The short stature homeobox gene SHOX is involved in skeletal abnormalities in Turner syndrome

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INTRODUCTION

Turner syndrome (or Ullrich–Turner syndrome) is a common disorder associated with the complete or partial loss of an X chromosome that affects 1 in 2500 live-born females worldwide (1–3). Although significant clinical variability exists in Turner syndrome, it is characterized by ovarian failure, leading to infertility, and short stature. Short stature in Turner syndrome is characterized by a tendency for intrauterine growth retardation, as well as growth failure during childhood and reduced adult height. Growth retardation is usually described as ‘slightly disproportionate’, indicating that the lower leg and arm are slightly shorter than the corresponding upper leg and arm. In addition to the key features of short stature and ovarian failure, Turner patients exhibit a great variety of somatic features, such as lymphoedema, webbed neck, cardiac and renal abnormalities, and several different skeletal defects (4). The skeletal defects include an abnormal upper-to-lower leg/arm ratio, micrognathia, cubitus valgus, short neck, high-arched palate, short metacarpals, genu valgum, scoliosis and Madelung deformity. These, as with the other somatic features, are diagnosed with a variable incidence in only a subfraction of Turner females (5).

The wide range of developmental abnormalities in Turner syndrome and the prevalence of the 45,X0 karyotype suggest that a number of different X-located loci are responsible for the complete Turner phenotype. It is widely accepted that dosage (haploinsufficiency) of specific X-Y homologous genes that escape X inactivation causes the characteristic Turner somatic features and associated cognitive defects (6–8). With regard to short stature, it has been suggested that the homeobox gene, SHOX, in the pseudoautosomal region is the major player and that haploinsufficiency of this gene leads to the growth failure seen in Turner syndrome (9,10). Furthermore, it has been shown...
that some patients with idiopathic short stature have mutations in the SHOX gene (10). Interestingly, SHOX mutations have also been described as causative for the Leri–Weill syndrome, a mesomelic short stature syndrome (11,12). Here, the lower leg or arm is considerably shorter than the upper leg or arm, hence it was termed 'disproportionate' or 'mesomelic' growth failure. In addition, the Madelung deformity, a characteristic skeletal abnormality of the forearm, is a consistent finding in Leri–Weill patients (13). In addition, homozygous SHOX mutations have been shown to cause Langer-type mesomelic dwarfism (11,12).

These different clinical findings, regarding bone deformities and growth, prompted us to ask whether SHOX causes, or contributes to, additional features of the clinical spectrum in Turner syndrome. Since SHOX has a close human relative, SHOX2 (formerly SHOT) (14), located on chromosome 3, and no direct mouse orthologue, it was decided to approach these questions directly by performing expression studies on human embryos. To evaluate further the relationship of the two human genes, SHOX and SHOX2, their expression pattern was also

Figure 1. Zoom blot analysis of the SHOX(a) and SHOX2(b) genes. SHOX fragment 640–724 and the corresponding SHOX2 fragment 624–728, sharing 71% sequence identity, were used as hybridization probes. DNAs were of the following species: fish (Tautoga onitis), frog (Xenopus laevis), chicken (Gallus domesticus), cow (Bovis domesticus), mouse (Mus musculus), hamster (Mesocricetus auratus), rat (Rattus norvegicus), rabbit (Oryctolagus cuniculatus), dog (Canis familiaris), sheep (Ovis aries), pig (Sus scrofa) and tamarin (Leontopithecus saginus).

Figure 2. Comparison of the expression of Og12x and SHOX. (a) In situ hybridization of an E11 mouse whole mount with Og12x antisense probe. Expression is seen in dorsal root ganglia (DRG), third pharyngeal arch (3rd PA), heart (He), upper limb (UL) and nasal process (NP). (b) Dark-field microscopy of a lateral, sagittal section of a CS16 human embryo (37 d.p.c., murine E11.5), following hybridization to SHOX antisense probe. Expression is seen in the central part of both upper and lower limbs (UL and LL), and in the first pharyngeal arch (1st PA). HB, hind brain; E, eye; NP, nasal process; FB, forebrain.
compared with the embryonic expression profile of the murine SHOX2 orthologue Og12x (15). Here, we present data on SHOX expression in the mesenchymal cells of the first and second pharyngeal arches and in the ulna/tibia, elbow and wrist (and equivalent bones of the leg), strongly suggesting that SHOX-related growth impairment leads to the high-arched palate, abnormal auricular development, cubitus valgus, genu valgum, Madelung deformity and short metacarpals that are seen in many Turner patients. In addition, we show that SHOX nonsense mutations can lead to some of these skeletal features with a rich intrafamiliar phenotypic variability.

RESULTS

Evolutionary relationship between SHOX and SHOX2/Og12x

To find out about the evolutionary relationship between the human genes SHOX and SHOX2 and the mouse gene Og12x, we carried out cross-species hybridization experiments. These experiments under low-stringency conditions have shown that the SHOX2 orthologue, Og12x, represents the closest mouse SHOX homologue (data not shown). We also carried out zooblot hybridizations and both SHOX and SHOX2 seem to be absent in invertebrates and first emerge in vertebrates (Fig. 1), suggesting a putative role of both genes in the development of body structures related to the skeleton. The absence of SHOX and SHOX2 homologous sequences in invertebrates was confirmed by database comparison with the complete sequence of the Caenorhabditis elegans genome. SHOX and SHOX2 are highly conserved and detectable in fish, the lowest vertebrate investigated. SHOX2 orthologues are detectable in all species studied throughout vertebrate evolution, except in dogs (Fig. 1). In contrast, SHOX is highly conserved in fish and chicken, yet was not detected in the frog, in the rabbit or any of the rodents studied (mouse, hamster, rat), raising the question of functional redundancy between SHOX and SHOX2.

Og12x expression in wild type, limb deformity (ld) and extra-toes (Xt) mouse mutants

The common structure of SHOX and SHOX2, exemplified by an identical homeodomain, a highly conserved OAR domain (16) and conserved putative phosphorylation sites, suggests that data derived from the mouse gene, Og12x, may also give us direct or indirect clues regarding the biochemical and physiological role of SHOX and SHOX2. The high conservation between SHOX and Og12x (79% amino acid identity) and the extraordinary conservation between SHOX2 and Og12x (99% amino acid identity) clearly argue for an indispensable role of these genes during development. With regard to a future animal model for SHOX, we were also interested in the question of whether Og12x embryonic expression reflects properties of SHOX expression. For these reasons, expression of Og12x was examined during embryogenesis between E5 and E18.5 by in situ hybridization to sections and whole-mount embryos. High levels of Og12x transcripts are restricted to four main structures: the central nervous system (CNS), the heart, the craniofacial tissues, and the fore- and hind-limb bud (14,17). Particularly interesting is the dynamic Og12x expression in the limb bud. At the onset of limb bud emergence [embryonic day (E) 9.25], Og12x is initially expressed in the posterior region. Subsequently, Og12x expression extends to most of the limb bud, excluding a small anterior/proximal region (E10.75) (Fig. 2a). During limb bud outgrowth (E10.5–E11.75), Og12x expression becomes restricted proximally and is excluded from the forming handplate. By E12.5, Og12x is highly expressed in the proximal limb.

The early anterior/posterior (A/P) pattern of expression led us to examine whether Og12x could be a downstream target of sonic hedgehog (Shh) (18,19). We therefore examined Og12x expression in two mutants where Shh signalling and A/P polarity are affected. In the limb deformity mutation (ld), several targets of Shh signalling are either downregulated or absent in the limb bud (20). In contrast, in the extra-toes mutation (Xt), ectopic Shh signalling is detected in the limb bud (21). Since no changes in Og12x expression were detected in either of the homozygous mutants Xt/Xt and ld/ld (data not shown), we suggest that Og12x is not directly implicated in the Shh-dependent A/P patterning of the limb bud. Accordingly, Og12x either acts genetically upstream of Shh or is not part of the Shh pathway at all.

SHOX and SHOX2 expression in human embryos

To investigate directly and differentiate the growth-related functions of SHOX from those of SHOX2 and Og12x, we carried out in situ hybridizations in human embryos from Carnegie stage (CS) 12 to CS21 [between 26 and 52 days post-conception (d.p.c.)]. This is equivalent to mouse E9.5–E13.5 (Theiler stages 15–22), a period in which Og12x expression has been analysed (14,17 and this paper). These stages encompass the early development of the limbs and genesis of the long bones, including condensation of mesenchyme (from CS16, murine E11.5) and chondrification from CS17 (murine E12). The end of the embryonic period (CS23, murine E14) is approximately the time when calcium deposition and ossification begin in the humerus. Lower limb development lags the upper limb by one CS.

SHOX2 expression was found to be comparable to that of Og12x in mouse, both spatially and temporally. SHOX2 expression, which was found in several organ systems, including the limb and pharyngeal arches, is summarized in Table 1. In contrast, SHOX expression is confined to the limb and pharyngeal arches (Fig. 2b). The expression of both genes in these two regions was examined in detail.

SHOX2 expression is first detected in the upper limb at CS13. This is initially on the dorsal aspect and then becomes more diffuse across the limb at CS14 (murine E10.5). As the limb develops, SHOX2 is expressed medially and proximally, predominantly in connective tissue (Fig. 3a and d). In the craniofacial region, in addition to expression in the nasal process, there is expression in the first, second and third pharyngeal arches (Fig. 4a). As with Og12x, the expression is in the outer mesenchyme layer.

SHOX is first seen in the developing limb bud at CS14, when it appears as a broad band (extending from ectoderm to endoderm), just proximal to the progress zone (Fig. 3b). Unlike several other genes important in limb development, a similar broad band of expression is seen in sections cut in either a sagittal or transverse plane. This suggests that there is no dorsoventral or anterior–posterior axis to this early expression (data not shown). As mesenchyme condenses to form pre-cartilaginous primordial bone, between CS15 and CS16 (murine
E11–E12), SHOX expression remains in the middle part of the developing limb, outlining the pre-cartilaginous structure of the bone (Fig. 3c). The signal is strongest in the perichondrium and condensing mesenchyme, but is also present in the overlying ectoderm. During later stages of development (CS18 and CS21, murine E12.5–E13.5), the various bones of the arm become identifiable. SHOX expression remains confined to the middle portion of the arm, around the distal end of the humerus, the radius and ulna, and in some of the bones of the wrist (Fig. 3c and e). There is no detectable expression in the phalanges, although some signal can be seen in the overlying ectoderm of the fingers (Fig. 3e). A similar pattern of expression, both temporal and spatial, was seen in the lower limb (data not shown).

Interestingly, SHOX expression is not restricted to the developing limbs, but was also detected in the mesenchymal core of the first and second pharyngeal arches (Figs 2b and 4b). Expression was first seen at CS15 and intensifies through the stages to CS21 as the mesenchyme condenses. The first pharyngeal arch mesenchyme goes on to produce the maxilla and mandible as well as some of the bony elements of the external and middle ear. The mesenchyme of the second branchial arch contributes to several bones, including those of the middle ear. A summary of SHOX, SHOX2 and Og12x expression is given in Table 1.

### Table 1. Comparison of SHOX, SHOX2 and Og12x expression

<table>
<thead>
<tr>
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<th>SHOX</th>
<th>SHOX2</th>
<th>Og12x</th>
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<tbody>
<tr>
<td><strong>First and second pharyngeal arch and derived structures</strong></td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>CS15 onwards, mesodermal core</td>
<td>CS13 onwards, surrounding mesenchyme</td>
<td>E10.5 onwards, surrounding mesenchyme</td>
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<tr>
<td><strong>Third pharyngeal arch and derived structures</strong></td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>CS13 onwards</td>
<td>+</td>
<td>E10.5 onwards</td>
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<tr>
<td><strong>Nasal process</strong></td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>CS15, and later, CS19, vomeronasal organ</td>
<td>E9.5, and later, E11, vomeronasal organ</td>
<td></td>
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<tr>
<td><strong>CNS basal plate</strong></td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>Forebrain to spinal cord</td>
<td>Forebrain to spinal cord</td>
<td></td>
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<tr>
<td><strong>Dorsal root ganglia</strong></td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td><strong>Cardiac inflow tract</strong></td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td><strong>Limb</strong></td>
<td>Initial expression</td>
<td>CS14</td>
<td>CS13</td>
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<tr>
<td><strong>Bone</strong></td>
<td>+</td>
<td>+/-</td>
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<tr>
<td><strong>Muscle</strong></td>
<td>–</td>
<td>+</td>
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<tr>
<td><strong>Connective tissue</strong></td>
<td>+/-</td>
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<tr>
<td><strong>Genital tubercle</strong></td>
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Clinical features in patients with SHOX nonsense mutations

Mutation analysis by PCR–SSCP was carried out in several hundred short stature patients of both genders with a normal karyotype and without Turner syndrome (unpublished data). Two short index patients of European and Australian descent, one with a familiar and one with a de novo SHOX mutation at R195X, were clinically re-examined for the presence or absence of additional clinical features. Patient B3, from an Australian family, is the only affected child of a sibship of nine. She has a de novo SHOX nonsense mutation. Clinically, she presented a bilateral Madelung deformity, cubitus valgus and hypoplastic short fourth metacarpals. Patient A1 derives from a German family with multiple affected short members. All five short members were investigated previously regarding their final height, which varies between −2.0 and −3.8 standard deviation score (SDS) (10). A detailed clinical re-evaluation of all short members with SHOX mutations now reveals that the grandmother of this family has a short neck and scoliosis, the mother has a short neck, scoliosis and Madelung deformity, the aunt has scoliosis, and the son a short neck and genu valgum. In contrast, the daughter with an identical SHOX mutation is free from any additional signs. This pedigree exemplifies a striking clinical intrafamilial variation and strongly supports the involvement of the SHOX gene in the development of additional skeletal Turner stigmata, as predicted by the human embryo expression studies.

**DISCUSSION**

A combination of expression studies and genetic analysis has shown that homeodomain proteins play critical roles in controlling embryonic development in vertebrates. We have investigated the evolutionary and functional relationship between SHOX, SHOX2 and the closely related murine gene Og12x. SHOX and SHOX2 represent the only two human members, and Og12x the only mouse member, of this small family of paired-related homeobox genes. Zooblot and database analyses show that the orthologues of both human genes, SHOX and SHOX2, first emerge in primitive vertebrate species. The strong evolutionary conservation of SHOX is highlighted by an 87% identical SHOX orthologue in the medaka fish (22). Both SHOX and SHOX2 seem to have co-evolved, rather than SHOX being a descendant of SHOX2, as previously suggested (14). The apparent lack of SHOX in frog and all rodent species investigated raises the possibility of functional redundancy between SHOX and SHOX2 in humans.

To address this question directly, we analysed the expression of SHOX, SHOX2 and Og12x in comparable stages of human and mouse embryonic development. Future work will also...
include the use of antibodies to compare the mRNA and protein expression patterns. All three genes exhibit dynamic and complex, yet specific, expression patterns with Og12x closely resembling the expression of SHOX2 in humans (Table 1). Og12x and SHOX2 are expressed in a diverse range of tissues and organs, both of mesenchymal and neuronal origin. From their expression pattern, we would predict that Og12x-deficient animals would be likely to exhibit a phenotype similar to that generated by SHOX2 (mutations) or a combination of SHOX and SHOX2. To date, SHOX2 has not been identified as the causative gene for a human disorder. In this context, the observation that Og12x expression in the limb does not seem to be directly connected to A/P patterning and may be independent of Shh is particularly interesting. Shh has been identified as a signal that can influence the expression of Hox genes in the limb (18). Hoxd9 to Hoxd13 can be ectopically activated by the combined influence of Shh and fibroblast growth factors, indicating that these factors play an endogenous role in the regulation of Hoxd genes involved in limb development. The unchanged expression of the mouse gene Og12x in Shh deregulated animals, therefore, also suggests a HOXD-independent regulation for the human SHOX2 gene.

There are two major regions in which SHOX is expressed: the limb and the pharyngeal arches (Fig. 2b). Abnormal development in both these areas underlies skeletal features of Turner’s syndrome. In both of these regions there is also SHOX2 expression. Within the limb, SHOX is initially expressed in undifferentiated mesenchymal tissue. When the mesenchyme condenses, and chondrification takes place, SHOX is expressed most strongly in the perichondrial layer. In this layer, chondroblasts (and subsequently osteoblasts) differentiate, prior to laying an envelope of cartilage surrounding the mesenchyme.

Figure 3. SHOX and SHOX2 expression in the developing limb. (a) Dark-field microscopy of a transverse section through the lower abdomen/lumbar region of CS13 human embryo (28 d.p.c., murine E10.25) hybridized to SHOX2 antisense probe. SHOX2 expression was shown in the dorsal region of lower limb (LL); US, umbilical stalk; SC, spinal cord. (b and c) Dark-field microscopy of transverse sections through upper limbs of human embryos, CS14 (b) and CS18 (c), hybridized to SHOX antisense probe. SHOX expression was initially seen as a band across the limb, and then became strongest around the pre-cartilaginous structure of the bones of the elbow joint. UL, upper limb; L, liver; SC, spinal cord; H, humerus; U-R, proximal ulnar-radial condensation; He, heart. (d and e) Dark-field microscopy of transverse sections through the thorax of CS21 human embryo (52 d.p.c., murine E13.5) hybridized to SHOX2 (d) and SHOX (e) antisense probes. SHOX2 expression was shown more proximal than that of SHOX. SHOX expression was confined to the middle portion of the arm, most highly in the perichondrial tissue. Note the lack of expression of SHOX in the ribs and vertebral body. Expression of SHOX2 can also be seen in the dorsal root ganglia. Apparent expression in the region of the heart and lungs is due to artefact caused by residual blood in the sections and is also seen in the control sense probe hybridizations (data not shown). (f) Bright-field microscopy of haematoxylin- and eosin-stained adjacent section to (d) and (e). H, humerus; R, radius; C, carpal bone; MC, metacarpal; AM, invading abductor muscles of the arm; He, heart; DRG, dorsal root ganglion; VB, vertebral body; Ri, rib. Bar, 500 µm.
SHOX expression is more intense in the outer mesenchymal layers. Furthermore, SHOX expression is intense laterally and diminishes medially, whereas the reverse is true of SHOX2 (data not shown). In summary, in both the limb and the pharyngeal arches there are regions of overlapping expression of SHOX and SHOX2. Overall, however, SHOX expression is limited to few places and tissues, whereas SHOX2 is expressed in a much more diverse range of tissues. This argues against a total functional redundancy for these genes.

The strongest expression of SHOX is in the middle portion of the limb, in particular the elbow and knee. This would help to explain the bowing and shortening of the forearms and lower legs as seen in Leri–Weill patients and some Turner females, and the characteristic cubitus valgus and genu valgum (or ‘increased carry angle’) in Turner females. In a similar way, SHOX expression in the distal ulna/radius and wrist (Fig. 3e) can explain the Madelung deformity and shortened metacarpals seen in Turner females (23). This conclusion is supported by the finding that SHOX mutation patients also show some of the skeletal features of Turner patients. Studies of other genes have already provided evidence of phenotypes that closely correlate with their respective expression patterns. For example, Pax3 is expressed in the somite, neural tube and neural crest, and defects in these structures are found in Splotch (Sp) mice and Waardenburg syndrome. Similarly, Pax6 is expressed during eye development and leads to eye abnormalities in Drosophila (Eyeless), mice (Sey) and humans (aniridia) (24).

The variability of the manifestations of the skeletal features even within the same family is reminiscent of situations seen in Leri–Weill and Turner syndrome (6,7,11–13). Phenotypic heterogeneity and intrafamilial variability are frequently found in haploinsufficiency syndromes (25), suggesting that multiple interactions of the participating determinants are particularly sensitive to disturbance, leading to imbalance. Modifier genes, epigenetic interactions and stochastic effects have been hypothesized to explain these phenomena (26). In addition, mosaicism frequently presents in females with Turner syndrome and has been shown to contribute to the observed phenotypic variability.

The finding of SHOX expression in the first and second pharyngeal arches is of special interest with regard to other features common in Turner syndrome. Some Turner females have a high-arched palate, microglossia and sensorineural deafness (3,27,28). They also have a higher propensity for middle ear infections (3). The associated otitis media and hearing defects in Turner females may represent secondary effects of the skeletal anomaly of the external ears. All of the structures involved in these disorders, the palatine maxillary shelves, mandible, auricular ossicles and the external auditory meatus, are formed from the mesenchyme of the first and second pharyngeal arches. This strongly supports the proposed role of SHOX in the morphology and growth of certain bone, or mesoderm-derived structures. SHOX expression, however, was not detected in the axial skeleton (Fig. 3e) or developing skull (data not shown). In addition, we have not detected SHOX expression during cardiac or renal organogenesis, or during angiogenesis, suggesting that SHOX is unlikely to have a role in the (embryonic) development of the non-skeletal somatic features of the Turner phenotype.

The XO mouse was initially considered to be a model for Turner syndrome. However, the XO effect in mouse is much less...
severe than in the human. XO mice are fertile, although fertility is reduced, and in utero loss is lower compared with in utero loss in Turner females. Furthermore, there is no obvious evidence of any physical abnormalities, including altered growth (29–33). The lack of a direct murine SHOX orthologue can now explain some of the discrepancies between human and murine XO phenotypes. Our study thus also highlights the importance of human embryonic work, and the comparison of its results to murine expression data, in particular in the characterization of human developmental genes lacking direct mouse orthologues (34).

In summary, our results strongly suggest that the function of SHOX extends far beyond its most obvious and immediate role in final height (short stature). Our data have uncovered its important role as a regulator of normal and abnormal development in specific tissues that correlate with the skeletal defects seen in Turner syndrome. The identification of patients with a normal karyotype, but with some of the skeletal features of Turner syndrome, will now provide useful clinical criteria for mutational analysis of SHOX within the heterogeneous group of patients with idiopathic short stature.

MATERIALS AND METHODS

Patient description

Complete physical examination and radiographic studies of all five members of the German family A previously reported to be affected by the point mutation C674T of the SHOX gene (R195X) (10) revealed the following signs besides short stature. The female proband, her brother and the affected grandmother had a short neck. The brother additionally presented a genu valgum. Using radiography of the forearms, we detected bowing of the radius and ulna in the mother, who did not exhibit dislocation of the ulna or any reduction in mobility of the affected joints. In addition, the mother, the affected sister of the mother and the affected grandmother suffered from lumbar scoliosis.

The female Australian proband B3 of 29 years and 150 cm (–2.3 SDS) is the third child in a sibship of nine and the only affected member of the family. She presents a de novo SHOX R195X nonsense mutation, which is not seen in her parents [mother 165 cm (+0.2 SDS), father 178 cm (+0.2 SDS)] nor adult sisters [160 cm (–0.6 SDS), 170 cm (+1.1 SDS), 168 cm (+0.7 SDS)] and one sister 10 years of age [142 cm (+0.3 SDS)] nor adult brothers [178 cm (0.2 SDS), 188 cm (+1.7 SDS), 196 cm (+2.9 SDS) and 185 cm (+1.2 SDS)]. At the time of the initial clinical assessment due to bilateral wrist pain at 12 years of age, proband B3 had not grown over the three preceding years, according to her parents. Her height was 135 cm (–2.8 SDS) (no other growth records available) and she had mesomelic shortening of the limbs with a bilateral Madelung deformity and bilateral hypoplastic fourth metacarpals. She had a normal exercise response to growth hormone. X-ray analysis confirmed bilateral Madelung deformity, showing epiphyseal distortion of the radial head, premature fusion of the lateral aspects of the distal epiphyses of the radii and proximal displacement of the carpal row of bone related to the wrist joint. There was radial bowing/shortening, plus an increased carrying angle of both elbows. The bone age was 11.2 years at chronological age 12.16 years. At 12 years of age, she underwent bilateral partial radial epiphysiolysis (the Vickers Procedure) to correct the Madelung deformity, with a good result. At 13.4 years, her height was 150 cm (–1.8 SDS) and at 29 years it was 150 cm (–2.3 SDS).

RNA hybridization on human embryo sections

Ethical permission was obtained from the Joint Ethics Committee of the Newcastle Health Authority for the collection and use of human embryos. They were collected following either surgical or medical termination (35). After developmental staging using a stereomicroscope, the embryos were fixed in 4% paraformaldehyde and embedded in paraffin wax. Tissue sections were cut in 5 µm intervals. Sense and antisense riboprobes, labelled with [35S]UTP, were prepared from linearized plasmids containing cDNA subclones of SHOXa [600 bp sequence from position 997 of the coding sequence and containing 500 bp of the 3′-untranslated region (3′-UTR)] or SHOX2 (400 bp entirely from the 3′-UTR), transcribed with the RNA polymerases T7 and SP6. SHOXa and SHOXb are differentially spliced products of the SHOX gene, which differ only in the 3′-UTR and a very small region of the coding sequence. The SHOX 3′-UTR contains extensive repetitive sequence. SHOXa and SHOXb have identical 3′-UTR sequences and differ only in the extent of their 5′

Figure 5. Comparison of SHOX and SHOX2 expression domains in the developing limb. Digitally superimposed expression patterns (Adobe Photoshop) of SHOX (green) and SHOX2 (red) in the upper limb at CS18 (a) and CS21 (b). SHOX2 expression is more medial and proximal than that of SHOX. SHOX expression is more intense around the developing bone. Note that there is a degree of overlap in the connective tissue surrounding the developing bone. SOX9 (yellow) was used as a positive control for early bone development (38).
coding region. The SOX9 probe is generated from IMAGE clone ESTW95308, which corresponds to nucleotides 2488–3125 of the SOX9 cDNA (GenBank accession no. Z46629). Tissue in situ hybridization was performed at 52°C using established protocols (36), with either sense or antisense probes at a concentration of 1 × 10⁶ c.p.m./ml. Hybridizations with experimental antisense and control sense probes were carried out on adjacent sections. No signal was seen with the control sense probes except as an artefact to residual blood in the sections (see legend to Fig. 3d and e) (data not shown). After stringent washes, slides were coated in Ilford G5 photographic emulsion and exposed for 10 days. Sections were viewed using darkfield illumination and video captured using the KS2000 system (Imaging Associates, Thame, UK).

RNA hybridization on whole-mount mouse embryos

Sense and antisense probes were generated from an Og12x cDNA fragment corresponding to cDNA positions 926–1533 (identical between Og12xa and Og12xb). Whole-mount in situ hybridization was performed as described (37).

Southern zooblot hybridizations

Southern zooblots (Genetic Model no. 353 and Mammalian Model no. 329) were purchased from BIOS Laboratories (New Haven, CT). Hybridizations were carried out in 5x SSPE, 10x Denhardt’s, 2% SDS, 10 µg/ml salmon sperm DNA at 60°C and washed in 2x SSC/0.1% SDS at 58°C.

SSCP analysis

SSCP analysis was performed on genomic amplified DNA from short stature patients as described (10). Primers and reaction conditions were as described (10).

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