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The basic helix-loop-helix transcription factor Hand1 regulates mouse development as a homodimer

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ABSTRACT

Hand1 is a basic helix-loop-helix transcription factor that is essential for development of the placenta, yolk sac and heart during mouse development. While Hand1 is essential for trophoblast giant cell (TGC) differentiation, its potential heterodimer partners are not co-expressed in TGCs. To test the hypothesis that Hand1 functions as homodimer, we generated knock-in mice in which the Hand1 gene was altered to encode a tethered homodimer (TH). Some *Hand1*$^{TH/-}$ conceptuses in which the only form of Hand1 is Hand1TH are viable and fertile, indicating that homodimer Hand1 is sufficient for mouse survival. ~2/3 of *Hand1*$^{TH/-}$ and all *Hand1*$^{TH/TH}$ mice died *in utero* and displayed severe placental defects and variable cardial and cranial–facial abnormalities, indicating a dosage-dependent effect of *Hand1*TH. Meanwhile, expression of the Hand1TH protein did not have negative effects on viability or fertility in all *Hand1*$^{TH/+}$ mice. These data imply that Hand1 homodimer plays a dominant role during development and its expression dosage is critical for survival, whereas Hand1 heterodimers can be either dispensable or play a regulatory role to modulate the activity of Hand1 homodimer *in vivo*.

Introduction

The basic helix-loop-helix (bHLH) transcription factor genes encode two highly conserved and functionally distinct domains; a basic DNA binding domain and a HLH dimerization domain that make up a region of approximately 60 amino-acids (Jones, 2004). The basic DNA binding domain binds to DNA at a consensus hexanucleotide sequence known as the E box, while the HLH domain mediates the dimerization with different HLH partners, which together contribute to the transcription specificity *in vivo* (Massari and Murre, 2000). In general, the tissue-specific class II bHLH transcription factors function by forming heterodimers with the ubiquitously expressed class I bHLH transcription factors, the E proteins (Massari and Murre, 2000). During development, many tissue specific basic helix-loop-helix (bHLH) transcription factors, such as MyoD, Twist, Ascl and Hand, play critical roles in specification of various cell lineages (Castanon et al., 2001; Firulli et al., 1998; Megeney and Rudnicki, 1995; Philogene et al., 2012; Riley et al., 1998; Tanaka et al., 1997). Basic HLH factors can either form homodimer or heterodimers, and dimerization partner choice becomes a key factor in regulating the activities of tissue specific bHLH transcription factors (Jones, 2004; Massari and Murre, 2000). The function of specific dimer complexes has been directly approached by using a 'tethered' strategy through linking two monomer partner sequences with a flexible linker sequence such as to study the role of MyoD:E47 in inducing myogenesis (Neuhold and Wold, 1993) in nonmyogenic cells *in vitro* and the role of tethered Twist homodimers and heterodimer in somatic myogenesis when over-expressed in mesoderm of *Drosophila in vivo* (Castanon et al., 2001).

Hand1 is a tissue specific bHLH factor that is expressed in placenta, extra-embryonic membranes, heart and many neural crest derivatives during murine embryogenesis (Cross et al., 1995; Cserjesi et al., 1995). Studies of *Hand1* knockout models (Firulli et al., 1998; Maska et al., 2010; Morikawa and Cserjesi, 2004; Riley et al., 1998, 2000; Scott et al., 2000) indicate that Hand1 plays an essential role during various developing processes including trophoblast giant cells (TGC) differentiation, yolk sac vasculature and heart morphogenesis and lateral mesoderm development. Hand1 is a class II tissue specific bHLH transcription factor and was first cloned

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independently by several groups by virtue of its ability to interact the E-protein E47 (Cross et al., 1995; Cserjesi et al., 1995; Hollenberg et al., 1995). *In vitro* biochemical studies have shown that Hand1 can heterodimerize with class I E-factors (Itf2, Alf1) and other closely related class II factors such as Hand2 (Firulli et al., 2000; Scott et al., 2000), but can also form homodimers (Firulli et al., 2000; Scott et al., 2000). Hand1 heterodimerization *versus* homodimerization can be regulated through different mechanisms such as the phosphorylation status of Hand1 (Firulli et al., 2003) and by tertiary interaction of Hand1 with the non-bHLH factor FHL2 (Hill and Riley, 2004). We have previously found that none of the various putative Hand1 heterodimer partners (Alf1, Itf2, and Ascl2) are expressed in the TGC layer of the placenta (Scott et al., 2000). Moreover, we have found that co-transfection of an E-protein expression vector inhibited the ability of Hand1 to promote differentiation of Rcho-1 trophoblast cells to the TGC fate and to transactivate a TGC-specific promoter (*Prl3d2* gene) (Scott and Cross, unpublished). Based on this evidence, we hypothesized that Hand1 may function as a homodimer *in vivo* to regulate TGC differentiation. To test this hypothesis, we generated a knock-in allele that produces a tethered homodimer (TH) form of Hand1 and generated mice that express either only tethered homodimer (*Hand1*$^{TH/-}$ and *Hand1*$^{TH/TH}$) or both the monomer and tethered homodimer of Hand1 (*Hand1*$^{TH/+}$). We find that while some *Hand1*$^{TH/-}$ mice are viable and fertile, there is a dosage-dependent effect of Hand1TH and overabundant Hand1TH leads to placental defects and embryonic lethality. Meanwhile, presence of the Hand1TH did not have negative effects on viability or fertility in all *Hand1*$^{TH/+}$ mice.

Material and methods

Plasmids

Site-directed mutagenesis was carried out according to manufacturer's instructions using the Altered Sites *in vitro* mutagenesis kit (Promega). pCMV-FLAG Hand1Δb, containing a RRR to GSG substitution in the basic domain, was constructed using site-directed mutagenesis of pCMV-FLAG Hand1 (Scott et al., 2000) (AS oligo 5′-aat gct ctc tgt gcc gga tcc ctc ctt ctt ggg tcc-3′). For construction of tethered expression cassettes, EcoRI sites were first introduced by site directed mutagenesis 5′ to the Hand1 (AS oligo 5′-gtt cat gtt gga GAA TTC cct ggc ctg tgc tg-3′) and Itf2 (S oligo 5′-ctt ggt ttg tgt GAA TTC tcc aac atg cat cac caa cag-3′) initiation methionine sequences (shown in bold). In a similar manner, PstI sites were introduced immediately 3′ to the same Hand1 (AS oligo 5′-ggc ggc gtt ggc ctC TGC AGa ctg gtt tag ctc-3′) and Itf2 (S oligo 5′-atg gga cag tCT GCA Ggt cca agt tgc-3′) coding regions, thereby removing the STOP codons (shown in bold). A PstI site was also introduced 5′ of a second Hand1 cDNA (AS oligo 5′-gct gcc cac gag gtt caC TGC AGa gag gct cct ggc ctg-3′). EcoRI/PstI (both containing Hand1 and Itf2) and PstI/XhoI (containing Hand1) fragments from these SDM reactions were ligated together in pBluescript SK+ and digested with PstI. Annealed oligos with PstI overhangs, encoding a $(GGGS)_n$ linker (S 5′-ggg ggt tcc ggc ggg ggt tct gga ggt ggg agc ggc gga ggg tcc ggc gga gga act gca-3′, AS 5′-gtt cct ccg ccg gac cct ccg ccg ctc cca cct cca gaa ccc ccg ccg gaa ccc cct gca-3′) were then ligated into the PstI site. Clones where the oligos had incorporated in the correct orientation were verified by sequencing. Finally, EcoRI/XhoI fragments of these were ligated into EcoRI/XholI-digested pCMV-FLAG-Hand1 to yield pCMV-FLAGHand1:Hand1 and pCMV-FLAGItf2:Hand1. For the construction of pCMV-FLAG Hand1:Hand1 Δb the same approach was used. The mammalian expression vectors pCMV-Ascl2 and pβActin-LacZ (Cross et al., 1995) and pCMV-Itf2 (Chiaramello et al., 1995) have been previously described.

Trophoblast cell culture and transfection

Rcho-1 cells were cultured in RPMI-1640 medium (Sigma) supplemented with 20% fetal calf serum (Hyclone), 50 μM β-mercaptoenthanol, and 1 mM Na-pyruvate, as previously described (Cross et al., 1995). For TGC differentiation assays, Rcho-1 stem cells were transfected using Lipofectamine PLUS (Gibco BRL) 5 h after plating to coverslips. In initial experiments, 250 ng of pβActin-LacZ and 375 ng of expression vector were added per 35 mm well, with empty expression vector (pcDNA3) added to a 1.0 μg total. Cells were fixed 48 h post-transfection in 4% paraformalehyde and permeabilized with methanol. Following incubation with mouse anti-FLAG (1/200 dilution, IBI) and rabbit anti-β-galactosidase (1/400 dilution, Cappell) primary antibodies, and anti-mouse-FITC and anti-rabbit-TRITC (Sigma, 1/50 dilution) secondary antibodies, cells were stained with bisbenzimide (Sigma) and examined by fluorescent microscopy. In subsequent experiments, cells were transfected with 250 ng pCMV-IRES: EGFP (Clontech) and 375 ng of the indicated expression vectors (to 1.0 μg total with pcDNA3). Cells were lightly fixed 48 h post-transfection in 4% paraformaldehyde and stained with bisbenzimide. Giant cell differentiation was scored as the percent of TRITC- or GFP-positive cells that had the enlarged nuclei characteristic of TGCs (Cross et al., 1995). Percent TGC differentiation values represent the mean +/− SE for 25 fields examined for each treatment group using a 40× objective, and were similar in 2–3 separate experiments.

Generation of Hand1TH *knock-in mice*

For cloning purpose, a Bg1II site was introduced 5′ to the Hand1 transcription initiation site by PCR, as previous described (Riley et al., 2000). Targeting vectors consisted of a 6.0 kb KpnI/Bg1II 5′arm (upstream of transcription start site) and a 2.6 kb SalI/Bg1II 3′ arm (part of intron 1, exon 2, and sequence 3′ to exon 2) in a pUC8 vector backbone containing HSV-*thymidine* kinase cassette. Bg1II/SalI fragments containing tethered homodimer Hand1 sequences were ligated between the two vector arms. For the tethered homodimer Hand1, this fragment was constructed as described above, except that the second *Hand1* monomer consisted of genomic sequences. A PGK-*puro*^{res} cassette flanked by LoxP sites was subsequently inserted into the SalI site found in intron 1. Constructs were linearized via digestion with KpnI and transfected into the *Hand1*$^{+/-}$ ES cell line 14A3 (Riley et al., 2000) via electroporation. Following positive–negative selection using gangcyclovir and puromycin (1.5 μg/mL), ES cell clones were isolated and screened for proper homologous recombination following Southern blotting as previously described (Joyner, 1993). The genotyping results are shown by PCR. Chimeras were generated via aggregation of targeted *Hand1*$^{TH(puro)/+}$ ES cells and 8-cell stage wildtype embryos, as previously described (Joyner, 1993). One male chimera (8D11) transmitted the *Hand1*$^{TH(puro)}$ allele through the germline, and was outbred to CD1 females. Progeny were crossed with CAGGS-nlsCre mice (a gift from A. Nagy), and pups were genotyped via PCR for excision of the PGK-*puro*^{res} cassette. *Hand1*$^{TH/+}$ mice were obtained and further bred with *Hand1*$^{+/-}$ mice, the genotype of the progeny was determined by PCR. Experiments were done in accordance with the guidelines of the Canadian Council on Animal Care and the University of Calgary Animal Welfare Committee (Protocol No. M01025).

Histological analysis of placental tissues

Placentas and embryos were dissected and harvested from timed matings and fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS). Following fixation, embryos were