cDNA Cloning and Expression of HIP, a Novel Cell Surface Heparan Sulfate/Heparin-binding Protein of Human Uterine Epithelial Cells and Cell Lines*

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Heparan sulfate proteoglycans and their corresponding binding sites have been suggested to play an important role during the initial attachment of murine blastocysts to uterine epithelium and human trophoblastic cell lines to uterine epithelial cell lines. Previous studies on RL95 cells, a human uterine epithelial cell line, had characterized a single class of cell surface heparan sulfate (HP/HS)-binding sites. Three major HP/HS-binding peptide fragments were isolated from cell surfaces by tryptic digestion, and partial amino-terminal amino acid sequences for each peptide fragment was obtained (Raboudi, N., J ulian, J., Rohde, L. H., and Carson, D. D. (1992) J. Biol. Chem. 267, 11930–11939). In the current study, using approaches of reverse transcription-polymerase chain reaction and cDNA library screening, we have cloned and expressed a novel, cell surface HP/HS-binding protein, named HP/HS interacting protein (HIP), from RL95 cells. The full-length cDNA of HIP encodes a protein of 159 amino acids with a calculated molecular mass of 17,754 Da and pl of 11.75. Transfection of HIP full-length cDNA into NIH-3T3 cells demonstrated cell surface expression and a size similar to that of HIP expressed by human cells. Predicted amino acid sequence indicates that HIP lacks a membrane spanning region and has no consensus sites for glycosylation. Northern blot analysis detected a single transcript of 1.3 kilobases in both total RNA and poly(A) RNAs. Examination of human cell lines and normal tissues using both Northern blot and Western blot analyses revealed that HIP is expressed at different levels in a variety of human cell lines and normal tissues but absent in some cell lines and some cell types of normal tissues examined. HIP has relatively high homology (∼80%) both at the levels of nucleotide and protein sequence to a rodent ribosomal protein L29. Thus, members of the L29 family may be displayed on cell surfaces where they may participate in HP/HS binding events. Heparin/heparan sulfate (HP/HS) proteoglycans (HSPGs) expressed by different cells are able to interact with HP/HS-binding effector proteins and perform important roles in extracellular matrix structure and function, cell adhesion, growth, and differentiation (Ruoslahti and Yamaguchi, 1991; Jackson et al., 1991). HP/HS-binding effector proteins comprise a variety of proteins that include growth factors (Ruoslahti and Yamaguchi, 1991), extracellular matrix components (Kallunki and Tryggvason, 1992; Vlodvasky et al., 1991), cytokines (Bernfield et al., 1992), and cell adhesion molecules (Cole and Glaser, 1986). Our laboratory has been interested in studying the mechanism of embryo implantation. We have found that HSPGs and their corresponding binding sites on cell surfaces may be important in the initial stage of mouse embryo attachment to uterine epithelium. Upon hatching from the zona pellucida, the embryo initially attaches to the uterus through the adhesion of the apical surfaces of the trophectodermal cells of the blastocyst. HSPGs are expressed by mouse embryos at the two-cell and post-implantation stages (Dziadek et al., 1985). Expression of HSPGs on trophectodermal cell surfaces of mouse blastocysts increases 4-5-fold at the peri-implantation stage (Carson et al., 1993; Farach et al., 1987), and HS expressed on mouse embryo surfaces is required for embryo attachment to isolated mouse uterine epithelial cells, fibronectin, and laminin (Farach et al., 1987). Similarly, studies have shown that the initial attachment of JAR cells, a human trophoblastic cell line, to RL95 cells, a human uterine epithelial cell line, is HP/HS-dependent, and enzymatic removal of HS from cell surfaces of both JAR and RL95 cells markedly inhibits JAR-RL95 cell-cell adhesion (Rohde and Carson, 1993). Therefore, HSPGs and their binding proteins also may play an important role in the initial attachment of human trophoblast cells to uterine epithelial cells. Specific, saturable cell surface HP/HS-binding sites have been identified on mouse uterine epithelial cells and human uterine epithelial cell lines (Wilson et al., 1990; Raboudi et al., 1992). Since mouse uterine epithelial cells have only been available by primary cell culture, there is a practical limitation to isolating HP/HS-binding sites from this source. Effort has been placed on the study of HP/HS-binding proteins expressed on the cell surfaces of a human uterine epithelial cell line, RL95 (Raboudi et al., 1992). Mild tryptic digestion of RL95 cell sur-

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† The abbreviations used are: HP/HS, heparan/heparan sulfate; bp, base pair(s); HPI, heparan sulfate/heparin interacting protein; HS, heparan sulfate; HP, heparan; HSPG, heparan sulfate proteoglycan; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; SSC, sodium chloride sodium citrate; BSA, bovine serum albumin; Pipes, 1,4-piperazinediethanesulfonic acid; kb, kilobase.
faces removed most of cell surface HP/HS-binding activity. Three major tryptic peptide fragments, ranging in M., between 6,000 and 14,000, were released from cell surfaces and retained HP/HS-binding activity. Partial amino-terminal amino acid sequences from each of these three peptides were obtained (Raboudi et al., 1992).

We have employed an approach of reverse transcription-polymerase chain reaction (RT-PCR) to identify transcripts encoding cell surface HP/HS-binding peptides. Predicted peptide sequence from one of the RT-PCR products revealed an antigenic sequence that also has features of a HP/HS-binding motif suggested by others (Cardin and Weintzraub, 1989). Polyclonal antibodies directed against the synthetic peptide corresponding to this motif recognize a novel HP/HS-binding protein, named HP/HS interacting protein (HIP), expressed on RL95 cell surfaces with an apparent M, of 24,000 determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Rohde et al., 1996). This peptide selectively binds HP/HS, recognizes certain forms of HP and cell surface HS expressed by JAR and RL95 cells, and supports the attachment of human trophoblast cell lines and a variety of other mammalian adherent cell lines.2

Complete cDNA sequence of HIP has been isolated by screening cdNA libraries using the partial cdNA sequence of RT-PCR product of HIP. HIP cdNA sequence contains a single open reading frame encoding 159 amino acids with a calculated molecular mass of 17,754 Da and a predicted pI of 11.75. This cdNA was cloned into the Lambda gt11 vector from Stratagene (La Jolla, CA) and Clontech (Palo Alto, CA) were used for cdNA library screening following the protocol provided by the manufacturer. Briefly, nitrocellulose filters (Schleicher & Schuell) lifted off the plated cdNA library were prehybridized in 0.8 M NaCl, 20 mM Pipes, pH 6.5, 50% formamide, 0.5% (w/v) cdNA, 100 µg/ml denatured, sonicated salmon sperm DNA for 4 h at 68°C. The filters were then washed using a 2× SSC, 0.1% (w/v) cdNA with several changes during 1 h at 25°C and washed once again using 0.5× SSC and 0.1% (w/v) cdNA for 2 h at 42°C before exposure to Kodak XAR film with an intensifying screen at -70°C.

Northern Blot Analysis—Northern blot analysis of RNA was performed using method described by Sambrook et al. (1989). Total RNA was extracted (Xie and Rothblum, 1991), and poly(A+) RNA was isolated using Oligotex-dT mRNA kit (Qiagen Inc., Chatsworth, CA). Both total RNA and poly(A+) RNA were separated on 1% (w/v) agarose gel and transferred to a nylon membrane (Hybond-N, Amersham Corp.). A cdNA probe (Clone 23-1 in Fig. 1A depleted of poly(A) tail) was labeled with 32P by utilizing the random oligonucleotide primer method (Sambrook et al., 1989). Hybridization was performed at 42°C overnight in a solution containing 50% (w/v) formamide, 5× SSC, 5× Denhardt's, 50 µg/ml dNTP, 0.1% (w/v) cdNA, and 100 µg/ml denatured, sonicated salmon sperm DNA for 4 h at 68°C. The filters were then washed using a 2× SSC, 0.1% (w/v) cdNA with several changes during 1 h at 25°C and washed once again using 0.5× SSC and 0.1% (w/v) cdNA for 2 h at 42°C before exposure to Kodak XAR film with an intensifying screen at -70°C.

cDNA Library Screening—HeLa cell cdNA libraries constructed in the Lambda gt11 vector from Stratagene (La Jolla, CA) and Clontech (Palo Alto, CA) were used for cdNA library screening following the protocol provided by the manufacturer. Briefly, nitrocellulose filters (Schleicher & Schuell) lifted off the plated cdNA library were prehybridized in 0.8 M NaCl, 20 mM Pipes, pH 6.5, 50% formamide, 0.5% (w/v) cdNA, 100 µg/ml denatured, sonicated salmon sperm DNA for 4 h at 68°C. The filters were then washed using a 2× SSC, 0.1% (w/v) cdNA with several changes during 1 h at 25°C and washed once again using 0.5× SSC and 0.1% (w/v) cdNA for 2 h at 42°C before exposure to Kodak XAR film with an intensifying screen at -70°C. Positive plaques were isolated and subjected to subsequent screenings under the same conditions.

Southern Blot Analysis and cdNA Sequencing—Positive phase cdNA from cdNA library screening was purified from the phage lysate (Sambrook et al., 1989) and digested with EcoRI. The cdNA inserts were separated on 1% (w/v) agarose gel, and transferred to a nylon membrane by standard method (Sambrook et al., 1989). Blots were hybridized in a fresh solution of 32P-labeled probe (2-4 × 106 cpm/ml) for library screening, at 42°C overnight. After hybridization, the blots were briefly washed with 0.1× SSC, 0.1% (w/v) cdNA at room temperature and then washed with same mixture at 65°C for 1 h. The blots were exposed to Kodak XAR films with an intensifying screen at -70°C overnight. Positive plaques were isolated and subjected to subsequent screenings under the same conditions.

Construction of cdDNA Expression Vector and Transfection—The entire HIP cdDNA (clone 36–1 in Fig. 1A) was digested from HIP cdDNA-containing pBluescript using NotI, separated on 1% (w/v) agarose gel, purified by phenol/chloroform extraction and ethanol precipitation, and then subcloned into the EcoRI site of pBluescript II SK-(Stratagene). Subcloned inserts were further analyzed using Southern blot analysis as described above, and positive inserts were identified and both strands of the cdNA sequences were determined.

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A Novel Cell Surface Heparan Sulfate-binding Protein

RESULTS

Characterization of RT-PCR Products—RT-PCR products obtained from each pair of primers were isolated, subcloned, and sequenced. Sequences of all of the RT-PCR products were determined. Among all of the RT-PCR products, one product (RT-PCR 224 in Fig. 1A) displayed several interesting features as follows. 1) There was a single open reading frame. 2) The amino acid sequence used to design the primer was contained in the predicted peptide sequence. 3) A polyadenylation signal was found adjacent to the poly(A') tail. 4) The predicted polypeptide sequence of the cDNA contained an antigenic peptide sequence, CRPKAKAKAKADQ TK, with features associated with HP/HS-binding motifs (Cardin and Weintraub, 1989). Computer analysis predicted that this motif was α-helical and hydrophilic and likely to be exposed on the external surface of the protein where it may bind HP/HS. The sequence of this 270-bp RT-PCR product was compared with available sequences in GenBank. The highest similarities found by this analysis were 64% (in 265-bp overlap) similarity to Ratus norvegicus (rat) mRNA for ribosomal protein L29 and 77.6% identity in 157-amino acid overlap to R. norvegicus (rat) mRNA for ribosomal protein L29.

Isolation and Characterization of a cDNA Encoding HIP—A HeLa cell cDNA library was screened using the cDNA of HIP RT-PCR product (RT-PCR 224 depleted of poly(A') tail in Fig. 1A) as a probe. As a result, 20 positive clones with identical inserts to that of 23–1 (or 42–1) (Fig. 1A) were obtained from approximately 5 × 10⁵ plaque-forming units. The cDNA sequence of inserts of 23–1 and 42–1 was determined by primer walking. The determined cDNA sequence contains an incomplete open reading frame encoding 117 amino acid residues.

Northern Blot Analysis of RNA from RL95 Cells—The expression of HIP mRNA was examined by Northern blot analysis using a 32P-labeled clone 23–1. A single predominant transcript of 1.3 kb was detected either using total RNA or poly(A') mRNA. A BLAST homology search using GenBank revealed two human nucleotide sequences, designated as a putative human ribosomal protein L29, in GenBank (accession number U10248 and Z49148) showing the same nucleotide sequence as that of HIP cDNA; however, these sequences are not published and no further information is available. The predicted amino acid sequence of HIP has 80.5% identity in 549-bp overlap to a rat mRNA for ribosomal protein related to yeast ribosomal protein YL43.

The exons of the HIP cDNA were 145 bp, 48 bp, 62 bp, and 117 bp in length, containing 26 more bp of 5'-untranslated region than 35–2; 2) 35–2 contains an extra 6 bp of nucleotides (marked with brackets in Fig. 1B) at the 3' end of 3'-untranslated region; and 3) there is a nucleotide replacement (C replaced with T, marked with [ ] in Fig. 1B) at nucleotide position 145. This nucleotide replacement does not affect the coding of the amino acid, leucine. The nucleotide sequence of HIP cDNA including differences between different clones and predicted amino acid sequence of HIP are shown in Fig. 1B (accession number U10248).
the HIP peptide and to verify cell surface expression of HIP, clone 36–1 was subcloned into a Rous sarcoma virus-based mammalian expression vector and used to transfect NIH-3T3 cells. Transiently transfected NIH-3T3 cells were fixed with paraformaldehyde, and expression of HIP was detected using anti-HIP. Fig. 3 shows the immunostaining of a representative field of paraformaldehyde-fixed intact NIH-3T3 cells transiently transfected with HIP cDNA. Staining demonstrated cell surface expression of transfected HIP protein in a portion of transfected cells, whereas other cells that presumably were not transfected during the transient assay were negative. Negative staining also was observed using paraformaldehyde-fixed parental NIH-3T3 cells (data not shown). Similar controls as that presented in the accompanying paper (Rohde et al., 1996; Fig. 7) using antibodies directed against cytokeratins were done for the cell surface staining of the transiently HIP-transfected NIH-3T3 cells, and no reactivity was observed under the fixing conditions performed (data not shown). Therefore, the immunostaining of the transfected cells was cell surface staining. Western blot analysis of total protein extracted from transiently transfected NIH-3T3 cells using anti-HIP-peptide detected a newly expressed protein with an apparent M_\text{r} of 24,000, i.e., the same molecular weight as that of HIP detected in RL95 cells. In contrast, the M_\text{r} 24,000 component was not detectable in the parental NIH-3T3 cells (Fig. 4). Collectively, these data demonstrate that the isolated cDNA sequence encodes the protein recognized by the anti-HIP peptide antibody and that this protein can be expressed on cell surfaces.

Expression and Distribution of HIP—Expression and distribution of HIP in different human cell lines and normal tissues were examined using both Northern blot analysis and Western blot analysis. Northern blot analysis using the HIP cDNA probe detected a single transcript of 1.3 kb in most human cell lines tested. HIP is expressed highly in most human epithelial cell lines including RL95, JAR, HeLa, HEC, and Ishikawa, as well as AFb-11, human fibroblastic cells, moderately in HUVEC (endothelial cells), and relatively low in HL60, a human leukemic cell line. HIP mRNA was not detectable in MDA-231, a human breast cancer cell line, and NCI-H69, a human lung epithelial cell line, or mouse NIT-3T3 cells (Fig. 5). Western blot analyses on several human cell lines also revealed a similar distribution pattern of HIP as that shown in Northern blot analyses.
analyses (Fig. 6 and Table I). Again, consistent with Northern blot analysis, HIP was not detectable in cell lines of MDA-231 (Table I) and high (480 x) magnifications, respectively. In both A and B, arrows point to the HIP-transfected cells, and examples of cells not transfected during transient transfection and lacking staining are indicated with arrowheads.

**Fig. 3. Expression of HIP on cell surfaces of HIP cDNA-transfected NIH-3T3 cells.** NIH-3T3 cells were transiently transfected with HIP expression vector as described under “Experimental Procedures.” Transfected cells were grown on cover slips for 24 h and fixed with 2.5% (w/v) paraformaldehyde and immunostained with anti-HIP antibodies as described under “Experimental Procedures.” A and B are photographs taken at low (96 x) and high (480 x) magnifications, respectively. In both A and B, arrows point to the HIP-transfected cells, and examples of cells not transfected during transient transfection and lacking staining are indicated with arrowheads.

**Discussion**

In the present study, we have isolated and sequenced a full-length cDNA encoding HIP, a novel human HP/HS-binding protein expressed on cell surfaces. This cDNA encodes a protein of 159 amino acids with high content of basic amino acids. There is no a potential transmembrane domain present in the predicted amino acid sequence of HIP. HIP is associated with the cell surface (Rohde et al., 1996 and present article). Therefore, it is likely that HIP is a peripheral membrane protein, perhaps bound to other proteins, lipids, or polysaccharides. The predicted amino acid sequence of HIP does not contain a classical hydrophobic amino-terminal signal peptide (Blobel and Dobberstein, 1975); however, there are multiple reports of the lack of a signal peptide in the sequences of membrane or secreted proteins (Kikutani et al., 1986; Bettler et al., 1989; Brown et al., 1987). The predicted protein sequence of HIP predicts a molecular mass of 17,754 Da. This is significantly less than expected for M, 24,000 protein recognized by anti-HIP-peptide on SDS-PAGE. The anomalous molecular mass may be due to the highly basic character of HIP (predicted pI = 11.75). Other highly basic proteins, e.g. histones, migrate relatively slowly on SDS-PAGE (von H perpetrators et al., 1989; Weber and Osborn, 1975), apparently due to an inordinately high amount of SDS binding. Alternatively, post-translational modifications may increase the size of HIP. No consensus sites for glycosylation are evident in this sequence, but other modifications are possible. Transfection of full-length cDNA of HIP into NIH-3T3 cells resulted in the expression of a protein with M, 24,000 determined by SDS-PAGE, further demonstrating that the cloned cDNA sequence encodes the same protein recognized by the antibody and contains the predicted peptide sequence. Northern blot and Western blot analyses revealed that both the 1.3-kb mRNA and HIP, 24,000 protein are expressed coordinately in a variety of human cell lines. Differential expression of HIP protein also has been observed in normal human tissues examined (Rohde et al., 1996).
eral lines of evidence indicate that HIP does not function sim-
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genes or pseudogenes (Rudert et al., 1993). It is not clear what
functions, if any, these sequences serve in rodents. Several
lines of evidence indicate that HIP does not function simply
as a ribosomal protein. First, HIP can be detected on cell
surfaces of cells transfected with HIP cDNA or RL95 cells
(Rohde et al., 1996). Second, HIP is expressed in a nonconsti-
tutive fashion in different human cell lines and normal tissues.
Constituent ribosomal proteins would be expected to be ex-
pressed at stoichiometric levels in different cells and with re-
spect to the cellular content of rRNA species. While there is
precedent for limited modulation of ribosomal proteins in some
cases (Nomura et al., 1982; Rudert et al., 1993), these proteins
are never essentially absent as in the case for both HIP mRNA
and protein in cells like MDA-231 and NCI-H69. Collectively,
these data strongly argue that HIP is not critical to ribosomal
function.

Cell surface localization (Rohde et al., 1996) and HP/HS-
binding activity suggest that HIP may play a role in HP/HS-
involved cell-cell or cell-matrix interactions or have other func-
tions yet to be determined. In the studies of rodent L29, the
identification and localization of this protein was based on
sequence homology analysis and standard procedures of ribo-
smal protein isolation (Ostvold et al., 1992; Svoboda et al.,
1992; Rudert et al., 1993). The distribution of the protein was
only examined in one study by Northern blot analysis and in
situ hybridization (Rudert et al., 1993). No studies of the ex-
pression of the L29 protein are reported. Thus, it is of interest
to re-examine the expression of rodent L29 considering the
possibility that it may not be a “housekeeping” protein. Con-
sidering the existence of the high number of sequences closely
related to L29 (Rudert et al., 1993), it will be important to use
probes specific for each gene in such studies.

HIP may be expressed both at cell surfaces and intracellu-
larly. Several reports indicated that some proteins are present
both at cell surfaces or secreted as well as inside the cell
(Terada et al., 1995). These examples include certain growth
factors (Abraham et al., 1986; Jaye et al., 1986), cytokines
(Matsushima et al., 1986), and lectins (Cooper and Barondes,
1990). Why these proteins are expressed in both locales is
unclear. In the present case, it is not known if intracellular HIP
is contained within vesicles or organelles or in the cytoplasm.
The following paper (Rohde et al., 1996) demonstrates that
almost all of the cell-associated HIP is formed in a 100,000 \times g
sedimentable fraction and, therefore, is not present in a freely
soluble form. Several mechanisms for sorting of cytoplasmic
and secreted proteins have been postulated, including that cell
lysis, death, or leakage might be responsible for the release
of these proteins (D’Amore, 1990) or that the release might
be induced by plasma membrane evaginations (Cooper and
Barondes, 1990).

Previous studies in our laboratory have suggested that
HSPGs and their corresponding binding sites may play an
important role in the initial attachment of mouse embryo to
uterine epithelium. In the present study, a novel cell surface
HP/HS-binding protein from a human uterine epithelial cell
line has been cloned and expressed. The accompanying article
(Rohde et al., 1996) describes expression of this protein in
normal human lumenal epithelium, a location where HIP could
participate in embryo attachment. Rigorous examination of a
role for HIP in human embryo attachment is not possible;
however, such studies can readily be performed in rodents.
Therefore, it should be possible to identify the murine func-
tional homologue of HIP and study the expression and physio-
logical functions of this protein in the mouse in order to test its
potential role in embryo implantation.

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helpful suggestions in selecting predicted peptide sequence for antibody
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assistance in preparation of this manuscript.

### Table I: HIP Expression in Human Cell Lines

| Cell type       | Cell line          | Protein | RNA |
|-----------------|--------------------|---------|-----|
| Neural          | Neuroblastoma      | ++      | ND  |
| Glioblastoma    | ++                 | ND      |     |
| U251            | ++                 | ND      |     |
| U343            | (+)                | ND      |     |
| Muscular        | Rhabdomyosarcoma   | ++      | ND  |
| Epithelial      | (+)                | ND      |     |
| Choriocarcinoma | (+)                | ND      |     |
| Teratoma 9113  | (+)                | ND      |     |
| AR               | (+)                | ND      |     |
| JEG              | (+)                | ND      |     |
| Belo              | (+)                | ND      |     |
| Uterine         | RL95               | (+)     | +   |
| HEC              | (+)                | ND      |     |
| Ishikawa        | (+)                | ND      |     |
| Breast          | MCF-7              | ++      | ND  |
| MDA-231         | ++                 | ND      |     |
| ZR75-1          | ++                 | ND      |     |
| Prostate        | A513       | ++      | ND  |
| Intestine       | +/-                | ND      |     |
| Cervical        | HeLa              | ++      | ND  |
| Ovary           | 2774              | +/-     | ND  |
| Lung            | NCI-H69           | –       | –   |
| Endothelial     | HUVEC             | +/-     | –   |
| Fibroblast      | AFb-11            | +/-     | –   |
| Lymphoid        | HL60              | +/-     | –   |
| Embryonic       | Terato 9117        | ++      | ND  |
| Terato 9113-Clone 1 | ++      | ND      |     |
| Terato 9113-Clone 6 | (+)    | ND      |     |

Sequence comparison with available data bases revealed that
HIP has a relative high similarity (80%) to rodent L29, a
ribosomal protein, at both the nucleotide and protein sequence
level. It is possible that HIP is the human homologue of rodent
L29. It is noteworthy that in the mouse, L29 is a member of
15-18 genes or pseudogenes (Rudert et al., 1993). It is not clear
what functions, if any, these sequences serve in rodents. Several
lines of evidence indicate that HIP does not function simply
as a ribosomal protein. First, HIP can be detected on cell
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normal human lumenal epithelium, a location where HIP could
participate in embryo attachment. Rigorous examination of a
role for HIP in human embryo attachment is not possible;
however, such studies can readily be performed in rodents.
Therefore, it should be possible to identify the murine func-
tional homologue of HIP and study the expression and physio-
logical functions of this protein in the mouse in order to test its
potential role in embryo implantation.

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