The Post-Translational Phenotype of Collagen Synthesized by Saos-2 Osteosarcoma Cells

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Abstract

The human osteosarcoma-derived cell line SAOS-2, exhibits many of the phenotypic characteristics of osteoblasts including the deposition of types I and V collagens in an extracellular matrix. Lesser amounts of collagen XI chains were also detected. The cell layer collagen contains hydroxylysyl pyridinoline cross-links but without the accompanying lysyl pyridinoline typical of human bone collagen. This indicates that the lysine residues at the two helical cross-linking loci are fully hydroxylated. The isoform of lysyl hydroxylase, LH1, known to be required for full hydroxylation at these sites, was shown to be highly expressed by SAOS-2 cells. Our findings provide insight on the mechanism of post-translational overmodification of lysine residues in collagen made by osteosarcoma tumors, and may be relevant for understanding a similar overmodification observed in osteoporotic bone.

Keywords

Bone; type I collagen; type V collagen; osteosarcoma; pyridinoline; lysyl hydroxylase; protein mass spectrometry

Introduction

Bone type I collagen can be distinguished from type I collagen of other tissues by its characteristic post-translational chemistry [1]. In particular, it has a distinctive profile of cross-linking amino acids that reflects the degree of hydroxylation of specific lysines in the telopeptide and helical cross-linking domains of the α1(I) and α2(I) chains. Hydroxylysyl pyridinoline (HP) cross-links are formed from two telopeptide and one helical hydroxylysine (Figure 1). Lysyl pyridinoline (LP) cross-links are a post-translational variant seen most prominently in bone collagen that are formed from two telopeptide hydroxylysines and a helical lysine [2,3].

Lysines are hydroxylated in collagen by the lysyl hydroxylase (PLOD, LH) family of enzymes [4]. The human genome has three genes PLOD1, PLOD2 and PLOD3 that encode three different isoforms, lysyl hydroxlyase 1, 2 and 3 (LH1, LH2, LH3) [5-8]. Early studies showed

that LH1 could hydroxylate lysine residues in the triple helical domains of α1(I) and α2(I) chains of type I collagen [4]. There is evidence that LH2 (which has 2 splice forms, LH2a and LH2b, [9]) hydroxylates cross-linking lysines in collagen telopeptide domains [10]. LH3, the most recently identified isoform [7], is essential for lysine hydroxylation in basement membrane type IV collagen [11]. LH3 also has glucosyl and galactosyl transferase activity, and so could add sugars to specific hydroxylysine side-chains [12]. Expression of lysyl hydroxylase isoforms is thought to be a factor in regulating tissue-dependent differences in cross-linking of collagen.

Lysyl hydroxylases are essential for normal development. Null mutations in PLOD 1 cause Ehlers Danlos syndrome type VIA (EDS-VIA) in which hydroxylysine is absent from skin collagen and deficient in other tissues [13-15]. In normal bone collagen the ratio of LP (lysyl pyridinoline) to HP (hydroxylysyl pyridinoline) is much higher than in other collagens (cartilage, tendon, ligament, etc.) that use the pyridinoline cross-linking pathway (0.25 to 0.5 versus <0.1). This is because the helical lysines, which donate the ring-nitrogen arm of the pyridinoline structure, are only partially hydroxylated in bone collagen [16,17]. Mutations in PLOD2 cause Bruck Syndrome, a bone disorder resembling osteogenesis imperfecta [18], in which the bone collagen lacks pyridinolines and the other cross-links based on hydroxylysine aldehydes. Since the telopeptide lysines are not hydroxylated, this implies that LH2 is the telopeptide lysine hydroxylase [10]. Mice in which *plod3* is null have defective basement membranes due to underhydroxylation of collagen type IV [11]. These genetic and molecular studies indicate that the three isoforms of lysyl hydroxylase act in a tissue-specific and collagen site-specific manner in the post-translational processing of collagens.

The cross-linking lysine residues of bone collagen appear to be partially hydroxylated in a distinctive pattern, resulting in the characteristic HP:LP ratio and the prominence of pyrrole cross-links in bone collagen [2,3,19]. How this is controlled is not fully clear but differential expression of LH1, 2 and 3 by osteoblasts seems to be involved. The overall content of hydroxylysine in human bone collagen has been shown to vary greatly with stage of bone maturity [20], being higher, for example, in osteosarcoma bone [21,22], osteoporotic cancellous bone [23] and woven repair bone [24] than in normal mature bone. Elevated levels of hydroxylysyl pyridinoline (a marker of bone degradation) and a high HP:LP ratio in the urine of osteosarcoma patients [25,26] is likely to be due to the degradation of bone collagen with a high degree of lysine hydroxylation.

Here we report on the post-translational quality of collagen deposited extracellularly by SAOS-2 osteosarcoma cells [27]. These cells express an osteoblastic phenotype [27-31], mineralize their matrix in the presence of β-glycerophosphate [32-35] and have been used extensively in bone biology research [36-40]. The SAOS-2 cell line synthesizes primarily types I and V collagens and deposits them in a copious extracellular matrix [35,41]. We show here, the collagen contains pyridinoline cross-links at a level typical of human bone collagen but the ratio of HP:LP cross-links is high. The demonstrated high expression level of the LH1 enzyme can explain the high HP:LP ratio.

Materials and methods

Cell culture

The SAOS-2 cell line (ATCC # HTB 85) was maintained as monolayer cultures in a humidified 37°C incubator, 5% CO_2 in air. McCoys media containing 10% FBS and 50 μg/ml ascorbate was exchanged every other day and the cells were cultured for 4 weeks. The harvested medium from the entire culture period was pooled and frozen for collagen analysis.

Isolation of collagen from the cultured cell extracellular matrix

After a month in culture, cell layers were washed with PBS and then extracted with 1M NaCl, 50 mM Tris, pH 7.5, containing 1 mM phenyl methyl sulfonyl fluoride (PMSF), 1 mM n-ethyl maleimide (NEM), 5 mM ethylenediaminetetraacetic acid (EDTA) for 2 days at 4°C. The NaCl extract (N) and the insoluble residue were separated by centrifugation at 18000 RPM (25000g). The residue was further digested with 0.1 mg/ml pepsin in 0.5M acetic acid for 24 hours at 4° C to solubilize the cross-linked collagen. Type I collagen was purified from the pepsin digest as a precipitate by centrifugation after adding NaCl to 0.8M. The insoluble residue was not analyzed further.

Collagen in the NaCl extract was precipitated by adding NaCl to 4.5 M. A portion was the treated with 0.1 mg/ml pepsin in 0.5M acetic acid and run on SDS-PAGE. The medium was acidified, digested with 0.1mg/ml pepsin and collagen was precipitated at 0.8M NaCl.

Gel electrophoresis

Collagen types in the medium and cell layer were identified by electrophoresis of the pepsin-digested protein. All samples were dried, dissolved in Laemmli sample buffer without reducing agent and heated at 100°C for 3 min. Collagen chains were electrophoresed on 6% gels [42] and visualized by Coomassie blue staining. Human bone type I collagen was used as standard. Gels were scanned densitometrically and the NIH Image software program with the gel electrophoresis macro was used to determine approximate ratios of collagen types in the extracts [43].

Mass spectrometry

Collagen in the 1M NaCl extract was separated by SDS-PAGE under reducing conditions and stained with Coomassie Blue. Individual bands were cut from the gel and subjected to in-gel trypsin digestion. Electrospray MS was performed on the tryptic peptides using an LCQ Deca XP ion-trap mass spectrometer equipped with in-line liquid chromatography (LC) (ThermoFinnigan) using a C8 capillary column (300μm × 150mm; Grace Vydac 208MS5.315). For protein identification, MS/MS spectra were searched again the NCBI nrfasta database using the SEQUEST database search algorithm.

Collagen cross-link analysis

Aliquots of pepsin-extracted collagen were hydrolyzed in 6M HCl, 110°C for 24 hours. Hydroxylysyl pyridinoline (HP) and lysyl pyridinoline (LP) cross-linking residues were resolved and quantified by RP-HPLC and fluorometry [44,45]. An aliquot of the hydrolysate was colorimetrically assayed for hydroxyproline as a measure of collagen content [46]. Pyridinoline content was expressed as moles per mole of collagen. Human bone type I collagen (fetal and adult) was run in comparison.

Preparation of polyclonal antibody

Recombinant LH1 antigen was generated in *E. coli* as a maltose-binding protein (MBP)-LH1 fusion product. The coding sequence of LH1 was amplified from SAOS-2 cells by RT-PCR using primer CAT ACC GAA TTC ATG CGG CCC CTG CTG CTA CT (92-111 of PLOD1 cDNA, containing an *EcoRI* site) and (AAG AAA GGT CCA AGA GGGTC 2307-2288 of PLOD1 cDNA, adjacent to a *StuI* site), then digested with *EcoRI* and *StuI*, and cloned into the EcoRI-EcoRV sites of Blue Script (Stratagene, La Jolla, CA) as clone "SAOS-LH1". The LH insert was transferred from SAOS-LH1 into the pMAL-cRI vector (New England BioLabs) as an *EcoRI*-HindIII fragment, placing LH in frame with MBP, expressed in *E. coli* strain XL1-blue (Stratagene), and gel-purified from the inclusion bodies by SDS-PAGE. In-frame fusion

was confirmed by N-terminal sequencing [47] of the LH peptide after release from MBP by factor Xa digestion.

Antibodies were generated in rabbits (R and R Rabbitry; Stanwood, WA) by repeated subcutaneous injections of the MBP-LH suspension (100 μg initial injection, and 5 boosts of 50 μg each at two week intervals). LH1-directed antibodies were enriched from crude serum by adsorption to a poly-histidine-LH1 (His-Tag-LH1) fusion protein, bound to a nickel column made with His-Bind Resin (Novagen). The His-TagLH1 fusion construct was made in pET-15b (Novagen).

Immunoblot analysis

Total lysates from plates of cultured cells were prepared by draining the plate for 2-3 min, shaking out all excess liquid, adding 0.5ml SDS sample buffer (2% w/v SDS, 5% v/v b-mercaptoethanol, 10 mM EDTA, 50 mM Tris, 0.1% w/v bromophenol blue, 10% v/v glycerol, pH 7.0), scraping the plate contents into a microfuge tube, and incubating at 95-100°C for 5 min with occasional vortexing. Lysates were cleared of insoluble material by centrifugation at 10,000 g. Total tissue lysates were prepared by powdering frozen tissue under liquid nitrogen in a SPEX 6700 Freezer/Mill (SPEX Industries), suspending it in about 10 volumes of SDS sample buffer, and heating as above. LH1 protein was enriched from placental homogenates by differential precipitation with ammonium sulfate and concanavalin-A affinity chromatography (4). Equal aliquots of protein in samples were separated by SDS-PAGE on 8% polyacrylamide gels, and transferred to PVDF membranes using a semi-dry electrotransfer apparatus (Millipore). Blots were blocked, and probed according to standard procedures (48), using 500X diluted purified LH1 antibody (see above), and 5000X diluted goat anti-rabbit IgG-horse radish peroxidase (BioRad) as secondary antibody. Bound horseradish peroxidase was detected by chemiluminescence using enhanced Luminol reagent (New England Nuclear) and Hyperfilm-MP (Amersham).

RNAase protection assays

A riboprobe template was generated by cloning the 208 bp PstI-NspI fragment of LH1 cDNA (nucleotides 947-1154) [5] between the PstI and EcoRV sites of the plasmid, pBC KS (Stratagene). The cDNA was generated by RT-PCR from the human osteosarcoma cell line, SAOS-2. An antisense RNA probe, including 46 bases of vector sequence and 208 bases of cDNA sequence, was synthesized from the T7 promoter, using the Riboprobe T7/T3 Combination kit from Promega. Probe was hybridized to cellular RNA and unbound probe was digested, using the RPII RNAase Protection kit from Ambion. Protected probe was resolved by acrylamide gel electrophoresis and detected by autoradiography and quantified by densitometry.

Northern blot analysis

RNA from SAOS-2 cells, normal fetal human skin and Swarm Rat chondrosarcoma (RCS-LTC) chondrocytes [47] was electrophoresed (5 μg/lane) and probed following the protocol described in [48]. Blots were prehybridized and the membrane bound RNA was hybridized to ^{32}P-labelled LH1 cDNA. Detection was by autoradiography.

RT-PCR analysis of lysyl hydroxylase gene expression

RNA was extracted using TriZol (Invitrogen) and purified according to the manufacturer's protocol. cDNA was synthesized using the Thermoscript RT-PCR system (Invitrogen). A co-RT-PCR assay was developed that yielded 748, 879, 434 and 378 bp products specific for LH1, LH2, G3PDH and COL1A1, respectively, in a single reaction. The primers used were as follows: LH1; Fwd: GTTTCCAGCCCCTGCGAGCGCCGC; Rev:

CAGGTTCCTCGCTCTCACATGGC LH2; Fwd:
ATGGGGGGATGCACGGTGAAGCCTC Rev:
ATCTACTGCAGACAAGTCGACTGTATCG G3PDH; Fwd;
AGCTTGCTCAAGAAGACAGACC Rev: GAGGACATCCCCTCTTCTCAC COL1A1;
Fwd GTGGACCTCCGGCTCCTGCTCC Rev GAAGTCCAGGCTGTCCAGGGATGC. The
primers designed for LH2 do not discriminate between LH2a and LH2b splice forms. During
each mRNA amplification reaction, each cycle of PCR included 1 min of denaturation at 94°
C, 1 min of annealing at 60°C and 3 min of extension at 72°C for 35 cycles.

The assay was applied to mRNA extracted from SAOS-2 cells, human foreskin fibroblasts,
CH1 human chondrosarcoma cells (a gift from Dr. Linda Sandell), mature human osteoblasts
and mRNA from fetal human bone, cartilage, skin and various other fetal human tissues. To
keep the amplification within the linear range for all the four gene products in the same reaction,
the cycle number was reduced to 25, and the sensitivity increased by ^{32}P-labelling. To assess
relative levels of LH1 message, dCT^{32}P labelled RT-PCR products were separated on 6%
polyacrylamide gels and semi-quantified by densitometry using NIH Image software [49-51].
G3PDH, COL1A1 or LH2 was used as reference.

Comparative genome analysis of LH1

Regions of the PLOD1 (LH1) gene and COL10A1 gene (reference) were amplified by
multiplex-PCR using 300 ng genomic DNA from human fetal liver or SAOS-2 cells as
described in [51]. Using primers for LH1: Fwd AGGACTGGAAGGAGAAGTACATCC, Rev
GGTCCCACCTTGTTGTTGCCCAG and COL10A1: Fwd
GGCCCAGCTGGCATAGCAACTAAGGG Rev
CTCCCTGAAGCCTGATCCAGGTAGCC yielded products of 800bp and 530 bp
respectively. PCR was carried out at 1 min of denaturation at 94°C, 1 min of annealing at 60°
C and 3 min of extension at 72°C for 35 cycles. After electrophoresis, products were quantified
by densitometry.

Results

Characterization of collagen

On pepsin digestion the medium and cell layer gave chains of types I and V collagens on SDS-
PAGE by Coomassie staining (Figure 2A). The α1(I) and α2(I) chains ran slightly slower than
pepsin-extracted control preparations from human bone, suggesting a higher level of post-
translational modification.

Of the total collagen synthesized, 65% was present in the medium, 2% in the 1M NaCl extract
and 33% in the pepsin extract of the cell layer. The insolubility of the cell-layer collagen without
pepsin digestion indicated extensive covalent cross-linking.

Type I collagen was the major collagen synthesized by the cell line accounting for 80% of the
total medium plus cell layer collagen. Most of the collagen found in the medium was type I
(Table 1). About equal amounts of types I and V collagen were recovered in the pepsin extract
of the cell layer (Table 1), indicating a preferential deposition of type V collagen in cross-
linked extracellular collagen fibrils. Type III collagen was not detected.

In-gel trypsin digestion and mass spectroscopic analysis of individual bands resolved by SDS-
PAGE from the non-pepsin-treated 1M NaCl fraction identified higher molecular weight forms
of α1(V) and α2(V) shown in Fig. 2B. The band indicated by the arrow in Figure 2B was a
pro-form of the α1(XI) chain. Presumably these components of the cell-layer matrix are
partially processed pro-forms that are not yet cross-linked. This cell line is known to express
the COL11A2 gene and export α2(XI) chains of type XI collagen [41].

Cross-link analysis

RP-HPLC analysis of pyridinoline cross-links in the cell layer collagen showed HP alone in contrast with HP:LP ratios in the range of 2 to 4:1 typical of human adult bone collagen and human fetal bone collagen (Fig. 3, middle and lower panels) [3]. Cross-linking lysines at the triple-helical sites in the SAOS-2 collagen appear therefore to be fully hydroxylated, whereas in bone collagen they are partially hydroxylated. The total pyridinoline (HP+LP) concentration in the SAOS-2 collagen (0.28 mol/mol of collagen) was similar to that of adult human bone (0.24 mol/mol collagen) [1]. On further analysis, the pyridinolines were shown to be present primarily in type I collagen (data not shown).

Expression of lysyl hydroxylase

The high HP:LP cross-link ratio in SAOS-2 cell collagen implied a high level of LH1 activity. In order to determine if LH1 expression was unusual in SAOS-2 cells, a co-RTPCR assay was developed (Fig. 4a). The abundance of LH1 RNA was compared to those of three other RNAs encoding COL1A1, G3PDH, and LH2 in human bone osteoblasts, skin fibroblasts, CH1 cells and skin. Semi-quantitative comparison of LH1 mRNA to each of the other RNAs (Fig. 4b, LH1/COL1A1and 4c, LH1/LH2) showed that LH1 expression in SAOS-2 cells was unusually high. Regardless of the RNA chosen as standard, LH1 RNA was 10-30 fold higher in SAOS-2 cells than in bone or skin. Since cultured cells derived from other connective tissues (fibroblasts from skin, and a human cartilage chondrosarcoma cell line [CH1]) did not exhibit this elevated LH1 mRNA the effect is unlikely to be a culture artifact. LH2 mRNA levels in SAOS-2 cells were not decreased when compared to skin fibroblasts and bone tissue, suggesting that the high LH1 mRNA levels are not due to a compensatory mechanism in these cells (data not shown).

A wider survey of LH1 mRNA levels in human fetal tissues including bone and skin (Fig 5a), standardized to G3PDH, showed considerable variation, with particularly high levels in cartilage and in muscle (Fig. 5b). However, none of the tissues exhibited levels approaching that of SAOS-2 cells.

The high level of LH1 mRNA in SAOS-2 cells relative to skin fibroblasts was confirmed by Northern blot analysis (Fig. 6a), and quantified by RNAase protection assay (Fig. 6b), which indicated a 30-fold higher level of LH1 in the SAOS-2 cells than in human fetal skin. Since the relative levels of LH1 in bone and skin were comparable and because LH1 is a crucial enzyme for lysyl hydroxylation in skin [13-15], human skin fibroblasts and human fetal skin were used for this comparison rather than osteoblasts. Mutations in PLOD1 result in loss of lysine hydroxylation in skin type I collagen [14]. The RCS-LTC chondrocytes showed a reduced level of LH1 when compared to skin fibroblasts. The collagen from this cell line is known to have a lower HP:LP ratio when compared to normal cartilage [45].

Immunoblot analysis

Western blot analysis was used to determine if LH1 protein is expressed in SAOS-2 cells. As shown in Fig. 7, an antibody raised against recombinant LH1 binds the predicted 82-85 kDa LH1 band in SAOS-2 cell lysates. SAOS-2 cells gave several fold higher levels than skin fibroblasts of this LH1 band. The high mRNA levels in SAOS-2 cells were therefore reflected in higher protein levels.

Comparative genome analysis

One possible mechanism for the elevated LH1 expression could be amplification of the PLOD1 gene in SAOS-2 cells, due either to karyotype abnormalities or to tandem duplication of the gene. To address this possibility, a multiplex-PCR assay was developed, which compared the abundance of the PLOD1 sequence in genomic DNA to that of a reference gene, COL10A1.

The relative abundance of PLOD1 and collagen X PCR products was the same in SAOS-2 DNA as in normal human fetal liver DNA (Fig. 8), indicating a normal PLOD1 copy number in SAOS-2 cells. The high levels of LH1 RNA and protein are due to elevated expression and/ or stability of these products in SAOS-2 cells.

Discussion

The results of protein analysis show that human SAOS-2 osteosarcoma cells in monolayer culture deposit and cross-link type I and type V collagen as the principal components of the extracellular matrix (Figs. 2, 3), as in human bone [52,53] and osteosarcoma tumors [21]. No type III collagen was detected. Type III collagen can be a minor component of bone [52], where it is concentrated at soft tissue insertion sites [54]. Shapiro and Eyre found type III collagen in the soft tissue regions of human osteosarcoma [21].

About four-fifths of the synthesized collagen was type I and one-fifth type V. Mass spectrometry after SDS-PAGE and in-gel trypsin digestion of extracted matrix collagen confirmed that the dominant chains were $\alpha1(I)$, $\alpha2(I)$, $\alpha1(V)$ and $\alpha2(V)$. However, the $\alpha1(XI)$ chain of type XI collagen was also identified (Fig. 2B). By sensitive Western blot analysis, $\alpha2$ (XI) chains were previously detected in extracts of SAOS-2 cell layers, but the level was much lower than Coomassie Blue-stainable collagen I and V chains [41]. The $\alpha1(XI)$ and $\alpha2(XI)$ chains are presumably incorporated into trimers, perhaps in the form of hybrid V/XI molecules as suggested by findings on bovine bone matrix by Niyibizi and Eyre (1989) [52]. The lack of any $\alpha3(XI)$ chain (i.e., $\alpha1(II)$) rules out a typical type XI collagen molecule of composition $[\alpha1(XI)][\alpha2(XI)][\alpha3(XI)]$). Interestingly the COL11A2 gene has been found to be expressed in low amounts by normal mouse osteoblasts [55] and by osteochondrogenic tumors [56]. Together these findings indicate that the collagen phenotype of SAOS-2 cells is similar to that of human bone, but with a higher level of lysyl hydroxylation. Indeed the SAOS-2 cells express abundant osteoblast markers [27] and appear to have a more osteoblast-like phenotype than other human osteosarcoma cell lines [28].

The presence of hydroxylysyl pyridinoline (HP) cross-links and essential absence of the lysyl pyridinoline (LP; Fig. 3) that features in normal bone collagen [3] implies an essentially complete hydroxylation of Lys 87 and Lys 930 in $\alpha1(I)$ and Lys 87 and Lys 933 in $\alpha2(I)$ chains. RNAase protection assays (fig. 6B) and Western blot analysis (Fig. 7) showed higher LH1 expression in SAOS-2 cells compared with normal human skin fibroblasts. This may explain the complete hydroxylation of the cross-linking lysines in SAOS-2 type I collagen. In Ehlers Danlos Syndrome Type VI, the lack of hydroxylation of these lysines is due to absent LH1 activity [14], which in bone collagen results in a low HP:LP ratio [13,17].

Of the tissues surveyed by semi-quantitative RT-PCR (Figs. 4, 5), cartilage had the highest level of LH1 expression relative to G3PDH, but still less than that of SAOS-2 cells (Fig. 5). Both cartilage and SAOS-2 matrix collagens had a similarly high ratio of HP:LP indicating that the helical-domain cross-linking lysines were fully hydroxylated [3].

The findings support a role for LH1 in efficiently hydroxylating cross-linking lysine residues in the triple-helical region of collagen. Although elevated levels of LH1 have not yet been associated with any form of human disease, high levels of hydroxylysine and of glycated-hydroxylysine residues have been found in osteoporotic bone collagen, diabetic bone and skin collagen (reviewed in [57]), and osteosarcoma bone collagen [21,22]. Resorption of bone collagen with a high degree of lysine hydroxylation can explain the high HP:LP ratio in urine of osteosarcoma patients (25,26). Upregulation of LH1 activity in osteosarcoma cells can explain this. SAOS-2 cells may be useful for studying the mechanism of regulation of LH1 gene expression, which is unclear.

Finally, it is worth noting that lysyl oxidase (LOX) was recently implicated in tumor metastasis [58]. This enzyme initiates collagen cross-linking and it has been shown that the mechanical properties of a collagen substratum can modulate the behavior of osteoblasts and osteosarcoma cells *in vitro* [59]. A stiff matrix directs human mesenchymal stem cells to an osteoblast lineage [60]. It is possible, therefore, that variations in the post-translational chemistry of collagen that alter cross-linking and matrix stiffness can affect the attachment and migration of bone forming cells.

## ACKNOWLEDGEMENTS

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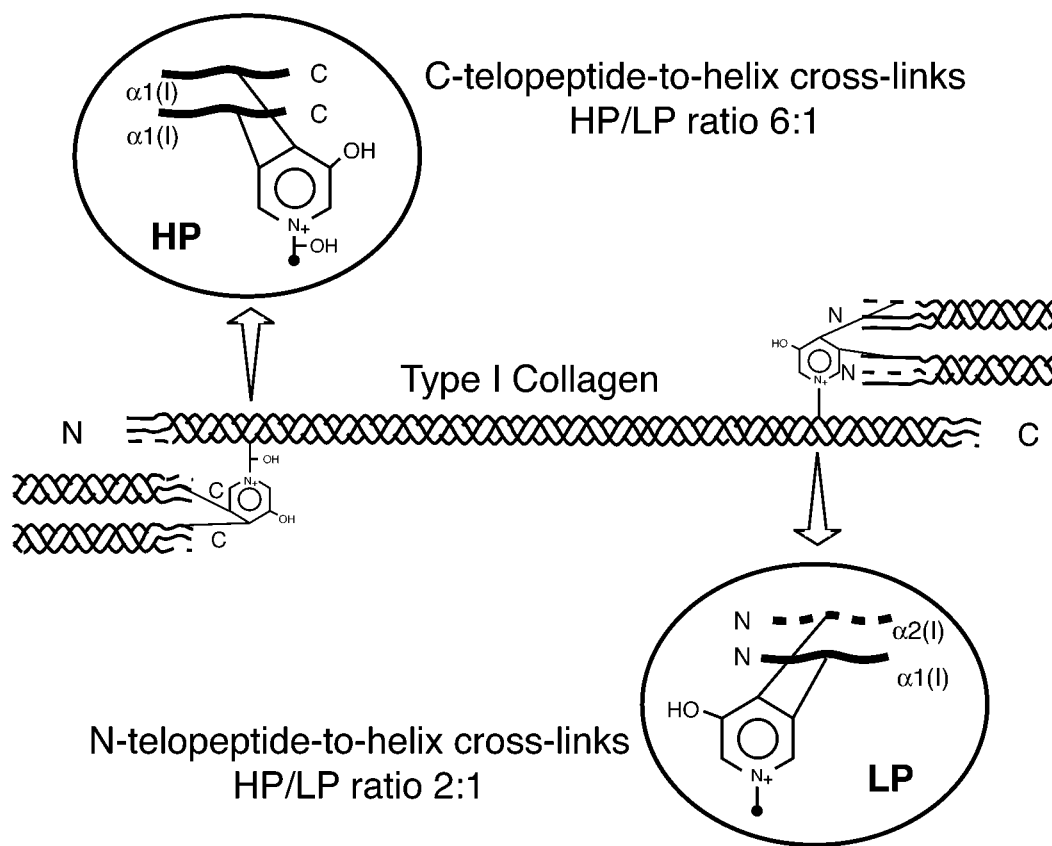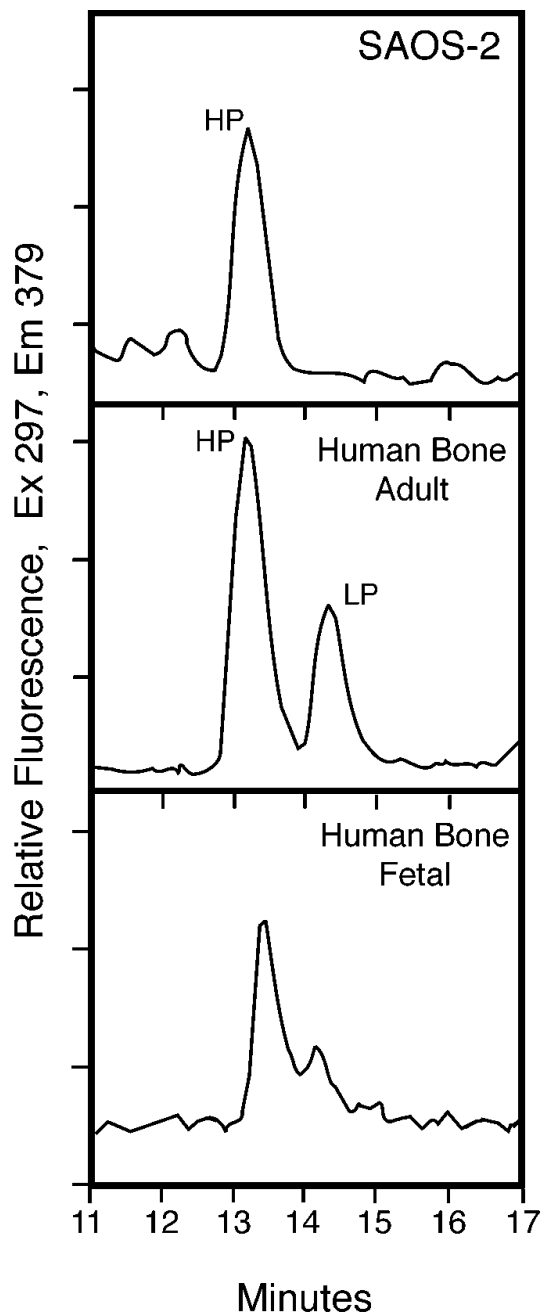
Figure 1.
Intermolecular cross-links in type I collagen
Diagram showing the sites, type and ratio of pyridinoline cross-links in bone type I collagen. Pyridinolines stabilize type I collagen fibrils of human bone at both ends of the molecule. HP is more abundant at the C-telopeptide-helix cross-link site (HP/LP ratio, 6:1) and LP is more abundant at the N-telopeptide-helix site (HP/LP ratio, 2:1) [19].
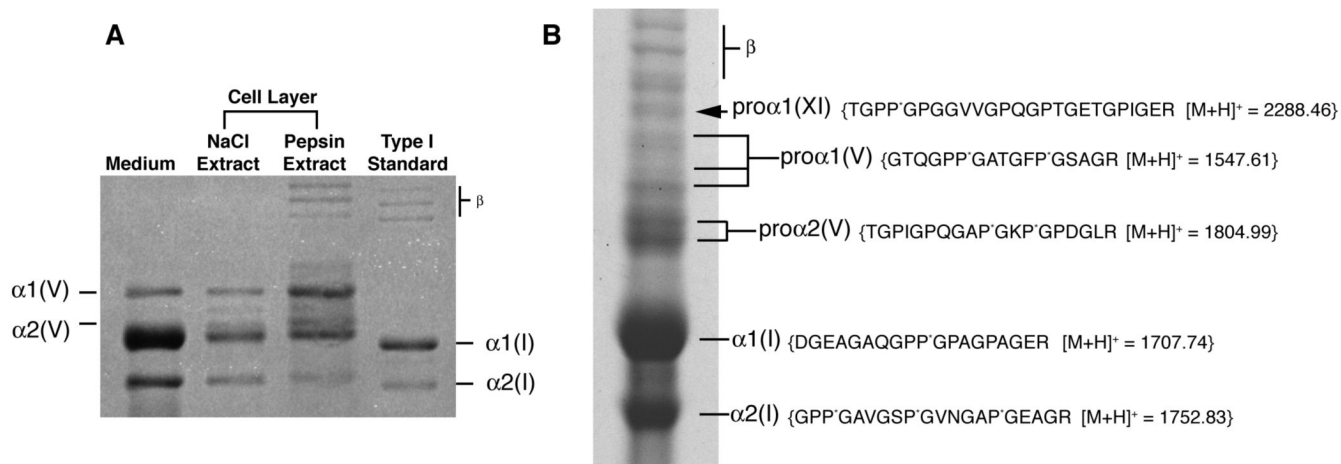
Figure 2.
Biochemical analysis of collagen
A) Collagen types synthesized by the SAOS-2 cell line in culture
The medium and cell layer on pepsin digestion showed types I and V collagen α-chains on
SDS-PAGE. The α1(I) and α2(I) chains ran slightly slower than pepsin extracted control
preparations from human bone, suggesting post-translational over-modification. Mass
spectrometry identified the band between the α1(V) and α2(V) to be a degradation product of
the α1(V) chain. Bands marked "β" are dimers of collagen chains.
B) Identification of α1(XI) collagen chains by mass spectrometry
SDS-PAGE (6%) of collagen extracted by 1M NaCl from the extracellular matrix of SAOS-2
cells (+DTT). Arrow indicates a band identified as a pro-form of the α1(XI) collagen chain.
The sequence of a tryptic peptide from this band unequivocally identified by mass spectrometry
is shown. Mass spectrometry also identified pro-forms of α1(V) and α2(V), as well as processed
α1(I) and α2(I) from this gel. Bands marked "β" are dimers of collagen chains.

Figure 3.
Pyridinoline cross-links in SAOS-2 cell layer collagen compared with human bone
RP-HPLC analysis of pyridinoline cross-links in the cell layer collagen showed HP alone
(upper panel) in contrast with the ratio of about 2:1 for HP:LP typical of adult human bone
(middle panel). This result indicates that the triple-helical domain cross-linking lysines were
fully hydroxylated in the SAOS-2 collagen, whereas in bone collagen they are only partially
hydroxylated.
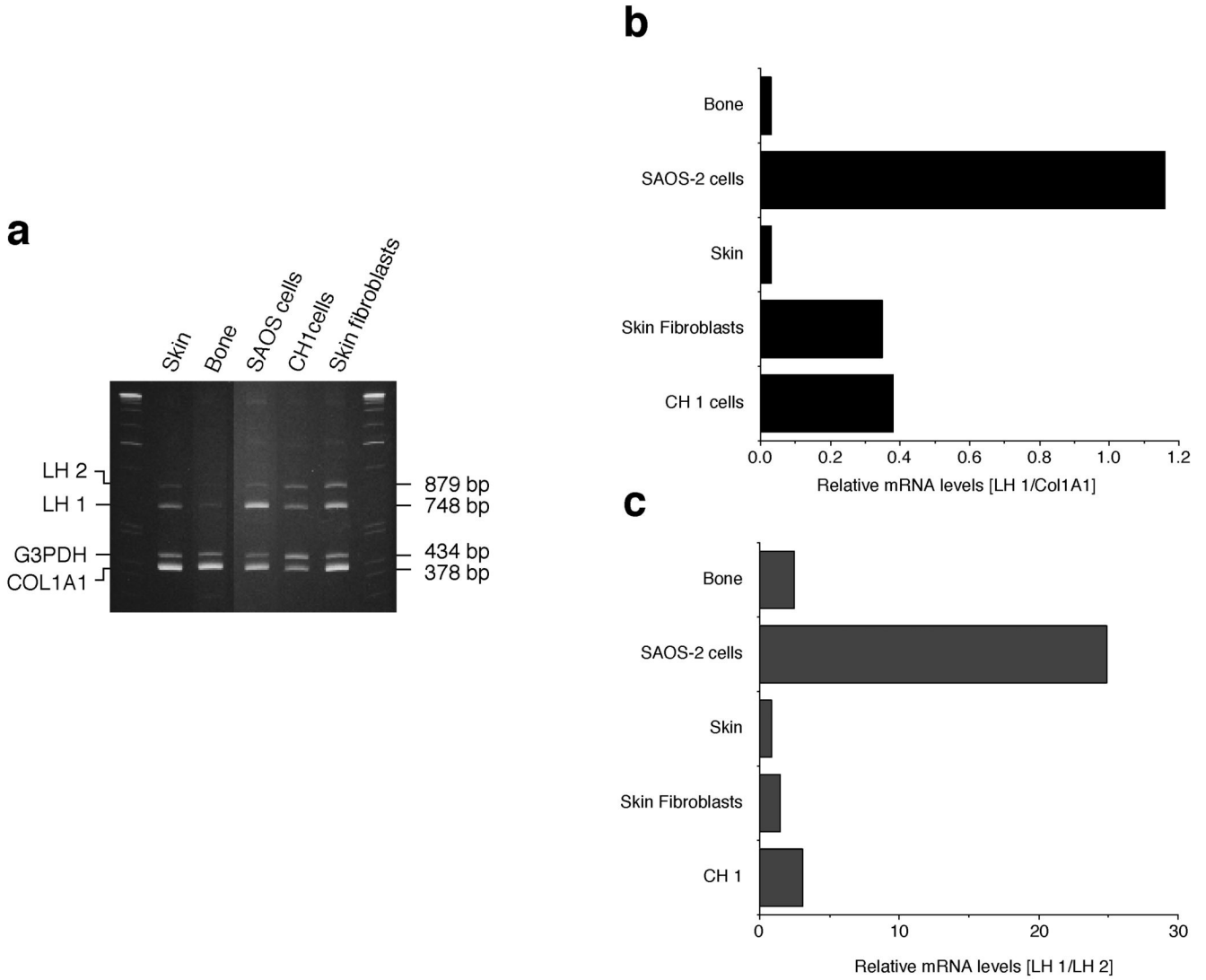
Figure 4.
Expression of lysyl hydroxylase mRNA
a) 6% polyacrylamide gels showing the ethidium bromide-stained RT-PCR (35 cycles) products of LH 1, LH2, G3PDH and COL1A1 from human skin, mature human osteoblasts, SAOS-2 cells, cultured human chondrosarcoma cells (CH1) and cultured skin fibroblasts.
b, c) A similar RT-PCR (25 cycles) experiment was performed after labelling with dCT^{32}P to assess relative levels of LH1 message. For each tissue or cell culture, RNA was amplified and resolved on a 6% gel. Autoradiograghs were scanned to compare bands quantitatively. As shown in the bar graphs, regardless of the RNA chosen as reference (COL1A1 or LH2), LH1 mRNA was much higher (10 to 30 fold) in SAOS-2 cells than in osteoblasts, skin and cultured cells.

a

b
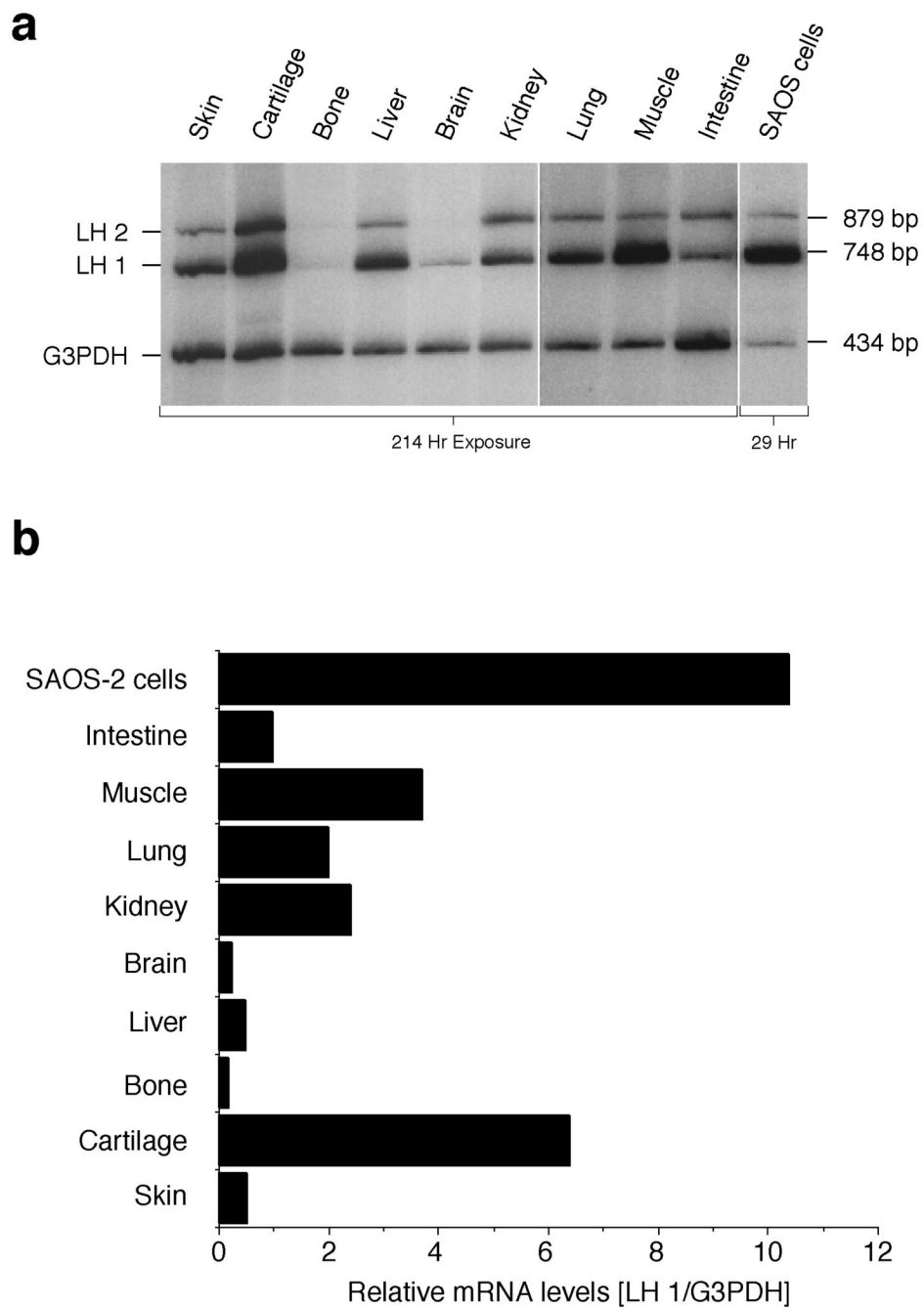
Figure 5.
Co-RT-PCR survey of LH1 mRNA levels in human tissues
LH1 and G3PDH RNAs were co-amplified from fetal human tissues, using $dCT^{32}P$ labelling conditions.
a) Products were separated by electrophoresis on 6% polyacrylamide gels, and detected by autoradiography.
b) Values represent the LH1 mRNA levels normalized to G3PDH as internal standard, as determined by quantitative scans of each lane. Large differences of LH1 mRNA levels were seen between the tissues, with particularly high levels in cartilage and muscle. None, however, exhibited levels approaching that of the SAOS-2 cells.
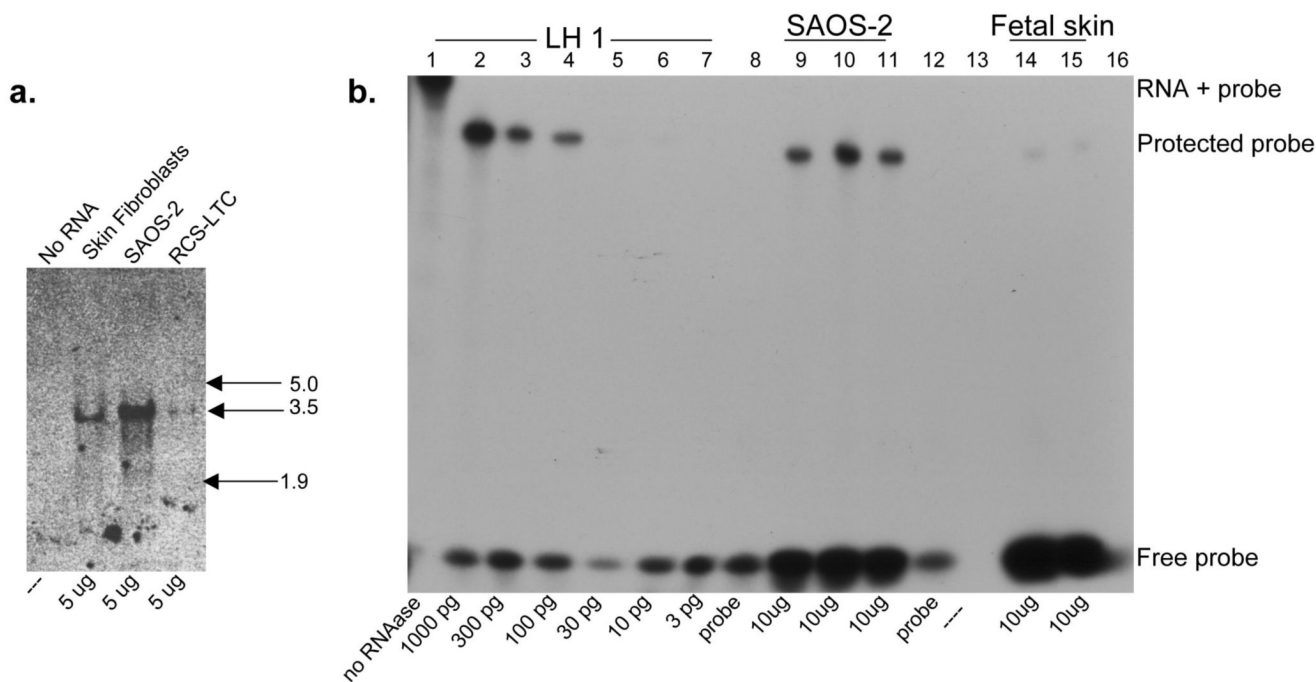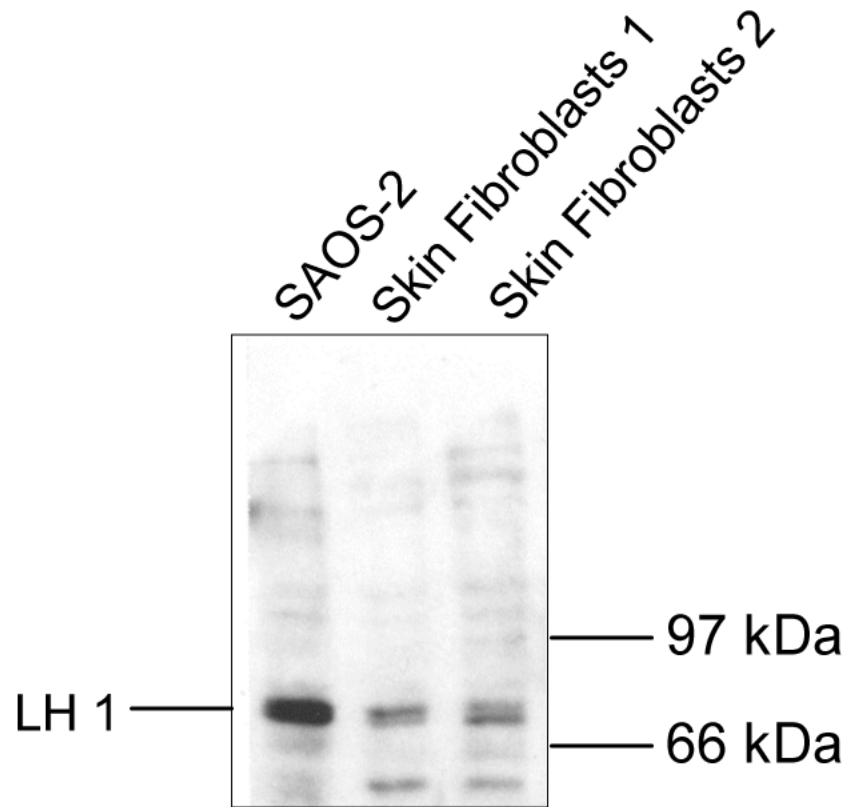
Figure 6.
a: Northern Blot analysis of LH1 mRNA in SAOS-2 cells and skin

5 μg of SAOS-2, human fetal skin and RCS-LTC chondrocyte cell line total RNA was resolved on a 1.2% agarose-formaldehyde gel, blotted to nitrocellulose membrane, probed with a ^{32}P-labelled LH1 cDNA, and detected by autoradiography. RNA kilobase markers are shown at right.

*b: Quantitation of LH1 mRNA in SAOS-2 cells and skin by RNAase protection.*
RNA was hybridized to a ^{32}P-labelled LH1 antisense probe, digested with nuclease to remove the non-homologous portion of the probe, resolved on a denaturing 6% acrylamide gel, and detected by autoradiography. Lane 1, Full-length probe prior to nuclease digestion. Lanes 2-7, Control LH1 RNA reactions containing 1000, 300, 100, 30, 10, and 3 pg LH1 RNA respectively. Lanes 9-11, Replicate reactions containing 10 μg SAOS-2 RNA. Lanes 14 and 15, Replicate reactions containing 10 μg human fetal skin RNA. Lane 8 and12, Probe only. Lanes 13 and 16 are empty.

Figure 7.
Expression of LH1 protein in cultured SAOS-2 cells
SDS-soluble protein from SAOS-2 cells (lanes 1) and cultured human skin fibroblasts (lanes 2, 3) were separated by SDS-PAGE, blotted to PVDF membrane, probed with an antibody to LH1, and detected with a chemiluminescence based secondary antibody assay. The background bands below 66 kDa are unidentified.
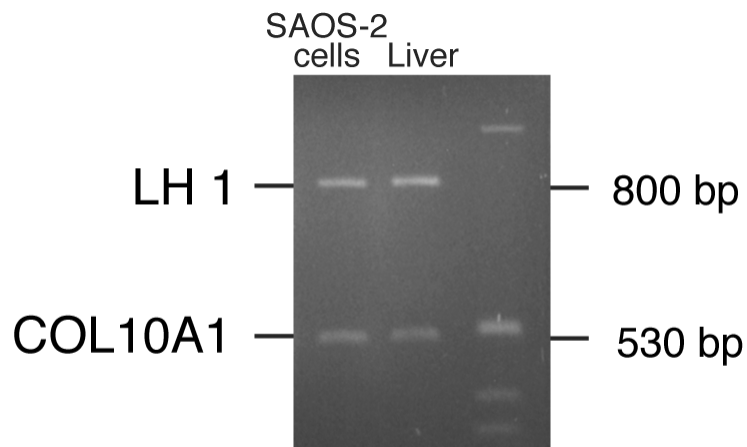
Figure 8.
LH1 gene copy number in SAOS-2 cells and human liver cells
A 6% polyacrylamide gel showing multiplex-PCR products of the LH1 gene (PLOD1) and type X collagen gene. The relative abundance of PLOD1 and type X collagen PCR products was similar in SAOS-2 DNA and in normal fetal liver DNA, indicating a normal LH1 copy number in SAOS-2 cells. Thus, the high levels of LH1 mRNA are due to elevated expression and/or stability of these products in SAOS-2 cells.

Table 1

Distribution of collagens type I and type V in the SAOS-2 cell culture extracts[a].

	Type I collagen	Type V collagen
Medium	90.2	9.8
NaCl extract	75	25
Pepsin extract	47.7	52.3

[a]Values expressed as percentages of total collagen in each extract