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The Acrosomal Protein Dickkopf-Like 1 (DKKL1) Facilitates Sperm Penetration Of The Zona Pellucida

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Abstract

Objective—To determine the role of *Dkk1* on mouse development, viability and fertility.

Design—Prospective experimental study

Setting—Government research institution

Animals—Mice of C57BL/6, B6D2F1/J, and 129X1/SvJ strains, as well as transgenic mice of mixed C57BL/6 and 129X1/SvJ strains were used for the studies.

Intervention(s)—Expression of the *Dkk1* gene was characterized during early mouse development, and the effects of *Dkk1* ablation on reproduction and fertility were characterized *in vitro* and *in vivo*.

Main Outcome Measure(s)—*Dkk1* RNA expression was determined by Northern blotting hybridization as well as quantitative Reverse Transcriptase-Polymerase Chain Reaction assays (qRT-PCR). *In vitro* fertilization (IVF) assays were used to assess fertility of sperm from male mice lacking functional *Dkk1*

Result(s)—*Dkk1* is a gene unique to mammals that is expressed primarily in developing spermatocytes and its product localized in the acrosome of mature sperm. Here we show that *Dkk1* also is expressed in the trophectoderm/placental lineage. Surprisingly, embryos lacking DKKL1 protein developed into viable, fertile adults. Nevertheless, the ability of sperm that type lacked DKKL1 protein to fertilize wildtype eggs was severely compromised *in vitro*. Since this defect could be overcome either by removal of the zona pellucida or by the presence of wild type sperm, *Dkk1*, either directly or indirectly, facilitates the ability of sperm to penetrate the zona pellucida. Penetration of the zona pellucida by *Dkk1*[−] sperm was delayed *in vivo* as well as *in vitro*, but the delay *in vivo* was compensated by other factors during preimplantation development. Accordingly, *Dkk1*^{−/−} males offer an *in vitro* fertilization model for identifying factors that may contribute to infertility.

Conclusion(s)—DKKL1 is a mammalian-specific, acrosomal protein that strongly affects *in vitro* fertilization, although the effect is attenuated *in vivo*.

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Capsule: *Dkk1* facilitates the ability of sperm to penetrate the zona pellucida, a problem that is overcome to some extent by subsequent events in the female reproductive tract.

Keywords

Fertilization; Acrosome; Sperm; Zona Pellucida; DKKL1; IVF

INTRODUCTION

Dickkopf (DKK) proteins function either as WNT antagonists or agonists by either inhibiting or activating the WNT co-receptor LRP6. However, except for DKK1 (an embryonic head inducer), the precise role of these proteins in animal development and maintenance has remained elusive [reviewed in (1)]. As for Dickkopf-Like 1 (DKKL1), other than weak homology to DKK3, the presence of a classic signal peptide at its N-terminal end, and its highly restricted expression pattern (2), little can be said that is relevant to its function. The NCBI/HomoloGene web site categorizes *Dkk1* as a “gene conserved in eutherians,” and a NCBI/Blast search against lower organisms detected only DKK3 homologues. Thus, *Dkk1* is found only in mammals, suggesting that its functions are unique to mammals. Here we show that *Dkk1* is expressed during placental development as well as during spermatogenesis, and that DKKL1 facilitates the sperm's ability to penetrate the zona pellucida.

MATERIALS AND METHODS

Construction of *Dkk1* deficient mice and the methods used to characterize them are described in (2) and references cited. *In vitro* fertilization (IVF) was carried out as described (3). For *in vivo* fertilization, 6–12 week old B6D2F1/J females were mated naturally with males. Presence of a vaginal plug indicated Day 1 of pregnancy (i.e. assume fertilization took place at 12 a.m. previously). Embryos were isolated at 12 p.m. on Day 2 of pregnancy (equivalent to E1.5) by flushing the oviduct (4), and the number of 2-cell embryos was scored. In order to assess whether non-2 cell embryos could eventually become 2-cells, embryos were cultured *in vitro* in KSOM media (Millipore).

RESULTS

Dkk1 Is Selectively Expressed In The Trophoblast/Placental Lineage During Implantation

Dkk1 mRNA appears soon after the onset of zygotic gene activation, but its expression in blastocysts is localized to the trophectoderm (5). This suggested that *Dkk1* might be expressed selectively during placental development *in utero*, as well as during spermatogenesis in adults. To test this hypothesis, *Dkk1* expression in trophoblast stem (TS) cells [the placental progenitor (6,7)] and trophoblast giant (TG) cells (product of TS cell differentiation that is required for embryo implantation) was compared directly with *Dkk1* expression in embryonic stem (ES) cells [the progenitor of all embryo and non-trophoblast-derived extra-embryonic tissues (8,9)] and embryoid bodies (product of ES cell differentiation *in vitro*).

As previously reported (5), little if any *Dkk1* mRNA was detected in either ES cells or embryoid bodies (Fig. 1B). In contrast, *Dkk1* was expressed strongly in TS cells and further up-regulated in TG cells (Fig. 1B). *Dkk1* mRNA levels in TS cells were comparable to those in EL4 cells, a T-lymphocyte cell line that expresses *Dkk1* (5,10). As TS cells differentiated into TG cells, *Dkk1* mRNA levels increased to at least 20-fold greater than in ES cells, but still about 9-fold less than in testis (10). *Dkk1* expression is maintained in post-implantation placental tissues *in utero*; *Dkk1* mRNA increased 15-fold from E7.5 trophoblast to E12.5 placenta (Fig. 2). *Dkk1* expression remains baseline in post-implantation embryonic tissues.

Tead2 is a gene 3.8 kb upstream of *Dkk1* (Fig. 1A). In contrast to *Dkk1*, *Tead2* mRNA levels in TS cells were comparable to those in ES cells or in the Leydig-derived TM3 cell line [Fig.

1C; (5,10)], and they remained so during differentiation of TS cells into TG cells. *Tead2* mRNA increased only when ES cells differentiated into embryoid bodies. Thus, ES cells and the differentiated cells derived from them selectively expressed the *Tead2* gene, while TS cells and the differentiated cells derived from them selectively expressed the *Dkk11* gene. This result was consistent with previous studies (5) in which either *Dkk11* or *Tead2* was expressed in differentiated cells, but never both. These results suggest that *Dkk11* plays a role in placental development as well as in fertilization. However, initial analyses of mice lacking *Dkk11* show that they are viable and show no obvious placental defect (2).

DKKL1 Protein Facilitates Sperm Penetration Of The Zona Pellucida

To determine whether or not *Dkk11* expression affected fertility, wild-type C57BL/6 males and females were continually mated with either wild-type or *Dkk11* deficient mice. Surprisingly, ablation of either one or both *Dkk11* alleles did not affect either the number of pups born per litter or the number of litters produced within six months, regardless of whether the mother or father was wild-type or *Dkk11* deficient (2). Therefore, *Dkk11* is not required for fertilization *in vivo*. However, *in vitro* fertilization (IVF) yielded a very different result.

Capacitated sperm were mixed with cumulus-intact eggs, cultured for 4 hours in IVF medium, and then washed and cultured in KSOM medium to allow development of fertilized eggs (3). Sperm from wild-type males yielded 2-cell embryos at a rate of 67% compared to only 2% for sperm from *Dkk11* nullizygous males a difference of 9-fold (Fig. 3A). Given that *Dkk11*^{-/-} mice are fertile as assayed by continuous mating, these results revealed that DKKL1 facilitates, but is not required, for fertilization.

Since the number, morphology, and motility of *Dkk11* wild-type and nullizygous sperm in these preparations were indistinguishable by visual inspection, DKKL1 appeared to affect the ability of sperm to enter the egg. Mammalian sperm must penetrate the layer of cumulus cells that surrounds each egg, attach to the egg's zona pellucida, and then one of these sperm must penetrate the zona and form a male pronucleus within the egg (11,12). Since the eggs appeared to have equivalent numbers of sperm attached to their zona pellucida, regardless of whether *Dkk11*⁺ or *Dkk11*⁻ sperm were used in the IVF, DKKL1 did not appear to affect either sperm penetration through the cumulus cell layer or sperm attachment to the zona pellucida (data not shown). Therefore, *Dkk11*⁻ sperm appeared to be defective at some step between penetration through the zona pellucida and production of a 2-cell embryo.

To determine whether or not the defect in *Dkk11*⁻ sperm could be compensated by the presence of wild-type sperm, equal numbers of sperm from wild-type and *Dkk11*^{-/-} males were mixed together and then tested for their ability to fertilize wild-type eggs, scored at the 2-cell embryo stage. Those embryos that reached the 2-cell stage were allowed to develop to blastocysts, which were then genotyped by PCR to determine whether they had been fertilized by a *Dkk11*⁺ or a *Dkk11*⁻ sperm. Remarkably, 40% of the blastocysts produced in these experiments were heterozygous for *Dkk11* (Fig. 3B, 3D). Therefore, *Dkk11*⁻ sperm were capable of efficient fertilization *in vitro* when wild-type sperm were present, suggesting that either wild-type sperm or their seminal fluid facilitated penetration of the zona pellucida.

To determine whether or not the barrier to fertilization by *Dkk11*⁻ sperm was penetration of the zona pellucida, zonae were removed from eggs before adding sperm. Whereas in the presence of zonae, only 2% of eggs were fertilized by *Dkk11*⁻ sperm; in the absence of zonae, 46% of the eggs were fertilized (Fig. 3C). The presence of the *Dkk11* nullizygous allele was confirmed by PCR genotyping (data not shown), effectively ruling out the possibility of parthenogenesis or other spurious events.

Taken together, the results described above revealed that DKKL1 affects the ability of sperm to penetrate the zona pellucida during IVF, a result that appeared to contradict those obtained during continuous mating (2). However, continuous mating experiments do not reveal the outcome of individual mating events. In addition, the genetic background of females used for continuous mating (C57BL/6) differed from those used for IVF (B6D2F1/J). Therefore, to compare directly the fertility of *Dkk11*^{-/-} sperm *in vivo* with their fertility *in vitro*, *Dkk11* wild-type, heterozygous and nullizygous males were mated to wild-type B6D2F1/J females, and the resulting embryos were isolated from pregnant females on day two (~36 hours post-coitus, equivalent to 2-cell embryo stage) of pregnancy (13). The results revealed that *Dkk11*^{-/-} males fertilized B6D2F1/J eggs *in vivo* at efficiencies significantly less than those from either wild-type or heterozygous males (Fig. 4A). When 1-cell embryos from these nullizygous matings were cultured *in vitro*, most of them eventually developed into two or more cells, but were delayed by at least 6 hours (Fig. 4B). This result was consistent with the conclusion that *Dkk11*^{-/-} sperm could not penetrate the zona pellucida during IVF without help (in that case, from *Dkk11*⁺ sperm), which was reflected *in vivo* by delayed fertilization.

To determine whether or not this delay is carried through preimplantation development, *Dkk11*^{-/-} males were mated to wild-type B6D2F1/J females that were then sacrificed four days post-coitus when wild-type embryos are at the blastocyst stage in development. These females contained a normal number of blastocysts that were indistinguishable in their development (data not shown). Given the fact that the number of pups born per litter and the number of litters produced in six months were essentially unaffected by the presence or absence of a functional *Dkk11* gene, this result suggests that a delay in fertilization caused by the absence of DKKL1 is compensated by other factors during preimplantation development.

DISCUSSION

Previous studies indicated preferential expression of *Dkk11* during preimplantation development in trophoblast (5). Here, we confirm and extend this observation to show that *Dkk11* is expressed in TS cells and their placental derivatives, and is induced upon differentiation of TS into TG cells. Hence, based on expression patterns, *Dkk11*'s reproductive function may involve both placentation and fertilization. However, when *Dkk11* is deleted, mice are viable and fertile (2). More extensive placental analyses of *Dkk11*^{-/-} mice may be required to gain insight into its role in placental development. Using these mice, this study conclusively shows that *Dkk11* is required for efficient fertilization.

The role of DKKL1 in fertilization was not immediately evident, because *Dkk11*^{-/-} mice are not only viable and fertile (2,14), but both male and female *Dkk11*^{-/-} mice produced offspring at efficiencies comparable to wild-type animals (2). However, sperm from nullizygous *Dkk11* males had substantially reduced capacity to fertilize eggs *in vitro*, an impediment that could be overcome either by mixing them with sperm from wild-type males, or by removing the zona pellucida prior to incubation of sperm with eggs. Thus, *Dkk11*^{-/-} sperm were capable of fertilization, but their ability to penetrate the zona pellucida was defective. Apparently, attachment of large numbers of wild-type sperm to the zonae has the effect of permeabilizing zonae to sperm from *Dkk11*^{-/-} males, a phenomenon reminiscent of the ability of large numbers of bacteriophage to permeabilize bacterial cell walls simply by attaching to them ['lysis from without' (15)]. Alternatively, components of seminal fluid [such as "capacitation factors" (16)] from wild-type males may aid *Dkk11*^{-/-} sperm in efficient penetration of the zonae.

Notably, a similar albeit less pronounced affect of *Dkk11* on fertilization was also detected *in vivo*. Matings between wild-type females and *Dkk11*^{-/-} males resulted in some 1-cell embryos that were delayed in their development (Fig. 4), but by the time of blastocysts formation, the number and appearance of embryos produced by matings with *Dkk11*^{-/-} males were

indistinguishable from those produced by matings with wild-type males. Taken together, the results presented here demonstrate that DKKL1 is required for efficient penetration of sperm through the zona pellucida.

What role might DKKL1 play in fertilization? A direct role in the interaction between sperm and egg is unlikely, because live sperm from wild-type mice did not show specific staining with anti-DKKL1 polyclonal antibody (data not shown). This antibody detects both full length and truncated forms of DKKL1 as well as glycosylated and non-glycosylated forms of the protein (2). Therefore, while DKKL1 protein was clearly present within the acrosome of mature sperm (2), none of its epitopes were accessible on the surface. More likely is the possibility that DKKL1 plays an indirect role in fertilization, either by facilitating proper assembly of the acrosome during spermatogenesis, or by modification of other acrosomal proteins.

Alternatively, DKKL1 may be involved in either stabilization of the acrosome to prevent untimely acrosome reaction, or efficient promotion of acrosome reaction upon binding to the zona pellucida of the egg. For example, DKKL1 may facilitate the process of sperm 'capacitation' (17,18). In most mammals, capacitation occurs in waves (19) such that only certain populations of sperm become fully fertile at any given time, assuring the likelihood that some of the capacitated sperm will be able to fertilize the egg during its window of receptivity (20). Since acrosome reacted sperm cannot fertilize eggs, the acrosomal reaction must occur under appropriate cues (21). For example, males lacking acrosomal polycystin-1 exhibit reduced fertility under controlled conditions due to compromised timing of capacitation (22).

Previous studies have revealed that most examples of sperm-dependent infertility are associated with dramatic defects either in sperm number, morphology or motility (23). Hence, there are a number of examples in which deletion of genes suspected to play major roles in male fertility do not result in infertility (24-27). For example, Spam1/PH-20 has been suggested to be the major cell-surface hyaluronidase required by sperm to penetrate the cumulus layer. However, when this gene was deleted, the only fertility defect detected was a delay in penetration through the cumulus layer during IVF (25). Subsequent studies revealed that another hyaluronidase that can dissociate cumulus cells, Hyal5, is present in spermatids (28). Even in mice lacking three different proteins thought to serve male germ cell function, no fertility defect was detected (29).

The ability to detect infertility or subfertility also can be masked by experimental factors, such as genetic background (30-32). For example, males lacking 'sperm mitochondria-associated cysteine-rich protein' (Smcp) do not exhibit fertility problems in a mixed C57BL/6X129/Sv background, whereas they are completely infertile in the 129/Sv background (30). Given that the studies reported here were performed with mice of mixed background, future studies should be carried out in a pure 129/Sv inbred background. In addition, it is well known that in the wild, female mice mate multiple times during their estrus cycles, thereby ensuring that only the most efficient sperm will be selected for fertilization (22,27,33); such evolutionary advantage will not be evident under most laboratory mating conditions.

The results described here suggest that IVF provides an assay for detecting fertilization-related problems that are not apparent *in vivo*. Even with the incredible advances in IVF technology, *in vitro* conditions do not exactly replicate *in vivo* conditions. For example, media and conditions in which sperm capacitation is done *in vitro* only approximate the milieu of the female reproductive tract. There exists an intricate balance between reactive oxygen species and antioxidants during fertilization in the female reproductive tract (34) which may not be replicated *in vitro*. In addition, IVF protocols involve preovulatory hormonal stimulation (i.e. superovulation), which may result in "hardening" of the zona (35); hence, the *Dkk1*⁻ sperm defect may be greatly enhanced during IVF. Extensive biochemical analyses of components

in the female reproductive tract that allow *Dkk1l⁻* sperm to overcome such an *in vitro* block may provide useful insights into male infertility.

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Figure 1.

During preimplantation development, *Dkk1* mRNA was preferentially expressed in trophoblast stem (TS) cells and trophoblast giant (TG) cells. (A) The mouse *Dkk1* gene locus at 23cM on chromosome 7 resides within a ~30 kb region that includes the *Tead2* and *CD37* genes. Arrows indicate direction of transcription for each of the five genes. (B) Total RNA (15 µg) was prepared from TS cells (lane 1), from TS cells induced to differentiated into TG cells for 4 hrs (lane 2), 8 hrs (lane 3), 24 hrs (lane 4), 48 hrs (lane 5), 5 days (lane 6), and 7 days (lane 7), and from ES cells (lane 8), embryoid bodies (EB, lane 9), EL4 cells (lane 10), TM3 cells (lane 11), and testis (lane 12). RNA samples were then fractionated by agarose gel electrophoresis, transferred to a nylon membrane, and hybridized with radio-labeled *Dkk1* cDNA (5). The 0.24–9.5kb RNA ladder from Invitrogen was run in parallel to determine mRNA sizes. The amount of ³²P in each band was quantified using a phosphorimager (Fuji), and the data expressed relative to the amount detected in TS cells. (C) The blot from (B) was stripped and then reprobbed using ³²P-labeled DNA specific for *Tead2* (36). (D) 28S rRNA was used as loading control and stained with ethidium bromide.

Figure 2.

During the first 12 days of development, *Dkk11* was expressed primarily in the trophectoderm lineage. (A) Total RNA was isolated from the indicated tissues and embryos from appropriate age pregnant females, and analyzed for *Dkk11* RNA semi-quantitatively by RT-PCR as described (5). E7.5 embryos were separated from the trophectoderm but still contained the epiblast and extraembryonic ectoderm. At E9.5, chorion and amnion tissues were pooled for 'yolk sac'. The data were normalized to glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) RNA. A representative ethidium stained agarose gel is shown. (B) Total RNA from (A) was subjected to quantitative Real-Time RT-PCR and normalized to *Gapdh*, as described (37).

Figure 3.

Sperm from *Dkk1* nullizygous males were defective in penetrating the zona pellucidae. (A) Sperm isolated from male mice that were wild-type (+/+; green bar), heterozygous (+/-; yellow bar) or nullizygous (-/-; red bar) at the *Dkk1* allele (2) were combined with eggs from B6D2F1/J wild-type females and scored 24 hours later for the appearance of 2-cell embryos. (B) When equal portions of sperm from wild-type and nullizygous males were mixed prior to IVF, 40% of the fertilized embryos that developed to blastocysts (hatched bar) showed heterozygous genotype, revealing that these eggs had been fertilized by *Dkk1*⁻ sperm. (C) Sperm from nullizygous males were capable of fertilizing eggs from which the zona pellucidae had been removed prior to IVF (red bar). Eggs were treated with hyaluronidase to remove the cumulus cells and then with Acidic Tyrode's solution until the zona disintegrated. Eggs were promptly removed and allowed to recover in IVF medium for 2 to 3 hours before addition of sperm. Data were combined from four separate experiments. (D) Multiplex PCR genotyping of blastocysts from (B) showed that in the presence of wild type sperm, nullizygous sperm were capable of IVF.

Figure 4.

Sperm from *Dkk11*^{-/-} males exhibited a delay in fertilization *in vivo*. (A) Males that were wild-type (+/+) or heterozygous (+/-) for the *Dkk11* allele (2) (hatched bar) or nullizygous (-/-; red bar) were mated with B6D2F1/J wild-type females. Embryos were isolated on Day 2 of pregnancy (E1.5) and the fraction of 2-cell embryos present was compared. (B) Photographs of embryos isolated at Day 2 of pregnancy (E1.5) from a wild-type female that had been mated to a *Dkk11*^{-/-} male. The isolated embryos were cultured in KSOM media and examined at indicated times to determine whether or not development continued.