**DAZL** expression in human oocytes, preimplantation embryos and embryonic stem cells

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In humans, the **Deleted in Azoospermia Like (DAZL)** gene is believed to function in the development of primordial germ cells and in germ cell differentiation and maturation because the expression of **DAZL** is only found in the germ and non-germ lineage of the reproductive system and in embryonic stem (ES) cells. The present study examined the presence of **DAZL** transcripts in the last stages of oocyte maturation, in ES cells, and throughout the preimplantation development; the link between gametes and ES cells. The finding of **DAZL** transcripts in the last stages of oogenesis and during the first two cell cycles of the preimplantation development was expected, because **DAZL** is a germ cell marker and the transcripts present at that time are generally encoded by the maternal genome. During the third cell cycle, **DAZL** showed a variable expression pattern, which may point to the maternal to embryonic transition. After the third cell cycle, transcripts were again consistently detected, suggesting embryonic **DAZL** transcription. In blastocysts, **DAZL** transcripts were only detected in those of good quality and this as well in the inner cell mass (ICM) as in the trophectoderm (TE). The presence of **DAZL** transcripts in the ICM and in ES cells was not surprising since both can lead to the formation of germ cells, but TE cells cannot. The quality-related expression of **DAZL** in blastocysts, and especially its trophectodermal expression, might imply other functions for **DAZL** beyond germ cell development.

Key words: **DAZL**/expression/human embryos/preimplantation development

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**Introduction**

The **Deleted in Azoospermia Like (DAZL)** gene is a member of the **Deleted in Azoospermia (DAZ)** family, which consists of three genes: **BOULE**, **DAZ** and **DAZL**. The ancestor member of the family is **BOULE**, which gave rise to **DAZL** via duplication prior to the divergence of vertebrates and invertebrates. Later, during primate evolution, **DAZL** gave rise to **DAZ** by transposition, repeat amplification and pruning. The genes of the **DAZ** family encode proteins with a highly conserved RNA-binding motif and a unique **DAZ** repeat, and all show a germ cell-specific expression pattern. The **DAZ** proteins are believed to function in the post-transcriptional regulation of mRNA expression because they interact with RNA homopolymers through their RNA-binding motif and form complexes with themselves, with each other or with proteins involved in mRNA transport/localization or in mRNA translation (Yen, 2004).

The **DAZL** gene and its homologues are essential for germ cell development in several species. In mice, a knockout of the **Dazl** gene results in meiotic arrest of male and female germ cells (Ruggiu et al., 1998). At the onset of meiosis, all **DAZL** is translocated from the nucleus to the cytoplasm and so, in secondary spermatocytes, spermatids and spermatozoa, **DAZL** is only found in the cytoplasm (Reijo et al., 2000; Lin et al., 2002). Further, transcripts of **DAZL** are also detected in Sertoli cells (Lee et al., 1998; Lin et al., 2001; Kuo et al., 2004). The amount of **DAZL** transcripts is decreased in the testes of azoospermic men as compared with fertile men (Lin et al., 2001). A polymorphism (T54A) occurring in the RNA-binding domain of the **DAZL** protein confers susceptibility to severe spermatogenic failure in the Taiwanese (Teng et al., 2002) but not in the Caucasian population (Bartoloni et al., 2004; Becherini et al., 2004). During oogenesis, **DAZL** is expressed in the cytoplasm of oogonia and developing follicular oocytes in the fetal and adult ovaries (Dorffman et al., 1999; Nishi et al., 1999; Brekhanman et al., 2000; Tsai et al., 2000). Further, expression has also been found in granulosa cells of the primordial follicle (Dorffman et al., 1999; Brekhanman et al., 2000), cells of the theca interna of the maturing follicle (Nishi et al., 1999) and in granulosa–luteal cells of the corpus luteum (Pan et al., 2002). **DAZL** expression throughout different stages of gametogenesis and in both germ cell and somatic cell compartments of the reproductive organs may imply important functional roles in the regulation of male and female reproduction from the development of PGCs to the differentiation and maturation of germ cells from PGCs onwards. Recently, expression of **DAZL** has been reported in undifferentiated human...
embryonic stem (ES) cell lines and in embryoid bodies (EBs), suggesting a spontaneous differentiation of a subpopulation of ES cells into germ cells (Clark et al., 2004; Moore et al., 2004).

The link between gametes and ES cells is the preimplantation period during which a fertilized oocyte develops towards a blastocyst. Whether DAZL is also expressed throughout this period is unknown. The present study examined the presence of DAZL transcripts in the last stages of oocyte maturation, in ES cells and also in normally developing preimplantation embryos. Embryos at the stage of zygote, compacted embryo or blastocyst were individually examined as intact embryos, whereas embryos prior to full compaction were individually examined at the level of single blastomeres. A number of blastocysts were split into inner cell mass (ICM) and trophoderm (TE) samples in order to investigate a possible difference in DAZL expression between the pluripotent ICM and the differentiated TE.

Materials and methods

Oocytes and embryos

Thirty-nine oocytes and 138 preimplantation embryos were obtained for research at our Centre for Reproductive Medicine with the couples’ informed consent and with the approval of the institutional ethical committee. The oocytes used were immature, at the germinal vesicle stage (GV; n = 12) or the metaphase I stage (MI; n = 12), or were mature, metaphase II oocytes (MII; n = 15). The MII oocytes were either in vitro matured or were donated for research at the time of oocyte retrieval when no sperm was found. The oocytes were denuded from surrounding cumulus and corona cells using a combination of enzymatic (40 U/ml hyaluronidase type VIII; Sigma Aldrich, Bornem, Belgium) and mechanical (pipetting) methods (Van de Velde et al., 1997). The denuded oocytes were individually transferred into droplets of acidic Tyrode’s solution pH 2.4 using a mouth-controlled hand-drawn Pasteur pipette and observed under a stereomicroscope (40× magnification, Wild, Leica; Van Hopplynus, Brussels, Belgium) until the zona pellucida had just dissolved. They were washed three times in droplets of home-made Ca2+- and Mg2+-free medium (14 mM NaCl, 0.2 mM KCl, 0.04 mM NaH2PO4, H2O, 5.5 mM glucose, 1.2 mM NaHCO3, 0.02 mM EDTA, 0.01% (v/v) phenol red) supplemented with 4 mg/ml bovine serum albumin (BSA) (Sigma Aldrich). Embryos were obtained after conventional IVF or ICSI (Devroyé and Van Steirteghem, 2004). The embryos used were assessed as unsuitable for transfer or cryopreservation at the day of transfer (day 3 or day 5 of the preimplantation development according to the transfer policy for the couple), or were obtained by applying ICSI on MII oocytes donated for research. Preimplantation embryo development was evaluated daily (Staessen et al., 2003). Blastocyst evaluation relied on the scoring system described by Gardner and Schoolcraft (1999). Briefly, scores were given to the ICM and TE of the blastocyst by means of a first and a second character, respectively. These scores ranged from A (tightly packed, many cells), B (loosely grouped, several cells) to C (very few cells) for the ICM and from A (tightly packed, many cells), B (loosely grouped, several cells) to C (several cells) to C (very few cells) for the TE. An additional D (degenerative or doubtful) score was introduced for both the ICM and the TE. All 138 research embryos were derived from normally fertilized oocytes (2PN) and showed a normal morphology and a normal developmental timing at the moment of use. These embryos could be subdivided into zygotes (n = 9), cleavage stage embryos (2- to 9-cell stage) (n = 29), a compacting 13-cell embryo (n = 1), compacted embryos (n = 12) and blastocysts (n = 87). All embryos, 63 blastocysts excluded, were individually denuded from their zona pellucida and washed as described above. Embryos prior to full compaction were then individually dissociated into single blastomeres by repeatedly in-/outpipetting using a mouth-controlled pipette. Washing was repeated for each blastomere. Of the remaining 63 blastocysts, 11 were submitted to a mechanical method (laser) and 52 to enzymatic methods (immunosurgery) to separate the ICM from the TE. Using the mechanical method, ICSI blastocysts with clearly distinguishable ICMs were split into one sample containing only TE cells (TE sample) and another sample containing the ICM with a few surrounding TE cells (ICM sample). Briefly, blastocysts were stabilized with a micromanipulator on an inverted microscope (400× magnification; Diaphot 300; Nikon, Tokyo, Japan). A hole was created in the zona pellucida at the height of the ICM using a non-contact 1.48 µm diode laser system (Fertilitase; Octax, Herborn, Germany). Using a biopsy pipette, the ICM was pulled out and separated from the blastocyst by laser. Afterwards, the zona pellucida was removed from the TE sample by in-/outpipetting. The ICM and TE sample were separately washed in droplets of Ca2+- and Mg2+-free medium. For the enzymatic method, two techniques of immunosurgery were compared. Prior to both procedures, the blastocysts were denuded from their zona pellucida using 0.5% pronase (Sigma Aldrich). Twelve blastocysts were subjected to the technique described by Hardy et al. (1989). The blastocysts were incubated on ice for 10 min in 10 mM pircysulfonic acid (TNBS; Sigma Aldrich) in 10%polyvinylpyrrolidone (PVP) solution [T6; 25 mM HCO3−; 4 mg/ml PVP (Sigma Aldrich)] and washed three times in HEPES buffered human tubal serum (HTS HEPES; Canbrx, Verviers, Belgium) supplemented with 4 mg/ml BSA (HSBSA). Next, they were incubated for 30 min at 37°C in 0.1 mg/ml anti-DNP (Sigma Aldrich) in HSBSA supplemented with 3% inactivated normal human serum (iNHS). After washing in HSBSA, they were incubated for 20 min at 37°C in 30% guinea pig complement (Cederlane, Hornby, Ontario, Canada) in HSBSA supplemented with 6% iNHS. By in-/outpipetting using a mouth-controlled hand-drawn Pasteur pipette with a diameter slightly bigger than the ICM, the ICM was isolated from the TE cells. The ICM and clumps of not completely lysed TE cells were separately washed in Ca2+- and Mg2+-free medium. The other 40 blastocysts were subjected to immunosurgery based on the principles described by Solter and Knowles (1975). Blastocysts were exposed to 50% goat antihuman serum antibody (Sigma Aldrich) in HSBSA for 30 min at 37°C and washed three times in HSBSA. Then, blastocysts were incubated at 37°C in 5–20% guinea pig complement in HSBSA until the TE cells started to lyse. The ICM was isolated by in-/outpipetting. The isolated ICM and the remaining TE cells were washed separately in Ca2+- and Mg2+-free medium.

In all experiments, media samples were taken as negative controls.

Human ES cells

Human ES cells from two independent cell lines were used (Mateizel et al., 2004; I. Mateizel, personal communication). From the first ES cell line, individual undifferentiated and spontaneous early-differentiated colonies at passage 15 were mechanically isolated by the use of a blunt-edge pulled Pasteur pipette and washed three times in stem cell culture medium (80% knockout D-MEM (Invitrogen, Merelbeke, Belgium), 20% fetal calf serum (Hyclone, South Logan, Utah, USA)). EBs from the first cell line were collected at day 15 using collagenase IV (1 mg/ml; Invitrogen) and centrifuged. From the second ES cell line, cells from undifferentiated colonies at passage 8 were isolated in the same way as the EBs. As negative controls, samples of inactivated mouse embryonic fibroblasts, stem cell culture medium and Ca2+- and Mg2+-free washing medium were used.

Other samples

Clumps (~10 cells) of cumulus cells and corona cells were isolated from oocyte–cumulus complexes containing MII oocytes and washed three times in Ca2+- and Mg2+-free medium. Cultured amniotic fluid cells were collected by amniocentesis (Aytöz et al., 1998). The cytogenetic analysis showed a normal karyotype. Clumps of approximately 10 and 30 amniotic fluid cells were isolated and washed in Ca2+- and Mg2+-free medium.

cDNA synthesis

All samples, except the ES cells from the second ES cell line and the EBs from the first ES cell line, were individually transferred into separate RT-tubes containing 2.5 µl of ice-chilled lysis buffer [0.8% Igepal (Sigma Aldrich), 1 U/µl RNaseOUT® (Invitrogen), 5 mM dithiothreitol (Sigma Aldrich)] made in diethyl pyrocarbonate (DEPC)-treated H2O (Daniels et al., 1997). The samples were immediately snap-frozen in liquid nitrogen for 30 min to avoid endogenous RNase activity. RT was performed with the First-strand cDNA Synthesis Kit using the Not1-d(T)18 primer (GE Healthcare, Hoevelaken, The Netherlands). The manufacturer’s protocol was adapted for single cells. First, the samples were incubated at 80°C for 5 min.
to disrupt secondary and tertiary structures in the RNA and transferred to ice. Subsequently, the RT mix (RT reagents for a 15 μl reaction supplemented with 2 U RNaseOUT™ dissolved in DEPC-treated H2O) was added to the samples. RT was carried out at 37°C for 1 h in an Eppendorf Mastercyler Personal (VWR International, Leuven, Belgium). The samples were returned to ice.

Total RNA was extracted out of the ES cells from the second ES cell line and the EBs from the first ES cell line using the Spin Protocol of the RNeasy Mini kit (Qiagen, Venlo, The Netherlands) according to the instructions of the manufacturer. RNA concentrations were determined by spectrophotometry (GeneQuant II; GE Healthcare) and all samples were stored at −80°C until use. Using the First-strand cDNA Synthesis Kit with the NotI-d(T)18 primer, RT was carried out with 5 μg of total RNA in a 33 μl reaction according to the instructions of the manufacturer.

The completed first-strand cDNA reactions were heated to 90°C for 5 min to denature the RNA–cDNA duplexes and to inactivate the reverse transcriptase. Next, they were chilled on ice.

**PCR**

PCR for DAZL was carried out using specific primers consisting of the forward primer (5'-GGAGCTATGTGTTAQCCTCC-3') labelled with 5' indocarbocyanine (Cy5) and the reverse primer (5'-GGGGCCATTCGCGACG-3') (Eurogentec, Seraing, Belgium). These intron-spanning primers were located in exon 8 and exon 9 of the DAZL gene, respectively, and yielded a 128 bp product (Saxena et al., 1994). Four microlitres of cDNA was used as the template in a 25 μl final reaction volume comprising 1× PCR buffer (Applied Biosystems, Nieuwerkerk a.d. IJssel, The Netherlands), 10 pmol of each primer, 5% dimethylsulphoxide (DMSO; Sigma Aldrich) and 1.25 U AmpliTaq DNA Polymerase (Applied Biosystems). PCR was carried out in an Eppendorf Mastercyler Personal using the following cycling profile: 5 min denaturation at 95°C followed by 50 cycles of 30 s at 95°C, 30 s at 60°C and 30 s at 72°C, and a final extension for 7 min at 72°C.

Amplification of the HPRT cDNA sequence was carried out in parallel for each examined sample and served as a positive control for cell viability as well as for the efficacy of the RT procedure. The intron-spanning HPRT primers consisted of the Cy5-labelled forward primer (5'-GCCGCTCCGTTATGGCG-3') and the reverse primer (5'-AGCCTCCCTGACGCA-3') (Eurogentec) and amplified a fragment of 226 bp (Ao et al., 1994). The PCR conditions were the same as for DAZL, except that no DMSO was used and that annealing took place at 55°C.

The negative controls, taken during sample preparation, were included in each experiment and processed in the same way as the samples.

The PCR fragments were analysed on an ALF-express automated sequencer (GE Healthcare). Results were processed using the Allelelinks software provided by the manufacturer.

**Statistical analyses**

Statistical analyses were performed on the single cell experiments in the preimplantation embryos for the expression of DAZL and HPRT using Pearson’s chi-square test. In the case of the intact blastocysts and the blastocyst samples, the Fischer’s exact test was used. A P-value of <0.05 was considered statistically significant in each situation.

**Results**

After adaptation of the RT–PCR technique to the single-cell level, DAZL and HPRT mRNA expression was examined in 39 single oocytes, 9 single zygotes, 141 single blastomeres derived from 29 cleavage stage embryos, 13 single blastomeres of a compacting 13-cell embryo, 12 single compacted embryos and 24 single, intact blastocysts. Due to lysis, two of the initial 143 blastomeres of the cleavage stage embryos were lost during isolation. The results are summarized in Table 1 and Figure 1. DAZL and HPRT transcripts were detected in nearly all oocytes, in all zygotes and in all but one of the blastomeres of embryos up to and including the 4-cell stage. From the 4-cell stage onwards up to and including the 8-cell stage, a variable expression pattern was found. Transcripts of DAZL were
Eleven blastocysts, graded from AA to DB, were mechanically separated into ICM and TE samples. Among the 11 ICM samples, four ICMs had an A score, five a B, one a C and one a D. Among the TE samples, three were scored as A, six as B and two as D. Transcripts of DAZL were only detected in ICM (4/4) and TE samples (2/3) with an A score and not in the samples with lower scores (Figure 3.2 and 3.3). The DAZL expression in the ICM samples originates from the ICM itself and not from the few surrounding TE cells, because when DAZL was found in an ICM sample, DAZL was not always present in the original matching TE sample. The expression of DAZL differed significantly ($P = 0.001$) between the samples of an excellent quality (score: A) and the samples of a lesser quality (scores: B, C and D). HPRT transcripts were detected in all ICM samples and in all but one TE sample. No statistical difference was reached for HPRT ($P = 0.318$).

Fifty-two blastocysts were submitted to immunosurgery (data not shown). Using the protocol of Hardy et al. (1989), 11 ICMs and 9 samples of vital looking TE cells could be isolated out of 12 blastocysts. None of the ICMs or TE samples displayed transcripts of DAZL or HPRT. Using the protocol of Solter and Knowles (1975), 33 ICMs and 19 samples of vital looking TE cells could be isolated out of 40 blastocysts. Transcripts of DAZL and HPRT could be detected in 12% (4/33) and 39% (13/33) of the ICMs, respectively, and in 5% (1/19) and 11% (2/19) of the TE samples, respectively. Here, no relation could be made between the presence of DAZL transcripts and the score of the blastocyst.

No amplification of DAZL or HPRT transcripts were achieved from negative controls.

Regarding the human ES cells, transcripts of DAZL and HPRT were present in undifferentiated colonies of both cell lines, in spontaneous early-differentiated colonies and in EBs, but not in activated mouse embryonic fibroblasts, stem cell culture medium and Ca$^{2+}$- and Mg$^{2+}$-free washing medium (Figure 4).

Cumulus, corona and amniotic fluid cells did not display the presence of DAZL transcripts, whereas HPRT transcripts were detected in all (data not shown).

**Discussion**

This study demonstrates the expression of the germ cell marker DAZL during the last stages of oocyte maturation, throughout human preimplantation development and in undifferentiated and spontaneous differentiated human ES cells.

Although we found expression of DAZL in oocytes, we could not confirm expression of DAZL in the non-germ lineage of the ovary because we did not detect DAZL transcripts in cumulus and corona cells (granulosa cells) of mature ovulated oocytes. As DAZL is believed to have a role in germ cell differentiation and maturation, the discrepancy with the reported expression in granulosa cells of primordial follicles (Dorman et al., 1999; Brekhman et al., 2000) could be explained by the fact that the granulosa cells of primordial follicles are involved in growth and maturation but the granulosa cells of mature oocytes are not (Tanghe et al., 2002).

The preimplantation development comprises all stages from oocyte fertilization until blastocyst implantation. A major event during this period is the embryonic genome activation, which represents the transition from maternal to embryonic control and is known to occur during the third mitotic cell division (Braude et al., 1988; Taylor et al., 1997; Dobson et al., 2004). In general, mature oocytes contain sufficient maternal transcripts to support fertilization and the first two cell divisions. At the time of the third cell division, these transcripts are down-regulated and/or degraded and specific

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*Figure 1.* Graphical representation of DAZL (white curve) and HPRT (black curve) expression in single cells (oocytes, zygotes and blastomeres).

*Figure 2.* Detection of DAZL (d) (128 bp) and HPRT (h) (226 bp) transcripts in single blastomeres (1–6) of two 6-cell embryos (A and B). The variability in expression is shown within an embryo and between embryos of the same cell stage. (A) DAZL transcripts were detected in three of six blastomeres. HPRT transcripts could be detected in only one blastomere. (B) One blastomere expressed detectable levels of DAZL. HPRT transcripts were detected in two of six blastomeres. *50 bp ladder.*

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...determined in only 52% (48/92) of the blastomeres, and HPRT transcripts in 40% (37/92). In these embryos, mRNA expression varied between individual blastomeres from the same embryo and between embryos of the same cell stage (Figure 2). Blastomeres positive (or negative) for HPRT were not necessarily so for DAZL. Later in development, DAZL and HPRT transcripts were again consistently detected in all the blastomeres of the 9-cell embryo and of the compacting 13-cell embryo, and in all compacted embryos. Statistical analyses on the single cell experiments in preimplantation embryos showed a significant difference in expression of DAZL ($P = 0.001$) and HPRT ($P = 0.001$) between the first two cleavages (embryos up to the 4-cell stage), the third cleavage (5- up to 8-cell embryos), and the fourth cleavage (9- and 13-cell embryo). In the intact blastocysts, transcripts of DAZL were only detected in 71% (17/24), whereas HPRT transcripts were found in all but one. The presence of DAZL transcripts appeared to be related to the quality of the blastocyst. All blastocysts that did not express DAZL had a score D for the ICM or TE (Figure 3.1). The expression between blastocysts suitable for transfer (scores: AA, AB, BA, BB) and blastocysts not suitable for transfer (scores: AD, BD, CA, CB, CD, DA, DB, DD) differed significantly for DAZL ($P = 0.006$) but not for HPRT ($P = 1$).
embryonic mRNAs are synthesized. The transcripts present after the 8-cell stage are assumed to be encoded by the embryonic genome. Transcripts of DAZL were virtually consistently detected as well in oocytes, zygotes and blastomeres of embryos up to the 4-cell stage, as in embryos with more than 8-cells. This indicates that DAZL is not only restricted to the maternal control but is also transcribed from the embryonic genome. During the third cell division, DAZL transcripts were not consistently detected in all the blastomeres. Variability was demonstrated within an embryo and between embryos of the same cell stage. This phenomenon may reflect the dynamic changes in mRNA levels during the maternal to embryonic transition. As it seems unlikely that the process of down-regulation/ degradation and activation is highly synchronized, variability from embryo-to-embryo, and even from blastomere-to-blastomere, can be expected (Monk and Holding, 2001). In blastocysts, DAZL seems to be positively related to the morphological quality. This may put forward different hypotheses: (1) DAZL is a positive marker for the quality of blastocysts and DAZL might even have an influence on the implantation and survival chances of the embryo; or (2) the DAZL transcripts are present at a lower level than the transcripts of HPRT in blastocysts and a possible cellular malfunction in poor quality blastocyst leads to the degradation of the transcripts. DAZL is expressed in both cell lines of the blastocyst: the differentiated TE, which gives rise to the placenta, and the pluripotent ICM, which in vivo gives rise to the proper embryo and which in vitro can lead to the formation of ES cells. In order to find a good method to examine expression in the ICM and the TE,
immunosurgery and a mechanical separation technique, both in combination with RT–PCR, were compared. After immunosurgery, the transcripts of DAZL and the housekeeping gene HPRT were found at least 4.5 times less than after the mechanical separation technique. Although immunosurgery has been proven to be a good technique for isolating the ICM in order to derive ES cells (Sjogren et al., 2004), there seems to be a detrimental influence on the transcript of DAZL immediately after the technique is applied. However, transcripts of some other genes can still be detected after immunosurgery (Cauuffman, unpublished results). This might explain why transcripts of DAZL were not found in the study of Clark et al. (2004) in ICMs that were isolated by immunosurgery.

The same study (Clark et al., 2004) reports the expression of DAZL in human ES cells and EBs. Based on the expression pattern of DAZL it was concluded that ES cells have already started the initial programme for germ cell specification. In the present study, we confirm the expression of DAZL in human ES cells and EBs. However, we also describe DAZL expression in the ICM and the TE of good quality blastocysts. TE cells do not have the potential to form ES cells and cannot lead to the formation of germ cells. And so, the relation between the DAZL expression and the initiation of the germ cell programme in human ES cells can be questioned.

So far, DAZL expression was described in the germ and non-germ lineage of the male and female reproductive system and in human ES cell lines. This study shows DAZL expression not only in human oocytes and ES cells, but also throughout the human preimplantation period, an episode that is rooted in oogenesis and connects gametes with ES cells. The expression of DAZL in the last stages of oocyte maturation and during the first two cell cycles of early embryogenesis was expected, as DAZL is a germ cell marker and as the transcripts present at that time are generally encoded by the maternal genome. On the other hand, embryonic DAZL expression was rather unexpected. DAZL’s quality-related expression in blastocysts, and especially its trophectodermal expression, might imply other functions for DAZL beyond germ cell development.

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