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SHORT COMMUNICATION

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The Human *ASCL2* Gene Escaping Genomic Imprinting and Its Expression Pattern

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The mouse achaete-scute homolog-2 gene (*Ascl2* or *Mash2*) encodes a transcription factor playing a role in the development of the trophoblast. The *Ascl2* is an imprinted gene with maternal expression and assigned to an imprinting gene cluster region (ICR) at a distal region of mouse chromosome 7. We previously isolated a phage clone carrying the human homolog, *ASCL2*, and mapped it to human chromosome 11p15.5, a human ICR. In the present study, we demonstrate the expression patterns of the human *ASCL2* in the fetus at a stage between first and second trimesters and in the placental tissues. In addition, it has been shown that the human *ASCL2* gene escapes genomic imprinting.

KEY WORDS: *ASCL2*; imprinting; expression; placenta.

INTRODUCTION

The *Ascl* (*Mash*) genes, *Ascl1* and *Ascl2*, are vertebrate homologs of the achaete–scute genes required for neuronal determination in Drosophila (1). The *Ascl* gene products are members of the basic helix-loop-helix (bHLH) family of transcription factors. The mouse *Ascl2*(*Mash2*) is strongly expressed in the extraembryonic trophoblast lineage and plays a critical role in early development of trophoblast progenitors, but it does not directly involve the embryonic development itself (2). A gene targeting approach demonstrated that the mouse embryos homozygous for the mutant *Ascl2* died of a placental failure by 10 days postcoitum (2). The expression is almost wholly attributable to embryonal transcripts at the late two-cell stage (3). Around the time of implantation, *Ascl2* becomes specific to diploid trophoblast cells and around Day 10, its expression is limited to the labyrinth layer and the outer spongiotrophoblast layer. After Day 10, the level of expression is declining until barely detectable at Day 18.5 (3). The mouse *Ascl2* has been reported to be involved in genomic imprinting with maternal-allele expression and assigned to a distal region of mouse chromosome 7, which corresponds to a mouse imprinting cluster region (ICR) containing the imprinted genes, *Ipl*, *Impt1*, *Itm*, *Cd81*, *Kvlqt1*, *Ins2*, *Igf2*, *H19*, and *p57Kip2* (4–8). We previously isolated a genomic clone covering the human *ASCL2* and localized it to human chromosome 11p15.5 (9) within ICR close to *IGF2*, *H19*, and *p57KIP2* (10). In this study, we show the expression patterns of the human *ASCL2* in the fetus at a stage between first and second trimesters and in the placental tissues. In addition, it has been demonstrated that the *ASCL2* escapes genomic imprinting.

MATERIALS AND METHODS

Sequencing of the *ASCL2* Region

The DNA from a human genomic phage clone (phHAS2-1) isolated in our previous work (9) was *Sma*I-cut and separated on 1.2% NuSieveTM agarose gel (FMC BioProducts, USA), and Southern hybridization was carried out using the rat *Ascl2* probe as used previously (9). The *Sma*I-digests were also loaded in 4% polyacrylamide gel, the DNA fragments were cut out and cloned into pUC19. An about 600-bp insert (Probe-1) was subcloned, sequenced, and used for further experiments. The phHAS2-1 digests with various restriction enzymes were subjected to Southern blot analysis using the Probe-1 as a probe. A 6.6-kb *Eco*RI-fragment (TM1) of phHAS2-1 which was hybridized to Probe-1 was subcloned into PUC19 and sequenced in both directions.

3′ Rapid Amplification of cDNA Ends (RACE)

The total RNA (20 mg) was extracted from a 7-week-old human placenta, and cDNA was synthesized using an oligonucleotide, 3RACER as a primer. PCR (polymerase chain reaction) was carried out using the cDNAs as a template with a primer set, 3RACE1F/3RACE1R. A nested PCR was then performed for 10 cycles using another set, 3RACE2F/3RACE2R. Two nested PCR products, 145-bp NPC-1 and 349-bp NPC-2, were isolated, subcloned into pUC19, and sequenced to confirm to be derived from TM1. The oligonucleotides used to amplify were 5′-TGACCATCGCATGACGTGAGTACTG

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CAGGATGACTCGCCTTTTTTTTTTTTTTTTT-3′ (3RACER), 5′-GGGGCACCAACACTTGGAGA-3′ (3RACE1F), 5′-TGACCATCGCATGACGTGAG-3′ (3RACE1R), 5′-GGGGAATTCTACACATTAACTTGAGCTG-3′ (3RACE2F), and 5′-TACTGCAGGATGACTCGCC-3′ (3RACE2R).

cDNA Library Screening

With the aim of the isolation of the ASCL2-cDNA, we screened cDNA libraries in Uni-ZAPTM XR vector (Stratagene) constructed from 9-week-old human villus tissues. Probe was prepared by means of reverse transcriptase-based (RT)-PCR, using the villus-derived total RNA as a template and using For1/Rev1 as primers. A seminested PCR was then performed using primers For1/Rev2. The oligonucleotides used to amplify cDNA were 5′-GGGGAATTCGGCCCAGCCTGACCAATG-3′ (For1), 5′-CCCAGGTCAAGGGTTCTTTG-3′ (Rev1), 5′-TCCGTTTGCCAGCAGTGTC-3′ (Rev2). The resultant product (RT1) was [a-32P] dCTP-labeled and used as a probe for a screening on the library. Isolated clones were confirmed by sequence analysis.

Northern Blot Analysis

To detect expression levels in a variety of tissues, the isolated cDNA was hybridized to ready-made filters (an RNA panel from the heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas) for northern blot analysis (Multiple Tissue Northern Blot, Clontech).

In Situ Hybridization

Four fetuses obtained through therapeutic abortions at a stage between first and second trimesters and placental tissues at 8–40 weeks of gestation were used. Ethical approval for the use of the human tissue was granted by the Ethics Committee. Serial 5-mm thick sections were cut from formalin-fixed, paraffin-embedded archival materials and mounted onto 2% aminopropyl triethoxysilane (Sigma) in acetone coated slides. The cDNA isolated was cloned into pBluescript KSII (-). Sense and antisense RNA transcribed from linearized templates using T7 and T3 RNA polymerases (Promega, USA) were labeled with 35S-CTP (Amersham, UK) to be used as probes. In situ hybridization and autoradiography were performed as described previously (11).

RFLP Analysis

Genomic DNA was prepared from placentae with various stages of development, and parental DNA was extracted from their peripheral blood leukocytes. To search for an RFLP, PCR was performed using For1/Poly1 (5′-GGGGGAATTCACGCTCCCTTGAAGAGG-3′) as a primer set, and PCR products cloned into pBluescript KSII (-) were sequenced. Total RNA was extracted from the same placentae with the guanidine thiocyanate method. The placental RNA was converted to the first-strand cDNA with oligo-dT 13–18 primer by M-MuLV reverse transcriptase. All the placental and maternal genomic DNA as well as placental RNA and cDNA were subjected to PCR with a primer pair, For1/ASC3. PCR products digested with *Sac*II were electrophoresed on 8% polyacrylamide gel to detect parent–fetus transmissions of alleles.

RESULTS

Structure of the Human ASCL2 Region

We first constructed a restriction map of the insert of a human ASCL2-covering phage clone (phHAS2-1), and sequenced the 6.6-kb EcoRI fragment (TM1) of the insert. From the sequence result, two possible poly–adenylation-like signals were found 1458 and 1662 bp downstream of the bHLH domain, respectively. The findings suggested that TM1 contains the whole ASCL2 gene.

Isolation of the Human ASCL2 cDNA

The 3′-RACE amplified approximately 300- and 120-bp fragments. Since sequence analysis revealed that both RACE-products corresponded to a 3′-portion of TM1, the transcript might have two alternative poly A signals. As a highly amplified product (RT1) was obtained by RT-PCR, using placental RNA as a template and a primer pair of 3RACE1F/Rev1, irrespective of gestational ages of the placenta used. The screening of the 9 weeks' library using RT1 as a probe gave seven positive clones and all clones were sequenced. All of them include the 3′UTR of the ASCL2 cDNA.

Expression Analysis of the Human ASCL2

To know the expression levels of the *ASCL2* in a variety of tissues and developmental stages, northern

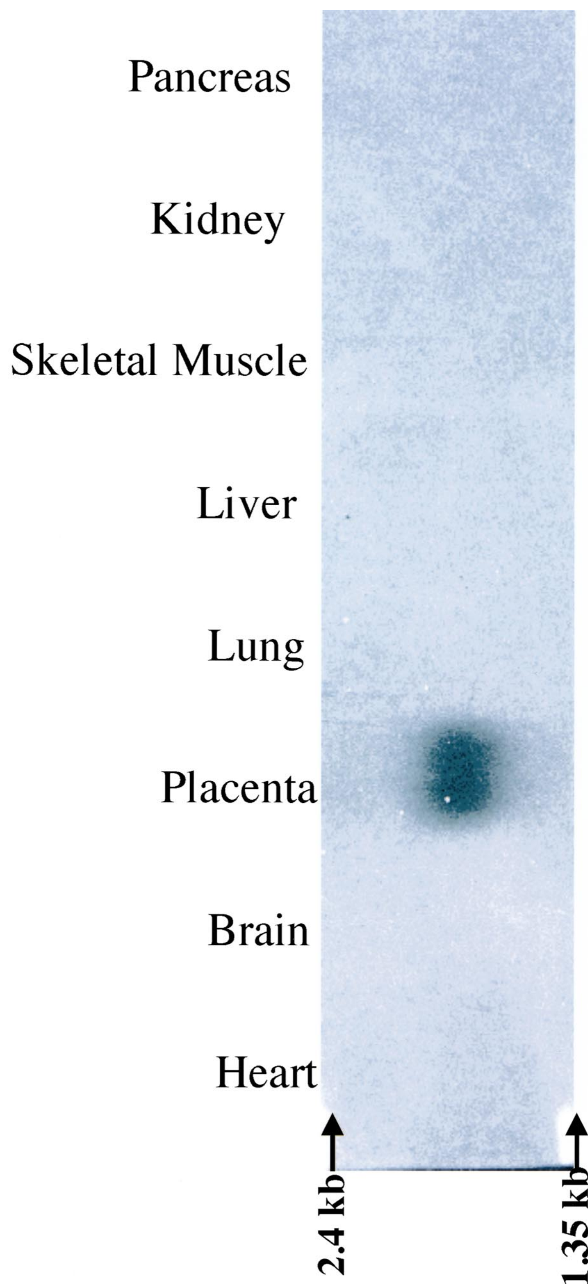

Fig. 1. Northern blots on a tissue panel using the 3′UTR of ASCL2 cDNA as a probe. Expression is seen only in the placenta.

blot analysis and in situ hybridization analysis were carried out. Northern hybridization revealed 1.6-kb band in the mature placenta (Fig. 1). The in situ hybridization demonstrated a high level of the transcripts in the placentae at a stage between the first and second trimesters (Fig. 2). Abundant transcripts were localized in the intermediate trophoblasts, while the expression level was low in the cytotrophoblasts and stroma of chorionic villi. The signal was regarded as specific, since the tissues hybridized with sense probes did not show any detectable signals. On the other hand, syncitiotrophoblasts showed only trace signals (data not shown). The hybridization using a whole mount fetus from the second trimester demonstrated that the *ASCL2* appeared to be localized specifically in the ectoderm but not in the mesoderm or endoderm. A moderate amount of the *ASCL2* was detected in photoreceptor, bipolar, and ganglion cells of the retina, and in primitive skin cells (Fig. 2). Primitive neuronal cells of the brain expressed a low-to-moderate amount of the transcripts (Fig. 3). A similar amount of signals was also detected in neuronal cells in the spinal cord, but not in the peripheral nerve.

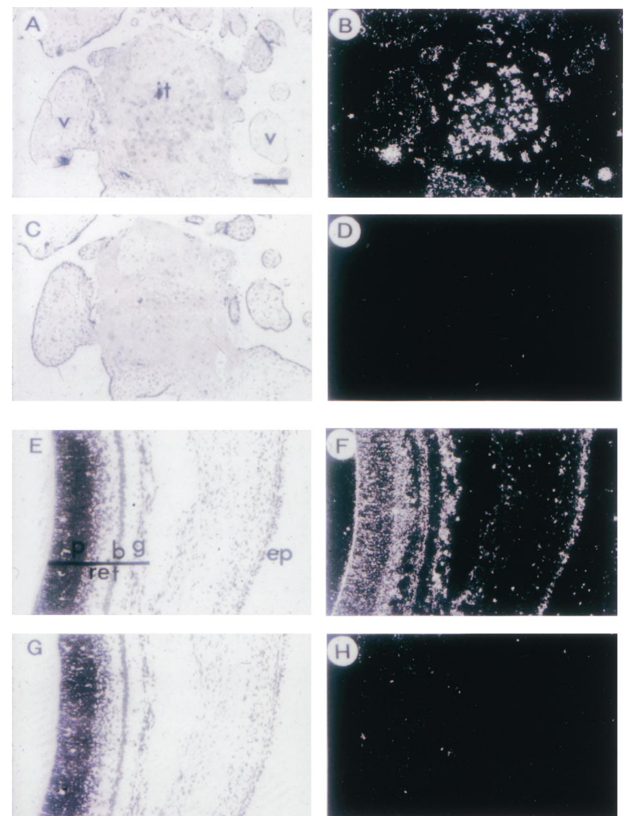

Fig. 2. In situ hybridization signals for the *ASCL2* by bright-field (A, C, E, and G) and their respective dark-field (B, D, F, and H) photomicroscopy. Signals are seen in the intermediate trophoblast (it) and villus (v) tissues of the placenta, the photoreceptor neuron (p), bipolar cell (b) and ganglionic cell (g) of the retina (ret), and in the epidermis (ep) hybridized with the antisense probe (A, B, E, and F), while signals are not observed in the same tissues with the sense probe (C, D, G, and H). Bar in A corresponds to 100 μm.

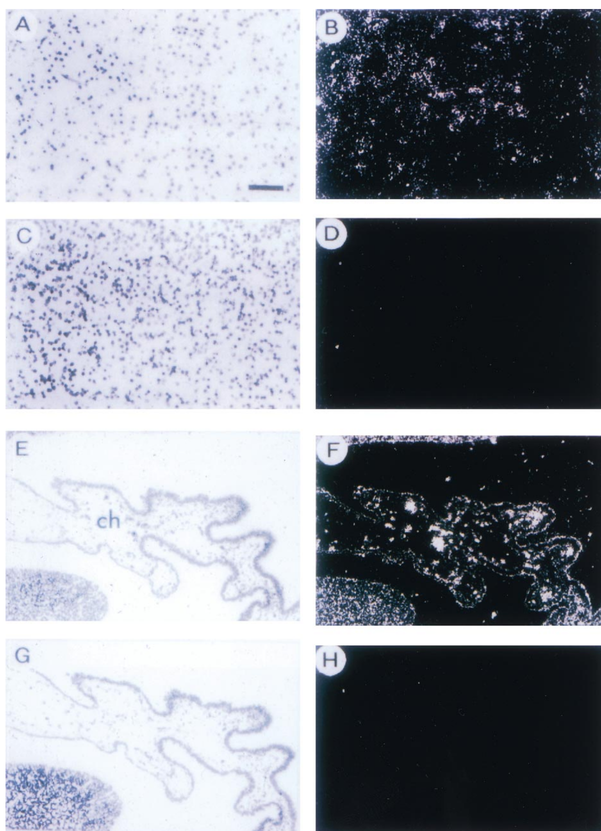

Fig. 3. In situ hybridization for the *ASCL2* in the brain (A–D) and choroid plexus (ch) (E–H). Bright-field photomicrographs are shown in A, C, E, and G and their respective dark-field photomicrographs in B, D, F, and H. Tissues hybridized with the antisense probe is shown in A, B, E, and F and those with the sense probe are in C, D, G, and H. Bar in A corresponds to 100 μm.

In addition, the choroid plexus show strong signals, the level of which was comparable to that of the placenta.

Biallelic Expression of the ASCL2 in the Placenta

We searched for a polymorphic site in *ASCL2* cDNA to discriminate between its parental alleles (12). Each of the genomic DNAs derived from 12–39-week's placentae was amplified by PCR using For1/Poly1 as a primer pair and then sequenced. In 16 of 55 placentae examined, a G to C substitution, a silent mutation, was found at the *ASCL2* 3′UTR region. Since the substitution loses a *Sac*II recognition site, PCR products from the placental DNAs were digested with the enzyme, and the *Sac*II RFLP was confirmed (data not shown). We then examined on whether each parental allele was expressed in the

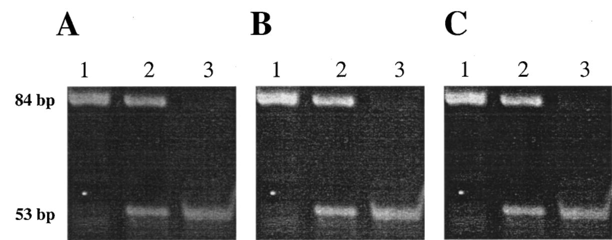

Fig. 4. Biparental expression of the *ASCL2* in the selected 3 placentae. A, B, and C are PCR products for 12-, 25-, and 37-week-old, respectively. Lanes of 1 are RT-PCR products, lanes 2 the RT-PCR products digested with *Sac*II, and lanes 3 the PCR products of DNA from the mother bloods digested with *Sac*II. Fragments with 84 and 53 bp indicate two alleles.

ASCL2 from the 16 placentae which were heterozygous for the polymorphic alleles. All of the SacII digests of RT-PCR products from the 16 placentae showed biparental expression (Fig. 4).

DISCUSSION

The mouse *Ascl2* is the first transcription factor shown to play a critical part in the development of the mammalian trophoblast lineage. At postimplantation stage the mouse *Ascl2* is abundantly expressed in the ectoplacental cone and extraembronic ectoderm of the Day 7.5. At Day 8.5 expression persists at high levels in all the diploid trophoblast cells (3). At that time, lower levels of expression are also detected in the embryo (2). At Day 9.5–10.5, expression persists in both the labyrinthine layer and outer spongiotrophoblast layer. Beyond Day 10, expression levels began to decline. In this study, we showed the expression patterns of the human *ASCL2* in placentae at a stage between the first and second trimesters (Fig. 2). Abundant transcripts were detected in the intermediate trophoblasts, while the levels were low in the cytotrophoblasts and stroma of chorionic villi. On the other hand, the *ASCL2* was localized specifically in the ectoderm by hybridization using a whole mount fetus from the second trimester (Fig. 3). The expression patterns resembling those for the mouse *Ascl2*. It remains to be seen what roles the human *ASCL2* plays in the development of the placenta and ectoderm.

At the present, there are two hypotheses on whether the human *ASCL2* is imprinted or not. The first one is following: It was suggested that the *ASCL2* is also imprinted in man by in situ hybridization methods, using the trophoblast cells of moles (13). The other one is following: The *ASCL2* is expressed

biallelicaly by analysis using only early placental tissues (weeks 5–10 of gestation) (14). The expression patterns of imprinting genes are sometimes different depending on the embryonic stage. For example, the mouse *Ascl2* is biallelically expressed from 6.5 to 7.5 days postcoitum (5). In this study, we analyzed the imprinting with a variety stage of placentae (Weeks 12–39 of gestation). All of them displayed biallelic expression patterns. Then we can exclude the possibilities of stage-dependent imprinting. In addition, we analyzed a total of 16 samples and all of them escaping genomic imprinting. Then we can deny the possibility of the imprinting polymorphism (15).

In conclusion, we showed the human *ASCL2* expression patterns in detail and established that the human *ASCL2* escapes from genomic imprinting.

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