Table 1

| Gene name | Function | Modification | Bone phenotype | Characterization of bone |
|---|---|---|---|---|
| BubR1 | Spindle assembly checkpoint | Hypomorph | Normal (kyphosis) | DXA |
| Klotho | Oxidative stress response, mineral metabolism? | Hypomorph | Osteoporosis | SXA, microCT, histological analysis cell culture |
| Ku86 | DNA repair, transcription | KO | Osteopenia | (not indicated) |
| MsrA | Oxidative stress response | KO | N/D | |
| mTR | Telomere maintenance | KO[a] | Normal | X-ray analysis, histological analysis |
| PASG | DNA methylation | Hypomorph | Osteopenia | X-ray analysis, histological analysis |
| PolgA | Mitochondrial DNA replication | Knock-in | Osteoporosis | X-ray analysis |
| Prdx1 | Oxidative stress response | KO | N/D | |
| Top3b | DNA replication and repair | KO | N/D | |
| TRp53 | Cell cycle | Deletion mutant | Osteoporosis | X-ray analysis, histological analysis |
| | | Mutant Tg | Osteoporosis | X-ray analysis |
| | | Short isoform Tg | Osteoporosis | X-ray analysis, histological analysis |
| XPD | DNA replication and repair | KO | Osteoporosis | X-ray analysis |
| Wrn/Terc | Telomere maintenance | Double KO | Osteoporosis? | microCT |

N/D, not described. DXA, dual energy X-ray absorptiometry; SXA, single energy X-ray absorptiometry; microCT, microcomputed tomography.

[a] The phenotype was observed in the 6th generation from mTR knockout mouse matings.

Errors in cell duplication, such as those mis-programmed by the above-mentioned mutations, can be detected and corrected by arresting cell cycle (Fig. 1). Checkpoint signaling systems play critical roles in those programs (Hartwell, 1992; Nurse, 1997). Checkpoint kinase cascades are involved in DNA replication and other cell cycle events. ATM is a PI3K family kinase involved in DNA repair and oxidative response (Rotman and Shiloh, 1998). The gene encoding the kinase has been identified as a gene mutated in ataxia telangiectasia, recognized as one of the human premature aging syndromes (Lavin and Shiloh, 1997). Knockout mice for *Atm* exhibit a similar phenotype to the human disease, including hyperradiosensitivity and ataxic defects (Barlow et al., 1996; Elson et al., 1996; Xu et al., 1996). The osteopenic phenotype has also been observed in these mice. Differentiation of osteoblastogenesis and osteoclastogenesis, however, is rather normal. Colony formation assays revealed that the phenotype was the result of the proliferative defect in bone marrow mesenchymal stem cells or progenitors (AH et al., submitted). The gain-of-function mutations in p53, a downstream effecter of ATM kinase, also result in a premature aging phenotype with osteoporosis (Tyner et al., 2002; Maier et al., 2004). Among them, p44 transgenic mice show a low turnover with a significant decrease in osteoblast number, along with a slight reduction of osteoclasts (Maier et al., 2004). Although fur-

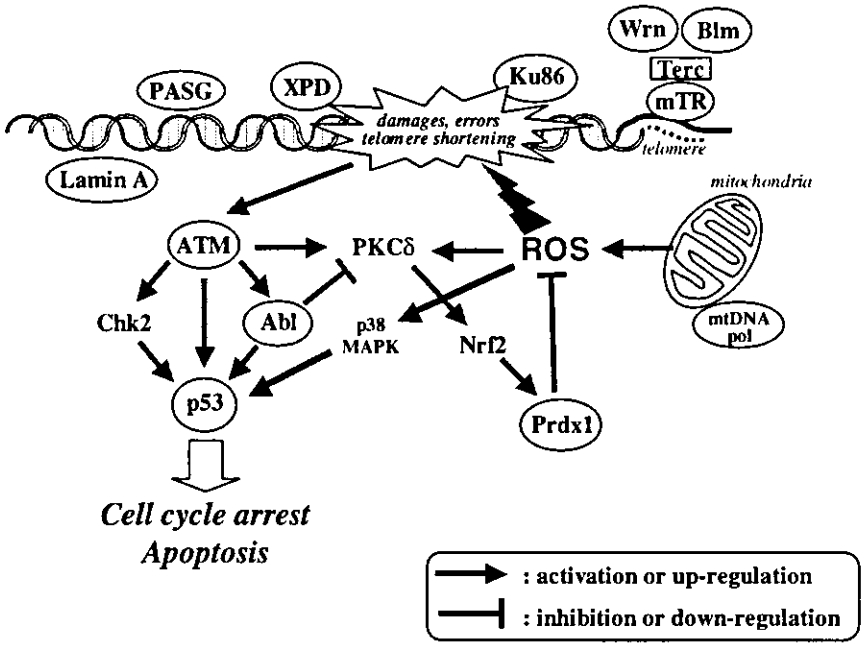


Fig. 1. Predicted pathways connecting the gene products responsible for the premature aging mutant phenotype. Most of the mouse models for premature aging are caused by mutations in the genes involved in genomic integrity and subsequent cell cycle regulation. Errors and damage to the genome or telomere shortening, which also affects DNA integrity could, in theory, be detected and corrected. Mutations in the genes responsible for genomic stability cause accumulation of phenotypic abnormalities. Genomic disorganization activates cell cycle-regulating pathways involving checkpoint kinases and p53. Oxidative stress is among the triggers that elicit genomic instability via DNA damage. Elevation and excess of ROS affect downstream signaling, including PKCδ, which subsequently stimulates the anti-ROS pathway, including transcriptional activation of Prdx1.

ther evaluation of each model is still required, these studies suggest that stem cell defects resulting from DNA damage and subsequent cell cycle arrest or other cell cycle abnormalities, at least in part, may account for the decreased bone formation and subsequent osteopenia observed in these premature aging models.

Other models for accelerated aging exist for which the responsible genes are apparently not directly involved in genomic integrity. For example, mice carrying hypomorphic mutations of the gene called Klotho show multiple aging phenotypes (Kuro-o et al., 1997). In Klotho mice (*kl/kl*), both bone formation and resorption were reduced, indicating a low turnover of bone metabolism resembling human osteoporosis (Kawaguchi et al., 1999). Although neither osteoblasts nor osteoclasts expressed the *kl* gene, ex vivo cultures for osteoblastogenesis and osteoclastogenesis showed reduced differentiation independently in both lineages. Thus, this model is unique in its correlation with senile osteoporosis in humans, in contrast to the canonical progeroid models.

It is also worth considering the contribution of longevity mouse models to our understanding of the pathophysiology of bone metabolism in normal aging (Migliaccio et al., 1999; Flurkey et al., 2001; Holzenberger et al., 2003). In that regard, IGF is an important factor for bone metabolism, especially bone formation. The critical role of its receptor, IGF1R, in bone modeling has been demonstrated using a conditional knockout strategy (Zhang et al., 2002). Interestingly, heterozygous knockout of IGF-1R results in 33% and 16% increases in the mean life-span of female and male animals, respectively (Holzenberger et al., 2003). Together, these results demonstrate that, while a correlation between bone phenotype and life-span is suggested, the issue is complicated and awaits further definition.

## 3. Bone formation defects mimicking pathogenesis in senile osteoporosis

Low turnover rate or uncoupling between bone resorption and formation in aged bones is often associated with decline in osteoblast function (Bilezikian et al., 2002). Indeed, reduced bone formation is one of the characteristic hallmark features of models for senile osteoporosis. By now, a number of the genes playing critical roles in bone formation have been described in genetically modified mice (Davey et al., 2004). Sca1 is a GPI-anchored membrane protein that is expressed in hematopoietic stem cells, and in a subset of bone marrow stromal cells (Yeh et al., 1986; Stanford et al., 1997). Whereas Sca1 knockout mice have normal bone development, the aged animals (15 months of age) show significant bone loss (Bonyadi et al., 2003). Progenitor and differentiation assays of bone marrow cells in these mice revealed that decreased bone mass is caused by impairment in the self-renewal of mesenchymal progenitors. Stem cell defects in hematopoietic lineages have also been reported in Sca1 knockout mice (Ito et al., 2003). Although multiple aging phenotypes in Sca1 knockout mice have not been reported, this is a good correlative model for senile osteoporosis in humans.

c-Abl, a downstream protein kinase of ATM, functions in DNA repair and oxidative stress response (Kharbanda et al., 1997; Hantschel and Superti-Furga, 2004). Mice deficient for the *Abl* gene also develop osteopenia with reduced bone formation (Li et al., 2000). Ex vivo cultures of osteoclastogenesis were not affected, and the number of osteoclasts was similar to that of wild-type littermates. However, the number of progenitors in bone marrow were decreased, and the differentiation of osteoblasts from *Abl* knockout mice was significantly impaired (Li et al., 2000). Using osteoblast cultures, distinct roles in the oxidative stress response between c-Abl and ATM, have been demonstrated (Li et al., 2004). Although decreased expression of peroxiredoxin 1 due to downregulation of PKCδ was observed upon arsenate-induced oxidative stress in osteoblasts from ATM knockout mice, expression of the redox protein, through the upregulation of PKCδ, was increased in the cells derived from Abl knockouts. The opposing roles of Abl and ATM gene products in the oxidative stress response, as shown in these knockout mice, may cause similar but slightly different bone phenotypes. Nagai et al. demonstrated that oxidative stress plays a crucial role in the aging symptoms of Klotho mice as well (Nagai

et al., 2003). Life-span shortening and age-related defects have been reported in mice lacking MsrA or Prdx1, which encodes methionine sulfoxide reductase or peroxiredoxin 1, respectively (Moskovitz et al., 2001; Neumann et al., 2003). Both genes play important roles in the oxidative stress response, by possessing anti-reactive oxygen species (ROS) activity. Oxidative stress-induced ROS activity often causes damage to DNA, suggesting that genomic stability and the oxidative stress response may share some common pathways in the aging phenotype. While the bone phenotype in these mutant mice has not been described, it is interesting to speculate that loss of these gene products could also play a role in bone pathogenesis.

## 4. Concluding remarks

Skeletal malformation and severe developmental defects in bone modeling have not been pronounced in the models of premature aging, suggesting that cell differentiation is not seriously arrested. Regeneration from stem cells is an essential step in tissue metabolism and maintenance, including bone remodeling, and the defects in self-renewal of stem cells cause degenerative diseases due to the resultant deviation of tissue metabolism (Fig. 2). The possibility cannot be ruled out that the aging phenotype is caused by dysfunction in these programs, which become active postnatally or after maturation. As in the case of *Abl* knockout mice, the defect in osteoblastic

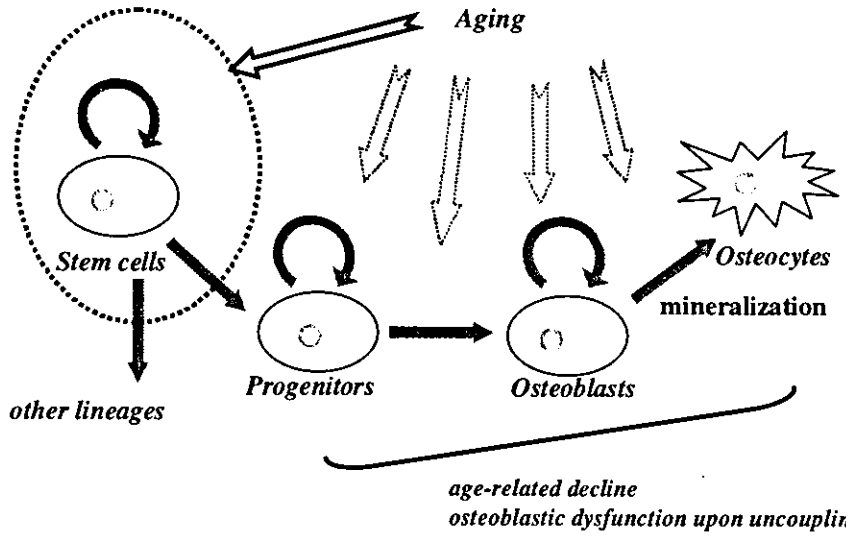


Fig. 2. Schematic presentation of osteogenesis and aging. Observations in naturally aged laboratory animals and ATM knockout mice suggest that the key to understanding aging and premature aging pathogenesis may be self-renewing stem cells. In these models, the pathway involving p53 (Fig. 1) upregulates the genes responsible for cell cycle arrest and/or apoptosis, lowering the regenerative potential necessary for homeostasis and tissue repair. While the mechanisms responsible for aging are largely unknown, the existing models suggest that there are common pathways, which may help in our understanding of the aging phenotype.

differentiation, probably due to deregulated response to oxidative stress, also causes symptoms mimicking senile osteoporosis. On the other hand, a number of age-dependent declines in tissue function, such as mineral homeostasis, also affect bone metabolism. Since the characterization of metabolic parameters and tissue functions are often limited in studies in mice, there is a possibility that the apparent age-related decrease in bone mass is secondary to metabolic dysfunctions, rather than from cell autonomous defects. In conclusion, while much information from these mouse models has been gathered over the last decade, studies on senile osteoporosis using these models may be just beginning.

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*Mini review*

# Progeroid syndrome as a model for impaired bone formation in senile osteoporosis

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**Abstract** Senile or age-related/dependent osteoporosis is caused by reduced bone formation, rather than increased bone resorption as in postmenopausal osteoporosis. Here we review genetically engineered mouse models with defects in osteoblastic proliferation or differentiation with focus on IGF signaling and stem cells. Model mice for human progeroid syndromes may provide useful tools for studying the pathogenesis of senile osteoporosis.

**Key words** premature aging syndrome · knockout mouse · osteoblast · differentiation · IGF

## Introduction

Osteoporosis is among the most severe problems affecting the quality of life for the elderly. An understanding of the pathophysiology and mechanism of the development of the disease is essential for diagnosis and treatment. Mechanisms of postmenopausal osteoporosis have been studied using animal models and clinical epidemiology or drug testing in human subjects. In contrast, the mechanism of senile osteoporosis has not been well characterized, mainly because of a lack of experimental models. It is generally recognized that senile osteoporosis is characterized by a decrease in bone-forming capacity. Defects in bone formation may be due to a decrease in the number or the function of osteoblasts, or both. Here, we review potential mouse models of senile osteoporosis with focus on osteoblastic function.

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## Proliferation and differentiation of osteoblasts

To date, important genes for bone development, such as Runx2 and Osterix, have been identified [1–3]. These transcription factors play key roles in osteoblast differentiation [3,4]. Differentiation and maturation of osteoblasts consist of a multistep sequence (Fig. 1). Osteoblasts are thought to derive from mesenchymal stem cells (MSCs), as are other lineages such as myocytes and adipocytes [5]. MSCs self-renew within an appropriate niche, maintaining their pluripotency. This first step (step I, Fig. 1) is critical for tissue maintenance, metabolism, and regeneration and is thought to be sensitive to aging. Once leaving the niche, MSCs start differentiation by responding to a variety of signals such as cytokines, hormones, adhesion molecules, or the extracellular matrix (ECM). Bone morphogenetic proteins (BMPs) are well known to induce ectopic bone formation and to accelerate osteoblastic differentiation, but BMPs also regulate development and regeneration of tissues other than bone [6]. For example, BMP can induce adipogenesis of stromal cell lines in vitro [7,8]. IL-1 signaling through the TAK1/TAB1/NIK pathway inhibits peroxisome proliferator-activated receptor γ (PPARγ) function, resulting in suppression of adipogenesis and a switch to osteoblastic differentiation [9]. Thus, many factors converge on osteoblastic differentiation cooperatively or antagonistically. When regulatory mechanisms in this second step (step II, Fig. 1) fail, osteoblastic differentiation is impaired, concomitantly with hypo- or hyperinduction of other lineages.

"Matching of seeds and soil" is an essential step in osteoblastic differentiation. Osteoblasts produce a large amount of collagens and other ECM molecules to build bone while sensing the matrix to stimulate osteoblastogenesis. A mitogen-activated protein (MAP) kinase, extracellular signal-regulated kinase (ERK), responds to extracellular signals and phosphorylates RUNX2, which is known to regulate genes encoding ECM mol-

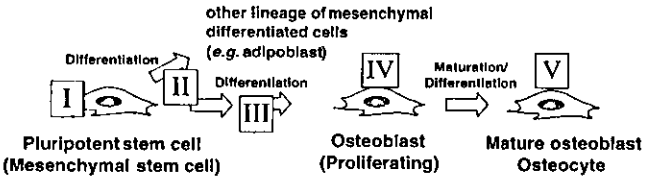


**Fig. 1.** Multistep differentiation of osteoblast lineage. The process of osteoblastic differentiation can be divided into five regulatory steps. The first step (*I*) corresponds to pluripotent mesenchymal stem cells in the stem cell niche as a source of osteoblasts. In the second step (*II*), lineage selection takes place. Commitment to osteoblastic differentiation is completed, and a checkpoint for progress in maturation may occur at step *III*. Step *IV* corresponds to osteoblast proliferation and activation of mature osteoblasts, e.g., responding to mechanical stress. The last decision as to whether osteoblasts undergo terminal differentiation into osteocytes or apoptosis is made at step *V*

ecules, to activate its transcriptional function [10,11]. This positive feedback step (step III, Fig. 1) may be one of the checkpoints for proceeding to osteoblastic differentiation before deposition of minerals.

Many stimuli have been reported to activate osteoblastic proliferation. Among the most recognized molecules that increase the number of osteoblasts are insulin-like growth factors (IGFs) [12,13]. The Wnt pathway, as a signal that stimulates proliferation, is another candidate [14–16]. Although the authentic ligand that activates the receptor on osteoblasts has not been identified, the coreceptor, LRP5, plays an essential role in bone formation and in controlling the number of osteoblasts [14–16]. Cyclin D1, one of the most potent mitogenic molecules, is a well-known downstream effector of β-catenin, which is a signal mediator in the canonical pathway of Wnt signaling [17], suggesting that Wnt/β-catenin is a pathway leading to increased numbers of osteoblasts and stimulated bone formation.

In the last step shown in Fig. 1, osteoblast differentiation is completed. At this step, osteoblasts produce molecules that regulate mineralization, such as osteocalcin, and release matrix vesicles [18]. Composition of phospholipids also changes in this step, suggesting that lipid metabolism is involved in mineralization [19]. This window may also determine whether osteoblasts undergo apoptosis or terminally differentiate into osteocytes. Although osteoblasts in this step are important for bone quality [20], the mechanism of mineralization or terminal differentiation is largely unknown.

## Knockout mice for signaling molecules

As already described, a number of signals are involved in the regulation of osteoblast differentiation. Here, the focus is on genetically modified mouse models showing impaired bone formation. It has been demonstrated that Sca-1, a cell-surface molecule that is expressed in hematopoietic stem cells, is required to maintain self-renewal of mesenchymal stem cells during step I [21]. Sca-1 knockout (KO) mice develop bone normally, but bone mass decreases as they age [21]. IRS-1, a major substrate of insulin receptor (IR) and IGF-1 receptor (IGF-1R) that transduces signals by interacting signaling molecules in a phosphorylation-dependent manner, is expressed in osteoblasts but not in osteoclasts [22]. IRS-1 KO mice exhibit low bone mass compared with controls, and cultured osteoblasts from KO mice are impaired in IGF-induced proliferation and differentiation, whereas differentiation induced by BMP is not altered [22]. In contrast, BMP-induced differentiation was markedly lowered in osteoblasts from Abl KO mice, which are osteopenic due to decreased bone formation [23]. ABL is known as a downstream mediator of integrin signaling, and may function during step III integrating signals from BMP and ECM.

Proliferation of osteoblasts is also impaired in Abl KO mice [23]. LRP5 KO has been reported as a model for osteoporosis and pseudoglioma syndrome, pointing to the involvement of Wnt signaling [24]. Those defects are mainly in the osteoblastic lineage, but do not have a cell-autonomous effect in osteoclastogenesis. On the other hand, knockout mice for another insulin substrate, IRS-2, also showed an osteopenic phenotype with reduced bone formation and accelerated bone resorption [25]. Osteoblasts from IRS-2 KO mice exhibit impaired differentiation but possess accelerated osteoclastogenesis-supporting activity with increased expression of RANKL. This model may serve as an "uncoupling" phenomenon of bone formation and resorption [25]. In view of the unexpected finding of increased bone mass in calcitonin (CT) KO mice, CT may have antibone formation activity in vivo despite the fact that it has long been thought to have antiresorptive activity [26]. It would be interesting to elucidate CT signaling on osteoblasts in relation to parathyroid hormone (PTH) signaling, both of which transduce signals through the G-protein-coupled receptor. In most cases, the development of new anabolic drugs depends on a priori clarification of the means by which these signals regulate bone formation.

## Mouse models for premature aging syndromes

Human progeroid syndromes include genetic diseases such as the Werner, Cockayne, and Hutchinson–Gilford syndromes, for which responsible genes are involved in maintenance of genomic stability. Mice with mutations of the responsible genes have not always shown the

expected aging phenotype [27]. However, most of these mice exhibit osteopenia as a hallmark of the pathology. Contrary to expectations, mice deficient in Werner syndrome helicase (WRN) or telomerase RNA component (mTR) did not show signs of accelerated aging, and this may be due to the difference in telomere length between humans and mice [28,29]. Evidently, the phenotype of premature aging develops after four generations in mTR KO mice [30]. It is possible that WRN KO mice may also exhibit an aging phenotype once their telomere length becomes critically short.

Although a classic model of senescence-accelerated mouse (SAM)-P6 is known to show osteopenia, the responsible gene(s) have not been identified [31]. Mice expressing mutant p53 proteins exhibit premature aging [32,33]. In mice expressing the N-terminally deleted p24 isoform or A135V mutant protein of p53, the osteopenic lesion is significant, with the result that both bone formation and resorption are greatly suppressed in these mice [32]. Klotho mice, which are produced by inactivation of the kl gene, exhibit a variety of age-related symptoms, and represent the first established case in which the gene encodes an extracellular protein rather than a nuclear molecule [34]. The pathogenesis of low-turnover osteopenia in the klotho mouse is well documented, resulting in a substantial decrease in osteoprogenitor pools [35]. A knockout mouse for the ataxia telangiectasia mutated (ATM) gene is also recognized as a model of premature aging, and shows a severe osteopenic phenotype with decreased bone formation [36].

It has been reported that IGFs inhibit p53 function by destabilizing the protein [37]. Interestingly, IGF-1R is downregulated in cells from ATM patients as well as those from ATM KO mice [36,38]. Loss of function of IRS-1 and IRS-2 leads to reduced bone formation [22,25]. Transgenic mice expressing IGF-1 in osteoblasts exhibit increased bone mass [39]. Although dysregulation of renal phosphate handling has been reported in klotho mice [40], bone formation seems to be resistant to growth hormone (GH) treatment [41]. It might be interesting to see whether the kl gene is involved in IGF signaling.

Defects in osteoblasts of these animals are often associated with a decrease in the number of osteoclasts as well as reduced hematopoiesis in bone marrow. It has been recently demonstrated that osteoblasts provide a stem cell niche in bone marrow [42,43]. This finding is in good agreement with the cell biological basis of low turnover as represented in klotho, ATMKO, and Sca1KO, but not only in relationship to osteoclastogenesis. Thus, failure in the maintenance of stem cell niche may underlie the pathogenesis of senile osteoporosis.

**Is strong bone contrary to longevity?**

Although the importance of IGF in bone is evident, IGF signaling does not favor long life span, at least in experimental animals [44]. Caloric restriction (CR) is the only established strategy that can prolong life span in various organisms spanning in diversity from yeast to mammals [45]. Decreased levels in growth hormone and insulin, as well as IGF-1, have been reported in CR animals. Mice defective in the GH–IGF axis or insulin signaling also showed expanded life span. Thus, there may exist a trade-off between longevity and strong bone. Is thicker bone contrary to longevity? Transgenic mice that express the p44 isoform of p53 exhibit an accelerated aging phenotype and short life span [33]. These mice showed decreased BMD, and histomorphometric analysis indicated a low turnover state of bone metabolism. In cells or tissues from the p44 mice, downregulation of phosphatase and tensin homologue deleted on chromosome 10 (PTEN) function and an increased level of phosphorylated Akt were observed, suggesting that hyperactivation of IGF signaling may occur [33]. This observation is in agreement with the GH/insulin/IGF hypothesis on longevity, but does not indicate exerting a favorable action on bone formation. Although it remains to be clarified whether IGF signaling is also hyperactive in the bones, it is suggested that tissue- or stage ('step' in osteoblast lineage)-specific response, or local feedback of IGF, needs to be considered in the action of the growth factor in bone formation.

Aging is a complex, unavoidable phenomenon, and many factors, not only genetic but also environmental, are involved. In terms of age-related changes in bone metabolism, we have to pay attention to neural control of bone formation [46] and consider age-related decline in mechanical stress response and mineral homeostasis by the kidney and/or intestine. Although we should recognize pathophysiological or structural differences between mouse and human skeletons, model mice provided valuable lessons that are useful for elucidating the etiology of senile osteoporosis.

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# はじめに
Introduction

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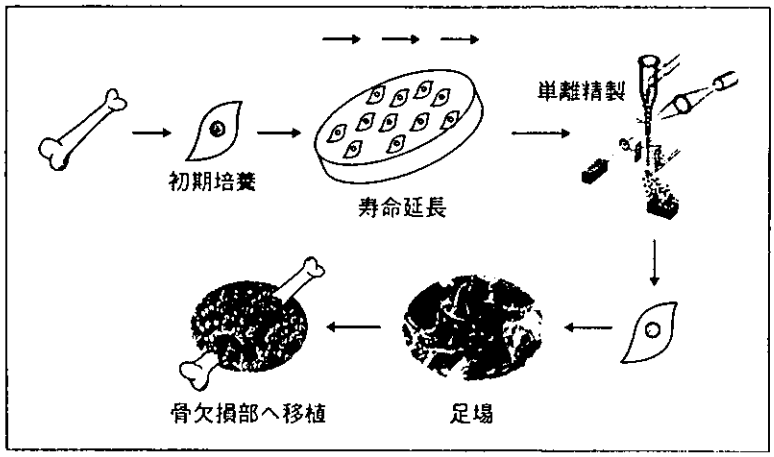
骨組織の維持にかかわる問題は社会的に注目されて久しい．骨粗鬆症，関節リウマチに代表される骨再生能の低下は，閉経後婦人，寝たきり(手術後を含む)，老化，宇宙医学(無重力状態)，ダイエットと多岐にわたる．また，悪性腫瘍などの治療技術の向上に伴い生じる術後骨欠損や，骨形成不全症など先天性の骨疾患に対する機能再建の必要性が増している．これらの社会的問題に対し骨組織の再生，維持は，国民の健康，医療，福祉の向上，政策医療の観点からも急務であり，欠くことのできない問題となっている．しかし，ヒト骨髄間葉系細胞は現在までの方法では必要な量の細胞数を得るには至らず，広範な欠損に対する再建や骨粗鬆症，関節リウマチに代表される自己の骨再生能の低下した患者に対する治療法としての限界があった．

このような，現代における骨再生医療の問題点に対する，サイエンティストの挑戦を本号で特集する．清野 透氏により発見された遺伝子を導入することによって，従来，困難であった腫瘍化を伴わないヒト細胞の寿命延長，増殖に戸口田淳也氏が成功する．また，牛田多加志氏が開発した生分解性ポリマーを足場とすることで，より整形性が高くなり，広範な骨欠損を再建することが可能となる．さらに，渡辺 研氏，東 佐由美氏，宮戸健二氏は骨形成における分子機構に関する，きわめて貴重な情報を提供する．これらの技術を応用することによって大串 始氏は骨髄間葉系細胞無機人工骨を用いての骨再生に多くの知見を有し医薬品GCP基準に合致する施設の立ち上げを終了しており，すでに早期から探索的な臨床研究を推進し，その結果をすべて紹介している．ヒト細胞の増殖をコントロールし移植への系を確立することは移植医療のあらたなパラダイムの獲得につながることになり，臨床応用を視野に入れた具体的なアプローチを本特集で追求する．



# 遺伝子導入によるヒト細胞寿命の延長

Extending life span of human cells by gene transfer

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◎ヒト組織幹細胞を体外で自由に殖やすことができれば細胞移植療法の可能性は大きく広がる．しかし，実際にヒト細胞を培養すると一定期間分裂した後分裂を停止し無限に増殖することはできない．この培養皿上での分裂可能回数を規定している機構にはテロメア依存性のものとテロメア非依存性の2つがある．テロメア依存性のものはテロメア短小化によりチェックポイント機構が働き増殖が停止するもので，狭義の分裂寿命(replicative senescence)とよばれる．しかし，皮膚線維芽細胞を除くほとんどの正常ヒト細胞の培養皿上での分裂可能回数を規定しているのはテロメア短小化によらない $p16^{INK4a}$/RB 経路の活性化による増殖停止である．したがって，ほとんどのヒト正常細胞を寿命延長するにはまず $p16^{INK4a}$/RB 経路の活性化を止める必要がある．ここでは遺伝子導入による細胞寿命延長法の現状と課題を概説し，今後のヒト細胞の寿命延長法の展望についても述べたい．

Key word：細胞老化，不死化，テロメラーゼ，$p16^{INK4a}$

## ヒト初代培養細胞の有限寿命

ヒト正常細胞を培養すると，一定の分裂を繰り返した後，分裂を停止しそれ以上増殖しなくなる．この広義の細胞老化(cellular senescence)は2つの異なる機構によってもたらされる．1つはテロメア短小化による増殖停止であり，狭義の細胞老化(replicative senescence)あるいはM1(mortality stage 1)とよばれる．もうひとつは $p16^{INK4a}$(以下単にp16と記す)の発現増加とRBの活性化により起こる増殖停止であり，ここではM0(mortality stage 0)という用語を使うことにする(「サイドメモ」，図1)．皮膚線維芽細胞にはM1のみがあり，テロメラーゼの触媒サブユニットであるTERTを発現させることで不死化させることができる．しかし，なにもしなくてもこの細胞は50〜70回程度分裂できるため，細胞移植に十分な細胞数が得られる．細胞移植に必要な細胞を体外で殖やす際に問題となるのはほとんどの場合，M0である．したがって，TERTを導入して不死化しようとしても不死化できない．まず，M0とM1がどのような機構で細胞老化を引き起こすのかを理解した後，具体的な寿命延長法について述べる．

## テロメア短縮による増殖停止(M1)

ヒトをはじめ脊椎動物のテロメアDNAは5′(TTAGGG)3′からなる数百から数千回の繰返し配列(ヒトでは約10 kb)からなる．テロメアはその繰返し配列とテロメア結合蛋白質(TRF1，TRF2)の結合によりt-loop，D-loopとよばれる特殊なクロマチン構造をとり，線状ゲノム末端をDNA断端として認識されることを防ぐとともに，DNA末端どうしの融合，組換えを防いでいると考えられる[1]．しかし，直鎖状DNAをゲノムとしてもつ生物にはいわゆる"末端複製問題"が生じる(図2)．テロメアが一定の長さにまで短縮するとDNA損傷チェックポイント機構とほぼ同じ機構が働き

ATM, p53 依存的に $p21^{WAF1/SDI1/CIP1}$(以下, 単に p21 と記す)の増加, 高リン酸化型 pRb の消失などが観察される. 通常の DNA 損傷によるチェックポイントの活性化は DNA が修復されると解除されるが, テロメア DNA 末端の露出によるものは修復されることがないため不可逆的な増殖停止が誘導される(図 2). また, テロメア結合蛋白質である TRF2 の機能を変異体の発現によって抑制するとテロメア長が長くてもそのクロマチン構造が保てず正常細胞では senescence や染色体末端融合が, また多くの癌細胞ではアポトーシスが誘導される[1,2].

**サイドメモ**

### 細胞老化(cellular senescence)

ヒト細胞が無限増殖できないことは培養の比較的簡単であった線維芽細胞を用い発見され Hayflick 限界とよばれた. 同じ現象論として M1(mortality stage 1)や細胞老化(cellular senescence)という用語が使われていたが, 原因論的にテロメアの短小化がこれらの現象を説明できることが推測され, 新生児皮膚線維芽細胞などでは証明されたため, Hayflick 限界＝テロメア短小化による増殖停止＝狭義の細胞老化としての分裂寿命(replicative senescence)＝M1(mortality stage 1)として使われることが多い. しかし, 現在では線維芽細胞でも皮膚由来のものを除き 2 つの異なる機構で細胞は増殖停止することが明らかになっている.

最初に明らかにされた乳腺上皮細胞では 2 つの増殖停止期がみられるが, 後期の増殖停止期が M1 とされ, 初期の増殖停止期は無視されていたため後に M0 と命名された. M0 は p16 の増加によって誘導される増殖停止期でテロメア長非依存性である. p16 プロモーターのメチル化により p16 の発現低下した一部の細胞がふたたび増殖するため 2 つの増殖停止期がみられる.

前立腺上皮細胞など一部の細胞では同様の 2 つの増殖停止期がみられるが, 多くの細胞種では p16 プロモーターのメチル化された細胞の出現頻度は低く 1 つの増殖停止期しか観察されない. 現象論的にははじめての増殖停止期なのでこれも M1 とよぶべきものであるが, 原因論的な立場からするとこの増殖停止期は M1 ではなく M0 と同じものである. ここではヒト細胞の通常の培養条件における p16 増加による増殖停止(細胞老化)を乳腺上皮細胞に限らず M0 とよぶことにする.

## テロメラーゼの発現調節

テロメラーゼ活性はおもに, *TERT* の転写レベルで調節されている. 正の調節因子としては Myc や Ets などが報告されているが, ほとんどのヒト正常体細胞では *TERT* の転写は抑制されており, 負の調節因子が重要であると予想される. 最近, Mad, SIP1, Menin, Rak1, BRIT1 など複数の因子がこの転写抑制にかかわっていることが報告されている[3]. 興味深いことにはその一部を不活化するだけで *TERT* の転写を活性化できるようである.

## CDK インヒビター p16 の増加による増殖停止(M0)

さきに述べたようにほとんどのヒト細胞ではおもに p16 の発現増加による p16/RB 経路の活性化により増殖が停止する. この増殖停止機構がテロメア非依存性であることは, 乳腺上皮細胞, 皮膚角化細胞を用いて明らかにされた. したがって, 多くのヒト細胞種を不死化するにはテロメラーゼの活性化のほかに p16/RB 経路の不活化が必要である[4].

## p16 の発現調節

*p16* の発現調節もおもに転写レベルで行われている(図 3). 正の転写調節因子として, Ets ファミリーや AP1 が報告されている. また, 転写因子ではないが, Polycomb-group genes(PcG)に属する bmi-1 や cbx 7 が p16 の転写を負に制御している. 外来性に bmi-1 や CBX7 を高発現してやると, p16 の発現増加は阻止され, 細胞増殖停止は抑制される[5,6]. 活性化癌遺伝子産物 Ras*(RasV12)が株化細胞を形質転換させることは古くから知られていたが, 初代線維芽細胞に対しては不可逆的な増殖停止を誘導する. この Ras*による線維芽細胞の premature senescence には, MAP キナーゼカスケードを介した Ets ファミリー蛋白質のリン酸化がかかわっているが[7], 下流の Erk が直接, 転写因子をリン酸化するのではなく p38 MAPK へのク

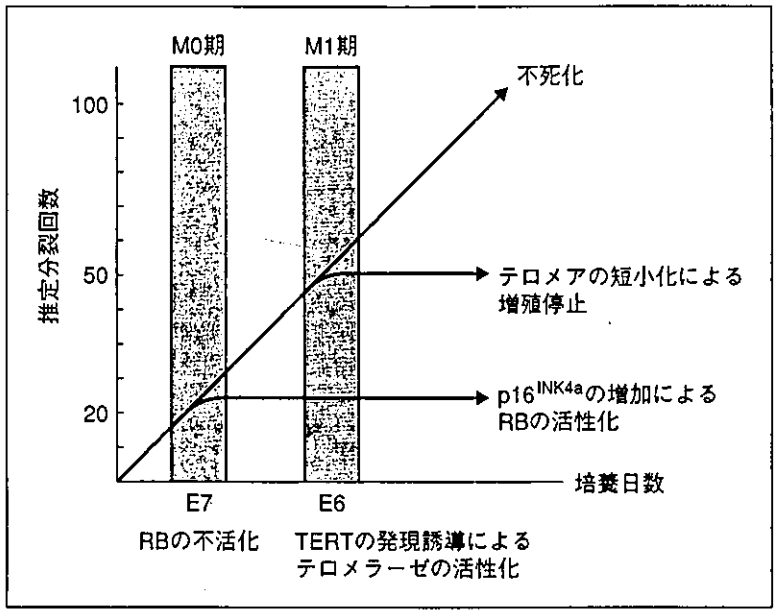


図 1　乳腺上皮細胞の不死化モデル(文献[13]より改変)

ヒト乳腺上皮細胞では約 20PD で最初の senescence(M0 期)を迎える．このとき $p16^{INK4a}$の発現増加がみられる．senescence を逃れたごく一部の細胞では $p16^{INK4a}$プロモーターがメチル化により発現が低下しており，増殖を続けるが，約 50PD で senescence(M1 期)を迎える．E7 を発現している細胞では E7 が RB を不活化することで M0 期をバイパスすることができる．一方，E6 あるいは TERT を発現している細胞ではテロメラーゼが活性化されているためテロメア長の短縮によって訪れる M1 期をバイパスすることができる．

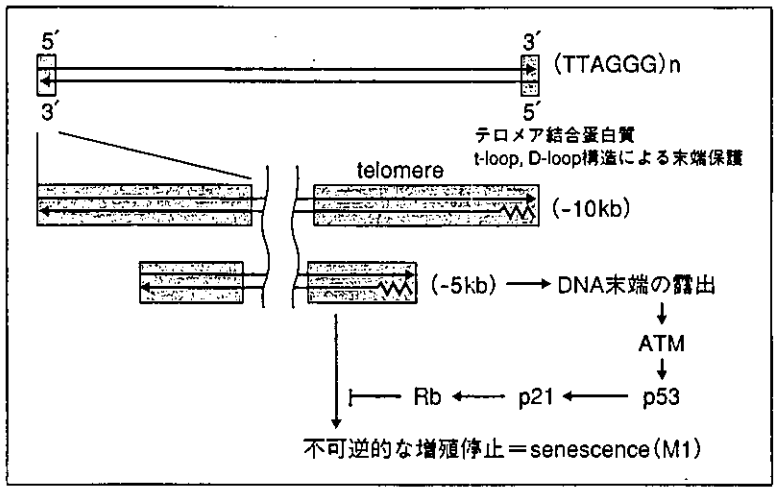


図 2　末端複製問題とテロメア/p53 経路(文献[13]より改変)

線状ゲノムをもつ生物ではゲノム複製の際，新生 DNA 鎖の 5′末端のプライマー RNA 部分およびそれより 5′末端側は DNA に置換されないため，テロメアは複製ごとに短縮される．したがって，テロメラーゼ活性のないヒト体細胞では 1 回の複製(分裂)あたりおよそ 100 塩基ずつテロメア長が短縮する．ヒト体細胞のテロメア長は約 10 kb あるが，50 回分裂して約 5 kb になるとテロメアのクロマチン構造を保てなくなり DNA 末端として露出され，通常の DNA 損傷チェックポイント機構が働くと考えられている．その結果，ATM，p53 が活性化しおもに p21 の増加によって RB のリン酸化が阻害され，細胞周期が停止する．

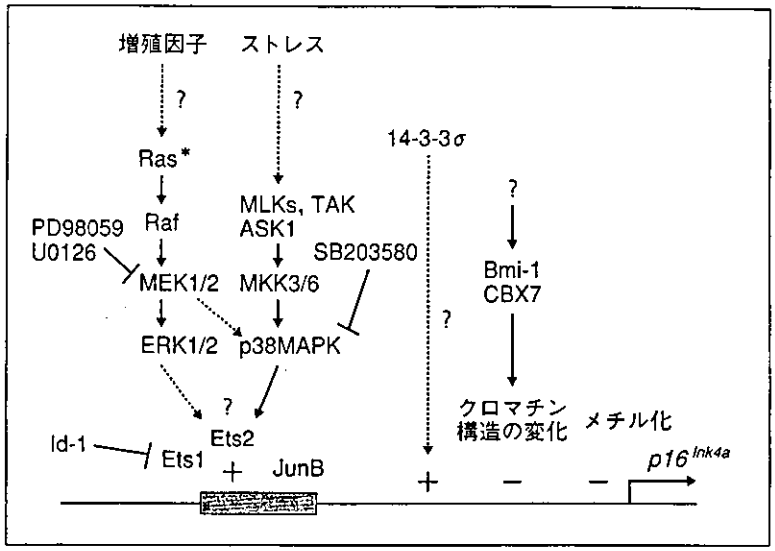


**図 3 $p16^{INK4a}$の転写調節**

p16 の発現調節もおもに転写レベルで行われている．正の転写調節因子として，Ets ファミリーや JunB が報告されている．一方，Id1 は Ets ファミリーと結合することで転写を負に制御している[5]．また，機構は不明であるが，14-3-3σ は p16 の発現を誘導することが報告されている．また，転写因子ではないが，Polycomb-group genes(PcG)に属する bmi-1 や cbx 7 が p16 の転写を負に制御している．活性型 Ras による線維芽細胞の premature senescence には MAP キナーゼカスケードを介した Ets ファミリー蛋白質のリン酸化がかかわっているが，下流の ERK が直接，転写因子をリン酸化するのではなく p38 MAPK へのクロストークを介しているらしいことが報告されている．また，p38 MAPK は種々の"ストレス"によって誘導されるストレスキナーゼとして知られており，SIPS のセンサーとして共通に働いている可能性が示唆されている[8]．

ロストークを介しているらしいことが報告されている．この機構は癌遺伝子活性化による細胞癌化を防ぐ監視システムとして近年注目されている．また，p38 MAPK は，過酸化水素水の添加や，二本鎖 DNA 切断を誘導する薬剤，UV 照射，放射線照射などのさまざまな"ストレス"によっても誘導されることから SIPS(stress-induced premature senescence)のセンサーとして共通に働いている可能性が示唆されている[8](図 3)．

## ヒト細胞不死化の現状

現在，著者らはヒト細胞の不死化を図 4 に示すアルゴリズムに従って行っている．その結果，①TERT の導入だけで不死化できるもの(皮膚線維芽細胞)，②TERT に加えて E7 など p16/RB 経路の不活化によって不死化できるもの(皮膚角化細胞など)，③さらに E6 など p53 経路の不活化が不死化に必要なもの(子宮内膜腺細胞など)，に分類される．p53 経路の不活化が必要なものは p16/RB 経路の不活化によって p53 経路が二次的に活性化されたとき感受性の高い細胞だと考えている．なお，それでも不死化に成功していないもの(胃腺細胞)もある．TERT の導入はまだしも，E6 や E7 などウイルス癌遺伝子を導入された細胞が移植医療に適さないのは明らかである．現時点では E7 などの替わりに RNA 干渉法による p16 の発現抑制と TERT 発現ベクターにより皮膚角化細胞や乳腺上皮細胞の不死化に成功している．

## ヒト細胞寿命延長の課題と展望

しかし，M0 期の p16 の発現誘導機構が解明されれば，よりよい寿命延長，不死化方法の開発が期待できる．もし M0 が SIPS と同じであるならばそのストレスを軽減する培養法の開発によって p16 の発現誘導そのものをなくすことが可能かもしれない．上皮系の細胞培養は低(無)血清培地が開発

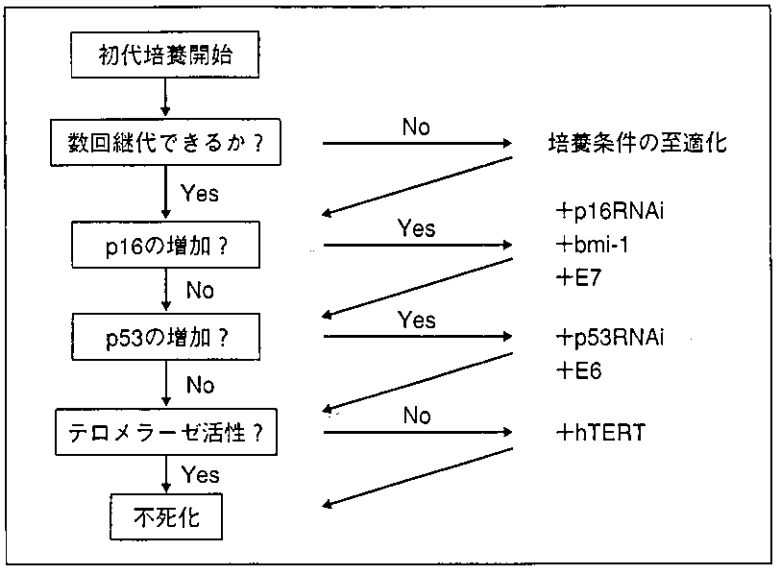


図 4　ヒト細胞不死化のアルゴリズム

まず，初代培養細胞が培養皿上で増殖するか，継代できるかが成否の鍵となる．また，図にはないが，初代培養細胞には目的以外の細胞種の混入は避けられず，培養条件が目的細胞により適したものであることが望まれる．上皮細胞を不死化しようとしたら線維芽細胞ばかり不死化したというようなことが起こりうる．目的の細胞種に特異的なマーカーなどがわかっていれば混在する細胞集団を不死化してから目的の細胞種をクローニングすることも可能である．

初代培養は困難であるが，数週間あるいは 1 カ月以上たってからコロニーが認められることもある．このような場合，p16 プロモーターのメチル化などの異常が起こっている可能性を検討する必要がある．p16/RB 経路を不活化するには，このなかでは E7 がもっとも強力であるが，その分 p53 経路を活性化させアポトーシスを誘導する可能性もある．材料が限られていて多少細胞の形質が変わってもとにかく不死化したいという場合には，E6 と E7 を同時に導入するのも現実的な選択である．通常テロメア短縮による増殖停止はかなり後期に起こるが，テロメラーゼ活性のないことが自明の細胞には TERT も早めに導入したほうが染色体の安定性が保たれやすい．

されてからプラスチック皿上での培養が普及した．しかし，線維芽細胞のフィーダー上で血清入り培地で培養することによって皮膚角化細胞や乳腺上皮細胞における p16 の発現誘導は軽減できるとする報告もあるがその機構は不明である[9]．ヒト初代培養細胞への遺伝子導入効率は一般に低いため TERT などはほとんどレトロウイルスベクターにより導入されている．しかし，レトロウイルスベクターは，細胞ゲノムにかなりランダムに組み込まれることから，癌遺伝子の活性化や癌抑制遺伝子の不活化などが起こる可能性がある[10]．また，TERT は癌抑制遺伝子の側面もあるが，逆に癌の 85％で発現していることから，癌遺伝子としての側面もある[11]．したがって，理想的には再生医療に用いる細胞には TERT の遺伝子導入さらにはレトロウイルスベクターの使用もできれば避けるのが望ましい．上述したように，多発性内分泌腫瘍 I 型(MEN-I)の原因遺伝子である menin などが TERT の転写を抑制しており，menin を RNA 干渉法でノックダウンすると TERT が発現し皮膚線維芽細胞を不死化できると報告された[3]．報告ではレトロウイルスベクターによる RNA 干渉法が使われているが，一過的な siRNA の導入でも活性化できる可能性もある．また，PTD(protein transduction domain)との融合蛋白質として種々の遺伝子産物を細胞に供給する方法が応用されはじめている[12]．遺伝子導入に替わる方法として期待できる．

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