Expression of Polycomb-group genes in human ovarian follicles, oocytes and preimplantation embryos

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Abstract

Mammalian oocytes possess unique properties with respect to their ability to regulate and reprogram chromatin structure and epigenetic information. Proteins containing the conserved chromodomain motif that is common to the Polycomb-group (Pc-G) proteins and the heterochromatin-associated protein HP1, play essential roles in these processes and more specifically, in X-chromosome inactivation in female zygotes and extra-embryonic tissues and in the regulation of genomic imprinting. To characterize the potential role of these proteins in the regulation of epigenetic events during early human development, we utilized a degenerate PCR priming assay to assess the expression of mRNAs of chromdomain proteins in cDNA samples derived from the human female germline and preimplantation embryos. Expression of mRNAs of HP1 genes was observed in ovarian follicles, (HP1\(^{\text{HSa}}\), HP1\(^{\text{HSb}}\), HP1\(^{\text{HSg}}\)), mature oocytes (HP1\(^{\text{HSa}}\), HP1\(^{\text{HSb}}\)), cleavage stage preimplantation embryos (HP1\(^{\text{HSa}}\), HP1\(^{\text{HSb}}\), HP1\(^{\text{HSg}}\)) and blastocysts (HP1\(^{\text{HSa}}\), HP1\(^{\text{HSg}}\)). Transcripts for three Pc-G genes, which are essential for early mammalian development (Yin Yang 1 (YY1), Enhancer of Zeste-2 (EZH2) and Embryonic Ectoderm Development (EED)) and that are essential for the regulation of X-inactivation and certain imprinted genes (EED) were revealed by gene-specific-PCR expression analysis of human ovarian follicles, oocytes and preimplantation embryos. YY1 and EZH2 transcripts were additionally detected in metaphase II oocytes.

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Introduction

The fertilized mammalian oocyte develops into many differentiated cell types in a process that requires epigenetic cellular memory systems to regulate gene expression patterns appropriately. The Polycomb-group (Pc-G) gene products regulate cellular memory in conjunction with the trithorax group (TrxG) and maintain the stable repression of homeotic (HOM-C) genes during development (Lewis 1978, Jacobs and van Lohuizen 1999, Hanson et al. 1999, Orlando 2003). Subsequently, the balance of expression of Hox transcription factors in a particular cell or tissue dictates the differentiated state (for a review, see McGinnis and Krumlauf 1992).

The polycomb proteins Yin Yang 1 (YY1), Enhancer of Zeste-2 (EZH2) and Embryonic Ectoderm Development (EED) are essential during the peri-implantation period and gastrulation (Faust et al. 1998, Donohoe et al. 1999, O’Carroll et al. 2001), indicating that these proteins may have additional roles in early development, prior to their essential functions in the regulation of homeotic genes. Accordingly, increasing evidence demonstrates that polycomb proteins are involved in the regulation of early epigenetic events. Thus, the murine Eed protein is essential for both imprinted X-inactivation in extra-embryonic tissues and also X-inactivation in the early embryo (Wang et al. 2001, Plath et al. 2003, Silva et al. 2003) in a mechanism mediated by the histone methyltransferase activity (H3-K27) of the sal(var), e(2) and trithorax (SET) domain within the Ezh2–Eed complex (Cao et al. 2002, Czermin et al. 2002, Kuzmichev et al. 2002, Muller et al. 2002). The mouse Eed protein is also essential for the appropriate epigenetic regulation of a subset of autosomal imprinted loci (Mager et al. 2003). The Ezh2–Eed Polycomb complex has recently been identified as being essential for the regulation of placental imprinting of the Kcnq1 domain on mouse distal chromosome 7 (Delaval & Feil 2004). Finally, the YY1 transcription factor binds to an insulator sequence within the imprinted mouse paternally expressed gene 3 (Peg3) gene in a methylation-sensitive fashion (Kim et al. 2003).

Some polycomb proteins share a conserved sequence motif, termed the chromodomain (chromatin organization modifier), with heterochromatin-associated protein, HP1 (Paro & Hogness 1991). This domain is essential for cell...
survival (Filesi et al. 2002) and is implicated in the regulation of nuclear organization and gene expression (Jones et al. 2000). The chromodomain of HP1 heterochromatin proteins recognizes the methylated Lys9 on histone H3 (H3-K9) (Bannister et al. 2001, Lachner et al. 2001), a putative imprinting signal that marks the alleles of imprinted genes (Xin et al. 2001, Fournier et al. 2002) although it can also bind to chromosomal DNA regardless of this mark (Cowell et al. 2002). Significantly, in mouse zygotes immediately after fertilization, heterochromatin protein HP1158 preferentially associates with the maternal genome that is rich in the H3-K9 modification (Arney et al. 2002, Cowell et al. 2002) and this interaction has been suggested to enhance the epigenetic asymmetry on the parental genomes in early development (for a review, see Surani 2001)).

In light of recent reports suggesting that assisted reproductive technologies (ART) may cause diseases of epigenetic origin (Cox et al. 2002, DeBaun et al. 2003, Moll et al. 2003), a greater understanding of the epigenetic and nuclear reprogramming events occurring in human oocytes and preimplantation embryos is required. We were therefore prompted to assess the expression of the Polycomb-group genes in the human female germline and during preimplantation development.

Materials and Methods

Complementary DNA (cDNA) samples were generated from pooled, staged human ovarian follicles from the primordial to the secondary stages and from individual mature metaphase II oocytes and preimplantation embryos utilizing the SMART amplification system (BD Clontech, Palo Alto, CA, USA) as previously described (Huntriss et al. 2002). All samples were obtained after informed consent under ethically approved and HFEA licensed protocols. cDNA samples were extensively verified with intron-spanning primers to exclude genomic DNA contamination and were characterized with positive controls and stage-specific markers prior to application in the assays described here. All primers used for the current report are presented in Table 1.

**PCR amplification of chromodomain sequences**

Degenerate PCR primers were used according to the protocol of Lessard et al. (1998) to amplify the chromodomains common to Pc-G and HP1 proteins. Heterogenous PCR products obtained from the cDNA samples were run on 1.5–2% agarose gels. The amplicons generated (chromodomain primer products, 115 bp), were isolated using a Qiagen gel-purification kit and were subcloned into the Invitrogen Topo TA sequencing vector. Samples were sequenced at the Biomolecular Analysis Facility, University of Leeds. Sequences of PCR products were obtained in both directions (M13 forward and reverse primers) and were confirmed by Basic Local Alignment Search Tool (BLAST) searching (National Centre for Biotechnology Information, National Institute for Health, USA).

**PCR amplification of Pc-G genes YY1, EED and EZH2**

Gene-specific PCR expression analysis was performed using 1 µl cDNA in a 25 µl volume PCR reaction mix (Bioline, London, UK). PCR primer sequences and annealing temperatures are given in Table 1. For EED, primers were designed to amplify both transcript variants (variant 1, NM_003797; variant 2, NM_152991). PCR was performed for 30 cycles for 1 min at each step at 94°C, the specific annealing temperature (see Table 1), and 72°C. Products were run on 1.5–2% agarose gels and visualized using ethidium bromide with reference to 100 bp DNA size markers (Invitrogen). All PCRs were repeated a minimum of three times. PCR product identity was confirmed by sequencing.

**Results**

**Degenerate PCR amplification of chromodomain sequences**

A developmental series of amplified cDNAs was generated from human ovarian follicles, mature oocytes and preimplantation embryos (Huntriss et al. 2002). Degenerate PCR primers were used which amplify the chromodomains that are common to both the polycomb proteins and heterochromatin-associated proteins (HP1) (Lessard et al. 1998). The 115 bp heterogenous PCR product that was generated from the cDNA samples derived from human oocytes and preimplantation embryos is shown in Fig. 1. The identities of the PCR products were confirmed by sequencing, with a minimum of ten clones being sequenced per developmental stage. The chromodomain

**Table 1** PCR primers used for the investigation of Polycomb-group gene expression in human ovarian follicles oocytes and preimplantation embryos.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer 5’ to 3’</th>
<th>Reverse primer 5’ to 3’</th>
<th>Annealing temperature (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT</td>
<td>aattatgacagcactaagctgc</td>
<td>ggcgtagtcaataaggactacatg</td>
<td>60</td>
<td>Gibbs et al. (1989)</td>
</tr>
<tr>
<td>EED</td>
<td>attgttagcgttcctgactgt</td>
<td>tggtagaataagccaccaaca</td>
<td>60</td>
<td>*</td>
</tr>
<tr>
<td>EZH2</td>
<td>atgtgtctgcaagatggctg</td>
<td>tctcgaggttcctgaagctcg</td>
<td>60</td>
<td>*</td>
</tr>
<tr>
<td>YY1</td>
<td>atctctcatcctataagggc</td>
<td>tgcagtagttccagactgct</td>
<td>60</td>
<td>Huntriss et al. (2002)</td>
</tr>
<tr>
<td>ZP3</td>
<td>gataatactacagcttgctcc</td>
<td>tcacttctctctctcagctgctg</td>
<td>60</td>
<td>Weisenberger et al. (2002)</td>
</tr>
</tbody>
</table>

*Primers newly designed for this study.*

primer expression results are summarized in Fig. 2 and the sequences of the isolated chromodomain transcripts are given in Table 2. Expression of different HP1 transcripts was detected in ovarian follicles, (HP1\(^{HSa}\), HP1\(^{HSb}\), HP1\(^{HSc}\)), mature oocytes (HP1\(^{HSa}\), HP1\(^{HSb}\)), early preimplantation embryos (HP1\(^{HSa}\), HP1\(^{HSb}\), HP1\(^{HSc}\)) and blastocysts (HP1\(^{HSa}\), HP1\(^{HSc}\)). Furthermore, we isolated an HP1\(^{HSa}\) variant that contained a single nucleotide mismatch (A to G transition) exclusively in the cDNA samples derived from primordial/early primary follicles.

**Gene-specific PCR analysis of expression of YY1, EED and EZH2 polycomb genes**

The expression of transcripts of three polycomb genes YY1, EED and EZH2, was assessed using gene-specific PCR analysis of the cDNA samples derived from key stages of human oogenesis and preimplantation development. These were compared with the expression of the control housekeeping genes hypoxanthine phosphoribosyl transferase (HPRT) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the zona pellucida glycoprotein 3 gene (ZP3), which is solely expressed in oocytes and early preimplantation stages. Expression of YY1, EED and EZH2 was observed in cDNA samples derived from ovarian follicles, including the primordial through to the primary stages follicles (Fig. 3). While YY1 and EZH2 transcripts were consistently detected in mature oocytes, we did not observe EED expression in cDNA derived from any mature oocytes tested (0/8 samples in Table 3). Expression of YY1, EED and EZH2 transcripts was observed in cDNA derived from early (two- to four-cell) and late (morula and blastocyst)-stage preimplantation embryos. The EED transcript present in ovarian follicles and preimplantation embryos was confirmed as transcript variant 1 by sequencing (NM_003797).

**Discussion**

This work reports that three genes which have been defined as being essential for early murine development – YY1, EED and EZH2 – are also expressed during human preimplantation development. Variability in expression was observed between some samples of the same stage but multiple sample testing has revealed some obvious trends. Although the variability may have a technological basis, especially as low sample numbers are being used (Holding et al. 2000), other causes such as oocyte/embryo quality and developmental differences may be considered. It has been shown that the epigenetic regulator EED, which in the mouse regulates X-chromosome inactivation and autosomal imprinting during early development (Wang et al. 2001, Plath et al. 2003, Silva et al. 2003), appears to be expressed after fertilization in humans. It remains possible that EED is expressed earlier during oogenesis and is stored as a protein within the ooplasm (Surani 2001) as it has to be accepted that it is possible...
Table 2  Chromodomain-containing HP1 sequences isolated from cDNAs derived from human ovarian follicles, oocytes and preimplantation embryos.

<table>
<thead>
<tr>
<th>Genes</th>
<th>cDNA sequences/translations amplified by degenerate chromodomain primers</th>
<th>No. isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP1 Hα</td>
<td>R V V K G Q V E Y L L K W K G F S E E H</td>
<td>45</td>
</tr>
<tr>
<td>HP1 Hα (A → G)</td>
<td>CGC GTG GTT AAG GGG CAA GTG GAA TAT C TA CTG AAG TGG AAA GGC TTT TCT GAG GAC</td>
<td>4</td>
</tr>
<tr>
<td>HP1 Hβ</td>
<td>CGA GTG GTA AAG GGC AAA GTG GAG TAC C TC A TA A GAG GGA TTC TCA GAT GAG GAC</td>
<td>30</td>
</tr>
<tr>
<td>HP1 Hγ</td>
<td>CGT GTA GTG AAT GGG AAA GTG GAA TAT TTC CTG AAG TGG AAG GGA TTT ACA GAT GCT GAC</td>
<td>16</td>
</tr>
</tbody>
</table>

The number of clones isolated is given in the right-hand column. HP1 Hα (A → G) represents the HP1<sup>Het</sup> nucleotide mismatch (A to G transition) variant sequences detected in the cDNA derived from primordial/early primary follicles.

Figure 3  Summary of expression profiles of Polycomb-group gene transcripts in cDNAs derived from human ovarian follicles, mature oocytes (MII), four-cell preimplantation embryos and blastocysts. Expression of Polycomb-group genes EED, EZH2 and YY1 in human ovarian follicles (lanes 1-5), metaphase II oocytes (lanes 6-10), early preimplantation embryos (lane 11, two-cell; lanes 12 and 13, four-cell cleavage stage embryos) and blastocysts (lanes 14-17). For ovarian follicle lanes, 1° represents primary follicles and 2° represents secondary follicles. Positive control PCRs for the same cDNA samples include HPRT, GAPDH and ZP3. Lane 18, negative control for PCR reactions; M, marker lanes (100 bp).

Table 3  Total sample numbers for specific Polycomb-group genes EED, EZH2 and YY1 expression across the human developmental series (includes extra data not shown in Fig. 3).

<table>
<thead>
<tr>
<th></th>
<th>Ovarian follicles</th>
<th>MII oocytes</th>
<th>2–8 cell embryos</th>
<th>Morulae</th>
<th>Blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td>EED</td>
<td>4/5</td>
<td>0/8</td>
<td>1/8</td>
<td>1/2</td>
<td>10/10</td>
</tr>
<tr>
<td>EZH2</td>
<td>3/5</td>
<td>7/8</td>
<td>4/8</td>
<td>2/2</td>
<td>9/10</td>
</tr>
<tr>
<td>YY1</td>
<td>3/5</td>
<td>6/8</td>
<td>5/8</td>
<td>2/2</td>
<td>10/10</td>
</tr>
</tbody>
</table>
that transcripts with shorter polyA tails may not be amenable to isolation using the oligo dT method described here. Heterochromatin-associated protein 1 transcripts were exclusively detected using the chromodomain primers, in agreement with previous reports such as those in human CD34 + bone marrow cells (Lessard et al. 1998). It seems likely that the HP1HS variant containing a single nucleotide A to G transition represents a novel human Polycomb member due to its repeated discovery within a highly conserved sequence such as that of the chromodomain. Accordingly, several HP1HS sequences have been found in expressed sequence tags (EST) databases and, furthermore, multiple bands result from the hybridization of mouse and human genomic DNA with a probe for HP1HS (Sanders et al. 1993).

Notably, HP1HS transcripts are detected in human mature oocytes and early preimplantation embryos. Due to the preferential association of the murine HP1HS protein with the paternal genome in mouse zygotes immediately after fertilization (Arney et al. 2002, Cowell et al. 2002), further experimentation is required to establish whether the corresponding chromodomain proteins are involved in nuclear reprogramming and epigenetic regulation in human oocytes and early embryos. Recent protein localization studies in the mouse have also demonstrated preferential binding of the Ezh2–Eed complex to the maternal pronucleus in the zygote (Erhardt et al. 2003). Depletion of maternal Ezh2 has been shown to disrupt this binding and the subsequent establishment of H3-K27 and H3-K9 modification on the two parental genomes. The Ezh2–Eed complex also co-localizes with the inactivated X-chromosome in blastocysts, an event that is disrupted by an Ezh2 mutant that also subsequently affects the establishment of H3-K27 methylation (Erhardt et al. 2003). The continued expression of EED and EZH2 genes in the present study may indicate that these histone methylation events also occur during human preimplantation development.

The consequences of epigenetic disruption during ART is an ongoing area of research (Cox et al. 2002, DeBaun et al. 2003, Moll et al. 2003). An association has recently been made between in vitro fertilization treatments and the occurrence of Beckwith Wiedemann syndrome (BWS) (DeBaun et al. 2003, Chang et al. 2005). Half of BWS patients have aberrant methylation and imprinting of long QT intronic transcript 1 (LIT1), an untranslated RNA within the potassium voltage-gated channel, KQT-like subfamily member 1 (K,LQT1) gene (Lee et al. 1999, Smilichnick et al. 1999). The equivalent imprinted control region in the mouse is the Kcnq1 domain on distal chromosome 7. Paternal repression in the murine domain depends on the methylation of H3-K27 and H3-K9 with Ezh2–Eed complexes being recruited to these regions early in development to regulate the methylation (Umlauf et al. 2004). It is essential to establish whether related processes involving the EZH2–EED complex are disrupted by ART in human early development.

To our knowledge, our study is the first to describe the expression of the Polycomb-group genes in human oocytes and preimplantation embryos. The data presented here serve as a basis for more detailed analysis of these epigenetic regulators using quantitative approaches and protein immunolocalization studies.

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