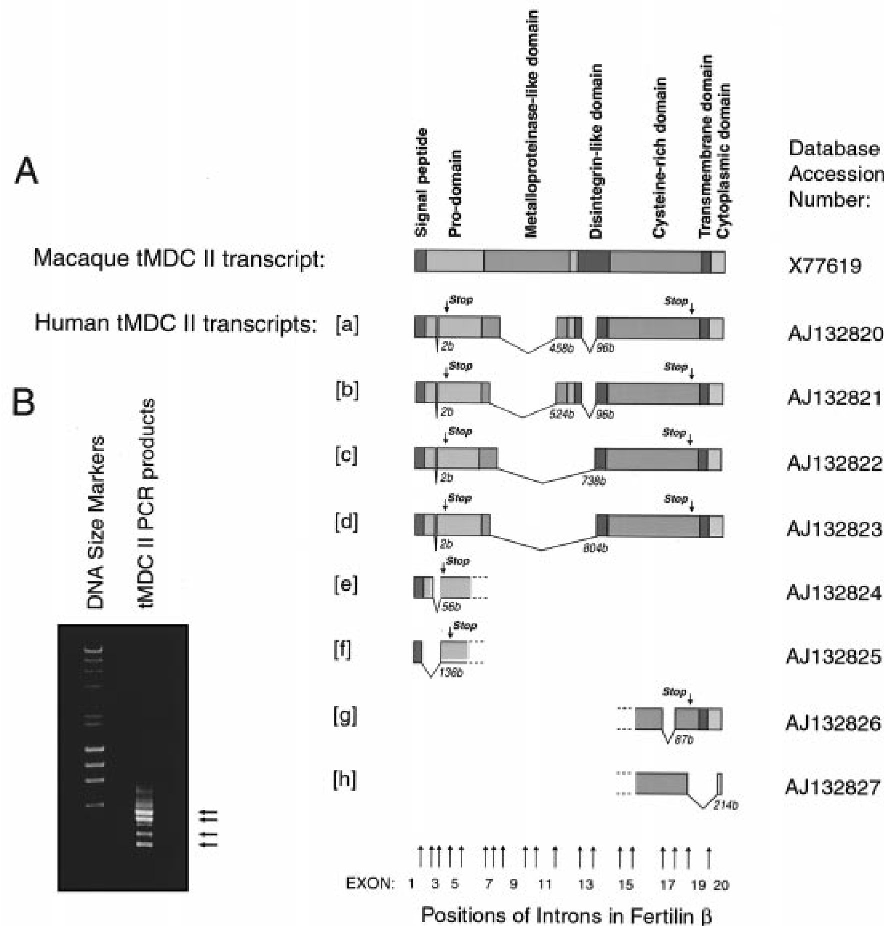

Figure 1 Diagrammatic representation of human tMDC II transcripts

(**A**) The domain organization of a series of human tMDC I transcripts is shown, with macaque tMDC II for comparison. Positions of deletions (with sizes in italics) and the first in-frame termination codon is indicated for each transcript. An additional termination codon within the expected reading frame (relative to the macaque orthologue), just prior to the transmembrane domain, is also indicated, together the putative exon/intron boundary positions based on the genomic organization of the mouse fertilin β gene [22]. Database accession numbers for each transcript isoform are indicated. (**B**) Agarose-gel electrophoresis of PCR products derived from human tMDC II cDNA using a pair of primers which flank the deletions. The four major tMDC II isoforms (corresponding to isoforms [a]–[d]) are indicated by arrows. The minor PCR products contained intronic sequences, indicative of incomplete or aberrant RNA splicing.

corresponded precisely to one or two complete exons (based on the mouse fertilin β genomic organization).

In addition to the multitude of large deletions associated with the human tMDC II transcripts, many of which interrupt the expected reading frame, the major transcripts ([a]–[d]) all contained a two-base deletion at position 158–159. This frameshift results in an in-frame termination codon soon after, at position 217–219, in human tMDC II isoforms [a]–[d]. In isoform [e], the inappropriate splicing out of exon 3 results in an earlier in-frame termination codon at position 163–165, and in isoform [f], the splicing out of exons 2 and 3 results in an in-frame termination codon at position 127–129. Overall, the predicted translation product for human tMDC II isoforms [a]–[d] would be 72 amino acid residues, for isoform [e] would be 54 residues and for isoform [f] would be 42 residues. These correspond to the signal peptide (21 residues) plus a very short stretch of the pro-domain. By analogy with its well-characterized snake-venom counterpart, the pro-domain region of an MDC protein is likely to be essential for correct folding of the nascent protein. Hence it seems highly unlikely that these truncated proteins, containing a signal peptide (and hence destined for secretion) and a partial pro-domain, could have any physiological function.

All human tMDC II transcripts also contained a termination codon in the expected reading frame (corresponding to histidine in the macaque tMDC II sequence) prior to the transmembrane domain (Figure 1A). This termination codon, regardless of the two-base deletion close to the 5′ end of the transcript (position 158–159), would ensure that any translation product would lack the C-terminal transmembrane domain and would not therefore be retained on the sperm surface.

Tissue distribution of human tMDC II transcripts

In rats, MDC II transcripts are exclusively expressed by spermatogenic cells and cannot be detected in tissues other than the testis, even using sensitive RT-PCR approaches [8]. In the macaque, tMDC II transcripts were also abundantly expressed in the testis but, somewhat surprisingly, very low levels of expression were also observed by RT-PCR in other tissues including skeletal

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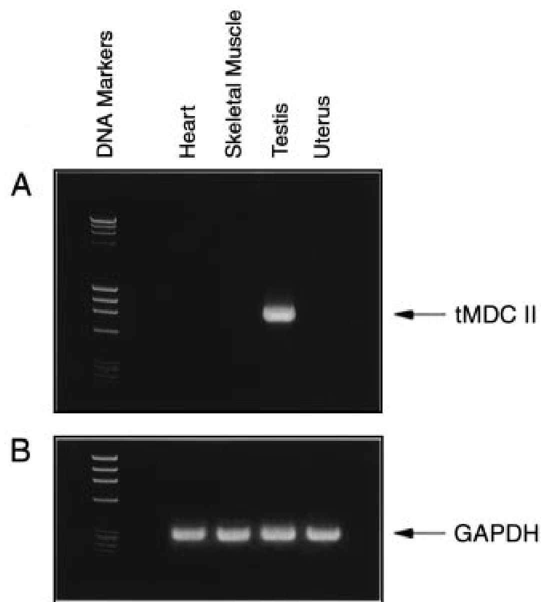

Figure 2 Tissue distribution of human tMDC II transcripts

Human tMDC II transcripts (**A**) were detected by RT-PCR as described in the Materials and methods section using total RNA from heart, skeletal muscle, testis and uterus as template and human tMDC II-specific primers. Parallel PCR reactions using glyceraldehyde-3-phosphate dehydrogenase primers (**B**) were carried out to ensure equivalence of cDNA concentration in each sample.

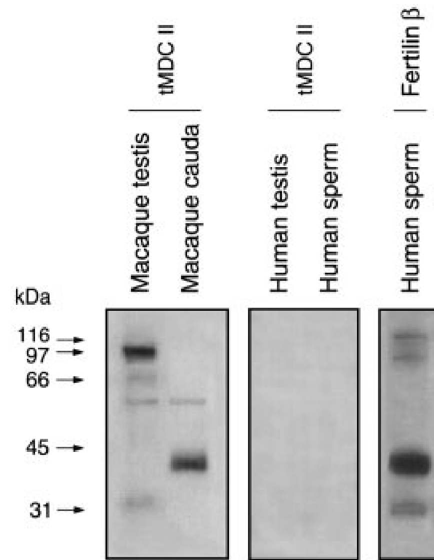

Figure 3 Western blot analysis of tMDC II

Macaque and human testis and sperm membrane extracts were separated on SDS/12%-PAGE gels, electroblotted and probed with a macaque anti-tMDC II polyclonal antiserum (J105). The human sperm membrane extract was also probed with a macaque anti-(fertilin β) polyclonal antiserum, to check its integrity.

muscle, uterus and ovary [10], the physiological significance of which, if any, is difficult to assess.

In the present study we have again used RT-PCR with specific primers designed to amplify a 795 bp PCR product of human tMDC II which spans a number of exon/intron boundaries (thereby eliminating possible problems associated with the amplification of contaminating genomic DNA), and does not encompass any of the deleted regions. As can be seen from Figure 2, human tMDC II transcripts were expressed abundantly in the testis, but could not be detected in the three other tissues examined (heart, skeletal muscle and uterus), suggesting that, at least within this limited range of tissues, tMDC II expression is highly tissue-specific in the human.

Western-blot analysis of tMDC II

The presence of the various deletions within all tMDC II transcripts sequenced, and the observed in-frame termination codon (relative to the macaque orthologue) prior to the region encoding the expected transmembrane domain, would all preclude the synthesis of a sperm surface integral membrane protein. To further substantiate this prediction, Western-blot analyses were performed on membrane preparations from human testis and ejaculated spermatozoa, using a tMDC II-specific antiserum (J105) raised against a middle/C-terminal region of the macaque tMDC II protein with approx. 90 % overall amino acid sequence identity with human tMDC II over regions common to both transcripts. No immunoreactivity was observed with human testis and sperm extracts (Figure 3), even using highly sensitive enhanced chemiluminescence. In contrast, this antiserum readily detected a protein of approx. 88 kDa in extracts of macaque testicular cells and of approx. 43 kDa in extracts of macaque cauda-epididymidal spermatozoa (Figure 3), representing precursor and processed mature forms of macaque tMDC II respectively. The integrity of the human protein extracts was verified by probing a parallel blot with an antiserum raised against a similar region of macaque recombinant fertilin β [10]. This antiserum detected a protein of the size expected for mature human fertilin β (Figure 3). Finally, Western blots were also probed with a polyclonal antiserum (H104) raised against a 20-residue C-terminal synthetic peptide derived from the cytoplasmic domain of macaque tMDC II which differed in only two residues from the human tMDC II sequence deduced from its cDNA. Once again, no immunoreactivity was observed with human testis and sperm membrane protein extracts (results not shown).

DISCUSSION

Screening a macaque cDNA library for tMDC II transcripts resulted in the isolation of more than ten independent, non-sibling clones, none of which possessed deleted regions [21]. Northern-blot analysis of macaque testicular polyadenylated RNA probed with a tMDC II cDNA clone [21] identified a single hybridizing species of 2.65 kb, indicating the presence of a single class of transcript. More recent RT-PCR-based analyses of macaque tMDC II transcripts with several primer pairs yielded single PCR products (results not shown), again indicative of a high degree of homogeneity in macaque tMDC II transcripts.

In contrast, DNA sequence analysis of human tMDC II transcripts has revealed a variety of isoforms, all of which possess multiple deletions and in-frame termination codons, rendering them non-functional. All of the large deletions map to the known positions of exon/intron boundaries in the related mouse fertilin β gene, implying that these deletions within the human tMDC II transcripts arise by the aberrant splicing of non-adjacent donor and acceptor splice sites, thereby excising two or more introns, and associated exon(s), in a single splicing event. We have recently reported very similar findings in the case of the human tMDC I gene, which is also non-functional in the human

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[17]. Taken together with the absence of a functional human fertilin α gene [18,19], it is now apparent that three of the five MDC proteins abundantly expressed in the testes of non-human primates and located on mature caudal sperm [10] are not expressed in the human, despite the substantial experimental data implicating two of these proteins (fertilin α and tMDC I) in sperm–egg interactions in rodent systems [5–7,13,14]. Such a finding is in keeping with our recently proposed hypothesis [23] that sperm–egg interactions involve many sperm proteins which act co-operatively, but are not absolutely essential. This paradigm, which attempts to reconcile the large number of sperm proteins which have been implicated in egg recognition, implies that the concerted effects of all these interactions will lead to 'maximum fertilizing ability', but that individual proteins are not indispensable. Nevertheless, the co-operative nature of these interactions would mean that progressive loss of individual proteins might be manifest as a reduction in 'fertilizing ability'. For most sperm surface proteins implicated in sperm–egg interactions (including MDC proteins), specific antisera, peptide mimics or recombinant proteins significantly decrease, but do not completely block, those interactions *in vitro*, implying that each does not play an essential role, thereby lending support to our hypothesis.

An alternative hypothesis might be that many of the MDC proteins expressed on the sperm surface are functionally redundant, with sperm–egg interactions requiring just one or two MDC proteins. However, whilst such a 'fail-safe' mechanism might be attractive, we feel that this is unlikely. A very large number of MDC transcripts have been sequenced from a wide range of non-human species with no evidence for loss of function. If many of these MDC proteins were functionally redundant we might expect to have identified at least one non-functional transcript in the rat, mouse or macaque, given the apparently high loss of functionality in the human, but this is not the case. There would therefore appear to be strong selective pressure to maintain the integrity of sperm surface MDC proteins in the non-human species which have been examined to date, and it is tempting to correlate this with the need to maintain a high 'fertilizing ability' for survival in many species, including rodents. In contrast, there is arguably less pressure to maintain 'maximum fertilizing ability' in humans, who do not need to, or strive to, produce such large numbers of offspring. The lower 'fecundability' of humans, when compared with many other species, may therefore reside in part in the non-functionality of sperm surface egg-binding proteins implicated in fertility.

Of the five characterized MDC proteins expressed on mature macaque sperm (fertilin α, fertilin β, tMDC I, tMDC II and tMDC III), three are not expressed in the human. One of the remaining two, fertilin β, we have shown to be expressed on human sperm by Western blotting [17]. Yet despite the considerable wealth of *in vitro* data implicating fertilin β as an important oolemma-binding protein in rodents, the decreased fertility of mouse fertilin β-null males was largely attributed to causes other than impaired oolemma binding. In particular, sperm were rarely observed in the oviducts of female mice mated with fertilin β-null males and the number of fertilin β-null sperm binding to the zona pellucida *in vitro* was significantly reduced. Whether this implies alternative or additional functions for fertilin β, or simply indirect effects associated with the lack of fertilin β on the membranes of the knockout sperm, remains to be established (see [24] for discussion). Nevertheless, the fact that fertilin β-null sperm are not completely infertile does raise the likelihood that additional sperm surface integrin-binding proteins are involved in sperm–oolemma interactions.

Very recently we have sequenced the human orthologue of the fifth characterized MDC protein expressed on the macaque sperm surface, namely human tMDC III, and found it to be potentially functional (E. A. C. Dimsey, J. Frayne and L. Hall, unpublished work). To date no functional studies have been performed on tMDC III orthologues from any species; indeed only the rat and macaque orthologues have previously been reported [8,10]. It therefore remains to be established whether tMDC III, possibly in collaboration with fertilin β, plays a major role in sperm–oolemma binding in the human.

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