Xiao, Z. S., M. Crenshaw, R. Guo, T. Nesbitt, M. K. Drezner, and L. D. Quarles. Intrinsic mineralization defect in Hyp mouse osteoblasts. Am. J. Physiol. 275 (Endocrinol. Metab. 38): E700–E708, 1998.—X-linked hypophosphatemia (XLH) is inherited as a dominant disorder and is characterized by hypophosphatemia, growth retardation, and rickets/osteomalacia (1, 16). The genetic defect underlying XLH rickets has been identified as mutations in the PEX gene product, or the phosphate-regulating gene Pex with homologies to endopeptidases on the X chromosome (1, 12, 14, 15, 33, 35). The Hyp mouse, a murine homologue of XLH, also has a loss of function Pex deletion associated with renal phosphate wasting and defects in osteoblast-mediated mineralization (2, 31). This murine homologue provides a model to study the molecular and biochemical events linking PEX gene expression and that attendant accumulation of putative endogenous synthesized substrates of the gene product lead to impaired mineralization in XLH.

METHODS

Reagents. α-Minimum essential medium (α-MEM), DMEM/F-12, penicillin-streptomycin-amphotericin (antibiotic-antimycotic) solution, Hanks’ balanced salt solution (HBSS), and Trizol Reagent for single-step isolation of total RNA from cells were obtained from Gibco (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Hydone Laboratories (Logan, UT). Pronase E, ascorbic acid, β-glycerophosphate, BSA, p-nitropheno{l, diethanolamine, and p-nitrophenolphosphate used for alkaline phosphatase assay were purchased from Sigma (St. Louis, MO). [1-32P]dCTP were purchased from Du Pont-NEN (Boston, MA). Bio-Rad reagent for protein assay was obtained from Bio-Rad Laboratories (Hercules, CA).

Isolation and culture of immortalized osteoblasts and clonal osteoblast cell lines from normal and Hyp mouse calvaria. Mice were maintained and used in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals, prepared by the Institute on Laboratory Animal Resources, National Research Council (DHHS Publ. NIH 86–23, 1985), and by guidelines established by the Institutional Animal Care and Use Committee of Duke University.

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We established immortalized osteoblast cell lines from calvaria obtained from male normal and Hyp mice transgenic for the large T antigen of simian virus 40 (SV40).

These transgenic animals were created by mating C57BL/6j males, heterozygous for the SV40 large T antigen, with female Hyp mice, as previously described (21). Progeny containing the SV40 transgene were identified by PCR amplification of a 500-bp product of SV40 from individual genomic DNA, using forward primer 5′-CAGAGCAGAATTGTGGAAGTGG-3′ and reverse primer 5′-GGCAACAAACCAACAATGAGTCGTG-3′. The normal and Hyp mice littermates were distinguished among the SV40-positive mice on the basis of serum phosphorus values that were measured by colorimetric techniques on a Roche COBRAS MIRA-S.

We used a nonenzymatic method for obtaining the initial osteoblast cell lines (11). A fragment of the frontal and/or parietal bone from a single calvaria was aseptically removed from a 6- to 7-day-old mouse. Suture lines and endosteum were dissected away, and the bone fragment was placed in a culture dish. One or two metal strips were positioned on the basis of serum phosphorus values that were measured by colorimetric techniques on a Roche COBRAS MIRA-S.

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fragments were cloned into pCR 2.1 (Invitrogen) and confirmed as Pex by direct sequencing.

In addition, we performed RT-PCR to characterize osteoblast gene expression in clonal TMObs derived from Hyp and normal mice. We used the following primer sets to amplify osteopontin (mapo-F 5′-ACACTTTCACTGCAAATGTCC-3′ and mapo-R 5′-TGCCCCTTGTTGTTGCC-3′), osteocalcin (moc-F 5′-CAAAGCCACAGCAGCTT-3′ and moc-R 5′-AAAGCGAGCTGCCAGAGT-3′), and α3(1) procollagen (mα3-F 5′-TCTCCTACCTCTGATGCTCT-3′ and mα3-R 5′-TTGGGTACCTTTACAGACTG-3′). We used mouse β-actin primers (mActinF 5′-GGGCGGCTCTAGGCAACA-3′ and mActinR 5′-GCTGCCAGAGTT-3′) to amplify a 245-bp fragment as a control for the amount and integrity of RNA in the PCR reactions. Gel-separated products were blotted on Nytran membrane (Schleicher & Schuell, Keene, NH) and immobilized on the membrane by ultraviolet (UV) cross-linking with a Stratagene (Stratagene, La Jolla, CA). In some studies, the identity of the bands generated by PCR was confirmed by hybridization with radiolabeled Pex and β-actin cDNA probes.

Northern blot hybridizations. Northern analysis was carried out as described (14). Briefly, ≈20 µg of total RNA were electrophoresed on a 1.2% formaldehyde agarose gel and transferred to Nytran membrane (Schleicher & Schuell), and the RNA was immobilized on the membrane by UV cross-linking with a Stratagene (Stratagene). The blot was hybridized overnight at 42°C in the prehybridization solution containing 10% dextran sulfate and 2 × 10^6 counts·min^{-1}·ml^{-1} of the random-labeled mouse osteocalcin and 28S probe. The blot was washed twice for 1 min at room temperature in a solution containing 2× standard sodium citrate (SSC) and 0.1% SDS, followed by washing two additional times for 15 min at 50°C in a solution containing 0.1× SSC and 0.1% SDS. The blot was air dried, and the bands were visualized by autoradiography.

Southern blotting. For Southern blot analysis, genomic DNA (~10 µg) from normal and Hyp mouse osteoblasts was digested with EcoRI. The digested DNAs were electrophoresed on 0.7% agarose gel and blotted to nylon membranes (Schleicher & Schuell) by alkaline transfer. Hybridizations were generally performed in a hybridization buffer containing 1.5× SSPE (15 mM NaH2PO4, pH 7.4, 225 mM NaCl, and 1.5 mM EDTA), 1% SDS, and 10% dextran sulfate at 65°C overnight. A probe containing mouse Pex exons 7–22 was

Fig. 1. Expression of a phosphate-regulating gene with homologies to endopeptidases on the X chromosome (Pex) in normal and Hyp mouse osteoblasts. A: Southern blot analysis of genomic DNA derived from normal (NL) and Hyp mouse osteoblasts. Genomic DNA was digested by EcoRI and hybridized with [32P]-labeled Pex cDNA probe, as described in METHODS. A size calibration was obtained by simultaneous loading of X DNA/Hind III fragments and a 1-kb DNA ladder. Bands <4.0 kb (corresponding to exons 16–22 (40)) are absent in Hyp mouse, consistent with deletion of 3′ end of Pex gene. B: RT/PCR amplification of Pex transcripts using 5′ and 3′ specific primer pairs. To amplify 5′ and 3′ ends of Pex, respective primer pairs M-5F/M1619R and M-5F/M1983R were used (see METHODS) with total RNA derived from normal and Hyp mouse osteoblasts cultured for 10 days. Amplified products were transferred to nylon membranes and probed with a radiolabeled Pex cDNA probe. As a control, β-actin was amplified and identified by hybridization. The 3′ end of Pex could not be amplified from RNA derived from Hyp mouse osteoblasts, and the 5′ end was present in low abundance. Both 5′ and 3′ ends of Pex could be amplified in normal osteoblasts. C: RT-PCR detection of break points in Hyp mutant Pex transcripts. Using the forward primer M+786F in combination with exon 15 primer M+1619R and exon 16 primer M+1680R and RNA from normal and Hyp mouse osteoblasts, we found evidence for a deletion beyond exon 15.
labeled by random hexamer priming, and washing was done in 0.1× SSC, 0.1% SDS at 65°C for 15 min.

Coculture experiments. Coculture experiments were performed using a 6-well culture plate (Becton-Dickinson, Franklin Lakes, NJ) that contained a 10-cm² lower plate well size and a 4.2-cm² upper well insert that incorporated polyethylene terephthalate track-etched membrane (pore size 3 µm) to permit diffusion of soluble factors into a lower well. We plated TMOb-Nl and TMOb-Hyp cells in either the lower or upper well at an initial density of 40,000 cells per well to achieve coculture. Controls consisted of coculture of TMOb-Nl with TMOb-Nl and TMOb-Hyp with TMOb-Hyp cells. After 14 days in medium containing ascorbic acid and β-glycerophosphate as described above, mineralization was assessed by alizarin red-S staining and quantified by modification of previously described methods (30). Briefly, the stained matrix was washed with water and PBS, the dye was diluted with 10% (wt/vol) cetylpyridinium chloride, and the alizarin red-S was quantified at 562 nm.

Statistics. We evaluated differences between groups by one-way analysis of variance (29). All values are expressed as means ± SE. All computations were performed using the Statgraphic statistical graphics system (STSC, Rockville, MD).

RESULTS

Characterization of the Pex mutation in Hyp mouse osteoblasts. To confirm the presence of a Pex mutation in Hyp mice, we performed Southern blot analysis of genomic DNA using a Pex cDNA probe (Fig. 1A). Consistent with previous observations, we identified a 3’ deletion of the Pex gene beyond exon 15 (31). Accordingly, in TMOb-Hyp cells, RT-PCR amplification of the 3’ end of Pex, with primers designed to amplify the gene segment extending to exon 19, failed to produce an RNA product, whereas this region was amplified in TMOb-Nl cells (Fig. 1B, top). In contrast, using primers designed to amplify exons in the 5’ end of Pex, we identified the predicted-size band from normal as well as from Hyp mouse osteoblasts. However, in four separate experiments, Pex was in lower abundance in the mutant cells (Fig. 1B, middle). Additional RT-PCR studies with reverse primers to sequences in exons 15 and 16, in combination with an upstream 5’ primer (Fig. 1C), further defined the deletion break point. Consistent with a deletion break point between exons 15 and 16, primer pairs, including exon 15, amplified the predicted Pex transcript, whereas no product was obtained using exon 16 primers in Hyp mouse osteoblasts.

Phenotype characteristics of immortalized osteoblast cultures. In subsequent studies, we examined whether the immortalized cells exhibited a temporal sequence of maturation characterized by an initial period of replication and subsequent postmitotic expression of osteoblastic characteristics. Similar to primary cultures (22) and other established cell lines (24), both normal and Hyp mouse osteoblasts underwent an initial period of rapid cell proliferation that was characterized by increments in cell number (Fig. 2A) and high levels of DNA synthesis (Fig. 2C). Additionally, in both cell lines we observed a disproportionate increase in protein content relative to cell number after day 10 of culture (Fig. 2B), which corresponded to confluence of the cultures, a concordant decrement in the growth rate, and the formation of collagenous ECM (24). However, there was no significant difference between normal and Hyp mouse osteoblasts with regard to parameters of cell growth and protein content.

During the period of rapid cell growth, both TMOb-Nl and TMOb-Hyp cells expressed low levels of alkaline phosphatase (Fig. 2D), consistent with their immature, preosteoblastic state. As anticipated, however, down-regulation of replication was associated with a signifi-
tant increase in the expression of alkaline phosphatase activity in both TMOb-Nl and TMOb-Hyp cells (Fig. 2D), although by 14 days of culture, activity was slightly greater in normal cells. Similarly, the process of osteoblast maturation in the immortalized cells was marked by the absence of osteocalcin transcripts in 4-day-old cultures but with high levels of osteocalcin in 14-day-old cultures (data not shown). In concert, we found that Pex expression increased in TMOb-Nl and TMOb-Hyp cells as a function of culture duration, a temporal increase corresponding to the osteoblast maturation stage (Fig. 3). Thus both immortalized osteoblast cell lines retain their capacity in vitro to undergo a normal temporal upregulation of osteoblast-related gene expression. Collectively, these observations indicate that the immortalized cells represent an excellent in vitro model system in which to study the bone mineralization defect in XLH.

Impaired mineralization in TMOb-Hyp osteoblast cultures. In ensuing experiments, we assessed mineralization in normal and Hyp mouse osteoblasts by use of 45Ca incorporation and alizarin red-S histochemical staining. In immature TMOb-Nl cells, we observed a marked increase in activity in both mice osteoblasts by use of 45Ca incorporation and alizarin red-S histochemical staining. In immature TMOb-Nl cells, we observed the absence of mineralization (data not shown), whereas marked increments in 45Ca incorporation (Fig. 4A) that corresponded to the presence of alizarin red-S-stained mineralization nodules were observed in these cells by day 14 of culture (Fig. 4B). In contrast, mature Hyp mouse osteoblasts exhibited significantly less 45Ca incorporation (Fig. 4A) after 14 days of culture. Moreover, alizarin red-S staining revealed only ill-defined patches with limited dye uptake and the absence of discrete mineralization nodules (Fig. 4B), consistent with impaired mineralization. The impaired mineralization was not related to differences in the amount of collagen produced in the normal and Hyp mice osteoblast cultures. In this regard, hydroxyproline content was similar between TMOb-Nl and TMOb-Hyp cell culture-derived matrix (0.125 0.001 vs. 0.126 0.001 mg/mg dry wt).

Persistence of defective mineralization in clonal osteoblasts derived from TMOb-Hyp cell cultures. In addition, we showed that the impaired mineralization in Hyp mouse-derived osteoblasts was not attributable to differences in cellular composition of the cultures, because clonal cell lines obtained from the TMOb cultures displayed identical results (Fig. 5). In this regard, clonal osteoblasts obtained from normal TMOb cultures exhibited maturation-dependent mineralization (Fig. 5, A and B) in association with increments in alkaline phosphatase activity (Fig. 5C) and normal Pex expression (Fig. 5D). In contrast, clonal osteoblasts obtained from TMOb-Hyp cultures manifest impaired mineralization (Fig. 5, A and B) in association with the 3' Pex deletion (Fig. 5D) and significantly greater alkaline phosphatase activity com-
pared with normal clonal osteoblasts (Fig. 5C). Moreover, we could identify no differences in osteopontin, osteocalcin, and type I collagen mRNA expression between clonal osteoblasts derived from Hyp and normal mice (Fig. 5D). These findings suggest that a nascent defect in osteoblast-mediated mineralization is a characteristic of osteoblasts with the Pex deletion.

Transfer of the Hyp mouse phenotype in coculture experiments between TMOb-Nl and TMOb-Hyp. To examine whether the abnormal mineralization in TMOb-Hyp cells is due to production of a factor(s) that inhibits mineralization, we cocultured TMOb-Hyp and TMOb-Nl cell lines separated by a semipermeable membrane. TMOb-Hyp cells displayed abnormal mineralization, whether cocultured with TMOb-Nl or TMOb-Hyp cells (Fig. 6B). In contrast, coculture of TMOb-Hyp with TMOb-Nl cells inhibited the mineralization of the normal osteoblasts, as evidenced by a failure to form discrete mineralization nodules (Fig. 6A) and significant reductions in alizarin red-S staining (Fig. 6B). Identical results were obtained in three replicative studies, consistent with the production of factors capable of inhibiting normal mineralization by TMOb-Hyp cells.

DISCUSSION

The bone mineralization defect in XLH may be due to inadequate circulating levels of mineral and/or hormonal/metabolic factors that influence osteoblast function or to nascent defects in osteoblast function that impair the mineralization process. Our studies indicate that the abnormal mineralization in Hyp mice is due, at least in part, to an intrinsic osteoblastic defect associated with abnormal Pex function. In this regard, we found that TMOb-Hyp cells manifest a 3’ Pex deletion (Fig. 1) and, in a setting remote from the in vivo Hyp mouse environment, fail to mineralize under culture conditions supporting mineralization in normal osteoblasts (Figs. 4 and 5). More importantly, we found that the Hyp mouse osteoblasts produce a factor(s) that is capable of regulating the mineralization of ECM. To this end, the mineralization defect observed in TMOb-Hyp cell lines is transferable to normal osteoblasts in
coclure experiments (Fig. 6). Such production of a mineralization inhibitor clearly represents a nascent defect in the osteoblasts from Hyp mice.

Because a physiologically relevant site of PEX expression is the osteoblast, it appears likely that production of this mineralization inhibitor is the result of the primary genetic abnormality underlying XLH, namely inactivating mutations of PEX. Indeed, dysfunction of the gene product may result in failure to degrade an endogenously synthesized but undefined inhibitor of mineralization that is a substrate of Pex. The alternate possibility, that Pex fails to activate a novel mineralization-promoting factor, is inconsistent with our coculture experiments in which the Hyp phenotype predominates (Fig. 6). In any case, further studies are necessary to identify the putative Pex substrates produced by osteoblasts and to determine their relationship to the osteoblast-synthesized factor(s). In these investigations, efforts to discriminate whether the mineralization inhibitor represents phosphatonin (17) or an additional putative PEX substrate will be essential.

Although Pex substrates appear to be present in osteoblasts expressing the 3’ Pex deletion, the mechanism whereby the accumulated Pex substrate causes the mineralization defect remains unknown. The impaired mineralization might be a direct consequence of a Pex substrate or might result from a multisteped cascade linking the Pex mutation and the accumulation of its substrate with impaired mineralization. Several observations suggest that a downstream event, rather than the putative Pex substrate, may be the mineralization inhibitor. In this regard, provided that Pex in the normal cells is not saturated and is located extracellularly (issues that require confirmation), the Pex endopeptidase in the normal cocultured cells should degrade any diffusible substrates, precluding a negative effect on mineralization. Given the results of our coculture experiments (Fig. 6), it is more likely that impaired mineralization results from a downstream kinase cascade that is regulated by the Pex substrate. Consistent with this possibility, additional studies have identified reductions in casein kinase and decreased phosphorylation of matrix proteins in Hyp mouse osteoblasts (16, 25).

The possible coproduction of Pex and its substrate in osteoblasts is supported by several studies in which an endopeptidase and its substrate are found in the same cell (35). However, when the identity of the substrate is determined, in situ and immunohistochemical studies will be necessary to establish its precise cellular localization. In any event, our study establishes that Pex effects on bone are likely mediated by its metabolism of local factors derived from cells that are within the osteoblast lineage or coisolated with osteoblasts from calvaria.

The current investigations also clarify the nature of the Pex mutation in Hyp mice. We found that TMObs derived from Hyp mice have a 3’ deletion of Pex (Fig. 1). Similar to prior Southern analysis of genomic DNA (31), we identified the absence of bands corresponding to the 3’ end of the Pex gene in Hyp mice (Fig. 1A) and localized the site of the deletion between exons 15 and 16 by RT-PCR (Fig. 1C). This deletion predicts the production of a protein lacking a portion of the extracellular domain containing the putative catalytic sites; consequently, this is likely to result in loss of Pex function. We were unable to identify the putative intronic sequence or retained 3’ end of the Pex transcript in TMOb-Hyp cells, as reported by Beck et al. (2). The reason for this apparent discrepancy is not clear but could be due to differences related to PCR conditions, lower abundance of the truncated message, and/or differences related to amplification from contaminating genomic DNA. Regardless, we found that Pex expressed a truncated 5’ transcript, albeit at lower levels compared with normal TMOb cells (Fig. 1B). Lower levels of Pex expression in Hyp mice osteoblasts suggest that the 3’ deletion may result in additional abnormalities of message stability. The possibility that message instability may also be clinically relevant is supported by the recent identification in certain families with XLH of mutations in the 5’- and 3’-untranslated regions of PEX that may be important in stabilizing messenger RNA (7).
Many questions remain regarding the pathogenesis of XLH, despite the identification of the PEX/Pex gene. Our results add to the growing body of evidence supporting the concept that osteoblastic cells are a physiologically relevant site of Pex expression and have significant implications regarding our understanding of the pathogenesis of the mineralization defect in XLH and Hyp mice. Further studies will be needed to determine the specific molecular abnormalities of ECM that are responsible for the impaired mineralization and whether these abnormalities are due to the accumulation of a Pex substrate itself or the downstream consequence of the Pex substrate. Our cell culture system also will permit molecular targeting and direct manipulation of Pex expression to prove a cause-and-effect relationship between Pex and the osteoblast phenotype. In turn, unraveling the pathogenesis of XLH and the function of Pex in osteoblasts may provide insights into novel factors that regulate bone mineralization.

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REFERENCES


