Allele-specific in situ hybridization (ASISH) analysis: a novel technique which resolves differential allelic usage of $H19$ within the same cell lineage during human placental development

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SUMMARY

Precursory studies of $H19$ transcription during human foetal development have demonstrated maternally derived monoallelic expression. Analyses in extra-embryonic tissues, however, have been more equivocal, with discernible levels of expression of the paternal allele of $H19$ documented in the first trimester placenta. By refining the in situ hybridization technique we have developed an assay to enable the functional imprinting status of $H19$ to be determined at the cellular level. This assay involves the use of oligonucleotide DNA probes that are able to discriminate between allelic RNA transcripts containing sequence polymorphisms. Biallelic expression of $H19$ is confined to a subpopulation of cells of the trophoblast lineage, the extravillous cytotrophoblast, while the mesenchymal stroma cells maintain the imprinted pattern of monoallelic expression of $H19$ throughout placental development. This data demonstrates that the low level of paternal $H19$ expression previously detected in normal human placenta is not due to a random loss of functional imprinting, but appears to result from a developmentally regulated cell type-specific activation of the paternal allele. In addition, biallelic expression of $H19$ does not seem to affect the functional imprinting of the insulin-like growth factor II gene, which is monoallelically expressed at relatively high levels in the extra-villous cytotrophoblasts. These results imply that the allelic usage of these two genes in normal human placental development may not be directly analogous to the situation previously documented in the mouse embryo.

Key words: genomic imprinting, $H19$, IGF2, trophoblast lineage, ASISH, human placenta

INTRODUCTION

Several human autosomal genes have been identified as being subject to genomic imprinting, i.e. monoallelic expression that is dependent on the parent of origin (reviewed in Franklin et al., 1995; Efstratiadis, 1994). The $H19$ and insulin-like growth factor II [$IGF2$ (human); $Igf2$ (mouse)] genes are two such examples: they are physically linked and map to an imprinted domain on human chromosome 11p15.5 and, in mouse, on the distal part of chromosome 7 (Zemel et al., 1992; Glaser et al., 1989). Their reciprocal patterns of monoallelic expression ($H19$ from the maternally, and $IGF2$ from the paternally inherited allele) and closely overlapping spatiotemporal expression patterns have led to the suggestion that their imprinting and expression may be co-ordinately regulated (Ohlsson et al., 1994; Bartolomei et al., 1993; Bartolomei and Tilghman, 1992). Support for these proposals has been provided in the mouse, where deletion of the maternally inherited $H19$ gene and a portion of surrounding sequences leads to an activation of the maternal $Igf2$ allele (Leighton et al., 1995a). This result has contributed to the hypothesis that both the $Igf2$ and $H19$ loci may compete for a common enhancer. Substantial support for this theory has been provided by the recent demonstration that the deletion of two enhancers located 3' of $H19$ affects in cis both $H19$ and $Igf2$ expression in mouse endodermal cell lineages (Leighton et al., 1995b).

There are a number of exceptions, however, where the attractive enhancer-competition model does not appear to be readily applicable. One example is the mouse choroid plexus/leptomeninges, where $H19$ is imprinted although $Igf2$ is biallelically expressed (Svensson et al., 1995; DeChiara et al., 1991). In another example, the plasticity of promoter-specific $IGF2$ allele usage in human postnatal liver is independent of the functional imprinting status of $H19$ (Ekström et al., 1995a). Biallelic expression of $H19$ has previously been documented in human extra-embryonic tissues. Studies using RNA prepared from samples of human placenta, representative of various gestational stages (Walsh et al., 1995; Jinno et al., 1995; Zhang and Tycko, 1992), have provided evidence for an active paternally inherited $H19$ allele, although in general, the paternal allele was found to constitute only a small fraction of the total $H19$ expression. A recent study has documented that initial biallelic expression of $H19$ is superseded by monoallelic expression during gestational develop-
ment (Jinno et al., 1995). It was proposed that this may reflect a progressive repression of the paternal allele, i.e. the functional imprinting status may be acquired during placental development.

The previous studies have not been able to establish whether ‘leakage’ of the paternal H19 allele is general and random or if it is restricted to a sub-population of expressing cells. To attempt to answer this question and resolve the functional imprinting status of H19 within the placental tissue we have developed an allele-specific in situ hybridization technique (ASISH). This method has enabled us to examine allele specific expression of both H19 and IGF2 at the cellular level. By exploiting polymorphic sequence differences, we have been able to design oligonucleotide DNA probes which can discriminate between the transcripts from each of the parental alleles in heterozygous human placental specimens of varying gestational ages. The results demonstrate that expression of the paternal H19 allele in normal human placenta occurs exclusively in a subset of cells, the extra-villous cytotrophoblasts, while the cells of the placental mesenchymal stroma express only the maternal H19 allele. These findings indicate the existence of a cell type-specific functional imprinting status for H19 in the human placenta. We have also used the ASISH technique to study IGF2 expression at the cellular level and find that all cells expressing IGF2 do so monoallelically, including the extra-villous cytotrophoblasts that display biallelic H19 expression.

MATERIALS AND METHODS

Placental samples

Placental samples were obtained from routine elective terminations or scheduled caesarean deliveries carried out at Karolinska Hospital, Stockholm. Placental tissue was immediately rinsed in ice-cold sterile isotonic saline, fixed in phosphate-buffered 4% paraformaldehyde at 4°C or snap-frozen. After fixation, samples were washed in sterile phosphate-buffered saline and sequentially dehydrated before embedding in ‘Histowax’ embedding medium (Histolab Products AB, Sweden). Sections 5 μm thick were mounted on ‘Super Frost®/plus’ pre-treated glass slides (Menzel-Gläser, Germany). Two adjacent sections were mounted on each slide, separated by a gap of approximately 2 cm. Before pretreatment the slides were heat treated at 65°C for 30 minutes, immersed in xylene and subsequently rehydrated in a sequential ethanol series.

Genotyping

H19 RsaI polymorphic status of DNA, extracted from the placental and the corresponding decidual samples, was established by DNA amplification and subsequent product digestion using primers and conditions as previously described (Walsh et al., 1995). The nucleotide sequences of each product were also determined either directly using ‘The fmol sequencing kit’ (Promega) or by cloning and plasmid sequencing (‘Sequenase, T7 Plasmid Sequencing Kit’, Amersham) to ensure complete complementarity of allelic sequences to those of the oligonucleotide probes used for in situ hybridization analyses. Amplification products were cloned (TA vector system, Invitrogen) and individual clones used in Southern analyses (Fig. 1) to determine the fidelity of hybridization of the oligonucleotide probes to their respective allelic counterparts. Southern hybridizations were carried out as previously described (Li et al., 1995), with the exception of the hybridization temperature (45°C). Filter washing was carried out using 0.1x SSC/0.1% SDS at 52°C (three times, each for 20 minutes). Oligonucleotide probe sequences (polymorphic sequences underlined) used for ASISH experiments were as follows:

H19 allele RsaI(-): 5’-CTC AAC ACT CGC ACC GAG ACT CAA GGC C.
H19 allele RsaI(+): 5’-CTC AAC ACT CGT ACT GAG ACT CAA GGC C.

IGF2 allele (A): 5’-TGT GAT TTC TGG GG C CCT TCT TT TT.
IGF2 allele (B): 5’-TGT GAT TTC TGG GG C CCT TCT TT TT.

Allele-specific in situ hybridization analyses

Pretreatment of tissue sections

Slides were incubated in 0.2 M HCl for 10 minutes followed by two 15 minute washes in 2x SSC. Sections were permeabilized with 2 μg/ml Proteinase K in pre-warmed 0.2 M Tris-HCl pH 7.2, 2 mM CaCl2 for 15 minutes at 37°C, followed by sequential ethanol dehydration, before placing slides in a baked Coplin jar for acetylation. Sections were acetylated by adding 200 μl acetic acid anhydride and 80 ml 0.1 M triethanolamine, pH 8.0, simultaneously and incubating for 10 minutes. Sections were then sequentially dehydrated and stored in 95% ethanol at ~20°C.

Probe preparation and in situ hybridization

Radiolabelled oligonucleotide probes were prepared by 3’ end-labelling with 35S-dATP (isotope, enzyme and buffers from Amersham) and purified using ‘Oligotex’ resin and spin column purification (Qiagen). Sections were allowed to air dry at room temperature before 4 hours of prehybridization at 33°C (H19) or 30°C (IGF2) in the following: 50% formamide, 3× SSC, 2 mg/ml BSA (grade V), 10 mM vanadyl ribonucleoside, 100 μg/ml Poly A, 1 mg/ml herring sperm DNA and 0.5 mg/ml yeast RNA. Hybridizations were carried out overnight at 33°C (H19) or 30°C (IGF2) in a solution differing from that of pre-hybridization by the additional components dextran sulphate (10%) and DTT (0.3 M). Prehybridizations and hybridizations were carried out in humidified conditions in boxes able to accommodate ten glass slides. Prehybridizations were carried out without coverslips, but hybridization cocktails were covered with Parafilm pieces, which had been presoaked in ethanol and allowed to air-dry before use. Hybridizations were carried out using 30,000 cpm/ml labelled oligonucleotide probe and 100μl excess cold competing oligonucleotide (that recognizing the transcript from the allele other than the one to be detected by the radiolabelled probe). As each glass slide contained two adjacent sections, a rubber cement barrier (‘Fastik’ – AB Thure Bünnger, Sweden) was used to prevent mixing of adjacent hybridization cocktails. One section from each slide was hybridized with the oligonucleotide DNA probe recognizing one allelic transcript [H19 allele RsaI(+) or IGF2 allele (A), respectively], with the second section on the same slide hybridized with the probe recognizing the opposite allele [RsaI(-) or (B), respectively]. Glass slides containing sections homozygous for each of the respective alleles were prepared and hybridized in an identical manner to that described for the glass slides containing heterozygous sections, i.e. one section on each glass slide was hybridized with the complementary allelic probe sequence, while the second section on each glass slide was addressed with the non-complementary oligonucleotide probe. Homozygous control sections were included in each humidified box used for every ASISH assay performed on slides adjacent to those containing the heterozygous sections.

Posthybridization treatment

Slides were washed in 2x SSC, 50% formamide, 10 mM DT in 37°C, once for 10 minutes, then twice for 45 minutes. In the case of the H19 ASISH experiments, this was followed by washing at room temperature, twice in 2x SSC for 15 minutes each, twice in
1× SSC for 15 minutes each and twice for 1 minute each in cold tap water, before sequential ethanol dehydration, removal of the rubber cement barriers and thorough air-drying. After the SSC/formamide wash steps, the IGF2 ASISH experiments were washed four times for 15 minutes each in 1× SSC at 55°C, before washing in cold tap water, ethanol dehydration, rubber cement barrier removal and air-drying. XAR5 autoradiograph or Fuji Phosphorimager Bas-1500 exposures were carried out before emulsion treatment in order to evaluate the length of time that the slides should be exposed with emulsion. Emulsion exposure was carried out with NTB2 (Kodak) and slides were exposed for 3 to 15 days before D19 (Kodak) development and fixation with sodium thiosulphate pentahydrate. Slides from any one experiment were all exposed for the same length of time. Staining was carried out using Mayers hematoxylin followed by 1% eosin, before mounting and image analysis. Digital images of the sections were collected using a ‘ColourCoolView’ integrating video camera (Photonic Science, UK) and a Leica DMRXE light microscope. The images were processed and mounted using ‘Adobe Photoshop 3.0’.

RESULTS

Fidelity of oligonucleotide hybridization

In order to examine expression of H19 by allele-specific in situ hybridization, we decided to design oligonucleotide DNA probes specific for a previously documented and well-characterised RsaI restriction enzyme polymorphism in exon 5 of the human H19 gene (Zhang and Tycko, 1992). Placental and corresponding decidual samples were initially examined for heterozygosity at this site by thermal cycle amplification of their DNA, followed by appropriate digestion and gel electrophoresis (Walsh et al., 1995). Amplified products were subsequently cloned and sequenced to ensure that the presence or absence of RsaI digestion correlated with a nucleotide sequence identical to that of each oligonucleotide probe sequence. In each case the alleles were found to differ by two C to T-transition mutations within the same 31 bp region of the fifth exon, shown in Fig. 1A. A third nucleotide polymorphism, previously reported to be occasionally associated with this region in the North American population (Zhang and Tycko, 1992), was not found in any of our samples. We also observed that samples homozygous for the RsaI non-cutting allele were relatively infrequent and may reflect a difference in allele distribution between populations. Genotyping of the decidual samples enabled the parent of origin of each placental allele to be determined in certain cases.

The fidelity of hybridization of each oligonucleotide probe to its corresponding allelic sequence was determined initially by Southern hybridization. A filter was prepared with individual lanes containing one of the two types of each cloned and sequenced allele product respectively (Fig. 1B). When this filter was addressed with one of the oligonucleotide probes, hybridization was seen only with the allele fully complementary to that of the oligonucleotide probe sequence. Strip-washing and hybridization of this filter with the oligonucleotide complementary to the second allele gave the expected reciprocal pattern (Fig. 1B).

Specificity of in situ hybridization signal

After determining that the oligonucleotide probes specifically recognized only their fully complementary allelic counterparts in Southern hybridizations, the next step was to examine their behaviour when used for in situ hybridization. In addition, riboprobe in situ hybridization analyses were performed for all samples subsequently used for ASISH experiments, to ensure that the hybridization signal observed using the oligonucleotide DNA probes corresponded to the expected expression patterns for both H19 and IGF2. Oligonucleotide probe in situ hybridizations were initially carried out on tissues homozygous for either H19 RsaI cutting [Rsa(+)1] or non-cutting alleles [Rsa(−)]. As previously mentioned, during the course of sample collection for this study we were unable to obtain

![Fig. 1. Specificity of oligonucleotide probe hybridization.](image-url)
placental samples homozygous for the \textit{RsaI} non-cutting \([\text{Rsa}(-)]\) allele. Instead, we have used sections taken from a juvenile ovarian teratoma, which has a high level of expression of \textit{H19} restricted spatially to particular epithelial structures (Fig. 2H-N). The homology of the oligonucleotide probes to the alleles of this tissue (as with all samples analysed in this report) was again determined by nucleotide sequencing. Fig. 2 shows that in situ hybridization analyses performed with the appropriate oligonucleotide probe gives a hybridization signal only with the expected homozygous tissue section. Control hybridizations carried out using the non-complementary oligonucleotide probe give no signal in each respective reciprocal case. In initial pilot experiments, a background level of inappropriate hybridization signal was observed when the non-complementary oligonucleotide probe was used. This was abolished by adding an excess of unlabelled oligonucleotide of

Fig. 2. \textit{H19} expression at the cellular level. Thin sections of 7-week human placenta homozygous for the \textit{Rsa(+)}\textit{H19} allele (A-G) and juvenile ovarian teratoma homozygous for the \textit{H19} \textit{Rsa(−)} allele (H-N), were hybridized with oligonucleotide DNA probes or riboprobes. (A) Bright-field view under 40\(\times\) magnification of \textit{+/+} homozygous placenta probed with \textit{Rsa(+)} oligonucleotide DNA probe. Villous trophoblast (vtb), mesenchymal stroma (ms) and extra-villous trophoblasts (evtb) are marked. (B,J) Dark-field views under the same magnification of \textit{+/+} homozygous placenta and \textit{−/−} homozygous ovarian teratoma, respectively, probed with the \textit{Rsa(+)} probe; (F,M) show the reciprocal hybridizations; dark-field views of \textit{+/+} homozygous placenta and \textit{−/−} homozygous ovarian teratoma, respectively, probed with \textit{Rsa(−)} probe. (L) Bright-field view of the \textit{−/−} homozygous juvenile ovarian teratoma probed with the \textit{Rsa(−)} oligonucleotide DNA probe. (C,N) The pattern of \textit{H19} expression obtained by antisense riboprobe analysis; (G,K) show the control sense riboprobe results. (D,E) The region indicated by the rectangle in A under 400\(\times\) magnification in bright-field. D is \textit{Rsa(+)} oligo-probed and E is \textit{Rsa(−)} oligo-probed. (H,I) The reciprocal pattern of expression for the ovarian teratoma, 252\(\times\) magnifications of the region indicated by the rectangle in L. H is \textit{Rsa(+)} probed and I is \textit{Rsa(−)} oligo-probed.
Fig. 3. Cell type-specific functional H19 imprinting status in the developing human placenta. Heterozygous placental specimens informative for the *Rsa*I cutting and non-cutting H19 alleles were hybridized with *Rsa*+ and *Rsa*− oligo-probes respectively. (A-L) The results of oligonucleotide DNA probe hybridizations to first trimester placental samples. (M-S) The results of hybridizations to heterozygous term placental samples. The results shown here are representative of the tissue sections as a whole and of the results obtained with all placental samples heterozygous for the H19 allelic sequences. (A,C,E,G,I,K,M,O and Q) Hybridization with the *Rsa*− oligonucleotide DNA probe, with the *Rsa*+ probe results depicted in the adjacent, alternate sections. Extra-villous trophoblast (evtb), mesenchymal stroma (ms), cytotrophoblast (ctb) and syncytiotrophoblast (stb) are indicated. Dark-field views are placed below corresponding bright-field views and are photographed at 40× magnifications. (E,F,Q) 252× magnifications of the boxed regions depicted in A,B and S, respectively. R is from a section adjacent to that shown in Q and S and is probed with the reciprocal oligonucleotide probe. The arrows in J (bright-field) and L (dark-field) indicate the gradual increase in activity of the (paternal) *Rsa*+ allele in comparison to the (maternal) *Rsa*− probe signal seen in I and K. G,H: 400× magnifications.
Extra-villous cytotrophoblasts (Fig. 3B,D,F,J and L), with cells sively confined to areas of structures termed the intermediate or riboprobe in situ analyses. Paternal allele expression was exclu- phoblastic cells seen to express allele probe. This was found in a sub-population of the cytotro- signal was also observed, however, from the reciprocal (paternal) allele expression (Fig. 3 I-L). To examine the relative hybridization sub-populations showed higher levels of maternal allele expression (Fig. 3M-P).

Expression of paternally derived H19 during human placental development

Previous reports have documented expression of both parental H19 alleles in first trimester placentae (Walsh et al., 1995; Jinno et al., 1995; Zhang and Tycko, 1992). The ASISH experimental approach enables us to discriminate the allele usage at the cellular level in human specimens and explore the possible existence of a mosaic imprinting status. Fig. 3 shows representative sections of informative first trimester placenta, which demonstrate wide- spread expression of H19 when ASISH analyses are performed with the oligonucleotide probe sequence complementary to the maternally derived H19 allele (Fig. 3A,C,E,G,I and K). A specific signal was also observed, however, from the reciprocal (paternal) allele probe. This was found in a sub-population of the cytotrophoblastic cells seen to express H19 at relatively high levels by riboprobe in situ analyses. Paternal allele expression was exclu- sively confined to areas of structures termed the intermediate or extra-villous cytotrophoblasts (Fig. 3B,D,F,J and L), with cells of the mesenchymal stroma expressing only the maternal H19 allele. Similar results were obtained with all heterozygous first trimester placental samples tested, which ranged from 7 to 11 weeks of gestation.

Functional imprinting of H19 within the trophoblast cell lineage

The hybridization signal observed in the ASISH experiments for the H19 paternal allele was found to vary between different sub-populations of extra-villous cytotrophoblasts. Some extra-villous cytotrophoblast populations appeared to express both parental alleles at similar levels (Fig. 3A-F), while other sub-populations showed higher levels of maternal allele expression (Fig. 3 I-L). To examine the relative hybridization signal in extra-villous cytotrophoblasts, we counted the silver grain density in representative areas of informative sections, hybridized under identical conditions, to the paternal- and maternal-specific oligonucleotide probes respectively. By measuring the number of silver grains per cell, we found areas of extra-villous cytotrophoblasts with comparable levels of expression of both alleles (data not shown). As other areas of extra-villous cytotrophoblasts predominantly display maternal allele expression, it is possible that H19 may still be functionally imprinted in some of these cells.

The villous cytotrophoblasts, from which the extra-villous population derives, appear to maintain monoallelic H19 expression, although at lower levels than were observed for extra-villous cytotrophoblast (Fig. 3G,H). These observations indicate that during placental development, the functional imprinting of H19 is progressively lost in the extra-villous cytotrophoblasts. This proposal is diametrically opposed to the previous suggestion that imprinted monoallelic expression of H19 is gradually established from an initial biallelic expression during early placental development (Jinno et al., 1995). By ASISH analysis, biallelic expression of H19 can be documented even in term placental samples. This is again limited to the extra-villous cytotrophoblasts, which can be found in the region of the decidua basalis (Fig. 3Q-S), although the bulk of term placental cells express H19 monoal- lically (Fig. 3M-P).

Allele usage of IGF2 in extra-villous cytotrophoblasts

Since the expression patterns of IGF2 and H19 extensively overlap in the human embryo (Ohlsson et al., 1994) and placenta (Jinno et al., 1995; Walsh et al., 1995) and their imprinting status has been suggested to be co-ordinately regulated in the mouse (Leighton et al., 1995a), we also applied the ASISH technique to IGF2. The ApaI polymorphism within exon 9 of IGF2 was exploited to distinguish between the alleles in this case (Tadokoro et al., 1991). Both types of alleles were cloned and sequenced as described for H19. The alleles in this instance differ by only a single base change, but despite this, allele-specific oligonucleotide probes were able to discriminate IGF2 allelic usage with high fidelity in placental control samples (Fig. 4A-F). In informative heterozygous specimens, it is clear that IGF2 is monoallelic in all expressing cell types discernible within the first trimester placenta (Fig. 4G-I), which corresponds to previous reports of IGF2 expression in placenta (Ekström et al., 1995b; Ohlsson et al., 1993). Analysis of adjacent sections of a placental specimen, which was heterozygous for both IGF2 and H19, resulted in the detection of only one IGF2 allele (Fig. 4G-I), whereas both parental H19 alleles were active (Fig. 4J- L). Since most extra-villous cytotrophoblast cells in adjacent sections display biallelic H19 expression but monoallelic IGF2 expression, we conclude that a functionally non-imprinted H19 is compatible with an imprinted IGF2.

DISCUSSION

In this report we have presented an adaptation of the in situ hybridization technique that has enabled us to determine the functional imprinting status of H19 and IGF2 at the cellular level in the developing human placenta. To accomplish this, we have utilised allele-specific sequence polymorphisms, which we have strategically positioned within defined discrimina- tory oligonucleotide DNA probe segments. The specificity of hybridization signal could be verified using tissue

Table 1. Summary of allelic usage of H19 and IGF2 in different placental cell types

<table>
<thead>
<tr>
<th>Placental cell types</th>
<th>Origin</th>
<th>Allelic usage</th>
<th>Comments</th>
</tr>
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<tbody>
<tr>
<td>Villous cytotrophoblasts</td>
<td>Trophodermal extraembryonic lineage</td>
<td>Mono, Mono</td>
<td>Dominating cell component during first trimester</td>
</tr>
<tr>
<td>Extra-villous cytotrophoblasts</td>
<td>Trophodermal; villous cytotrophoblasts</td>
<td>Mono, Mono and bi-</td>
<td>Appear predominantly during first trimester</td>
</tr>
<tr>
<td>Mesenchymal stroma</td>
<td>Epiblast-derived</td>
<td>Mono</td>
<td>Dominating cell component during final two trimesters</td>
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Functional imprinting of H19

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sections homozygous for each type of allele and was found to be specific for each of the allelic transcripts of H19 and IGF2 respectively. In addition, the relative intensity in the hybridization signal obtained for each allelic probe was found to be similar (verified by the analysis of silver grain density per cell; data not shown). In the investigations documented here, the hybridization reactions performed with discriminatory oligonucleotide probe pairs were carried out simultaneously at the same hybridization temperatures, respectively. We do not feel, however, that this is an absolute requirement for this technique and in other instances, for example where polymorphic differences involve the insertion or deletion of nucleotide sequences, the ASISH technique should be equally applicable if the hybridization of each oligonucleotide probe is optimised and the appropriate control hybridizations are carried out.

This novel ASISH approach was used to examine the previously reported biallelic expression patterns of H19 in the human placenta (Walsh et al., 1995; Jinno et al., 1995; Zhang and Tycko, 1992), as these studies had addressed only the allelic usage in the tissue as a whole. The observations of biallelic expression of H19 in the human placenta (or indeed of any imprinted gene in any tissue) could be the result of one of several underlying mechanisms. The paternally inherited allele could be transcribed initially at relatively high basal levels. In this situation, the gradual loss of biallelic expression can be explained by an overall and progressive temporal repression of the paternal H19 allele, as previously suggested (Jinno et al., 1995). Alternatively, the same results would be obtained if paternal H19 allele expression was restricted to a sub-population of placental cells, or to particular cell types in which H19 could be either bi- or mono-allelically expressed. This model would require the decline or disappearance of such cells or cell type during later stages of development, perhaps in association with the remodelling of the placenta at the end of the first trimester of pregnancy; a criterion which the extra-villous cytotrophoblasts fulfil (Hamilton and Hamilton, 1977).

The overall results of the allele-specific examination of H19 gene expression by the ASISH method are summarized in Table 1. In the cells of the placental mesenchymal stroma, which derive from the inner cell mass (Larsen, 1993), H19 appears to be functionally imprinted (with expression from the maternal allele only). The ectodermally derived villous cytotrophoblasts, which eventually give rise to the extra-villous trophoblast, also seem to maintain monoallelic H19 expression. The paternal allele of H19 does not therefore appear to be expressed at low levels in a global manner in the developing placenta, but seems to be specifically restricted in activity to sub-populations of extra-villous trophoblast. This population of cells gradually regresses during placental development, although isolated populations of extra-villous cytotrophoblasts can be found, for example in the decidua basalis region (placental/maternal interface) of term placenta (Holmgren et al., 1992). We have found that, even in the terminal phase of placental development, such extra-villous cytotrophoblasts exhibit biallelic H19 expression. This implies that the area of placenta sampled may be crucial to the correct interpretation of the functional imprinting status of H19 during placental development and may explain why paternal H19 allele expression has been observed sporadically at later stages of gestation in other studies. In this context, the methylation data previously documented for the H19 gene during the development of the human placenta should also be considered. A progressive allelic methylation has been reported to be associated with the 3′ portion of the paternal H19 allele (Jinno et al., 1995). The possibility exists that this observation could result from a lineage-specific allelic methylation, with the apparent increase in methylation reflecting the proportional increase in the amount of mesenchymal stroma cells as development progresses.

Since we show here that the paternal H19 allele is active in a cell type-specific manner in the extra-villous cytotrophoblast of the normal placenta, it is not surprising that H19 is active in complete hydatidiform moles, which lack the maternal genome (Walsh et al., 1995; Ariel et al., 1994; Mutter et al., 1993). In most complete hydatidiform mole specimens, the bulk of H19 expression occurs in extra-villous cytotrophoblasts (Walsh et al., 1995; Ariel et al., 1994), which are similar to those of the normal placenta. This situation is in contrast to mouse androgenetic trophoblasts, which also express H19 at high levels (Walsh et al., 1994). Both RNase protection analysis (Walsh et al., 1994) and ASISH analysis (K. Svesson, unpublished data) using mouse interspecific F1 hybrid conceptuses show no visible activity of the paternal H19 allele in mouse placenta from blastocyst implantation onwards. Such results support the suggestion that in mouse, in contrast to human, androgenetic trophoblasts may lack a maternally expressed repressor targeted for the paternal H19 allele (Walsh et al., 1994).

Our observations indicate that other potential differences may exist in the control of paternal H19 expression between mouse and man. This is of particular importance in the context of the hypothesised co-ordinate regulation of the H19 and IGF2 genes; the enhancer-competition model (Leighton et al., 1995b; Bartolomei et al., 1993; Bartolomei and Tilghman, 1992), which states that the transcriptional competence of the H19 gene regulates in cis the activity of the nearby Igf2 gene. This model has received considerable experimental support in the mouse, and the monoallelic expression observed for both these imprinted genes in the mesenchymal stroma cells of the developing human placenta is also in agreement with this model. Our finding of monoallelic IGF2 expression in conjunction with biallelic H19 expression in the same cells, however, is in conflict with such a model, since biparental H19 activity would be expected to lead to loss of IGF2 expression. Since IGF2 is expressed monoallelically at high levels in the same structures that display a functionally non-imprinted H19, this is obviously not the case. In this context, it is important to determine if H19 is functionally imprinted at all in the human trophodermal lineage. Villous cytotrophoblasts, when analysed by the ASISH method, appear to display a monoallelic expression pattern of H19. Perhaps more importantly, even some areas of the extra-villous trophoblast structures display monoallelic H19 expression, indicating that gradual activation of the paternal H19 allele occurs in the extra-villous trophoblast populations. This may indicate the existence of an independent pathway of activation for H19 during the generation of extra-villous cytotrophoblast. A second method of activation of H19 could operate concurrently with imprinted gene regulation, in which case the paternal allele would be
preferentially activated, or even replace the imprinted \textit{H19} regulation altogether. Precedents for potential rapid switching of signalling pathways in trophoblast have been provided by the integrins (Vicovac et al., 1995; Moss et al., 1994). It is conceivable that modulations of ligand/receptor interactions could be contributing factors in activating an alternative regulatory pathway, as could micro-environmental changes and differential cell/cell interactions.

The novel technique described in this report, enabling the imprinting status at the cellular level to be explored, could be applied to a number of other pertinent issues. Our understanding of the underlying mechanisms causing abnormal allelic usage (loss of imprinting) during tumourigenesis, such as Wilms’ tumours (Moulton et al., 1994; Steenman et al., 1994; Rainier et al., 1993; Ogawa et al., 1993), hepatoblastoma (Li et al., 1995), human lung cancer (Kondo et al., 1995), human testicular germ cell tumours (van Gurp et al., 1994) and trophoblastic diseases (Walsh et al., 1995; Ariel et al., 1994;
Mutter et al., 1993), for example, could be broadened by ASISH analyses. Other potential applications include the timing of the establishment of functional imprinting during pre- and postimplantation development. This is currently being examined in the mouse.

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