C h a p t e r  3

PTHrP Suppresses Chondrocyte Maturation via Cyclin D1 Induced Runx2 Degradation

Abstract

PTHrP is a growth factor which plays an important role in skeleton development. In skeletal system, PTHrP receptor is expressed at high level by prehypertrophic chondrocytes which relay the stimuli from PTHrP secreted by proliferative chondrocyte and PTH from circulation. Genetic evidence demonstrated that PTHrP delays chondrocyte maturation so that they could go through enough rounds of proliferation before entering terminal differentiation. In vitro study also demonstrated that PTHrP promotes chondrocyte proliferation. The detail molecular mechanism of how PTHrP regulates cartilage development by coordinating proliferation and differentiation is still elusive. In this thesis, we proposed a molecular mechanism of how growth plate chondrocytes couple differentiation and proliferation through cyclin D1-induced Runx2 degradation and further test the physiological significance of this regulatory mechanism using a cyclin D1 null mouse model.
Introduction

The growth of most bones engages endochondral bone formation, a process initiated by the condensation of mesenchymal stem cells and then differentiated into chondrocytes. In long bone, two ossification centers, primary and secondary ossification centers, are formed at different stages of bone development. The primary ossification center is formed in mouse at E14.5 of embryonic development, and the formation of secondary ossification center occurs at postnatal day 7 after birth. In between is a specialized group of chondrocytes, growth plates, where chondrocytes are organized from resting chondrocyte precursors, to undifferentiated chondrocytes with high proliferative potential, to fully differentiated hypertrophic chondrocytes. The continuous division of growth plate chondrocytes contributes to the longitudinal growth of long bones.

The precise organization of chondrocyte morphology and coordinated regulation of chondrocyte behaviors implicate exquisite interplay of extrinsic morphogens and intrinsic factors in chondrocytes. Indeed, previous research has demonstrated that many locally secreted factors, BMPs, Wnts, FGFs, PTHrP, hedgehog proteins, IGFs and retinoic acids are essential for chondrocyte development and organization (Kronenberg, 2003). And autocrine growth factors are joined by systematic factors such as growth hormone, thyroid hormone, estrogen, androgen, vitamin D and glucocorticoids to coordinately regulate cartilage development and bone formation.
Human PTHrP is encoded by a single copy gene located on chromosome 12. The amino acid sequence of PTHrP is homologous with the sequence of PTH at the amino terminus, which is associated with most of its known biological function. PTHrP and PTH bind with equivalent affinities to a common receptor, the PTHrP receptor 1, and consequently they have very similar ranges of biological activities. PTHrP receptor 1 belongs to a large family of G protein coupled receptors (GPCRs) feathered with seven transmembrane domains. The extracellular domains of GPCRs recognize small signaling molecules, which then cause the conformation change of receptor and mediate cytoplasmic signaling through activation of receptor-coupled small G protein complex, \( \alpha \), \( \beta \) or \( \gamma \).

Responding to different extracellular signaling molecules, different types of G protein subunits are activated. Among these, \( \alpha \) is the the G protein which couples to PTHrP receptor 1 and activates cAMP/Protein Kinase A (PKA) pathway (Schwindinger et al., 1998). \( \alpha \) is ubiquitously expressed in animal tissues.

PTHrP is widely expressed and present locally in many tissues, including cartilage, muscle, brain, kidney and placenta. The normal circulating level of PTHrP is considerably lower than the level of PTH, while it plays an important role in calcium homeostasis. Homozygous mutation of PTHrP gene results in embryonic lethal phenotype. It is speculated that PTHrP plays a more important role in early development than for maintaining daily calcium homeostasis.
Mice homozygous for the PTHrP null mutation died within minutes after birth possibly from respiratory failure (Karaplis et al., 1994). The overall body size and weight of mutants were similar to those of wild type and heterozygous littermates. But mutant mice did show some characteristic phenotypes, like "domed skull, short snout and mandible, protruding tongue, and disproportionately short limbs". Mice homozygous for the PTHrP receptor deletion were smaller than normal littermates from at least embryonic E9.5. Histological examination of mouse embryos at E9.5 and E12.5 reveals normal morphologies except the very smaller organ size in mutants. Most homozygous mutant died at E14.5 (Lanske et al., 1996). Histological examinations of PTHrP receptor null mice at different time points showed a very similar phenotype to that of PTHrP knockout mice. This includes a decreased growth plate proliferative zone, advanced or premature cartilage maturation or ectopic bone formation (Karaplis et al., 1994; Lanske et al., 1996). On the contrary, mice with cartilage specific over expression of human PTHrP gene or PTHrP constitutive active receptor exhibited a phenotype of inhibited chondrocyte maturation (Weir et al., 1996; Schipani et al., 1997).

At growth plates, PTHrP is secreted by proliferative chondrocytes and signals to prehypertrophic chondrocytes, where the PTHrP receptor 1 is highly expressed. PTHrP mainly maintains chondrocytes at proliferative stage, and prevents the chondrocytes from terminal differentiation, or hypertrophy. In PTHrP-/- or PPR-/- mice, chondrocytes
showed premature ossification and decreased population of proliferative chondrocytes in growth plate (Amizuka et al., 1994; Karaplis et al., 1994; Vortkamp et al., 1996; Lanske et al., 1996). This abnormality possibly contributes to the smaller body size of the mice by loss of PTHrP signaling. Conversely over expression of PTHrP in chondrocytes or expression of constitutively active PPR correlated with delayed chondrocyte maturation.

To study the downstream mediator of PTHrP signaling and the role of Gsa in cartilage development, a mouse model with chondrocyte specific knockout of Gsa was generated by breeding Gsa-loxP mice with chondrocyte specific Cre mice. Tissue specific disruption of Gsa in chondrocytes leads to immediate death after birth. Further histological analysis reveals defects in growth plate with shortening of proliferative zone and advanced differentiation of growth plate chondrocytes (Sakamoto et al., 2005). This is a typical phenotype found in mutant mice with PTHrP receptor gene deletion, suggesting that Gsa plays an important role in mediating PTHrP receptor downstream signaling during chondrocyte development.

*In vitro* studies using chondrocyte cell lines suggest that PTHrP activates cyclin D1 gene expression and promotes chondrocyte proliferation through transcription factor ATF2 or CREb, (Beier et al., 2001). Cyclin D1 encodes the regulatory subunit of the holoenzyme which interacts with and phosphorylates Rb protein to promote the transition of G1/S check point in cell cycle progression. Cyclin D1 is believed to be critical for normal
development since its important role in cell cycle progression. Surprisingly, Cyclin D1 null mice generated by homologous recombination show relatively normal development at early stage. Adult mice showed few negative consequences: reduced body weight, mild neurological impairment, and an underdeveloped retina (Sicinski et al., 1995).

Three different types of cyclin D have been identified and they are cyclin D1, D2 and D3. Each of these cyclin proteins interacts with certain CDK and regulates the cell cycle progression (Sherr CJ, 1995). The questions are whether there are differences in expression pattern and functional preference regarding to different cyclin D proteins and whether cyclin D2 or D3 compensates the loss of cyclin D1. Evidence from mouse genetics showed that mutant embryos with deletion of each cyclin D gene developed to terms. Cyclin D2 or D3 null mice were indistinguishable from their littermates, while cyclin D1 mutant mice showed obvious smaller body size and growth retardation (Pagano M and Jackson PK, 2004).

In the skeletal system, the molecular mechanism of PTHrP signaling in promoting chondrocyte proliferation and preventing chondrocyte maturation is still poorly understood. In this part of studies, we will investigate the mechanisms by which PTHrP balances cyclin D1 mediated proliferation and Runx2 driven maturation, through cyclin D1-induced Runx2 phosphorylation and protein dadradation. The present studies provide the first demonstration that cyclin D1 mediated activities prevent chondrocyte
differentiation by inducing Runx2 degradation.

Materials and Methods

Cyclin D1 knockout mice

Cyclin D1 knockout mice were obtained from the Jackson Laboratory. Genotyping was performed following the protocols provided.

Histologic analysis

For histologic analysis, mice were sacrificed, de-skined and fixed with 10% formalin and then 70% ethanol. Fixed skeletal samples were subjected to decalcification for 2 ~3 weeks, and checked with X-ray. Paraffin blocks were prepared by standard histological procedures. Paraffin sections were stained with hematoxylin and eosin.

Chondrocyte cell culture

The ATDC5 chondrocyte cells were cultured at a density of 12 000 cells per cm² in multi-well plates in a maintenance medium of DMEM/Ham’s F12 (Invitrogen) supplemented with 5% FCS (Invitrogen), 10 µg/ml human transferrin and 3x10⁻⁸ M sodium selenite (Sigma, Poole, Dorset, UK) until confluent. Thereafter, differentiation was induced by the addition of insulin (10 µg/ml; Sigma) and ascorbic acid (20 µg/ml) to the maintenance medium (differentiation medium). Incubation was at 37°C in a
humidified atmosphere of 95% air/5% CO₂ and the medium was changed every other day.

**Primary chondrocytes isolation**

Primary chondrocytes were isolated as described previously (Li et al., 2004). Briefly, sternae from Cyclin D1⁻/⁻, Cyclin D1⁺/⁻, mice were harvested, trimmed to remove any connective tissues and subjected to digestion for 1 hr with pronase (Roche; 2 mg/ml) in HBSS. Sternae then were washed with HBSS and subjected to digestion with collagenase D (Roche; 3mg/ml) in DMEM. After washing the digested sternae twice in HBSS, they were applied to digestion with collagenase D (3mg/ml) in DMEM to obtain purified primary chondrocytes.

**Results**

**Cyclin D1 is necessary for mouse normal development**

To examine the regulation of Runx2 protein stability and function in its physiological context, we took advantage of cyclin D1 knockout mice as an *in vivo* model. Since the mutant mice were from 129 and BL/6 background, the heterozygous mice were bred with BL/6 background for more than 10 generations before experiments. The experimental litters were from breeding of heterozygous mutants. Newborn and 2, 4 and 6-week-old mice were harvested for histological analysis. 4 days after birth, there were no obvious
differences observed between wild type, cyclin D1 heterozygous and homozygous mutants. At 2 weeks, cyclin D1 homozygous mutants showed dramatically reduced body weight (Fig. 3.1a) and body size as revealed by X-ray imaging (Fig. 3.1b). This growth retardation phenotype was sustained until 6 weeks at the time when almost all cyclin D1 homozygous mutants died. Heterozygous mutants did not show significant difference from wild type littermates and were used as control instead of wild type.

**Growth plate of cyclin D1 null mice showed premature chondrocyte hypertrophy and reduced proliferative zone.**

To determine whether loss of cyclin D1, or possibly gain of Runx2 based on the finding described in chapter 2, affects cartilage development, the growth plates of long bones were monitored to compare chondrocyte maturation. At day 4, histological examination of femoral growth plates revealed no distinguishable differences from heterozygous and homozygous mutants (Fig. 3.2a, 3.2b). At 2 weeks, the length and diameter of both femur and tibia of homozygous null mutant mice were smaller than heterozygous littermates (Fig. 3.2c-3.2f). The tibia growth plate sections showed a clear cartilage maturation delay at secondary ossification center, while it wasn’t obvious at femoral growth plate. These results suggest that there is a critical chondrocyte proliferation period driven by cyclin D1 at the postnatal stage which is necessary for proper cartilage maturation.

To better understand the role of cyclin D1 in cartilage development, 2, 4 and 6-week-old
mutant mice and littermate controls were harvested and growth plate phenotype was analyzed. At these time points, long bone samples from homozygous mutant mice were much smaller than heterozygous littermates. Histological examination of growth plates showed decreased length of growth plate in 2-week-old homozygous mutant mice (Fig. 3.3a and 3.3b). Further quantification of the number of proliferative chondrocytes in each growth plate column showed a dramatic decrease of proliferative chondrocytes in knockout mice, suggesting chondrocytes did not undergo enough rounds of replication prior to entry of hypertrophy (Fig. 3.3c and 3.3d). Surprisingly, the number of hypertrophic chondrocytes in each column was also much smaller than that in heterozygous littermates, which could be a direct effect of higher level of Runx2 driven chondrocyte apoptosis since loss of its negative regulator, cyclin D1. The numbers of proliferative chondrocyte in growth plate columns from homozygous mutants and heterozygous littermates became closer at 4 weeks (Fig. 3.3a’-3.3d’). And, they finally become indistinguishable at 6 weeks (Fig. 3.3a’’-3.3d’’).

**Loss of cyclin D1 correlates with osteopenia**

Runx2 plays an equally important role in osteoblast development and bone formation as well as in chondrogenesis and cartilage maturation. Loss of cyclin D1 would naturally lead to accumulation of Runx2 protein level, if cyclin D1 mediated regulation of Runx2 stability does exist in osteoblasts. 2 and 4-week-old mouse femurs were analyzed to access the trabecular bone density and cortical bone thickness. Micro-CT results showed
that homozygous mutants have much less trabecular bone compared to heterozygous
littermates at 2 weeks (Fig. 3.4 a and b) and the cortical bone thickness and diameter were
also much smaller (Figure 3.4 a’ and b’). These differences sustained till 4 weeks (Fig.
3.4c-3.4f’). These observations are consistent with previous publications, which showed
bone loss and osteopenia in transgenic mice with Runx2 overexpression in osteoblasts
(Liu et al., 2001; Geoffroy et al., 2002).

**Premature chondrocyte maturation in cyclin D1 knockout mice**

Histological examination and micro-CT analysis suggest that loss of cyclin D1 led to
defects in skeletal development. However, it is not clear if these defects are caused only
by the delay of skeletal development or the combination of delayed development and
accelerated chondrocyte maturation. To further dissect the phenotypic abnormalities in
cyclin D1 mutant mice, the length of long bones were compared between homozygous
and heterozygous mutant mice. X-ray image showed that 16-day-old heterozygous
mutants exhibit similar body size and long bone length with 4-week-old homozygous
mutants (data not shown). Histological analysis showed the number of proliferative
chondrocytes in growth plate column of cyclin D1-/- mice was much smaller than that of
heterozygous mutants. These results suggest that cyclin D1 is necessary for early
chondrocyte development, and loss of cyclin D1 leads to premature chondrocyte
maturation at femoral growth plates.
Cyclin D1 is the critical downstream component of PTHrP signaling in regulating chondrocyte maturation.

Genetic data have been well established that PTHrP promotes chondrocyte proliferation and prevents its maturation. *In vitro* studies showed PTHrP induced cyclin D1 gene expression in chondrocytes, while the direct interaction of cyclin D1 and Runx2 had not been reported until recently (Shen et al., 2006). To examine if there is an inverse relationship existed between PTHrP stimulation and Runx2 protein levels, we treated ATDC5 cell line with PTHrP and found that treatment with PTHrP indeed induced the downregulation of Runx2 protein levels and inhibited ALP activity (Fig. 3.6a and 3.6b), one differentiation marker of chondrocytes. To examine if cyclin D1 inhibits Runx2 level *in vivo* and if cyclin D1 is the critical intracellular transducer of PTHrP signaling, primary chondrocytes were isolated from heterozygous and homozygous cyclin D1 mutant mice to access changes in endogenous Runx2 protein levels and their responses to PTHrP stimulation. Without treatment, endogenous Runx2 protein levels were reduced in cyclin D1-/- cells compared with those in heterozygous mutant cells. Upon PTHrP treatment, Runx2 levels of heterozygous cells were down regulated. In contrast, it did not change in heterozygous mutant cells (Fig. 3.6c). In addition, results of ALP staining demonstrate that heterozygous mutant cells are more differentiated after 5 days culture than cyclin D1-/- cells (Fig. 3.5d).
Skeletal phenotype of cyclin D1 knockout mice is primarily due to the direct effects of loss of cyclin D1 function

The cyclin D1 knockout mouse has provided us a consistent inheritable model to test our *in vitro* finding, although some complexities of using a genetic model have to be considered. Cyclin D proteins have 3 family members and we then ask if expression of cyclin D2 or D3 is up regulated due to the loss of cyclin D1. Primary chondrocytes were isolated from heterozygous and homozygous mutant mice and Western blot was performed using cyclin D2 and D3 antibodies. The results showed no significant changes in protein levels of cyclin D2 and D3 in cyclin D1\(^{-/-}\) chondrocytes (Fig. 3.7a).

During cartilage maturation and bone formation, Runx2 promotes chondrocyte and osteoblast differentiation which is coupled with osteoclast activity. The osteoclast activity is primarily responsible for decomposition of the debris from apoptotic chondrocytes and bone matrix deposited by osteoblasts. To examine if osteoclasts contribute to the growth plate abnormalities in cyclin D1 null mutant mice, TRAP staining was performed to examine osteoclast formation (Fig. 3.7b). After quantification of TRAP-positive multinuclear cells in trabecular bone, no significant difference in osteoclast numbers was observed between heterozygous and homozygous mutant mice (Fig. 3.7c). These results further support the notion that enhanced maturation of chondrocytes is caused directly by the loss of cyclin D1 in cyclin D1\(^{-/-}\) mice.
Summary and Discussion

The significance of biochemical regulation of Runx2 by cyclin D1-mediated kinase activities was extended through further in vivo studies in chondrocytes and cyclin D1 null mouse model.

In this chapter, we demonstrated that PTHrP treatment inhibits the differentiation process of chondrocytes, and increasing time period of PTHrP treatment correlates with decreased endogenous protein level of Runx2. Loss of cyclin D1 in cyclin D1-/- mice results in smaller body size and weight compared to those of wild type or heterozygous littermates after birth. We further address the question if the smaller body size of cyclin D1-/- mice is due to the insufficient rounds of cell divisions or premature of terminal differentiation of chondrocytes. Further analysis of growth plate section and quantification of chondrocyte numbers in growth plate column reveals that both of these two aspects of development may co-exist and possibly they closely interplayed within the growth plate microenvironment.

To understand the difference that arised after birth in body size between normal and mutant mice, it is important to focus on the development of growth plate chondrocytes. While some concerns complicate the analysis of cyclin D1 KO mice. First, the cyclin D1 gene is globally depleted in every tissue in this knockout model, so the phenotypic
abnormalities could be the indirect effect from other tissues, which play important roles in regulating skeletal growth. Bone biology primarily focuses on the postnatal bone phenotype. Phenotype of cyclin D1 mice could also come from incompetence of breeding since smaller body size, improper muscle development since Runx2 is also important for tooth development. To fully exploit the cyclin D1 knockout mouse model, some of the drawback may be overcome. For example, tissue specific knockout of cyclin D1 could address the role of cyclin D1 in chondrocyte development. We may supply soft diet to avoid possible problems arising from defects of tooth development. We may separate cyclin D1 knockouts from their littermates to make sure they receive enough care from the breeding mother. Harvesting mouse littermates at earlier time after birth may also minimize the postnatal effects posted on cyclin D1 knockout.

We recognized that some detailed mechanisms will not be able to be addressed using this cyclin D1 knockout model. For example, this model could not provide any clues on if the critical amino acid residue of Runx2 identified by biochemical methods play important role in regulating Runx2 function in vivo. This issue may be addressed by generating the Runx2 (S472A), or Runx2 (S472E) knock-in mice. In addition, using this model alone couldn’t address if cyclin D1 is necessary for PTHrP-regulated chondrocyte maturation.

In summary, we showed that cyclin D1 is critical for normal skeletal development and loss of cyclin D1 function correlates with insufficient cartilage growth and premature
chondrocyte maturation, which explains some abnormalities observed in cyclin D1 KO mice, such as smaller body size and reduced body weight. This chapter together with chapter 2, the biochemical characterization of the interaction between Runx2 and cyclin D1, demonstrated that cyclin D1-induced Runx2 down regulation is an important event in chondrocyte development. Moreover this study provided a compelling example of combining of genetic and biochemical approaches together to understand the molecular mechanism of developmental processes.
a

![Graph showing weight (g) comparison between different genotypes at newborn, 2 weeks, and 4 weeks.]

b

![Images of mice at 2 weeks and 4 weeks of age, showing genotype differences.]

2 Weeks

4 Weeks
Figure 3.1. Smaller body size and body weight in cyclin D1 mutant mice.

(a) Cyclin D1<sup>−/−</sup> mice have reduced body weight. The changes in body weight were examined in newborn and 2 and 4-week-old WT, heterozygous (+/−) and homozygous (−/−) cyclin D1 knockout (KO) mice. No difference was found in newborn mice. However, the body weights of homozygous (−/−) KO mice were reduced about 50% compared to their WT or heterozygous (+/−) littermates.

(b) X-ray image of 2 and 4-week-old cyclin D1<sup>−/−</sup> mice showed that the sizes of homozygous (−/−) cyclin D1<sup>−/−</sup> mice are much smaller than their WT or heterozygous (+/−) littermates.
Figure 3.2. Growth plate phenotype of tibia and femur at early developmental stage.

(a and b) Femoral growth plates of 4 days cyclin D1 heterozygous mutant (a) and homozygous mutant (b). No cartilage difference was developed.
(c and d) Femoral growth plates of 2 weeks cyclin D1 heterozygous mutant (c) and homozygous mutant (d). No cartilage difference was developed.
(e and f) Tibia growth plates of 2 weeks cyclin D1 heterozygous mutant (e) and homozygous mutant (f). Cyclin D1\textsuperscript{-/-} mice showed maturational delay at secondary ossification center.
Figure 3.3. Quantitation of growth plate chondrocyte phenotype in cyclin D1 knockout mice.

(a-d) Femoral growth plates of 16 days cyclin D1 heterozygous mutant (a) and homozygous mutant (b). One representative area from each growth plate was showed at higher magnification (c). The left of (c) represents +/- mice, and the right of (c) represents -/- mice. Quantitation of proliferative chondrocytes from at least 6 growth plate columns were averaged and represented in (d). The growth plates from -/- mice (b) were much thinner than those of +/- mice (a). And the numbers of proliferative chondrocyte quantitated from +/- and -/- showed significant difference (d).

(a’-d’) Femoral growth plates of 4 weeks cyclin D1 heterozygous mutant (a’) and homozygous mutant (b’). One representative area from each growth plate was showed at higher magnification (c’). The left of (c’) represents +/- mice, and the right of (c’) represents -/- mice. Quantitation of proliferative chondrocytes from at least 6 growth plate columns were averaged and represented in (d’). The growth plates from -/- mice (b’) were much thinner than those of +/- mice (a’). And the numbers of proliferative chondrocytes quantitated from +/- and -/- showed significant difference (d’).

(a”-d”’) Femoral growth plates of 6 weeks cyclin D1 heterozygous mutant (a”) and homozygous mutant (b”). One representative area from each growth plate was showed at higher magnification (c”). The left of (c”) represents +/- mice, and the right of (c”) represents -/- mice. Quantitation of proliferative chondrocytes from at least 6 growth plate columns were averaged and represented in (d”). The growth plates from -/- mice (b”) were much thinner than those of +/- mice (a”). And the numbers of proliferative chondrocytes quantitated from +/- and -/- showed significant difference (d”).
Figure 3.4. Osteopenia in cyclin D1 knockout mice.

(a-b and a’-b’) Micro-CT analysis of 2 weeks old trabecular bone density (a and b) and cortical bone thickness (c and d) in heterozygous (Hts) mutant (a and a’) and knockout (KO) mutants (b and b’). Trabecular bone density of cyclin D1^{-/-} mice was lower than +/- littermates, and +/- cortical bones were also thinner than +/- littermates.

(c-f and c’-f’) Micro-CT analysis of 4 weeks old trabecular bone density (c-f) and cortical bone thickness (c’-f’). Both femur (c, c’, e, and e’) and tibia (d, f, d’, and f’) from heterozygous (Hts) mutant (c, d, c’ and d’) and knockout (KO) mutants (e, f, e’, and f’) were assayed. Trabecular bone density of cyclin D1^{-/-} mice was lower than +/- littermates, and +/- cortical bones were also thinner than +/- littermates in both femur and tibia.
Figure 3.5. Premature hypertrophy of chondrocytes in cyclin D1 knockout mice.

Growth plate phenotypes of 16 days wild type mice (a) and 4 weeks -/- mutant mice (b) were compared based on they share similar body sizes. Growth plates of -/- mice (c right) were much thinner than those of wild type mice(c left). One representative area of growth plate from each mutant was showed at higher magnification (c). Quantitation of proliferative growth plate was represented with significant difference (d).
**a**

ATDC5 Cell, Alkaline Phosphatase Assay

<table>
<thead>
<tr>
<th>BMP-2</th>
<th>TGF-β</th>
<th>PTHrP</th>
<th>Control</th>
</tr>
</thead>
</table>

![Image of cell cultures with labels and dimensions](image)

Day 0 1 3 5 7

**b**

![Image of western blot with labels and dimensions](image)

<table>
<thead>
<tr>
<th>Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
</tbody>
</table>

Runx2  Actin

![Image of western blot results](image)
C

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>+/</td>
<td>+/-</td>
</tr>
</tbody>
</table>

-  PTHrP  -  PTHrP

Runx2

Actin

d

-/-  +/-

ALP Staining

(a) ATDC5 cells were cultured and treated with BMP2, TGF-β, PTHrP and blank media for 7 days, and then assayed for alkaline phosphatase activity (ALP). ALP activity was upregulated upon BMP2 stimulation, and downregulated with or TGF-β treatment. PTHrP suppressed the ALP activity of ATDC5 over 7 days of treatment.

(b) ATDC5 cells were cultured and treated with $10^{-8}$M PTHrP for 3 days, and samples from each day were harvested to blot Runx2 expression. PTHrP treatment of ATDC5 cell correlated with downregulation of Runx2 protein level.

(c) Primary chondrocytes from cyclin D1$^{+/−}$ mice and +/- mutant mice were isolated to assay endogenous Runx2 level and its response to PTHrP treatment. Without treatment, Runx2 from knockout mice was a little higher than from +/- mice. With PTHrP treatment, loss of Runx2 abolished the downregulation effect of Runx2 induced by Runx2.

(d) Primary chondrocytes from cyclin D1$^{+/−}$ mice and +/- mutant mice were isolated to assay the differentiation potential. Cells from knockout mice are more differentiated than +/- mice after 5 days culture.
**a**

<table>
<thead>
<tr>
<th></th>
<th>-/-</th>
<th>+/-</th>
<th>+/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin D2</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>Cyclin D3</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>Actin</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

**b**

![Image]

**c**

![Image]

**d**

![Graph: Positive Staining/Genetic Profile]

- Hts
- KO
Figure 3.7. Skeletal phenotype of Cyclin D1 knockout mice is primarily due to the direct effects of loss of cyclin D1 function.

(a) Primary chondrocytes from cyclin D1 -/- mice and +/- mutant mice were isolated to examine if there were some indirect effects from loss of cyclin D1 gene. Primary cells were assayed for cyclin D2 and D3 gene expression, and cyclin D2 and cyclin D3 did not show differential expression in wild type, heterozygous and homozygous mutant mice. (b-d) 16 days femoral growth plates were stained with TRAP staining. Quantitation of TRAP staining showed no dramatic difference existed in osteoclast activities between knockout mice and +/- mutant mice (d).