Human Hand1 basic helix-loop-helix (bHLH) protein: extra-embryonic expression pattern, interaction partners and identification of its transcriptional repressor domains

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The basic helix-loop-helix (bHLH) transcription factor, Hand1, plays an important role in the development of the murine extra-embryonic trophoblast cell lineage. In the present study, we have analysed the expression of Hand1 in human extra-embryonic cell types and determined its binding specificity and transcriptional activity upon interaction with different class A bHLH factors. Northern blotting and *in situ* hybridization showed that Hand1 mRNA is specifically expressed in amnion cells at different stages of gestation. Accordingly, we demonstrate that the protein is exclusively produced in the amniotic epithelium *in vivo* and in purified amnion cells *in vitro* using a novel polyclonal Hand1 antiserum. Reverse transcriptase-PCR and immunohistochemical staining of blastocysts revealed the production of Hand1 mRNA and polypeptide in the trophectodermal cell layer. In the presence of E12/E47, Hand1 stimulated the transcription of luciferase reporters harbouring degenerate E-boxes, suggesting that E-proteins are potential dimerization partners in trophoblastic tumour and amnion cells. In contrast, Hand1 diminished E12/E47-dependent transcription of reporters containing perfect E-boxes by inhibiting the interaction of Hand1/E-protein heterodimers with the palindromic cognate sequence. Furthermore, we show that Hand1 down-regulated GAL–E12-dependent reporter expression, indicating that the protein can also act directly as a transcriptional repressor. Mutational analyses of GAL-Hand1 suggested that two protein regions located within its N-terminal portion mainly confer the repressing activity. In conclusion, human Hand1 may play an important role in the differentiation of the amniotic membrane and the pre-implanting trophoblast. Furthermore, the data suggest that Hand1 can act as a repressor by two independent mechanisms; sequestration of class A bHLH factors from E-boxes and inhibition of their transcriptional activity.

Key words: amniotic expression, E-protein dimerization, D-box interaction, repressor activity.

INTRODUCTION

Class B basic helix-loop-helix (bHLH) proteins, which exhibit a restricted expression pattern, play a major role in tissue-specific gene expression, cell lineage determination and differentiation. The transcription factors regulate diverse developmental processes, such as myogenesis, neurogenesis, haematopoesis, pancreas formation and cardiac differentiation [1–5]. Commonly, class B factors heterodimerize with family members of widely expressed class A bHLH proteins, e.g. E12/E47, HEB, or ITF-2 [6–8]. Interaction of their HLH regions allows the formation of a combined DNA-binding domain, which recognizes the palindromic sequence, CANNTG, termed the E-box [9]. Class A/B heterodimers as well as class A homodimers are thought to function as transcriptional activators [10], whereas other groups of bHLH factors act as repressors of bHLH function and promoter activity. The Id proteins, which lack the basic domain, form inactive complexes with both class A and B bHLH factors and prevent differentiation [11,12]. Members of the hairy and Enhancer-of-split proteins [13] inhibit transcription by interacting with N-boxes (CACNAG), a variant of the canonical E-box sequence motif [14].

Recent studies in mice [15–24] have demonstrated that tissues-specific bHLH factors are crucially involved in the differentiation of the placenta, an organ that is critical for growth and intrauterine survival of the embryo. In particular, bHLH proteins are required for the development of trophoblasts. These cells form the outermost cell layer of pre-implanting embryos, trigger implantation and finally build the different, specialized epithelial structures of the placenta [15,16]. Mash-2, a mammalian member of the Achaete–Scute family, which has virtually no function in embryonic development, is essential for survival and/or proliferation of spongiotrophoblasts, the late precursor cells of secondary giant cells [17]. On the other hand, differentiation of giant cells, which face the maternal decidua and form polyploid nuclei by endoreduplication, is promoted by the bHLH proteins I-mfa and Hand1 [18–20].

Murine Hand1, formerly known as eHand, Hxt or Th1, is expressed in developing heart tissue and derivatives of neural crest cells [21,22]. Extra-embryonic mRNA production was detected in the ectoplacental cone, in giant cells as well as in distinct regions of the spongiotrophoblast cell layer overlapping Mash-2 expression [19,22,23]. Mice lacking Hand1 arrest at E7.5 with severe abnormalities in differentiation of trophoblast giant cells, but also show failures in cardiac differentiation upon rescue of the placental defect [20,24]. *In vitro*, Hand1 promotes differentiation of Rcho-1 trophoblasts into giant cells, whereas Mash-2 and Id-1 inhibit this process [19]. Hand1 can compete for

dimerization partners, such as the E-proteins, which may in-activate Mash-2 function, but DNA-binding of the protein is also essential for giant cell formation *in vitro* [23].

In humans, an analogous set of tissue-specific bHLH proteins could be involved in the regulation of placental and trophoblast development. These factors are of particular interest, since failures in trophoblast differentiation are associated with gesta-tional diseases, such as pre-eclampsia or intrauterine growth retardation [25,26]. Human placenta, however, differ consider-ably from murine placenta with respect to morphology, cell types and differentiation pathways. Thus expression and function of class B bHLH proteins have to be studied in the appropriate human tissues and culture systems. The expression of the human Mash-2 homologue (Hash-2) and Id proteins, such as Id-2, which may influence cell migration/invasion of the primary trophoblasts, has been demonstrated [27–29].

To investigate the role of Hand1 in the differentiation of human extra-embryonic tissues, we have recently cloned the human Hand1 cDNA and gene [30,31]. Furthermore, we have shown that Hand1 is expressed in adult cardiac tissues and trophoblastic tumour cells, but absent from placentae of different gestational stages and purified trophoblast cultures, questioning its role in human trophoblast differentiation [30]. In the present study, we provide a detailed study of the expression pattern of human Hand1 in diverse extra-embryonic tissues. Furthermore, we demonstrate the production of potential dimerization partners in Hand1-expressing cells and show their interaction with Hand1 in a binding site-dependent and -independent manner. Using mutated GAL–Hand1 polypeptides in interaction/transcription assays, we have identified novel protein domains that are responsible for the transcriptional repressor activity of the bHLH factor.

MATERIALS AND METHODS

Oligonucleotide sequences

Oligonucleotide sequences utilized for plasmid construction, reverse transcriptase (RT)-PCR reactions and electrophoretic mobility-shift assays (EMSAs) are shown in Table 1.

Construction of plasmids

The coding sequence of Hand1, lacking 72 out of the 215 amino acid residues at its N-terminal end (Hand1NΔ72), was PCR-amplified from the full-length cDNA clone [30] using the primers Hand1-NΔ72S and Hand1-WTA1. Conditions for PCR (50 μl) containing Hand1 sequences [20 pmol of each primer, 0.02 unit/μl *Taq* DNA polymerase (Gibco BRL, Rockville, MD, U.S.A.) and 0.008 unit/μl *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA, U.S.A.)] were 30 s at 96 °C, 30 s at 58 °C and 45 s at 72 °C for 35 cycles. The PCR product was digested with *Bam*HI and subcloned into pQE30 (Qiagen, Hilden, Germany) for production of a His-tagged protein.

For construction of the binding-site-dependent reporters, the complementary oligonucleotides Th1Dbox-S and Th1Dbox-A [harbouring three copies of the degenerate E-box (Th1 D-box)] were annealed and subcloned into the pGL3 promoter vector [harbouring the proximal SV-40 promoter region (Promega, Madison, WI, U.S.A.), using *Sac*I/*Bgl*II restriction sites. Flank-ing regions were chosen as described previously [22]. Luciferase reporters containing palindromic E-boxes were constructed after annealing of the complementary sequences of Th1Ebox-S and Th1Ebox-A.

For expression of the 215-amino acid open reading frame (ORF) of Hand1 as a GAL4 fusion protein, PCR of cDNA was performed in the presence of Hand1-WTS1 and Hand1-WTA2. The PCR fragment was digested with *Bam*HI and subcloned into pMGAL4 (Clontech, Palo Alto, CA, U.S.A.). Deletion of C-terminal sequences was performed by PCR using Hand1-WTS2 and the antisense oligonucleotides Hand1-CΔ28, Hand1-CΔ65, Hand1-CΔ110 and Hand1-CΔ123 respectively. PCR-mediated deletions of N-terminal sequences were carried out with combina-tions of Hand1-WTA2 and Hand1-NΔ21, Hand1-NΔ48, Hand1-NΔ78, Hand1-NΔ93 and Hand1-NΔ105 respectively. Internal deletions of the basic domain (Δb), HLH (ΔHLH), bHLH region (ΔbHLH), residues 39–45 (Δ39–45), 48–78 (Δ48–78) and 79–93 (Δ79–93) were performed by a two-step PCR approach. The first cycles were performed in the presence of Hand1-WTA2 and the sense oligonucleotides Δb, ΔHLH, ΔbHLH and (Δ39–45) re-spectively. Subsequently, DNA products were eluted from gels and the PCR was repeated in the presence of 1 μg of the purified fragments and 20 pmol Hand1-WTS2 respectively. Alternatively, the first step of the PCR was performed in the presence of Hand1-WTS2 and the antisense oligonucleotides Δ48–78 or Δ79–93 respectively, and the second PCR was carried out with the purified PCR fragment and Hand1-WTA2. All constructs were digested with *Eco*RI and ligated into pM-GAL4 in-frame to the GAL4 DNA-binding domain, or into pCIneo (Promega) for untagged gene expression. Similarly, the pMGAL4-E12 construct was obtained after PCR of the E12 ORF and *Eco*RI cloning using E12ORF-S and E12ORF-A. GAL-HEB was ligated into pMGAL4 after PCR of HEB cDNA with HEBORF-S and HEBORF-A and restriction digestion with *Bam*HI. All PCR fragments were sequence verified using the non-radioactive ABI PRISM® Terminator Cycle Sequencing Ready Reaction Kit according to the manufacturer's instructions (Applied Biosys-tems, Foster City, CA, U.S.A.). Additionally, cDNAs encoding HEB, ITF-2, E12 and E47, obtained from Dr R. Kingston (Department of Genetics, Howard Hughes Medical Institute, Pennsylvania, PA, U.S.A.), Dr T. Kadesch (Department of Molecular Biology, Massachusetts General Hospital, Boston, MA, U.S.A) and Dr C. Murre (Department of Biology, Uni-versity of California, San Diego, CA, U.S.A.) respectively, were subcloned into pCIneo.

Cell culture and preparation of tissues

JEG-3 and JAR trophoblastic tumour cells were grown in Dulbecco's modified Eagle's medium and RPMI 1640 respect-ively, containing 10 % (v/v) foetal-calf serum and antibiotics [30]. The amnion tumour cells, WISH (CCL-25; A.T.C.C., Manassas, VA, U. S.A) and FL (CCL-62; A.T.C.C.), were cultured in Dulbecco's modified Eagle's medium containing 10 % (v/v) foetal-calf serum. Foetal membranes (week 11 of pregnancy) were obtained from legal abortions and snap frozen. Epithelia from amnion and chorion were mechanically separated after term delivery (week 40 of pregnancy) and either frozen in liquid N$_2$ for RNA preparation or embedded in paraffin for immunohistochemistry or *in situ* hybridization. Preparation of amnion cells was performed by digestion of the amniotic epi-thelium with trypsin as described previously [32]. Amnion cells were 100 % cytokeratin 7-positive and 95 % vimentin-positive, suggesting purification to homogeneity (results not shown). Pure villous trophoblasts were prepared by the method of Kliman [33] and differentiated *in vitro* as described previously [33,34]. All experiments with human embryos were performed at the School of Biological Sciences, University of Manchester, U.K. Material was obtained from patients of an *in vitro* fertilization programme at St Mary's Hospital, Manchester, U.K. Research was carried out with the permission of the local ethical committee, and in

Table 1   Oligonucleotide sequences used for construction of plasmids, RT-PCR reactions and EMSA

In the Th1Dbox and Th1Ebox sequences, bold letters indicate palindromic E-boxes or D-boxes, underlined sequences depict flanking regions of the binding elements, and linker sequences (GC) are presented in italic letters. In the Th1Dbox-A and Th1Ebox-A sequences, nucleotides that are not underlined at the 5′ and 3′ ends respectively are the overhangs/restriction sites for *Sac*I/*Bgl*II cloning. S, sense; A, antisense.

Oligonucleotides	Sequences
Hand1-WTS1	5′-GGCGGATCCACATGAACCTCGTGGGCAGCTACGCA-3′
Hand1-WTS2	5′-CGGAATTCACCATGAACCTCGTGGGCAGCTACGCA-3′
Hand1-WTA1	5′-GCGGGATCCATGCATTTATCACTGGTTTAACTCCAGCGC-3′
Hand1-WTA2	5′-GCGGGATCCATGCATTTATCACTGGTTTAACTCCAGCGCCC-3′
Hand1-NΔ21	5′-CGGAATTCACCATGCTCCACGAACCCTTCCTC-3′
Hand1-NΔ48	5′-CGGAATTCACCATGCCGGCTGACGCTGCCCCGGAC-3′
Hand1-NΔ72S	5′-GGCGGATCCACCATGTATGGTCCTGACGCCAGGCCTG-3′
Hand1-NΔ78	5′-CGGAATTCACCATGGGGCAGAGCCCCGGGCGGCTG-3′
Hand1-NΔ93	5′-CGGAATTCACCATGCGGCGGAAAGGCTCAGGACCC-3′
Hand1-NΔ105	5′-CGGAAT TCACCATGCGCACTGAGAGCATTAACAGC-3′
Hand1-CΔ28	5′-GCGAATTCTTATCAAAAACCTTCGTGCTGCTGCAG-3′
Hand1-CΔ65	5′-GCGAATTCTTATCAGGCCAGCACGTCCATCAGGTAG-3′
Hand1-CΔ110	5′-GCGAATTCTTATCAGCGTCTCCGCTCCTTCTTGGG-3′
Hand1-CΔ123	5′-GCGAATTCTTATCAGCCAAGA CGGCCGCCAAGCGC-3′
Δb	5′-GCGCTTGGCGGCCGTCTTGGCCGCACTGAGAGCATTAACAGC-3′
ΔHLH	5′-GGACCCAAGAAGGAGCGGGAGACGCGCCAAGGATGCACAGTCTGC-3′
ΔbHLH	5′-GCGCTTGGCGGCCGTCTTGGCGCGAAGGATGCACAGTCTGGC-3′
Δ39–45	5′-GCCTCGCGCTGTCATCAGGAACTGCTGAGCCCGGCTGACGCTGCC-3′
Δ48–78	5′-CCAGCCGCCCGGGGCTCTGCCCCAGCAGCCAGCTCTGGAAGTA-3′
Δ79–93	5′-GGGTCCTGAGCCTTTCCGCCGAGGCCTGGCGTCAGGACCATAGGC-3′
E12ORF-S	5′-GCGAATTCCGCATGGCGCCTGTGGGCACAGAC-3′
E12ORF-A	5′-GCGAATTCTTATCATGTGCCCGGCGGGGTTG-3′
HEBORF-S	5′-GGCGGATCCACCGCATGAATCCCCAGCAACAACGC-3′
HEBORF-A	5′-GCGGGATCCTTATCACATATGACCCATAGGGTAGGTAG-3′
Hash2-S	5′-TCGCAGATTTCGCCAGTTG-3′
Hash2-A	5′-TCTCACGCCCTCCTTCCTTC-3′
HEB-S	5′-ACTGAAAACAAAGAAAAGGATGAAAACC-3′
HEB-A	5′-CCCTTTCTATCTTCTGTTCAGGGTTC-3′
ITF2-S	5′-GGCAACCCAGGAACCCTTTC-3′
ITF2-A	5′-TGAAGATGGCAAACCTGGGG-3′
E12-S	5′-ACCAGCCCAGACGAGGACGAGGACGACC-3′
E12-A	5′-GGGCTTCTCGCTGTTGAGGTGCAG-3′
E47-S	5′-AGCAGTACGGACGAGGTGCTGTCCCTGG-3′
E47-A	5′-CGCTTTGTCCGACTTGAGGTGCAT-3′
Hand1-S	5′-TGCCTGAGAAAGAGAACCAG-3′
Hand1-A	5′-ATGGCAGGATGAACAAACAC-3′
βactin-S	5′-GACAGCAGTCGGTTGGAGC-3′
βactin-A	5′-CAGGTAAGCCCTGGCTGC-3′
Th1Dbox-S	5′-C<u>ATTGG</u>**CGTCTG**<u>GCATT</u>*GC*<u>ATTGG</u>**CGTCTG**<u>GCATT</u>*GC*<u>ATTGG</u>**CGTCTG**<u>GCATTA</u>-3′
Th1Dbox-S2	5′-<u>ATTGG</u>**CGTCTG**<u>GCATT</u>*GC*<u>ATTGG</u>**CGTCTG**<u>GCATT</u>*GC*<u>ATTGG</u>**CGTCTG**<u>GCATT</u>-3′
Th1Dbox-A	5′-GATCT<u>AATGC</u>**CAGACG**<u>CCAAT</u>*GC*<u>AATGC</u>**CAGACG**<u>CCAAT</u>*GC*<u>AATGC</u>**CAGACG**<u>CCAAT</u>GAGCT-3′
Th1Ebox-S	5′-C<u>ATTGG</u>**CATCTG**<u>GCATT</u>*GC*<u>ATTGG</u>**CATCTG**<u>GCATT</u>*GC*<u>ATTGG</u>**CATCTG**<u>GCATTA</u>-3′
Th1Ebox-S2	5′-<u>ATTGG</u>**CATCTG**<u>GCATT</u>*GC*<u>ATTGG</u>**CATCTG**<u>GCATT</u>*GC*<u>ATTGG</u>**CATCTG**<u>GCATT</u>-3′
Th1Ebox-A	5′-GATCT<u>AATGC</u>**CAGATG**<u>CCAAT</u>*GC*<u>AATGC</u>**CAGATG**<u>CCAAT</u>*GC*<u>AATGC</u>**CAGATG**<u>CCAAT</u>GAGCT-3′

accordance with the licence conditions of the Human Fertilization and Embryology Authority, U.K. Thawing, culturing to different cellular stages and lysis of embryos were performed as described previously [35]. Synthesis, amplification and normalization of cDNA libraries prepared from single oocytes or groups of 2–3 embryos were carried out as described previously [35,36].

Northern-blot analysis

Polyadenylated [poly(A)+] RNA was isolated from cultured cells or tissues according to the manufacturer's instructions (Dyna-beads mRNA DIRECT™ Kit, Dynal, Oslo, Norway). mRNA (2–3 μg) was glyoxylated, separated by electrophoresis on agarose gels, transferred on to nylon membranes and hybridized with a radiolabelled Hand1-specific cDNA fragment as previously described [30]. To control RNA loading, filters were stained with Methylene Blue prior to hybridization and photographed.

In situ hybridization

The Hand1 full-length cDNA was subcloned into pGEM-3 and pCR2.1 using *Eco*RI and linearized with *Hin*dIII respectively, for the production of sense and antisense mRNAs. *In vitro* transcription and digoxigenin (DIG) labelling were performed using 2 units of T7 RNA polymerase (Promega) and DIG RNA Labeling Mix according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). RNAse-free sections (3 μm) of paraffin-embedded foetal membranes (week 40 of pregnancy) were hybridized with the DIG-labelled probes for 16 h at 65 °C. Stringent washes of slides (55 °C), incubation with an anti-DIG alkaline phosphatase-conjugated antibody and detection with Nitro Blue Tetrazolium ('NBT') and 5-bromo-4-chloroindol-3-yl phosphate ('BCIP') were performed as described previously [37]. Slides were mounted and photographs were taken on Ektachrome 100HC film (Kodak, Vienna, Austria).
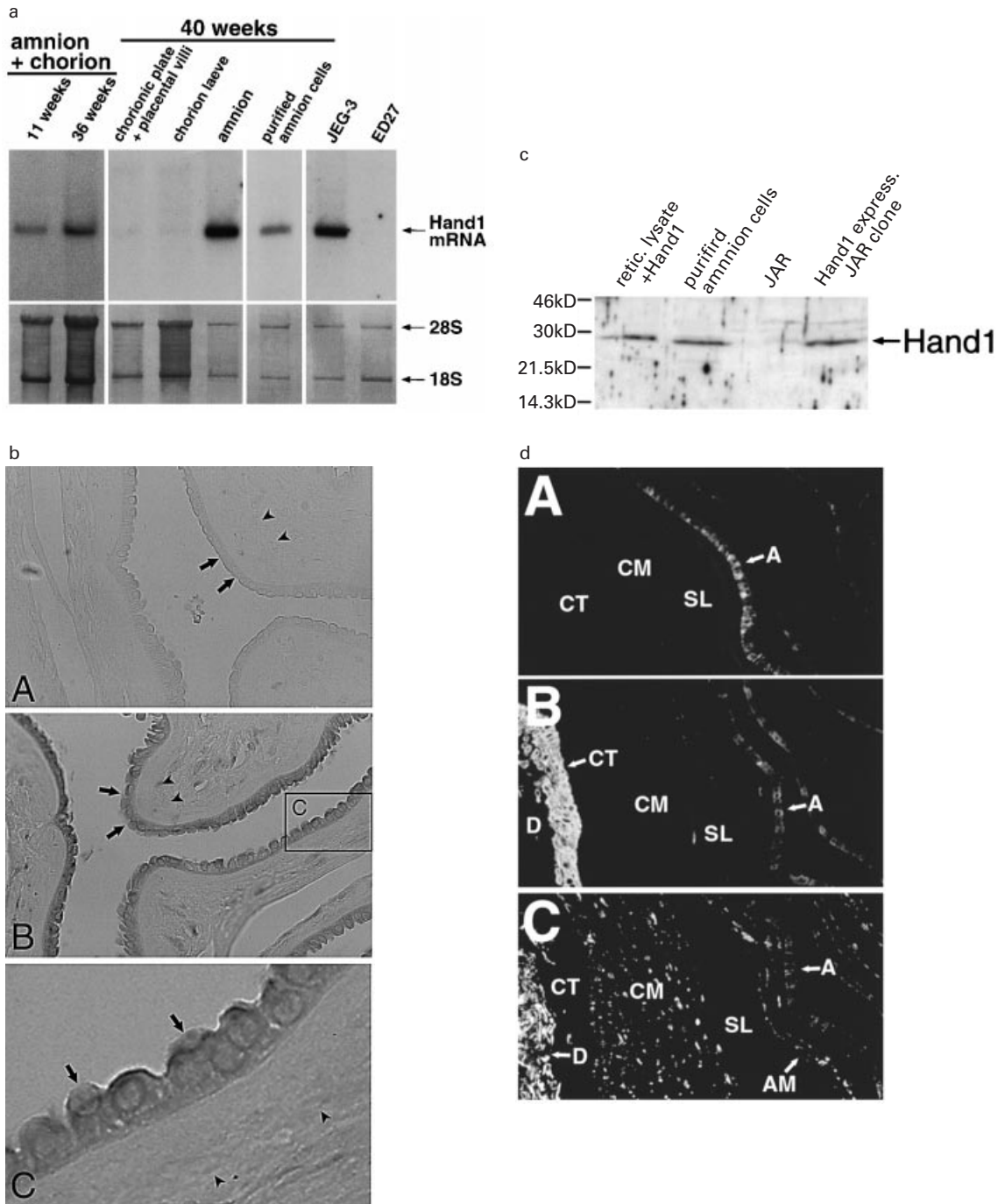
Figure 1   Expression pattern of Hand1 mRNA and protein in extra-embryonic tissues during human gestation

(**a**) Northern-blot analysis of Hand1 mRNA expression. Poly(A)[+] RNA was prepared from different tissues and cells, separated on gels and hybridized with a Hand1-specific radiolabelled cDNA probe as described in the Materials and methods section. JEG-3 choriocarcinoma cells and ED27, a placental cytotrophoblast cell line [50], represent positive and negative controls respectively. Methylene Blue staining of the filter (bottom) demonstrates the loading of RNA. Residual amounts of rRNAs (18S and 28S), left in the mRNA preparation and not completely removed during the poly(A)[+] selection process, are indicated by the arrows. (**b**) *In situ* hybridization of foetal membranes at week 40 of gestation. DIG-labelled probes were synthesized and hybridized to tissues as described in the Materials and methods section. Photomicrographs showing Hand1 signals (indicated by arrows) were taken (magnification × 200). The Hand1-negative amniotic mesenchyme is marked by arrowheads. (A) Hand1 sense probe. (B) Hand1 antisense probe. (C) Higher magnification (× 1000) of the section in (B) showing Hand1-specific signals in the amnion epithelium. (**c**) Western-blot analysis of Hand1 protein expression. Total cell extracts were isolated from purified amnion cells, JAR cells and a JAR clone constitutively overexpressing Hand1 (Hand1 express. JAR clone). After separation on polyacrylamide gels and transfer on to filters, immunodetection using the anti-Hand1 sera was performed. As a control, 1 μl of reticulocyte lysate (retic. lysate) containing Hand1 was utilized. The specific 29 kDa band is indicated. Molecular-mass markers (in kDa) are indicated on the left. (**d**) Immunohistochemical analysis of Hand1 in foetal membranes (week 40 of pregnancy). Serial sections of foetal membranes were immunostained using antibodies raised against Hand1 (A), cytokeratin 7 (B) and vimentin (C), analysed by fluorescence microscopy and photographed (magnification × 200). (A) Detection of Hand1 protein expression is restricted to the amnion epithelium. (B) Cytokeratin 7 specifically staining amnion and trophoblast epithelium. (C) Vimentin reacting with all fibroblast cell types. Labelled structures are: amnion epithelium (A), spongy layer (SL), amniotic mesenchyme (AM), chorionic mesenchyme (CM), chorion trophoblast (CT) and maternal decidua (D).
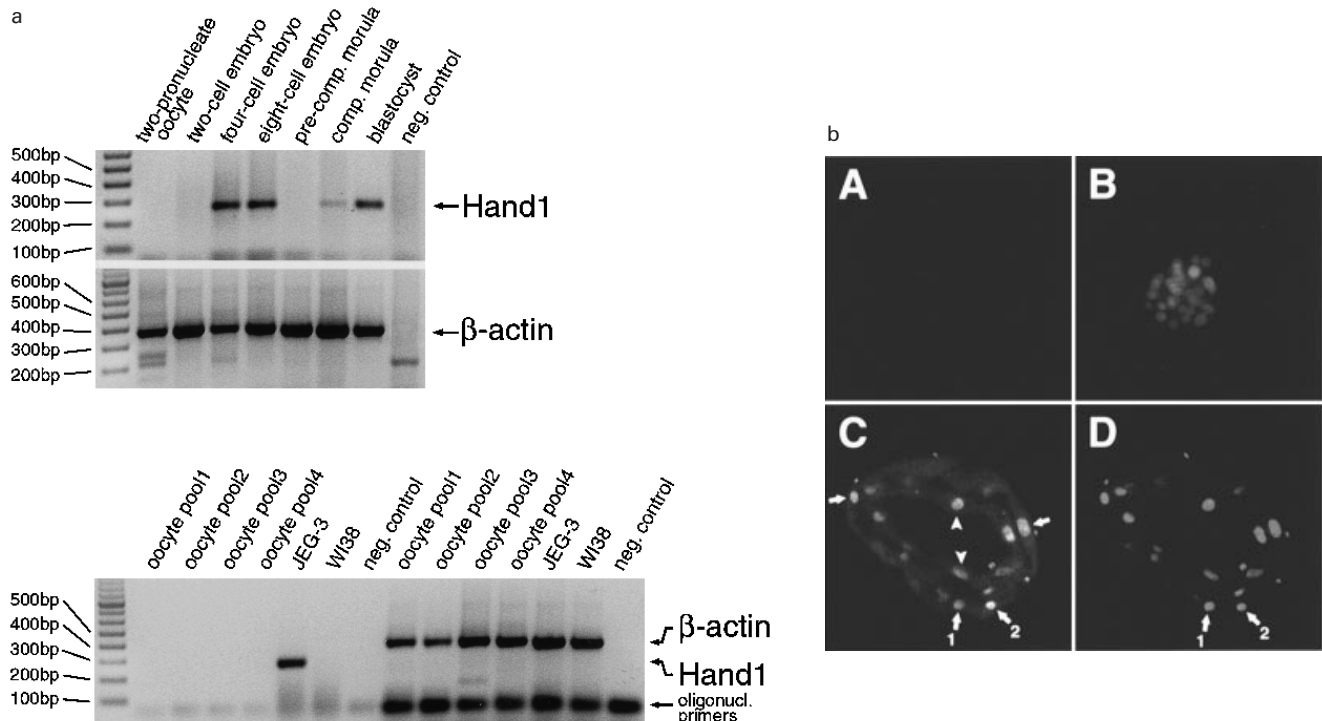
Figure 2 Hand1 expression pattern in human pre-implantation embryos

(**a**) Qualitative PCR detection of Hand1 mRNA in embryos and oocytes. The cDNA libraries, prepared from 2–3 pooled embryos at different stages and pooled oocytes [35], were amplified in the presence of Hand1-specific primers as described in the Materials and methods section. In parallel, PCR using β-actin-specific primers was performed to control for the abundancy and quality of different cDNA pools. The Hand1 (273 bp) and β-actin signals (398 bp) are indicated. JEG-3 and WI38 (human fibroblasts) represent Hand1 positive and negative cDNA controls respectively. To evaluate the specificity of the bands, RT was omitted in PCR amplifications of blastocysts (neg. control, negative control) as well as in the PCR reactions of JEG-3 cDNA and 4–8-cell cDNA pools (results not shown). A similar developmental expression pattern of Hand1 mRNA was observed in another set of human embryo cDNAs (results not shown). Comp. morula, compacted morula; pre-comp. morula, pre-compacted morula; oligonucl. primers, oligonucleotide primers. Sizes of the DNA marker bands (in bp) are indicated on the left. (**b**) Localization of Hand1 protein in human blastocysts. Embryos at the blastocyst stage were fixed, incubated in the presence of the affinity purified Hand1 antibody, visualized by immunofluorescent staining, analysed by confocal laser scanning microscopy and photographed. (A) Preimmune serum (negative control). (B) Nuclear staining of (A) using DAPI. (C) Hand1 localization in the nuclei and cytoplasm of the trophectoderm and cells of the ICM (magnification of a single confocal section × 650). (D) Nuclear staining of (C) using DAPI. Arrows mark the nuclei of trophectodermal cells, whereas arrowheads depict nuclei of the ICM. Differences in Hand1 expression between nuclei were noticed (compare 1 and 2 in C and D).

Semi-quantitative analysis of mRNA expression

Poly(A)$^+$ RNA (2 μg in a 20 μl final volume) was reverse transcribed after the addition of 500 ng of a hexanucleotide mix (Roche) and 10 units/μl of Superscript RT as described by the manufacturer (Gibco BRL). PCR (1 μl of RT reaction, 27 cycles) was performed as described above. Sequence-specific primers (Table 1) and optimized annealing temperatures were used to detect specific cDNA fragments of Hash-2 (160 bp, 52 °C), HEB (165 bp, 50 °C), ITF-2 (149 bp, 52 °C), E12 (173 bp, 58 °C), E47 (162 bp, 58 °C), Hand1 (273 bp, 64 °C) and β-actin (398 bp, 64 °C). Qualitative Hand1 mRNA expression was analysed by PCR (40 cycles) with 2 μl of embryonic/oocyte cDNA pools, using the primers Hand1-S and Hand1-A as described above. Fragments were separated on 3 % (w/v) agarose gels, visualized with ethidium bromide and photographed.

In vitro translation

In vitro transcription/translation was performed using TNT® Coupled Reticulocyte Lysate Systems according to the manufacturer's instructions (Promega). HEB cDNA in pBSATG [7] and Hand1 in pGEM-3 were transcribed using T3 and T7 RNA polymerase respectively. Transcripts of E12 and E47 in pSP64 [9] were obtained after incubation with SP6 RNA polymerase. The quality and quantity of *in vitro* translated proteins was assessed by SDS/PAGE on 15 % (w/v) gels after the incorporation of 40 μCi of [^{35}S]methionine (Amersham Biosciences, Little Chalfont, Bucks., U.K.).

EMSA

Oligonucleotides harbouring D- (Th1Dbox-S2) or E-boxes (Th1Ebox-S2) were ^{32}P-labelled and annealed with their complementary sequences. Binding reactions (20 μl) were carried out for 30 min at 20 °C in a mixture containing 1–2 μl of reticulocyte lysate, 12 mM Hepes (pH 7.9), 1 mM dithiothreitol, 100 mM KCl, 20 μg/ml BSA, 1 μg of poly[d(I-C)], 12 % (v/v) glycerol, 10 μM ZnCl$_2$ and 0.1 pmol ^{32}P-labelled double-stranded oligonucleotide. Annealing/labelling of oligonucleotides and separation of DNA-binding complexes on 5 % (w/v) polyacrylamide-Tris/borate/EDTA gels were performed as described previously [34].

Production of an immunopurified Hand1 antibody

After transformation of pQE30-Hand1NΔ72 in *Escherichia coli* M15[pREP4], induction in the presence of isopropyl β-D-thiogalactoside ('IPTG') as well as purification of the fusion protein by Ni^{2+}-nitrilotriacetate chromatography were performed using
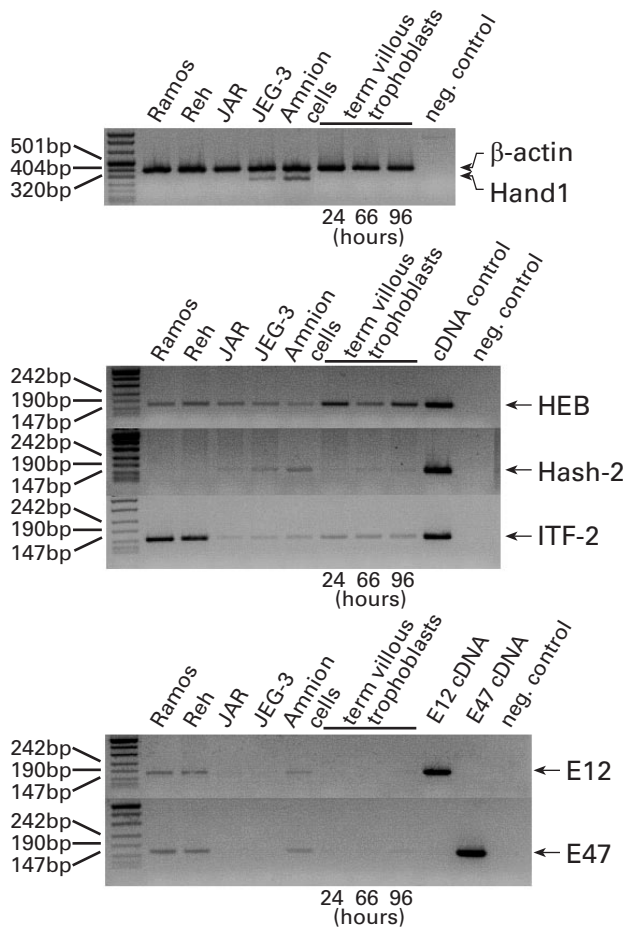
Figure 3   Semi-quantitative-expression analysis of different class A and B bHLH factors

Pure amnion cells (cultivated for 6 days) and villous trophoblasts (cultivated for 24, 66 and 96 h) were isolated as described in the Materials and methods section. Poly (A)$^+$ RNA was extracted from primary and tumour cells. Equal amounts of mRNA (2 μg) were reverse transcribed and subjected to PCR cycles within the linear range. To confirm equal abundancies of cDNAs, co-amplification of β-actin and Hand1 was performed. Subsequently, the same cDNA pools were utilized to detect HEB, Hash-2, ITF-2 and E12/E47 transcripts. Specific primer pairs, PCR conditions and length of the obtained DNA products are described in the Materials and methods section. The RT reaction was omitted in PCR negative controls (neg. control) of JEG-3 cDNA. In PCR positive controls, 1 ng of plasmid harbouring full-length bHLH cDNAs was added. DNA fragments were separated on 3 % (w/v) agarose gels. Sizes of the DNA marker bands (in bp) are indicated.

QIA*expressionist* according to the manufacturer's instructions (Qiagen). Subsequently, the His-tagged protein was further purified by SDS/PAGE on a 17.5 % (w/v) gel and electroeluted from the gel. Production of a polyclonal Hand1 antiserum was performed by Eurogentec (Seraing, Belgium). Briefly, freeze-dried Hand1 protein (100 μg) was injected four times into rabbits on day 0, 14, 28 and 56. Immune serum was collected after 38, 66 and 80 days. Preimmune serum was collected from the same animals prior to immunization. The antiserum (final bleeding) which gave the strongest signals with *in vitro* translated Hand1 protein on Western blots was chosen for immunopurification. His-tagged Hand1 protein (260 μg) was coupled to a solid support by azlactone chemistry using the UltraLink Immobilization Kit (Pierce, Rockford, IL, U.S.A.), and the antiserum was immuno-purified using ImmunoPure® Gentle Ag/Ab Buffers (Pierce).

Western-blot analysis

Protein extracts were prepared from amnion and trophoblastic tumour cell lines as described previously [34]. Equal amounts of protein extract (100 μg) were separated by SDS/PAGE on 12.5 % (w/v) gels and transferred on to Hybond-P or PVDF membranes (Amersham Biosciences). After blocking for 1 h in I-Block (Tropix, Bedford, MA, U.S.A.), filters were incubated overnight at 4 °C in the presence of immunopurified Hand1 antiserum (1 : 200 dilution). Subsequently, anti-(rabbit IgG) conjugated with horseradish peroxidase (Amersham Biosciences) was added at a 1 : 100 000 dilution for 1 h, and detection was performed using the ECL Plus® Western blotting detection system (Amersham Biosciences).

Fluorescence immunohistochemistry

Human blastocysts were fixed in 4 % paraformaldehyde for 10 min at 20 °C, treated with 1 % (v/v) Triton X-100 and incubated in 3 % (v/v) goat serum to block non-specific binding. Paraffin embedding of tissues, sectioning, pretreatment of slides and staining procedures were performed as described previously [34]. Mouse monoclonal antibodies raised against human cyto-keratin 7 IgG$_1$ (clone OV-TL 12/30; Dako, Glostrup, Denmark) and vimentin IgG$_{2a}$ (clone 3B4; Dako) were used at 0.3 and 0.5 μg/ml respectively. Immunopurified anti-Hand1 serum was used at a 1 : 5 dilution. Subsequently, horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1 : 100 dilution; Amersham Biosciences) was added. Signals were visualized using the Tyramide Signal Amplification (TSA™) kit for fluorescence immunohistochemistry, according to the manufacturer's instructions (NEN, Boston, MA, U.S.A.). No signal was identified when the sections were incubated with preimmune serum (1 : 5 dilution; results not shown). Blastocyst nuclei were counter-stained with 4,6-diamidino-2-phenylindole (DAPI).

DNA transfections and reporter assays

For analysis of luciferase reporter expression, JEG-3 cells were transfected in 24-well plates using the calcium phosphate ProFec-tion® Mammalian Transfection System (Promega). A mixture of plasmids (250 ng each) was added including, pGL3-promoter vector containing D- or E-boxes, Hand1-pCIneo and different E-proteins ligated into pCIneo and pCMV-βGal (in which CMV stands for cytomegalovirus; Clontech) for normalization. Two parallel transfections were performed per construct. Precipitates were left on the cells for 12 h, after which the medium was changed. After an additional 48 h, supernatants were aspirated and cellular protein lysates were prepared using reporter lysis buffer (Promega). Luciferase and β-galactosidase (β-Gal) assays were performed as described previously [38]. For binding site-independent interaction/transcription assays, the mammalian MATCHMAKER™ Two-Hybrid Assay Kit was used according to the manufacturer's instructions (Clontech). Cells were trans-fected in Petri dishes (60 mm × 15 mm) by adding precipitates containing 2 μg of pM-GAL4 (or different GAL4–Hand1 fusion proteins), 1.5 μg of pG5-CAT (the reporter gene), 1.5 μg of CMV-βGal and 2 μg of pCIneo encoding class A bHLH proteins. Cell lysates were prepared and the total amount of chloramphenicol acetyltransferase (CAT) was measured using a CAT ELISA according to the manufacturer's instructions (Roche Diagnostics).

Statistical analysis

Statistical analyses were performed with Sigma Stat Statistical Software (Jandl Corporation, Chicago, IL, U.S.A.) using a

Student's paired *t*-test. A *P* value of < 0.05 was considered statistically significant.

RESULTS

Expression of Hand1 and class A bHLH factors in human extra-embryonic cell types

To assess the relevance of Hand1 in extra-embryonic differentiation, we analysed mRNA expression of placental, chorionic and amniotic tissues from different gestational ages by Northern blot (Figure 1a). Hand1 mRNA production was observed in trophoblast-like JEG-3 cells and foetal membranes early and late in gestation. As previously observed [30], placental villi did not express Hand1 transcripts. A closer examination of the membranes revealed production of the mRNA in amnion tissues as well as in purified amnion epithelial cells, but not in different parts of the chorion. The amnion-derived tumour cell lines WISH and FL, however, did not synthesize the bHLH factor. In agreement, *in situ* hybridization specifically detected Hand1 mRNA in the amniotic epithelium, suggesting a role in differentiation and/or tissue-specific expression of the particular cell layer (Figure 1b). To confirm these results at the protein level, a polyclonal Hand1 antiserum was developed. To date, Hand1 protein expression has not been reported in mice or humans. The immunopurified antibody detected a single 29 kDa band in Hand1-programmed reticulocyte lysates as well as in extracts of purified amnion cells (Figure 1c). As a control, JAR cells, lacking

Hand1, and a G418-selected JAR cell clone, which constitutively expresses Hand1 mRNA, were utilized in the Western-blot analysis. Furthermore, immunohistochemical analysis showed that Hand1 expression was restricted to the amnion epithelium (Figure 1d). The polypeptide could not be detected in chorionic trophoblasts and was absent from mesenchymal cells of amnion, chorion and decidua. The discrepancy in expression between different cell types (trophoblast tumour cells versus chorionic and placental trophoblasts) prompted us to analyse Hand1 in trophoblasts of human pre-implantation embryos. RT-PCR analyses revealed Hand1 mRNA production in pre-implanting four- and eight-cell embryos as well as in blastocysts (Figure 2a). Transcripts were undetectable in pooled zygotes or in four different single oocytes. To study the localization of Hand1 in blastocysts, whole-mount immunohistochemical analyses were performed (Figure 2b). The protein was detectable in nuclei and cytoplasm of the outermost cell layer (i.e. trophectodermal cells) covering the inner cell mass (ICM), suggesting that Hand1 could have a function in early pre-implanting cytotrophoblasts. Murine Hand1 may interact with different bHLH factors depending on the cell type and experimental setting [22,23]. Since we were interested in identifying the most likely partner protein(s) in human Hand1-expressing cells, semi-quantitative RT-PCR of different class A and B bHLH proteins was performed (Figure 3). Hand1 mRNA was detected in purified amnion and JEG-3 cells, but was absent from the lymphoid cell lines Ramos and Reh and purified placental trophoblasts. HEB and ITF-2 were detectable
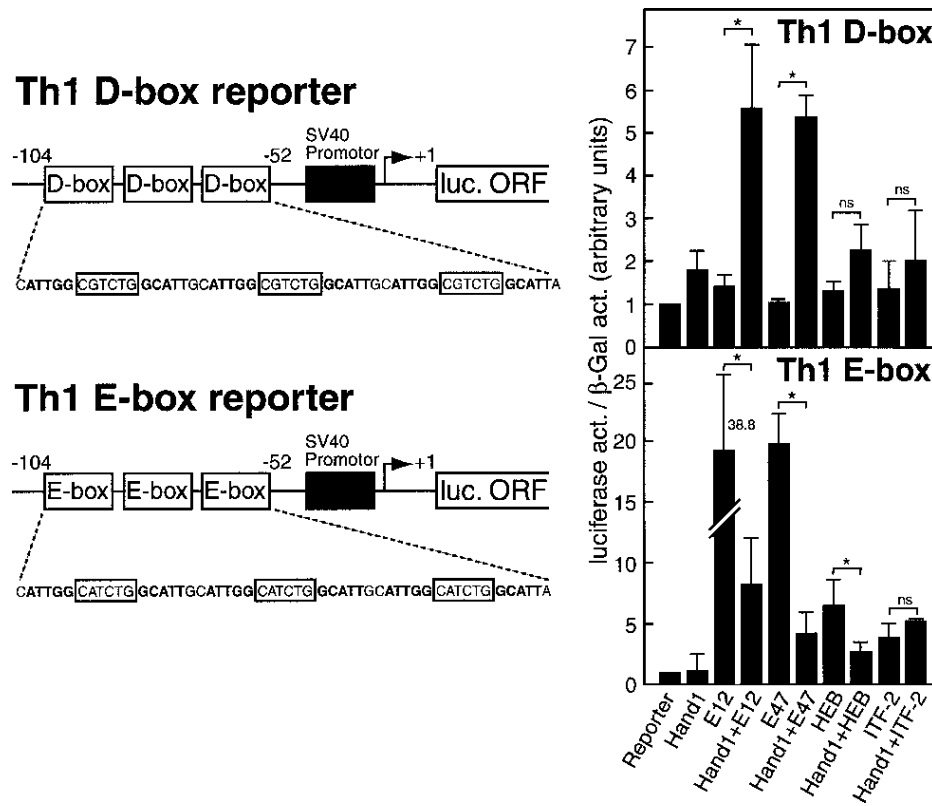

Figure 4   Hand1-dependent expression of luciferase reporters containing Th1 D- and E-boxes

JEG-3 cells were transfected with luciferase reporters harbouring either a trimeric Th1 D- or E-box (as shown on the left) and plasmids encoding different bHLH factors. All proteins were produced using the same expression plasmid (pCIneo). For normalization, pCMV-β-Gal was co-expressed. After 36 h, total protein was isolated and luciferase and β-Gal assays were performed as described in the Materials and methods section. To compare different experiments, transfections containing only the reporter plasmid were arbitrarily set to 1. Luciferase activity (luciferase act.) was normalized to β-Gal activity (β-Gal act.) and the results expressed as means ± S.E.M. from five independent transfections performed in duplicate. *P < 0.05; ns, not significant.
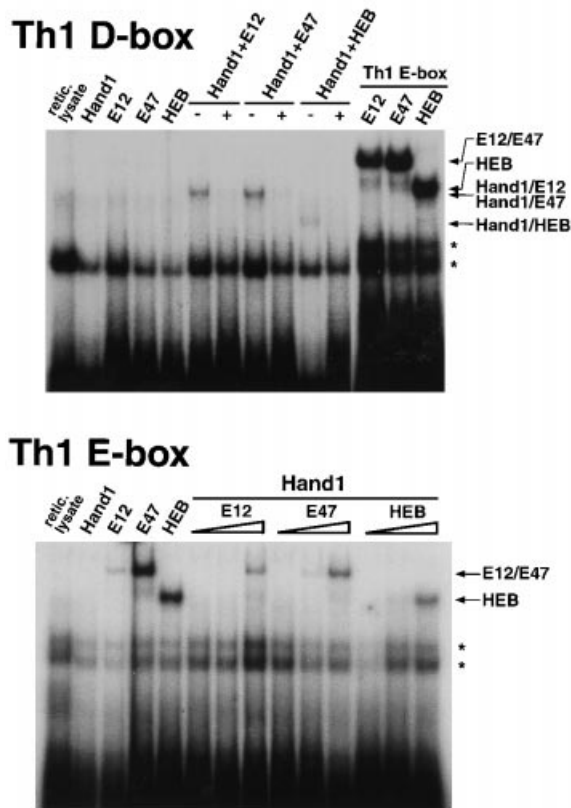
Figure 5   *In vitro* interaction of Hand1 with different class A factors and DNA-binding affinities of the Hand1 heterodimers

In the binding reactions, radiolabelled oligonucleotides harbouring trimeric Th1 D-box (upper panel) or Th1 E-box (lower panel) were incubated for 30 min with reticulocyte lysates in the absence (retic. lysate, negative control) or presence of bHLH factors. For combinations of different bHLH proteins (D-box binding), equal amounts of lysates (1 μl) were mixed. Reactions containing Hand1 and E12, E47 or HEB respectively were incubated in the absence ($-$) or presence ($+$) of a 20-fold molar excess of unlabelled D-box sequences. Incubations with Th1 E-boxes were performed in the absence (upper and lower panels) or presence (lower panel) of Hand1 and a 1-, 3- and 5-fold molar excess of E12, E47 or HEB respectively. DNA–protein complexes were separated on 5 % (w/v) polyacrylamide gels and subjected to autoradiography as described in the Materials and methods section. Different homo- and heterodimeric complexes are indicated by arrows. Non-specific DNA binding is indicated by an asterisk (*).

in all cell lines tested. Next, we designed specific primers that discriminate between E12 and E47 mRNA, according to nucleotide composition in their DNA-binding regions [39]. We observed expression of both transcripts in amnion cells. The Hand1-expressing JEG-3 cells produced little E12 and lacked E47. Interestingly, co-expression of Hash-2 and Hand1 was observed in JEG-3 and amnion cells, which might suggest a regulatory interplay of the two factors.

Interactions, DNA-binding specificity and transcriptional activity of Hand1 in the presence of different class A bHLH proteins

Although the target genes of Hand1 in trophoblasts are unknown, interaction of the murine factor with CGTCTG elements, representing a degenerate E-box (Th1 D-box), was demonstrated by PCR-mediated selection of *in vitro* binding sites [22]. We cloned three of these elements into luciferase reporters to investigate the interaction and Hand1-dependent transcriptional regulation in the presence of potential bHLH partners produced in JEG-3 cells
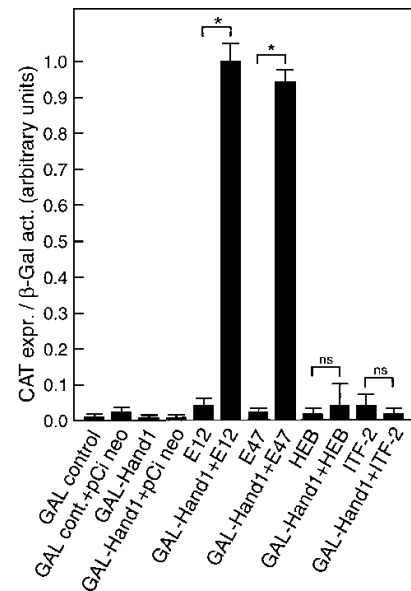
Figure 6   Interaction and transcriptional activation of GAL–Hand1 with different class A bHLH factors

JEG-3 cells were transfected with the GAL-Hand1 plasmid and the CAT reporter in the absence or presence of pCIneo harbouring E12, E47, HEB or ITF-2. For normalization, pCMV-βGal was co-expressed. Transfections containing only pM-GAL4 (GAL control) or pM-GAL4 and the different class A factors in pCIneo (E12, E47, HEB, ITF-2) were used as controls. After transfection for 60 h, CAT expression (CAT expr.) and β-Gal activity (β-Gal act.) were determined in protein lysates. Individual CAT values were normalized to the corresponding β-Gal values. Results are means $\pm$ S.E.M. of eight independent transfections performed in duplicate. *$P < 0.05$; ns, not significant.

(Figure 4). Expression of Hand1, E12, E47, HEB and ITF-2 alone was not sufficient to activate the Th1 D-box reporter. Co-expression, however, of Hand1 and E12 or E47 respectively, resulted in a significant increase (3.9-fold and 5-fold) in reporter expression. On the contrary, co-expression of Hand1 with HEB or ITF-2 did not significantly activate transcription. To investigate whether Hand1 may confer binding to E-boxes *in vivo*, a reporter harbouring three palindromic CATCTG elements flanked by the same sequences as the D-boxes was utilized. Activation of the Th1 E-box reporter was observed in the presence of the different class A factors (E12 > E47 > HEB > ITF-2). Upon co-expression of Hand1 with E12, E47 or HEB, transcription was significantly reduced, suggesting that the factor blocks E-box binding and/or E-protein-mediated transcriptional activation. To study further the binding affinity of Hand1, *in vitro* transcription/translation and EMSA with radiolabelled Th1 D- or E-boxes was performed (Figure 5). *In vitro* translated Hand1, E12, E47 or HEB alone failed to interact with Th1 D-box elements. After co-translation of Hand1 with E12, E47 or HEB, specific DNA–protein complexes were detectable, which migrated differently to HEB homodimers and E12/E47 complexes on Th1 E-boxes. D-box binding of the Hand1/E12, Hand1/E47 or Hand1/HEB heterodimers was abolished upon addition of a 20-fold excess of unlabelled D-box oligonucleotide. In EMSA with radiolabelled Th1 E-boxes, dimers of HEB and E12/E47 were readily detectable, whereas formation of Hand1/E-protein complexes were not observed. When equal amounts of Hand1 protein and E12, E47 or HEB respectively, were mixed, sequestration of the bHLH complexes from E-boxes was detected. Binding of class A protein dimers was restored when
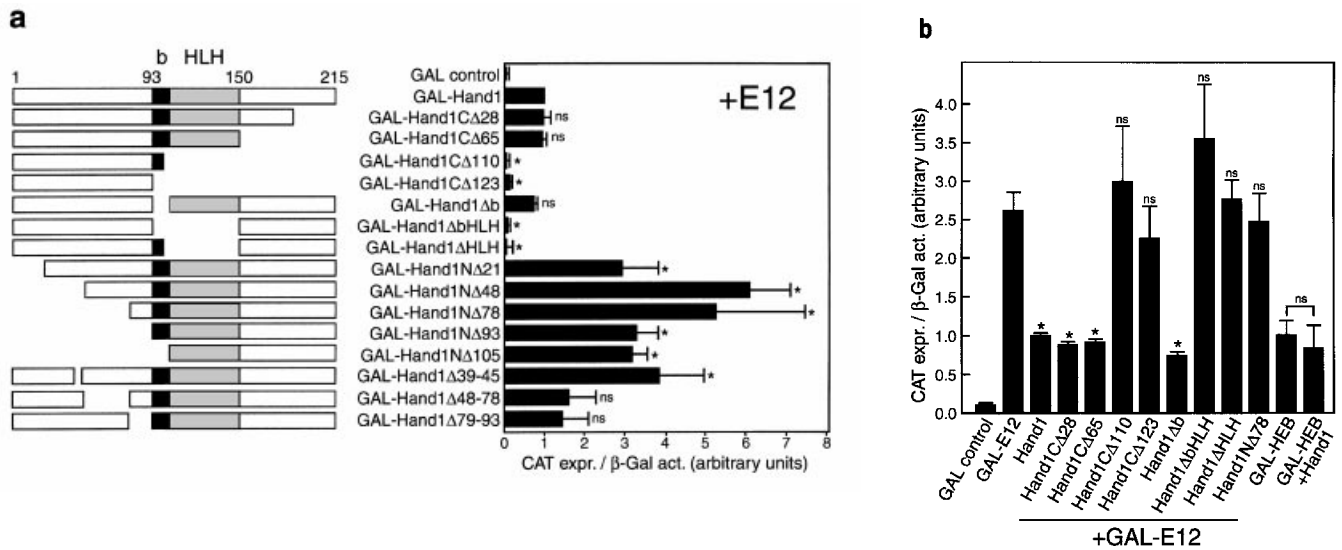
Figure 7   Characterization of functional Hand1 protein domains using the mammalian GAL system

Interaction/transcription of Hand1 mutants with E12 was investigated after transfection of JEG-3 cells with GAL fusion proteins. (**a**) The construction of the different Hand1 mutants was performed as described in the Materials and methods section and is shown schematically on the left. The basic and HLH region of the Hand1 protein are depicted as black and grey boxes respectively. These different GAL–Hand1 fusion proteins were co-expressed with E12 and CAT reporter activity determined. (**b**) Cells were co-transfected with plasmids expressing GAL-E12 or GAL-HEB and Hand1 or different Hand1 mutants subcloned in pCIneo. CAT production and β-Gal activity were determined as described in Figure 6. Normalized data (means ± S.E.M.) from eight (**a**) and five (**b**) independent transfections (each performed in duplicate) are shown. $*P < 0.05$; ns, not significant.

a 5-fold excess of reticulocyte lysate containing E12, E47 or HEB respectively, was added. The data suggest that Hand1 can act as a repressor of E-box-dependent transcription by inhibiting interaction of Hand1/class A heterodimers with the palindromic sequence. Additionally, we utilized a mammalian GAL4 system to study Hand1 interactions and transcriptional properties independently of its binding to DNA (Figure 6). Expression of a GAL–Hand1 fusion protein alone was not sufficient to activate the GAL binding-site-dependent CAT reporter. Compared with transfections with E12 and E47 alone, co-expression of GAL–Hand1 with the two class A factors resulted in a 24-fold and 35-fold increase in transcription respectively. Co-expression of Gal–Hand1 with HEB or ITF-2 did not significantly alter CAT reporter expression.

Identification of Hand1 repressor domains

Using the GAL4 system, a detailed mutational analysis of Hand1 was performed. The influence of different protein domains on interaction/transcription was investigated in the presence of E12 (Figure 7a). Deletion of amino acid residues 150–215 affected neither the binding of E12 nor the transcriptional transactivation of the heterodimer. Truncation or internal deletion of the HLH or bHLH regions resulted in the loss of both E12 interaction and thus transactivation. Deletion of the basic region alone did not significantly alter transcriptional properties of the Hand1/E12 heterodimer. However, truncation of the Hand1 protein from its N-terminus resulted in changes in CAT reporter expression. Deletion of the N-terminal 21 amino acid residues resulted in a 3-fold increase in transcription, whereas truncation of the N-terminal 48 amino acid residues resulted in an overall 6-fold elevation of reporter expression. After deleting 78 amino acid residues from the N-terminus, a 5.2-fold rise in transcriptional activity was observed. Subsequently, internal deletions of the N-terminal portion were analysed. Although mutant proteins

harbouring deletions from residues 48–78 and 79–93 respectively, did not significantly alter transcription, the Hand1 mutant Δ39–45 increased reporter expression 3.8-fold. The data therefore suggest that the transcriptional repressor activity of Hand1 resides mainly within the first 21 N-terminal amino acids and between residues 39–45. To confirm the transcriptional repressor activity in an independent experiment, a GAL–E12 fusion was co-expressed with different Hand1-mutants (Figure 7b). Compared with the control, GAL–E12 increased transcription 23-fold. Co-transfection with Hand1 reduced reporter expression 2.6-fold. The C-terminal mutants (Hand1CΔ38 and Hand1CΔ65) as well as the Hand1Δb mutant repressed transcription to a similar extent, indicating that these protein regions do not confer the repressor activity. On the contrary, deletion of N-terminal 78 amino acid residues abolished transcriptional repression by the Hand1 protein. Compared with GAL–HEB alone, co-expression of Hand1 and GAL–HEB did not significantly change the CAT activity induced by the fusion protein.

DISCUSSION

Several classes of tissue-specific transcription factors, which determine the development of distinct murine trophoblast cell layers, have been elucidated (reviewed in [40]). In particular, the bHLH factors Hand1 and Mash-2 play central roles in the formation/maintenance of giant- and spongiotrophoblasts respectively [17,20]. Since production of the Mash-2 counterpart, Hash-2, was described in human villous and extravillous cytotrophoblasts [27,28], one might assume that a similar interplay of bHLH factors triggers differentiation of the specialized human trophoblast cell types. Expression of Hand1, however, has not been detected in human cytotrophoblasts [30], which could be explained by the fact that the equivalent cell type of murine giant cells is lacking in human placentae. Instead, in the present study

we demonstrate that extra-embryonic expression of human Hand1 mRNA and protein is exclusively detected in the amniotic epithelium of the foetal membranes. In contrast with the expression pattern in mice, Hand1 was absent from different extra-embryonic mesodermal components of amnion and chorion [24]. Hand1 could be required for the expression of amnion-specific genes to maintain the differentiated state of the epithelium. It is noteworthy that, dedifferentiation of the tissue, represented by the amniotic tumour cells WISH and FL, may result in the loss of Hand1 expression.

Although Hand1 is absent from the diverse trophoblast cell types after implantation, a role of Hand1 in early trophoblast differentiation cannot be excluded completely. Unlike in mice [19], maternal Hand1 mRNAs are not present in human oocytes or in the fertilized zygote. Embryonic Hand1 expression, however, was already detected at the 4-cell stage concomitant with the initiation of embryonic transcription. At the blastocyst stage, Hand1 transcripts were present and the protein was abundantly expressed in the trophectodermal cell layer, which may suggest a role in specification of early trophoblast stem cells. With respect to this, it is also interesting to note that trophoblastic JEG-3 and BeWo tumour cells, which are widely used as a trophoblast model system, express Hand1 [30]. One may speculate that these choriocarcinoma cell lines are derived from precursor cells originating very early in trophoblast development.

Hand1 expression in trophectodermal cells may also provide insights into the development of the human amniotic epithelium. In mice, it is generally accepted that amniogenic precursor cells are derived from the primitive ectoderm shortly after implantation. Programmed cell death in this region of the ICM results in cavitation and separation of future amnion and ectodermal cells [41]. In humans, it was speculated that the amniogenic cells, which develop underneath the trophectoderm, may eventually arise by delamination of polar trophoblast cells [42]. Expression of Hand1 in trophectoderm and the amniotic epithelium may support this hypothesis. At later stages, down-regulation of the factor in the developing trophoblasts could be a prerequisite for early placentation and villous formation.

In mice, the class A bHLH factors ALF-1 and ITF-2 activate transcription in a GAL-Hand1-dependent reporter assay, suggesting that they could be potential interaction partners of Hand1 [23]. However, overlapping expression of Hand1 and the two factors is restricted to a region of the ectoplacental cone, whereas E-proteins are absent from differentiated giant cells [23]. Although Hand1 weakly interacts with the human homologue of ALF1, HEB, in reticulocyte lysates, modulation of transcriptional activity was not observed in the D-box-dependent luciferase- or GAL assays of JAR (results not shown) and JEG-3 cells. Based on these experiments, we may conclude that HEB is not a preferred dimerization partner in the choriocarcinoma cells. Indeed, differences between murine cell lines in Hand1 dimerization/transactivation have been observed previously [23]. However, the transfection data indicate that E12/E47 could be potential Hand1-dimerization partners in human trophoblastic tumour cells. Co-expression of Hand1 with E12 or E47 resulted in transcriptional activation of the Th1 D-box and GAL reporters, whereas E-box recognition of E12/E47 dimers was abolished in the presence of Hand1. Heterodimers of Hand1 with E12/E47 were also readily detectable *in vitro* (EMSA) and could be immunoprecipitated with the Hand1-specific antibody (results not shown). JAR and JEG-3 cells, however, do not express E47 and produce low amounts of E12. Thus it is also possible that, similar to murine giant cells, another bHLH protein which has not been discovered yet, is the preferential partner. We consider homodimerization unlikely since Hand1/Hand1 dimers

were unable to interact with D-boxes *in vitro* (G. Meinhardt, T. Loregger and M. Knöfler, unpublished work). In amnion epithelial cells, however, the full repertoire of class A bHLH factors was identified. Additionally, the cells express Hash-2. Therefore Hand1 may antagonize Hash-2 function by competing for E-proteins [23]. On the other hand, the target genes of Hand1 and Hash-2 in amnion cells are unknown. It is also possible that both class B factors interact with different bHLH proteins to maintain tissue-specific transcription/differentiation of the epithelium.

We (present study) and others [23] have shown that Hand1 can activate transcription upon recognition of a non-canonical Th1 D-box. DNA-binding specificity of Hand1/E-protein dimers is thought to depend on a high amount of positively charged amino acids and an unusual proline residue in the Hand1 basic region, which is common to the hairy/E(spl) family of bHLH repressor proteins [43]. Indeed, the repressor activity of Hand1 has been described previously [22]. In the present study, we confirm the observations of other workers [23,44] demonstrating that Hand1 can titrate class A bHLH factors from canonical E-boxes. However, Hand1 can also down-regulate transcription induced by tethered MyoD–E12 heterodimers, which may indicate a second mechanism of repression [44]. It was suggested that the repressing activity of Hand1 is localized in the basic and HLH regions, which could involve recruitment of yet another repressor [22]. Our present results do not support the role of the Hand1 basic region in transcriptional repression, since deletion of the domain did not relieve the repressing activity of Hand1 in both GAL–Hand1- or GAL–E12-dependent experiments. Instead, we found that two protein regions located in the N-terminal portion of Hand1 are required mainly for this particular function. It is likely that the unusual stretch of histidine residues (HHHHHH-PHPAH) within the first 21 amino acids represents one of the repressing activities. Although histidine-residue stretches have been identified in various transcriptional repressors, the particular role of the positively charged sequences have not been elucidated [45,46]. The region 39–45 of Hand1 contains the motif RPYFQSW, which is similar to repressing sequences in human hairy-related transcription factors [47], Hey1 (PYRPW), Hey2 (PYRPW) and HeyL (LYHSW). Classically, bHLH genes of the hairy family have the WRPW motif in common, which is necessary to recruit the co-repressor protein groucho [48]. Whether human homologues of groucho interact with the motif in the Hand1 coding sequence requires further analysis. On the other hand, it cannot be excluded that additional repressing activity is conferred upon HLH interaction with other repressors. *In vitro*, Hand1 has the capacity to bind the hairy-related transcription factors [49], but the biological significance of these interactions remains to be identified.

In summary, our present results suggest the function of Hand1 is complex. The protein modulates transcriptional activity of E12/E47 by influencing the binding to E-boxes and by repressing transactivation. Various Hand1 heterodimers may exist depending on the complexity of expression of other bHLH factors in a given cell. Further analysis with tethered Hand1/bHLH dimers is required to identify the function and target genes of the different Hand1 activities.

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