ROS-induced damage to DNA is associated with replication fork stalling in cooperation with mitogenic signals from oncogenes. Thus, it might further exacerbate unbalanced DNA replication.

Both pRb null cells and p107/p130 double null cells are resistant to p16-mediated arrest, suggesting that the latter is not mediated exclusively by pRB, but depends on the non-redundant functions of at least two pRB-family members [31]. On the other hand, as only triple knockout of the pRb family genes (pRb, p107 and p130) endows resistance to senescence and OIS, overlapping functions of the pRb family members appear to be required for induction of senescence in mouse cells [32]. However, acute loss of the *Rb* gene is sufficient for cell cycle re-entry of senescent MEF cells [33]. Thus, among the pRb family members, pRb has a dominant function in senescence when it is present, but the other two members have overlapping functions, as well as non-redundant functions under some conditions of genetic manipulation.

The bZIP transcription factor, CCAAT/enhancer binding protein β (C/EBPβ), which is activated by receptor tyrosine kinase and oncogenic Ras, cooperates with pRb and E2F in oncogenic Ras-induced senescence [34]. C/EBPβ-deficient MEF cells are resistant and proliferate, despite unimpaired induction of p19ARF and p53. Enforced C/EBPβ expression, however, inhibits proliferation of wild-type MEF cells and also slows proliferation of p19Arf$^{-/-}$ and p53$^{-/-}$ cells, but rather stimulates proliferation of MEFs lacking all three Rb family proteins or wild-type cells expressing dominant negative E2F-1, which does not bind pRb [34].

7. Senescence as a tumor suppression mechanism

Both telomere-dependent senescence and OIS are considered to be conceivable tumour suppression mechanisms. OIS has also been shown to take place *in vivo*. B-Raf or N-ras mutations are frequently found in both melanomas and nevi [35-37] and there are strong suggestions that the melanocytic nevus consists of melanocytes that have undergone OIS. Melanoma cells frequently demonstrate inactivation of the p16/Rb pathway, in addition to harboring B-Raf or N-ras mutations. A physiologic relevance of OIS as a tumor suppression mechanism is further supported by animal models, in which inducible KrasV12, E2F3 and Phosphatase and Tensin homolog deleted on chromosome ten (PTEN) knockout results in senescence within premalignant lesions [24,38,39].

Telomere-induced senescence also functions in mouse models as a tumor suppression mechanism, which is as effective as apoptosis in reducing cancer incidence. INK4a–Arf$^{-/-}$ mice crossed with late generations of Terc$^{-/-}$ mice are more cancer resistant, which coincides with severe telomere shortening and associated dysfunction [40]. Progeny of crosses of Emu-myc transgenic and Terc$^{-/-}$ mice become resistant to lymphoma development, due to p53-mediated senescence in response to short telomeres [41]. Spontaneous tumorigenesis in p53(R172P) knock-in mice, which are not susceptible to induction of apoptosis, but retain intact cell cycle arrest and cellular senescence pathways, is also suppressed by crossing with Terc$^{-/-}$ mice, whereas chemical induction of skin carcinomas is not affected [42].

As senescence and apoptosis are anti-tumour suppression mechanisms that are mainly mediated by p53, induction of apoptosis and/or senescence is a good therapeutic strategy against human malignancies. Restoration of endogenous p53 results in clearance of liver carcinoma and sarcomas accompanied by cellular senescence [43,44], also leading to apoptosis and regression of autochthonous lymphomas developing in Emu-myc transgenic mice [44,45].

8. Post-translational modification of p53 and senescence

p53 functions as a mediator of senescence and apoptosis, as well as transient cell cycle arrest and DNA repair, in response to stresses, such as DNA damage and oncogene activation. Types and strength of stresses determine outcome in each cell type. The associated functions are mainly regulated by a selection of genetic targets, including proapoptotic genes, p21 and repair genes. Post-translational modification of p53, including phosphorylation and acetylation, may also be important.

p38 stress-activated protein kinase, induced by many kinds of stresses and, therefore, expressed in senescing cells [46,47], is directly and indirectly involved in phosphorylation of p53 at multiple sites. In ras-induced senescence, p38 can activate its substrate MAPK activated protein kinase 5 (MK5), also known as p38-regulated/activated kinase, (PRAK), which in turn activates p53 through phosphorylation at Ser37, although MK5 is in fact not indispensable for UV-induced phosphorylation of p53Ser37 [48]. However, it is an essential mediator for ras-induced senescence in both primary mouse and human fibroblasts. In primary cells, inactivation of MK5 prevents senescence and promotes oncogenic transformation [48]. In the mouse, this pathway appears critical in OIS as a tumor suppression mechanism *in vivo*, as MK5 deficiency in mice enhances-induced skin carcinogenesis, coinciding with compromised senescence induction [48].

Mechanisms involving preferential activation of proapoptotic genes can be partly explained by phosphorylation of p53 at Ser46 by p38 and homeodomain interacting protein kinase 2 [49-51]; homeodomain interacting protein kinase 2 co-localizes with promyelocytic leukemia (PML) and p53 in nuclear bodies [51]. PML is also activated by oncogenic Ras and its overexpression acetylates p53 at lysine 382; this is sufficient to promote p53-dependent cellular senescence [52,53]. PML4-induced senescence involves stabilization and activation of p53 through phosphorylation at Ser46 and acetylation at Lys382 [54].

Table 1. The p38MAPK cascade and consequences related to senescence.

Direct target	Downstream target	Consequence
MK2	Cdc25	Inhibition of CDKs
	PRC1 (Bmi1 etc)	Induction of p16
MK3	PRC1 (Bmi1 etc)	Induction of p16
MK5	p53(Ser37)	Induction of p53-responsive genes
p53(Ser46)		Induction of p53-responsive genes
p21(Ser190)		Accumulation of p21
HBP1(Ser401)		Inhibition of Wnt signalling

CDK: Cyclin-dependent kinases; HBP1: HMG-box containing protein; MK: MAPK-activated protein kinase; PRC: Polycomb repressor complexes.

More recently, it was suggested that Tip60-dependent acetylation of p53 modulates the decision as to whether cell cycle arrest or apoptosis should occur [55,56]. The histone acetyl transferase Tip60 and the ATP-dependent chromatin remodeler p400, both of which belong to the NuA4 multimolecular HAT complex, are required for DNA damage-induced apoptosis. Tip60 participates in p53-dependent activation of some proapoptotic p53 target genes, as well as p21. In contrast, p400 represses p21 expression in unstressed cells by inhibiting Tip60 function, although this is abolished following DNA damage [57]. As the p53 K120R mutant, which is not acetylated by Tip60, can not accumulate on proapoptotic gene promoters, but still bind to p21 and HDM2 promoters [55,56], it seems likely that p400 inhibits histone acetyl transferase activity of Tip60, including acetylation of p53. HDM2 targets both p53 and Tip60 for degradation [58], so that p21 and HDM2 upregulation might form a negative feedback loop to inhibit apoptosis. It is noteworthy that Tip60, independent of the NuA4 complex, is also involved in DNA damage sensing by directly binding and activating ATM [59], although it remains to be demonstrated whether it directly acetylates ATM so as to activate its kinase activity ([60] for review).

9. Regulation of p21 expression

p21(SDI1/CIP1/WAF1) functions as a CDK inhibitor that is regulated by p53 and many other regulators, and expressed in terminally differentiating cells. However, mutations in the *p21* gene rarely appear in human cancers and p21-deficient mice develop normally without showing any predisposition to cancer. This may be explained by the fact that p21 not only induces growth arrest, but also often inhibits apoptosis induced by p53. In mouse fibroblasts, p21 is neither essential for senescence nor for Ras-induced senescence [61], indicating a p53 target other than p21 is the relevant downstream effector of senescence in rodent cells. In this context, it is of interest that plasminogen activator inhibitor-1 (PAI-1), known as a senescence-associated marker, is reported as a critical downstream target of p53 in the induction of cellular senescence of human and mouse fibroblasts (**Figure 1**) [27].

In addition to p53, many transcription factors are involved in the regulation of p21 expression. Miz-1 is required for upregulation of p21 after UV irradiation [62]. CSL (CBF1/Su(H)/Lag2) as an effector of the Notch signaling activates p21 transcription in terminally differentiating keratinocytes [63], and microphthalmia transcription factor in cooperation with pRb activates p21 in differentiating melanocytes [64]. However, as p21-deficient mice develop normally, p21 is not essential for normal differentiation of cells [65]. p53-mediated induction of p21 is inhibited by Myc binding to the promoter through interaction with Miz-1 [62]. This interaction blocks p53- and differentiation-mediated induction of p21 [66,67]. As Myc induces p14ARF-mediated accumulation of p53 and apoptosis [68], blockage of p21 induction appears to also involved in Myc-induced apoptosis. Accumulation of p400 on the p21 promoter in unstressed cells suppresses its transcription, possibly through interaction with Myc and Miz [60].

The Y box binding protein, YB-1, which belongs to a large family of proteins that feature the cold shock domain, normally represses transcription of p21 and p16, although the mechanism is not known. YB-1 is broadly expressed throughout development, but its level decreases steadily over time. YB-1-deficient cells, under oxidative stress, express high levels of p16 and p21 and senesce [69].

10. Regulation of p16 expression by polycomb repressor complexes

Expression of p16 is primarily regulated by transcription. In normal cells, this is repressed by polycomb repressor complexes (PRC), components of which were first found in Drosophila. PRC1 and PRC2 are known to function as repressors of homeobox genes so as to maintain differentiated phenotypes, and to play different roles in heterochromatinization of genes. PRC2 includes EZH2, which is a catalytic subunit of histone methyltransferase at H3K27. PRC1 is a very large protein complex, including human polycomb (HPC) proteins such as HPC3 (CBX8), which recognizes methylated H3K27, and ring finger protein 1, which is a ubiquitin ligase at H2A-K119 and recruits DNA methyl transferases (DNMTs). Thus, PRC2 initiates suppression of genes by marking them with H3K27 methylation and PRC1 maintains their heterochromatinization ([70] for review). A similar mechanism involves Suv39h and HP1 proteins, which also share homology with HPC proteins and are members of the CBX family (Table 2). In mammals, PRC members function as repressors of

Table 2. HP1 and polycomb group genes.

Gene	Drosophila melanogaster	Human	Protein domains	Functions
HP1	HP1a	HP1α (CBX5)	Chromodomain	H3K9me binding
	HP1b	HP1β (CBX1)		
	HP1c	HP1γ (CBX3)		
	Su(var)3-9h	Suv39h	SET domain	H3K9 methyl transferase
PRC1	PC	HPC1 (CBX2)	Chromodomain	H3K27me binding
		HPC2 (CBX4)		
		HPC3 (CBX8)		
		CBX6		
		CBX7		H3K27me and H3K9me binding
	PH	HPH1 (EDR1)		
		HPH2 (EDR2)		
		HPH3 (EDR3)		
	RING	RING1A (RNF1)	RING-finger domain	H3K119 ubiquitin ligase
		RING1B (RNF2)		
	PSC	BMI1		
		PCGF2		
		ZNF134		
PRC2	ESC	EED		
	E(Z)	EZH1	SET domain	H3K27 methyl transferase
		EZH2		
	SU(Z)12	SUZ12		

ESC: Extra sex comb; HPC: Human polycomb; HPH: Human poloyhomeotic; PH: Polyhomeotic; PC: Polycomb; PRC: Polycomb repressor complexes; PSC: Posterior sex combs; RING: Ring finger protein; SET: Supressor of variegation-enhanser of zeste-trithorax domain.

not only Hox, but also many other genes, including p16 [71]. A correlation between detachment of PRCs from the p16 gene and induction of its expression has been shown in senescence, induced either by activated ras or serial passage of cells [72]. MK3 and MK2, which are downstream targets of activated p38, have been found to interact with a component of PRC1, human poloyhomeotic 2 (HPH2) [73,74]. Furthermore, activation or overexpression of MK3 results in phosphorylation of Bmi1 and other PcG members, HPH1 and HPC2, and their dissociation from chromatin (**Figure 2A**) [74].

11. p38 and senescence

p38 is a member of the stress-activated protein kinase class of MAPKs, directly targeted by MKK3 and MKK6. Constitutive activation of p38 is sufficient to induce cellular senescence [46,47]. The enzyme itself activates three closely related MAPK-activated protein kinases, MK2, MK3 and MK5, by phosphorylation (Table 1) ([75] for review). As described earlier, MK2 and MK3 then phosphorylate components of PRC1 to cancel its transcriptional repression function. MK2 also inactivates CDC25 phosphatase by phosphorylation and, thereby, mediates traffic through the G2–M checkpoint [76]. MK5 has been described as an essential mediator of ras-induced senescence [48]. In addition, p38 in addition to ERK is involved in activation of transcription factors, JunB, Ets1 and Ets2, which activate transcription of p16 (**Figure 2B**) [77,78].

p38 phosphorylates p21 at Ser190 and the HMG-box containing protein (HBP1) at Ser401 so as to increase their protein stability. HBP1 binds to pRb and p130 through LXCXE and IXCXE motifs and to histone deacetylase 3 (HDAC3) through an Ataxin homology domain, functioning as a general suppressor of Wnt signaling and repressing transcription of cyclin D1 and c-Myc, which can be activated by another HMG box-containing transcription factor, T-cell factor (TCF), downstream of Wnt [79] for review. HBP1, in cooperation with pRb, is required for senescence induced by MKK3/6, p38 and activated Ras [80], whereas knockdown of HBP1 does not relieve replicative senescence of human fibroblasts. Thus, p38 plays a pivotal role in inducing senescence by various stresses. However, little is known about the mechanisms underlying its activation by different stresses. In accordance with the functions of p38 in senescence, inactivation of

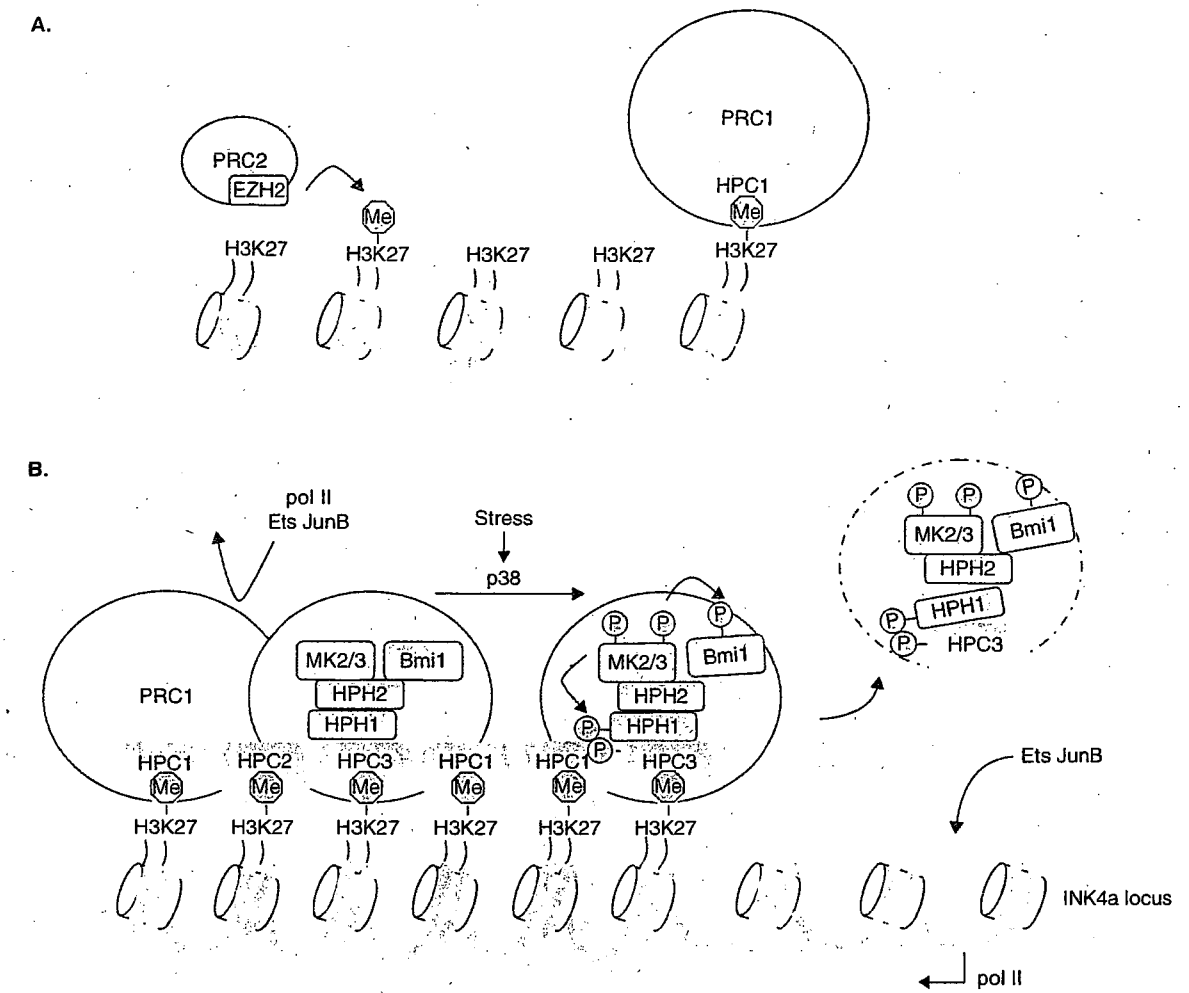

Figure 2. Polycomb repressor complexes-mediated repression and p38-mediated de-repression of the p16 promoter. **A.** PRC2-mediated histone H3 methylation on lysine 27 initiates PcG-mediated gene silencing. A PRC2 component, EZH2, has methyl transferase catalytic activity. PRC1 recognizes the trimethylated H3K27 through the chromodomain of HPC proteins (HPC1 in this figure), and might facilitate PRC2-mediated histone methylation of neighboring nucleosomes. **B.** The INK4a locus is normally repressed by PRC. MAPK-activated protein kinase 2 and -3 (MK2 and MK3) physically interact with HPH2, a component of PRC1, and phosphorylate other components, such as Bmi1, after activation by p38 MAP kinase. Phosphorylation of PRC1 components results in detachment of PRC1 from the locus and activation of gene expression enhanced by transcription factors, such as Ets and JunB.

EZH: Enhancer of zeste gene; HPC: Human polycomb; HPH: Human polyhomeotic; MK: MAPK activated protein kinase; pol II: Polymerase II; PRC: Polycomb repressor complexes.

p38α in the adult mouse lung leads to an immature and hyperproliferative lung epithelium that is highly sensitive to K-Ras(G12V)-induced tumorigenesis [81].

12. SNF5 and senescence

The mammalian homolog of SNF5 (also called INI1 or BAF47), a component of the SWI/SNF chromatin remodeling complex, is involved in transcriptional activation of p16 and p21, and thus senescence. Loss of the *hSNF5* gene occurs in most rhabdoid tumors, rare pediatric tumors, and germ line mutations predispose individuals to their development [82,83]. Though SNF5-deficient mice suffer embryonic death at E6.5, SNF5 hetero-insufficiency or conditional knockout allows survival with rhabdoid tumor development [84,85]. Re-expression of hSNF5 in rhabdoid tumor cell lines induces senescence associated with upregulation of p16 and p21 [86]; p16 is required for the hSNF5-induced senescence [87]. Thus, SNF5 normally functions as a rhabdoid tumor suppressor by inducing senescence through upregulation of p16 and p21. SNF5 is a mammalian homolog of *Drosophila* trithorax group (TrxG) proteins. PcG and TrxG silence or activate, respectively, many developmental genes, including HOX forms [88]. In mammals, p16 is an important gene that is regulated by PcG and TrxG proteins. Recent work suggests that

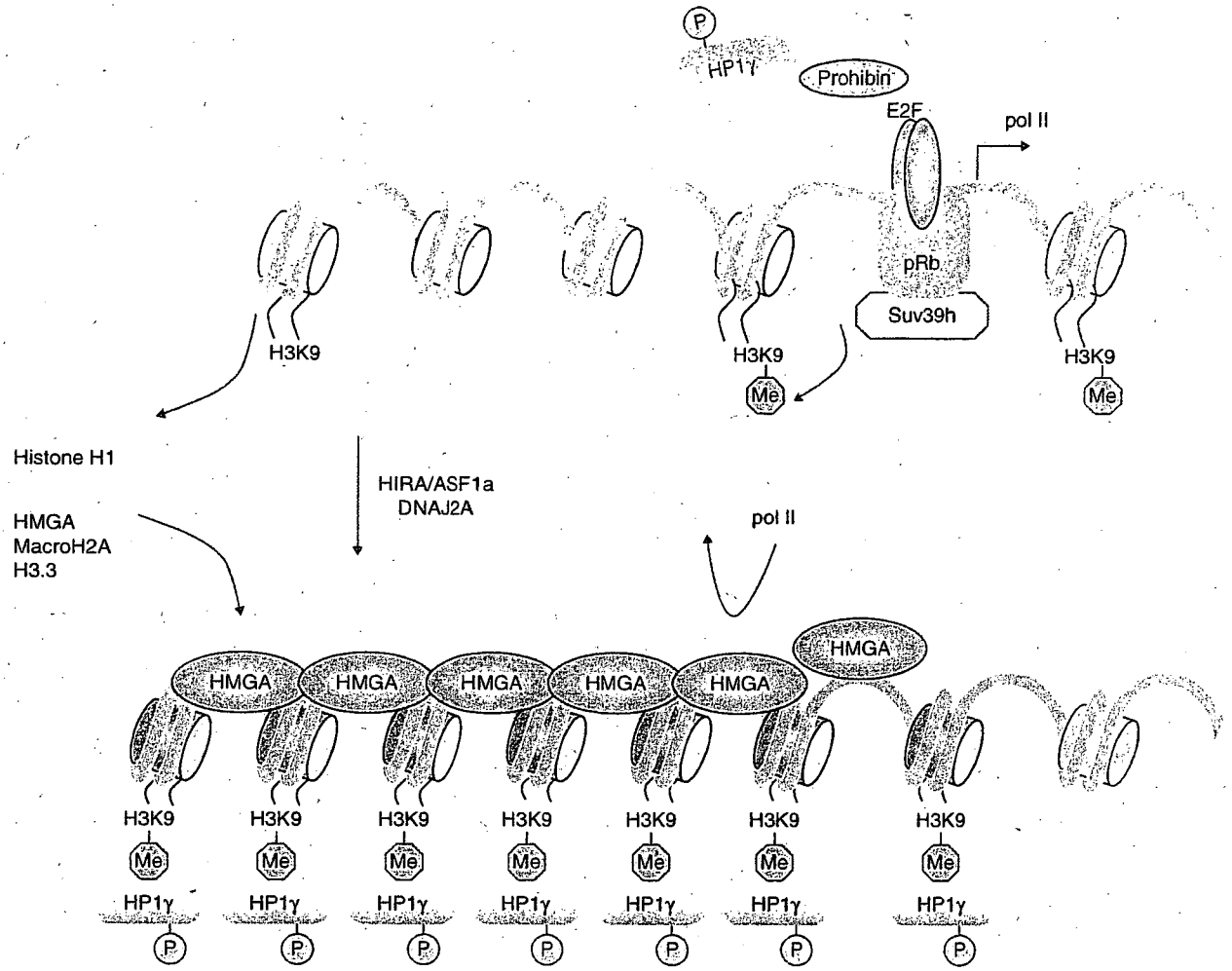

Figure 3. Molecular mechanisms of senescence-associated heterochromatic focus formation. E2F target genes are transcriptionally active in replicating cells. In senescent cells, repression of E2F-target genes is initiated by a multi-protein complex consisting of pRb, Suv39h, prohibin and HP1γ phosphorylated at Ser93. Stable repression of E2F target genes is maintained by SAHF formation, which is associated with alteration of histone and non-histone chromatin associated proteins.

ASF: Antisilencing function; HIRA: Histone repressor A; HMGA: High-mobility group A protein; HP: ; pol II: Polymerase II; pRb: Retinoblastoma protein.

PcG-mediated gene silencing, including X-inactivation, involves noncoding RNAs and the RNA interference (RNAi) machinery ([88] for review). However, it is not known how PcG proteins maintain silencing of specific genes, such as *CDKN2A* (p16) during the process of cell division and how specific genes are targeted by TrxG for activation.

13. Senescence-associated heterochromatic foci formation

Irreversibility of the senescent state can be explained by global heterochromatinization of the genome in the cells. Senescence-associated heterochromatic foci (SAHF) formation was originally found on chromosomes in senescent cells induced by activation of the p16/pRb pathway [89]. SAHF are enriched in high-mobility group A proteins (HMGA) and heterochromatin-associated proteins, such as histone H3 methylated at lysine 9 (H3K9me), HP1γ and macroH2A [89-91]. Increased levels of variant histones, such as H1.0 and H3.3, and decreased levels of canonical histones, such as linker histone H1 and H3.1, are also observed in senescent cells ([92-95] for review). Similar substitution of histone variants, both in senescence and differentiation, suggests overlapping mechanisms between the two processes. It is noteworthy that HBP1, a transcription factor stabilized by p38 and required for ras-induced senescence, positively regulates expression of H1.0 [80]. Histone chaperones, histone repressor A (HIRA) and antisilencing function 1a (ASF1a), which interacts with its deposition substrate histone H3 [90,96], are involved in chromatin remodeling at SAHF (Figure 3). Overexpression of either HIRA or ASF1a is sufficient for induction of SAHF formation and

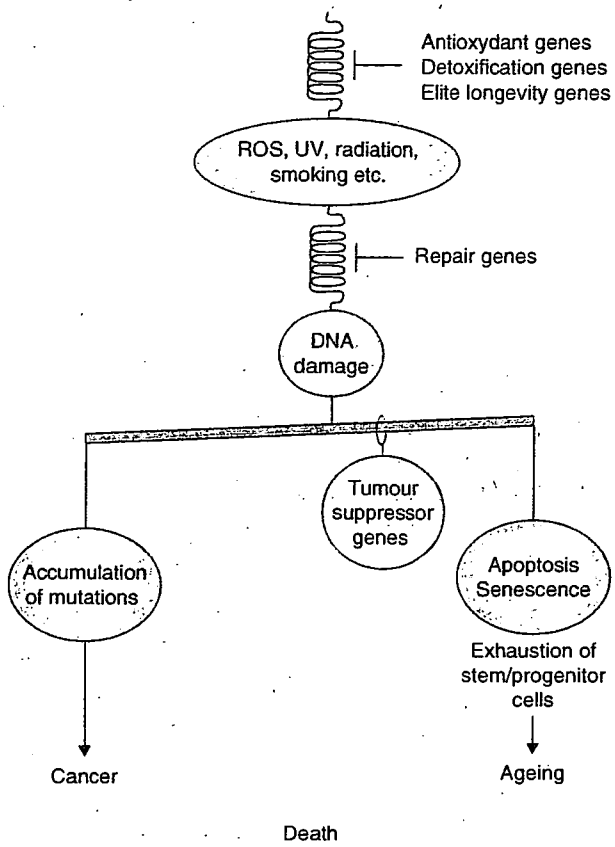

Figure 4. Genetic and environmental risk factors for cancer and ageing. Tumor suppressor genes evolved to balance risks of cancer and ageing. Although tumor suppressor genes are a burden to the balance, as some of the protective genes against DNA damage are regulated by a tumor suppressor gene p53, they could be either burden or balloon to the balance.
ROS: Reactive oxygen species.

senescence phenotypes, and ASF1a is required for oncogenic ras-induced senescence [90]. Although macroH2A is enriched in inactive X-chromosomes and functionally associated with heterochromatinization, H3.3 is generally associated with open chromatin, so that it is not clear whether incorporation of H3.3 by HIRA and ASF1a is associated with SAHF formation. It will be more important to determine the mechanism whereby macroH2A is incorporated into chromatin. Translocation of HIRA into PML nuclear bodies and transient colocalization with HP1γ precedes SAHF formation [90] and dominant negative HIRA mutants that block the translocation prevent SAHF formation [96]. Moreover, ectopic expression of DNAJA2, an Hsp40 member that physically interacts with both HIRA and pRb, is also sufficient to trigger SAHF formation [96]. Thus, DNAJA2 might integrate signals from the pRb and HIRA/ASF1a pathways [96]. In senescing cells, HP1γ is specifically phosphorylated on Ser 93 and efficiently incorporated into SAHF [96]. HP1γ recognizes and binds H3K9me, whose methylation in SAHF formation requires Suv39h [97]. HP1γ is specifically recruited to E2F1-regulated promoters in senescence, but not in quiescent cells. This process requires prohibitin, a growth inhibiting protein [98]. However, the total amount of H3K9me3 is unchanged before and after senescence [94], and a dramatic reduction in the amount of chromatin-bound HP1 proteins by expression of dominant negative mutant of HP1β does not affect SAHF formation [96]. These results suggest that Suv39h might be required for local suppression and heterochromatinization of E2F-regulated promoters by a multimolecular protein complex including E2F, pRb, HP1γ and prohibitin [98]. As individual SAHF form on individual chromosomes [94,96], spreading of heterochromatinization should occur as with X-inactivation. Knockdown of HMGA strongly inhibits SAHF formation induced by overexpression of p16 in normal human fibroblasts [91]. As HMG proteins compete with H1 for the same nucleosome site, it is probable that HMGA proteins substitute for histone H1 in SAHF. Although HMG proteins are considered to destabilize the higher-order chromatin structure, enrichment of HMGA proteins in SAHF suggests that HMGA-dependent changes in chromatin structure depends on other components and modification of chromatin proteins. Once SAHF are established, transcription factors, including E2F, have difficulty in accessing target genes involved in cell cycle re-entry and irreversibility of growth arrest is assured. Interestingly, acute depletion of p400 leads to cellular senescence of normal human fibroblasts associated with induction of p21, SA-β-gal and SAHF formation. In this setting, pRb is not required for SAHF formation, although it is possible that other pRb family members substitute the pRb function. It remains to be determined as to whether senescence can be established in the absence of all three pRb family members.

14. Immortalization of human cells

Most human cells, including human dermal keratinocytes, mammary epithelial cells and prostate epithelial cells [99,100] undergo cellular senescence independent of telomeres. Even fibroblasts derived from the lung, such as the WI38 line, cannot be immortalized with hTERT alone [101]. In these cases, senescence is often accompanied by p16 accumulation with or without accumulation of p53 and inactivation of the p16/pRb pathway by Bmi-1 or RNAi is often sufficient to suppress telomere-independent senescence [101,102]. Unlike the case with MEF cells, a low oxygen concentration does not prevent elevation of p16 levels in these cells (Kiyono unpublished observation [101-103]), although it reduces expression of the p53 target, p21 [101].

Some human somatic cells can be immortalized by viral and/or cellular oncogenes. Unlike mouse cells, however, spontaneous immortalization of human cells is a rare event. Although SV40 large T and the adenovirus E1 region, both of which can inactivate p53 and pRb, have been

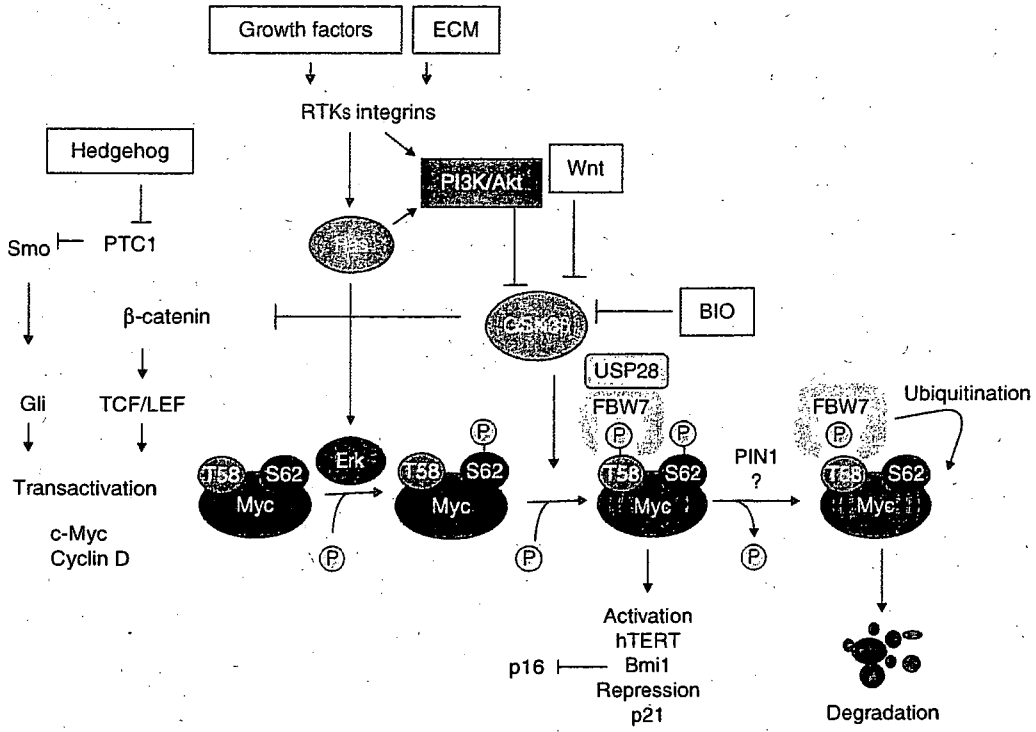

Figure 5. Regulation of Myc by various signaling pathways. Fine tuning of Myc activity by extracellular signaling molecules appears important for maintenance and expansion of stem and/or progenitor cells *in vitro*.

known to extend the life span of many human cells, these eventually undergo crisis or mortality stage 1 (M1) due to the fusion of extremely shortened telomeres. Until hTERT cDNA was cloned, efficient immortalization of human cells was only achieved by HPV16 E6 and E7, which were subsequently found to activate telomerase in addition to their inactivation of p53 and pRb. As activation of the p16/pRb pathway and/or the p53 pathway often limit the proliferation of primary human cells, temperature-sensitive mutants of SV40 LT and HPV E6/E7 in addition to hTERT are still used for immortalization of various human cells. However, they often change the differentiation potential and induce chromosomal instability. *PcG* genes, Bmi-1, CBX7 and CBX8, have been shown to repress *CDKN2A* loci and have been applied for immortalization [104-106]. Similarly, RNAi of p16 has been used [102]. As Cdk4–cyclin D can phosphorylate and inactivate pRb, overexpression of Cdk4 has been also applied to immortalize bronchial and small airway epithelial cells [103].

15. Conclusion

Cellular senescence, despite its name, is only one of many processes that contribute to senescent phenotypes in multicellular organisms, including humans. Therefore, even complete inhibition of cellular senescence does not promise perpetual youth and longevity. Rather, cellular senescence functions as a tumour suppression mechanism with consequent longevity. Sustained inhibition of CDKs is a major mechanism that can induce cellular senescence *in vitro*. However, organismal ageing is much more complicated. Many factors that induce exhaustion of tissue stem cells and/or degeneration of even differentiated cells may be involved ([107] for review).

Nevertheless, an understanding of cellular senescence can lead to improvements of human health in several aspects. As most genotoxic stresses that induce cellular senescence and/or apoptosis are also known causes of cancer, reducing putative stress is good for health in both ways (Figure 4). Therefore, some chemopreventive drugs for cancer could be good candidates for anti ageing drugs, and vice versa.

16. Expert opinion

Amplification of somatic stem or progenitor cells for cell transplantation therapy is often limited by cellular senescence. *In vitro* amplification of epidermal cells and corneal cells is relatively successful without genetic manipulation and has been applied for clinical use. However, hematopoietic stem cells, myoblasts, neural stem cells, pancreatic β-cell and hepatocytes are not easily amplified, not because of telomere shortening, but due to other factors including telomere-independent senescence. Understanding the mechanisms by which these primary human cells

enter senescence with serial passage will facilitate their amplification *in vitro*.

Accumulation of p16 during serial passage is observed in many human cell types, with exceptions such as human foreskin fibroblasts. It has been proposed that stress, imposed by inadequate culture conditions, induces senescence due to accumulation of p16 [108]. Indeed, accumulation of p16 can be alleviated by improved culture conditions with fibroblast feeder cells for human keratinocytes and mammary epithelial cells [108,109]. In these cases, it is suggested that culture on plastic and chemically defined serum-free media provide the stress, although the precise mechanism is unknown.

Mesenchymal–epithelial interactions are considered to be important in many circumstances, including development, differentiation and maintenance of stem or progenitor cells. Therefore, it is possible, by mimicking the *in vivo* environment or niche, that cultured cells on feeder cells can be stimulated to grow happily without sensing stress. As accumulation of p16 can be seen in adult tissue, cells in the *in vivo* environment in adults, especially in elderly individuals, appear to be exposed to stress. However, no p16 expression can be detected in the developmental stages of either the human or the mouse, with then exception of the thymus [110,111]. In accordance, mice lacking p16 exhibit normal development except for thymic hyperplasia [19]. Therefore, it might be a good idea to mimic certain aspects of the environment of the corresponding tissue at the developmental stage. During development, stem cells or progenitor cells are amplified until they reach sufficient numbers of cells to constitute the body. Subsequently, even in elderly individuals, stem cells help to build and maintain tissue homeostasis of the adult organism. Stem cell niches are also diverse, sometimes harboring multiple stem cell types [112] for review. The cellular environment consists of attached cells, elements of extra-cellular matrix (ECM) and humoral factors, which are regulated spatially and temporally. In some cases, attached cells, which correspond to feeder cells in culture, might be able to express cell surface ligands and secrete ECMs and/or humoral factors. Indeed some cell types, including embryonic stem (ES) cells and hematopoietic stem cells, largely depend on good feeder cells. Identification of essential factors to support the 'stem' nature and growth of these cells should be actively pursued. Although it is not easy to identify such factors for each tissue stem cell, Wnt and Hedgehog, as well as bone morphogenetic proteins, fibroblast growth factors and Notch ligands should be highlighted as potential candidates. Developmental signals also play important roles in regeneration [113,114], as well as carcinogenesis of certain organs ([115] for review). There are many examples in which stem cells and cancer cells derived from them are influenced by the same signals [114,116-120]. For instance, Wnt signaling is active in both colon epithelial stem cells and most colorectal cancers. Hedgehog signaling is active in both epidermal stem cells and basal cell carcinomas. Interestingly, these two appear to engage in crosstalk. Hedgehog signals can down-regulate Wnt signaling and proliferation of colon cancer cell lines [121], suggesting that such mutual inhibition might be applied to cancer therapy, although both Hedgehog and Wnt signals are simultaneously activated in some malignancies, including chronic myeloid leukemia [122].

Signaling pathways are thought to determine cell fate by inducing epigenetic changes in differentiation-related genes, including HOX forms, although the latter can also regulate expression of *Hedgehog* genes [123]. The pancreas develops in the absence of Hedgehog signals and the stomach develops under its influence. Interestingly, Hedgehog signaling is active in most pancreatic cancers that ectopically express gastric markers [124], indicating that these signals can induce further epigenetic changes in tissue progenitor cells in adults. Therefore, it is important to choose appropriate signaling molecules and also to optimize their concentrations to maintain the phenotypes and proliferation of progenitor cells *in vitro*. It is possible that progenitor or stem cells derived from adult tissues might have the capacity for growth like in the developmental stage *in vivo* without accumulating p16 if Wnt or Hedgehog signaling is appropriately modulated under optimal culture conditions.

More recently, overexpression of c-Myc alone was shown to activate hTERT expression, override the accumulation of p16 and immortalize prostate epithelial cells [125], without inducing apoptosis or senescence. Myc is an essential target of β-catenin in colon tumorigenesis ([126] for review), and is required for most Wnt target gene activation following Apc loss [127]. Myc is also an important target of Hedgehog signaling. Thus, Wnt and/or Hedgehog signals might be able to regulate the Myc function so as to induce immortalization of human cells. Small molecules such as the GSK3 inhibitor, 6-bromoindirubin-3′-oxime, are alternative regulators of Myc function (Figure 5).

Immortalized cells following genetic manipulation have been used for cancer research and basic enquiries into regenerative medicine, but they are far from clinical application due to putative risk of cancer development after implantation. New strategies to immortalize human cells without genetic manipulation are urgently required for the broad application of human cells in regenerative medicine.

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REGULAR ARTICLE

Characteristic phenotype of immortalized periodontal cells isolated from a Marfan syndrome type I patient

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Abstract The periodontal ligament (PDL) is situated between the tooth root and alveolar bone, thereby supporting the tooth, and is composed of collagen and elastic system fibers. Marfan syndrome type I (MFS1, MIM #154700) is caused by mutations in *FBN1* encoding fibrillin-1, which is a major microfibrillar protein of elastic system fibers. MFS1 is characterized by tall stature, aortic/mitral valve prolapse, and ectopia lentis and is occasionally accompanied by severe periodontitis. Since little is known about the biological functions of elastic system fibers in PDLs and the pathogenesis of the periodontitis in MFS1, PDL cells were isolated from an MFS1 patient with a heterozygous missense mutation in a calcium-binding epidermal-growth-factor-like domain of *FBN1*. Isolated PDL cells were immortalized by transducing a retrovirus carrying genes for the human Polycomb group protein, Bmi-1, and human telomerase reverse transcriptase. Immortalized PDL cells from the MFS1 patient (termed M-HPL1) and those of a healthy volunteer (termed HPDL2) both expressed various PDL-related genes. The growth and attachment of M-HPL1 and HPDL2 to hydroxyapatite particles were comparable. However, when M-HPL1 were transplanted with hydroxyapatite particles into immunodeficient mice, disorganized cell alignment and irregular microfibril assembly were noted. The activation of the signaling of transforming grwoth factor-β (TGF-β) is thought to cause the pathogenesis for lung and cardiovascular abnormalities in MFS1. Interestingly, M-HPL1 shows a higher level of activated TGF-β than HPDL2. Thus, M-HPL1 represent a powerful tool for clarifying the biological roles of elastic system fibers in PDL and the pathogenesis of periodontitis in MFS1. Our findings also suggest that *FBN1* regulates cell alignment and microfibril assembly in PDLs.

Keywords Elastic fiber · Fibrillin-1 · Marfan syndrome · Periodontitis · Periodontal ligament · Human

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Introduction

The periodontal ligament (PDL) is a specialized connective tissue situated between the cementum covering the root of teeth and the alveolar bone socket (Beertsen et al. 1997; Freeman 1998). PDLs consist of various kinds of cells and fibers. The cells include fibroblasts, epithelial cell remnants of Malassez, macrophages, undifferentiated mesenchymal cells, cementoblasts, osteoblasts, and osteoclasts. The fibers include collagen and elastic system fibers. PDLs are well adapted to support teeth in bone and to act as a sensory

receptor. To support teeth, collagen fibers are embedded both in the cementum and alveolar bone, and each collagen fiber works as a spliced rope to withstand the considerable forces of mastication.

Elastic system fibers provide elasticity and resistance to stretch and expansion forces (Mecham 1991). They are widely distributed in various tissues, e.g., skin, lungs, eyes, and blood vessels. Three types of elastic system fibers (oxytalan and elastic and elaunin fibers) are known; they differ in the content of elastin (Kielty et al. 2002). Oxytalan fibers solely consist of bundles of microfibrils, which are predominantly composed of glycoproteins and fibrillin-1 and -2. The elastic fibers are constructed of bundles of microfibrils peripherally associated with elastin. In the elaunin fibers, bundles of microfibrils are intermingled with small amounts of elastin. In the PDL, the main elastic system fibers are oxytalan fibers oriented in an occluso-apical direction (Fullmer et al. 1974; Beertsen et al. 1997). A small amount of elaunin fibers is also found in the apical region (Staszyk and Gasse 2004; Sawada et al. 2006). In contrast to collagen fibers, the biological functions of the elastic system fibers in PDLs are still obscure.

Marfan syndrome type I (MFS1, MIM #154700) is an autosomal dominant disorder affecting the elastic system fibers. Its prevalence has been estimated to be 2–3 per 10,000 (Nollen and Mulder 2004). MFS1 is characterized by various clinical manifestations primarily in skeletal, ocular, and cardiovascular organs, e.g., tall stature, aortic dissection, mitral valve prolapse, and ectopia lentis (Pyeritz 2000). The responsible gene for this syndrome has been identified as *FBN1*, which encodes the major microfibrillar protein, fibrillin-1 (Dietz et al. 1991; Maslen et al. 1991). In addition to anomalies in skeletal, ocular, and cardiovascular systems, MFS1 exhibits characteristic oral features including maxillary protrusion (Westling et al. 1998), high palate, and crowding and fragility of the temporomandibular joint (Bauss et al. 2004). Severe periodontitis, which has a serious impact on the quality of life of MFS1 patients, is occasionally associated with this syndrome (Straub et al. 2002).

To clarify the biological functions of fibrillin-1 in PDLs and the pathogenesis of the periodontitis in MFS1, PDL cells have been isolated from an MFS1 patient. The prepared immortalized PDL cells, which have a mutation in a calcium-binding epidermal-growth-factor-like (cbEGF) domain of fibrillin-1, might be a powerful tool to help answer these questions.

Materials and methods

Subjects

The patient was a 46-year-old female. She was 172 cm tall, weighed 58 kg, and had arachnodactyly. Her father and younger brother were also diagnosed as having Marfan syndrome. She had previously had dissecting aneurysm of the aorta and had had a surgical replacement of the aortic root (Bentall operation) at 42 years of age. She had suffered from mitral valve prolapse and had had a replacement of the mitral valve at 46 years of age. Since she had severe periodontitis in all teeth, all teeth were extracted before the mitral valve replacement to avoid infective endocarditis. The patient kindly provided these teeth to us with consent.

Extracted teeth were also provided by three healthy volunteers (volunteer A, 15-year-old male; volunteer B, 15-year-old male; volunteer C, 21-year-old female) during the course of orthodontic treatment, with consent. The experimental protocol was approved by the Ethical Review Committee of Tokyo Medical and Dental University.

Isolation and culture of primary PDL cells

Isolation and culture of human PDL cells were performed as previously described (Kapila et al. 1996; Shiga et al. 2003). In brief, the extracted teeth from the MFS1 patient and healthy volunteers were washed with α-minimum essential medium (α–MEM; Kohojin Bio, Japan) containing Antibiotic-Antimycotic (GIBCO, Calif.). The PDL attached to the middle part of the root was isolated with a surgical scalpel. The PDL was minced and placed in 35-mm tissue culture dishes (SUMILON, Japan). The explants were then covered with sterile glass coverslips and incubated in α–MEM with 10% fetal bovine serum (FBS; Japan Bioserum, Japan) at 37°C under 5% CO_2 and 95% air until cells outgrew from the explants. After the outgrowth of cells, coverslips were removed from the culture dishes. The culture medium was changed every 3 days. PDL cells from passages 3–7 were used for examing mineralization, measuring alkaline phosphatase (ALP) activity, and transduction.

Mineralization of the primary culture of PDL cells

To determine the mineralization of cultured PDL cells, cells were plated at 2.0×10^4 cells/cm^2 and cultured in α–MEM containing 10% FBS. After cells became confluent, the medium was changed to α–MEM containing 10% FBS with 50 μg/ml ascorbic acid (Wako, Japan), 10 nM dexamethasone (Sigma, Mo.), and 10 mM β-glycerophosphate (Sigma) in some cultures, as previously described (Cho et al. 1992; Nohutcu et al. 1997; Chien et al. 1999). The medium was changed every 3 days, and the cells were cultured for 3 weeks. Mineralized matrix in the culture were stained by Alizarin Red S (Wako, Japan) at the end of the culture (Saito et al. 2002). All experiments were performed in triplicate wells.

ALP activity

Cells were cultured under the same conditions as described above for mineralization. ALP activity was assayed in cell lysates by enzymatic conversion of the p-nitrophenylphosphate substrate to p-nitrophenol by using the LabAssay ALP kit (Wako) according to the manufacturer's instructions. The activity was recorded as millimoles per milligram per 15 min. The total protein amount in the cell lysates was measured by using the Bradford microassay (Bio-Rad, Calif.) according to the manufacturer's instructions.

Immunohistochemical staining of cultured PDL cells

Cultured PDL cells were immunohistochemically stained by using anti-human periostin rabbit polyclonal antibody (BioVendor laboratory Medicine, N.C.), anti-bovine collagen type XII monoclonal antibody (Clone 378D5, Kamiya Biomedical, Wash.), anti-active transforming growth factor-β (TGF-β) rabbit polyclonal antibody (LC1-30, provided by K. Flanders, National Cancer Institute, Md.; Flanders et al. 1989), or anti-human latency-associated peptide-β1 (LAP-β1) goat polyclonal antibody (R&D systems, Minn.). Cells (2.0×10^4 cells/cm^2) were cultured on poly-L-lysine-coated glass (Iwaki, Japan). They were then fixed with 4% paraformaldehyde (PFA) for 30 min, blocked with 1% bovine serum albumin (BSA), and incubated with each antibody for 1 h. Sections were then treated with Alexa Fluor 594 goat anti-rabbit IG (H+L; Invitrogen, Calif.), Alexa Fluor 488 goat anti-mouse IG (H+L; Invitrogen), or Alexa Fluor 488 donkey anti-goat IG (H+L; Invitrogen). As negative controls, primary antibodies were replaced with normal rabbit serum (Vector Laboratories, Calif.), mouse IgG (Jackson Immuno Research Laboratories, Pa.), or normal goat serum (Vector Laboratories). After washes with phosphate-buffered saline, fluorescence was observed by means of a fluorescence microscope (AF6000, Leica, Germany).

Mutational analysis

DNA from the MFS1 patient and healthy volunteers was extracted by using a DNA extraction kit (Bio-Rad, Calif.). Extracted DNA was amplified by using specific primers for *FBN1* and *TGFBR2* (encoding TGF-β receptor II, which is the responsible gene for Marfan syndrome type II; MFS2, MIM #154705; Mizuguchi et al. 2004). Primer sequences and polymerase chain reaction (PCR) conditions were as given on the website of "Multiple Malformation Syndromes (http://www.dhplc.jp/genetics/frame.html)" provided by the Department of Pediatrics, Division of Medical Genetics, Keio University School of Medicine. Mutations in each amplicon were analyzed by denaturing high-performance liquid chromatography (DHPLC), as described in previous studies (Kosaki et al. 2005; Udaka et al. 2005).

After DHPLC analysis, PCR products were purified on a desalting column and were sequenced by a dideoxy-sequencing method (BigDye Dideoxy sequencing kit, Applied BioSystems, Calif.) and an automated sequencer (ABI3100, Applied Biosystems; Udaka et al. 2005).

Retroviral vectors and infection

Primary PDL cells, obtained from a healthy volunteer and the MFS1 patient, were transduced with genes for human Polycomb group protein, Bmi-1, and human telomerase reverse transcriptase (hTERT) by using retrovirus-mediated gene transfer. The production and infection of LXSN-Bmi-1 and MSCVpuro-hTERT retroviruses were performed as described previously (Kyo et al. 2003; Saito et al. 2005). The infected cells were selected in the presence of geneticin (125 μg/ml) or puromycin (0.5 μg/ml). For combined retroviral infection, cells were sequentially transduced with LXSN-Bmi-1 and then with MSCVpuro-hTERT. Stably transduced cells were maintained in the medium described above.

Detection of telomerase activity

After infection, telomerase activity was determined by a telomerase repeat amplification assay by using the TRA-Peze Telomerase Detection Kit (CHEMICON International, Calif.), according to the manufacturer's instructions.

Cell proliferation in monolayer culture

To examine cell proliferation, cells were inoculated at 5.0×10^3 cells/cm^2 into 6-well dishes (Iwaki, Japan) and cultured in α–MEM containing 10% FBS. The medium was changed every 3 days, and cells were counted every 3 days up to day 15. All experiments were performed in triplicate wells.

Western blot analysis

The introduction of Bmi-1 was identified by Western blot analysis by using anti-human Bmi-1 monoclonal antibody (BD Pharmingen, San Diego, Calif.). Cells were cultured in α–MEM containing 10% FBS and lysed in buffer containing 50 mM TRIS-HCl (pH 7.4), 125 mM NaCl, 0.1% Nonident P-40 (NP-40; Sigma), and 1 mM each of EDTA and phenylmethylsulfonyl fluoride, followed by sonication. After electrophoretic resolution of the cell lysate (20 μg each protein) on 12.5% SDS-polyacrylamide gels, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Amersham, N.J.). The subsequent

detection procedure was performed as described previously (Saito et al. 2005).

RNA preparation and reverse transcription/PCR

Total RNA was isolated from primary PDL cells (both from healthy volunteer B and the MFS1 patient) and from immortalized PDL cells cultured in α–MEM containing 10% FBS, by using ISOGEN (Nippon Gene, Japan) according to the manufacturer's instructions. cDNA was synthesized from 1 μg total RNA by QuantiTect Reverse Transcription (QIAGEN, Germany), and each cDNA was used as the template for subsequent PCR amplification. Amplification was performed in a GeneAmp PCR System 9700 (Applied Biosystems). The reaction conditions were 94°C for 1 min, 60°C for 30 s, and 72°C for 30 s. The sequences of the used primers were: *POSTN* encoding periostin, sense 5′-ATTGATGGAGTGCCTGTG-3′, antisense 5′-CCTTGGTGACCTCTTCTTG-3′; *ASPN* encoding asporin, sense 5′-CGATACAAAGAACTACAAAGGCT GG-3′, antisense 5′-GCATTTCCCAGTATTTCA CCG-3′; *COL12A1* encoding collagen type XII, sense 5′-CGGA CAGAGCCTTA CGTGCC-3′, antisense 5′-CTGCCC GGGTCCGTGG-3′; *BGLAP* encoding osteoclacin, sense 5′-CCTTTGTGTCCAAGCAGGAG-3′, antisense 5′-TCA GCCAACTCGTCACAGTC-3′; *OPN* encoding osteopontin, sense 5′-TTGCAGTGATTTGCTTTTGC-3′, antisense 5′-TGTGGGG CTAGGAGATTCTG-3′; *BSP* encoding bone sialoprotein, sense 5′-GAACCACTTCCCCACCT TTT-3′, antisense 5′-TCTGACCATCATAGCCATCG-3′; *COL1A1* encoding collagen type I, sense 5′-CTGACCTT CCTGCGCCTGATGTCC-3′, antisense 5′-GTCTGGGGC ACCAACGTCCAAGGG -3′; and a human gene encoding β-actin, sense 5′-ATGAGGATCCTCACCGAGCGCGGCT ACAG C-3′, antisense 5′-ACACCACTGTGTTGGCGTAC AGGTCTTTGC-3′. Optimization of PCR cycle number to allow semi-quantitative analysis was performed by generating saturation curves of amplified product against cycle number. Saturation was seen with 33, 34, 31, 34, 41, 41, 25, and 25 cycles for *POSTN*, *ASPN*, *COL12A1*, *BGLAP*, *OPN*, *BSP*, *COL1A*, and β-actin, respectively. Thus, the semi-quantitative gene expression analysis by reverse transcription/PCR (RT-PCR) was performed with 30, 31, 28, 31, 39, 39, 23, and 23 cycles for *POSTN*, *ASPN*, *COL12A1*, *BGLAP*, *OPN*, *BSP*, *COL1A*, and β-actin, respectively.

A 151-bp fragment of *POSTN* (2220–2370 in NM_006475), a 292-bp fragment of *ASPN* (1031–1322 in NM_017680) (Yamada et al. 2001), a 180-bp fragment of *COL12A1* (7041–7220 in NM_080645), a 151-bp fragment of *BGLAP* (122–272 in NM_199173), a 166-bp fragment of *OPN* (173–338 in NM_001040058), a 201-bp fragment of *BSP* (876–1076 in NM_004967), a 300-bp fragment of *COL1A1* (4180–4479 in NM_000088), and a 327-bp fragment of the gene encoding β-actin (641–967 in NM_001101) were separated on 2% agarose gels (Nippon Gene, Japan) by electrophoresis. The gels were stained with ethidium bromide, photographed under ultraviolet excitation, and analyzed by using picture-imaging software (Scion Image, Scion, Md.).

Cell adhesion assay

To examine the adhesion of PDL cells, viz., HPDL2 from healthy volunteer 2 and M-HPL1 from the MFS1 patient, to hydroxyapatite particles (size 300–500 μm; OSferion, Olympus, Japan), both types of cells were labeled by using the PKH26 Red Fluorescent Cell Linker Mini Kit (Sigma) and incubated with hydroxyapatite particles for 18 h in α–MEM containing 10% FBS. The attached cells were observed by using a fluorescence microscope (AF6000, Leica, Germany).

In vivo differentiation assay

Fiber formation in the HPDL2 and M-HPL1 cells was assessed as described previously (Handa et al. 2002; Saito et al. 2005; Yokoi et al. 2007). Briefly, 1.5×10^6 cells were incubated with 40 mg hydroxyapatite particles and fibrin clot (mixture of mouse fibrinogen and thrombin; Sigma). They were transplanted subcutaneously into 5-week-old male CB-17 *SCID/SCID* mice (Nihon Crea, Japan). Mice were sacrificed after 4 weeks and implanted tissues were collected. Three transplants were prepared for each group, and experiments were repeated in triplicate.

To examine human (not mouse) vimentin-positive cells in transplanted tissues, tissues were fixed in 4% PFA for 1 day, decalcified with 10% formic acid for 3 days, and embedded in paraffin, and 5-μm-thick sections were prepared. To avoid non-specific staining by mouse monoclonal antibodies, sections were blocked by using the M.O.M. kit (Vector Laboratories, Calif.) as previously described (Handa et al. 2002). Sections were incubated, for 1 h, with anti-human vimentin monoclonal antibody (V9, DAKO, Calif.), which recognizes human but not mouse cells. After being washed, sections were incubated with biotinylated secondary antibody (M.O.M. kit) and avidin-peroxidase conjugate (M.O.M. kit). The reaction was visualized by using diaminobenzidine.

To examine human fibrillin-1-positive cells, transplanted tissues were embedded in carboxymethyl cellulose compound (Finetec, Japan), and 5-μm-thick frozen sections were prepared (Kawamoto and Shimizu 2000). Frozen sections were incubated with anti-human fibrillin-1 rabbit polyclonal antibody (Elastin Products, Mo.) for 1 h. After being washed, sections were incubated with Alexa Fluor 594 goat anti-rabbit IG (H+L; Invitrogen), and fluorescence

was observed with a fluorescence microscope (AF6000, Leica, Germany).

Statistical analysis

Student's *t*-test was used to analyze differences in cell numbers between M-HPL1 and HPDL2. Each difference was considered significant at a *P*-value of less than 0.05.

Results

MFS1 patient with a heterozygous mutation in cbEGF domain of FBN1

As shown in Fig. 1a–d, the 46-year-old female patient had severe periodontitis. All teeth had to be extracted before the surgical replacement of the mitral valve to avoid infective endocarditis. Mutational analysis of *FBN1* and *TGFBR2* was performed by using a genomic DNA sample. Since *FBN1* and *TGFBR2* have 65 and 7 exons, respectively, screening of gene mutations before direct sequencing was performed by DHPLC. In total, 65 and 8 amplicons of *FBN1* and *TGFBR2*, respectively, were amplified by PCR and subsequently analyzed by DHPLC. Among them, the peak in the amplicon of exon 52 in *FBN1* from the MFS1 patient shifted to the left compared with that of the wild-type sample (Fig. 1e). This demonstrated heteroduplex formation of the amplicon from the MFS1 patient. Direct sequencing of this product was performed (Fig. 1f). A heterozygous mutation (A to G) was seen at position 6431 (from the translation site in NM_000138). This missense mutation resulted in the replacement of Asn by Ser at amino acid position 2144 (N2144S) in the 32th cbEGF domain (Fig. 1g; see also in previous study of this MFS1 patient in Hewett et al. 1993). Thus, this is not a single nucleotide polymorphism (SNP) or a novel mutation. The elution profile of DHPLC for *TGFBR2* did not show differences between the wild-type sample and MFS1 patient.

Phenotype of primary PDL cells with N2144S mutation from MFS1 patient

Cells were isolated from the PDL of extracted teeth from the MFS1 patient and were cultured in vitro. In order to examine the cellular phenotype of these isolated PDL cells, ALP activity (Fig. 2a) and mineralization (Fig. 2b) were examined. In the cell differentiation medium containing

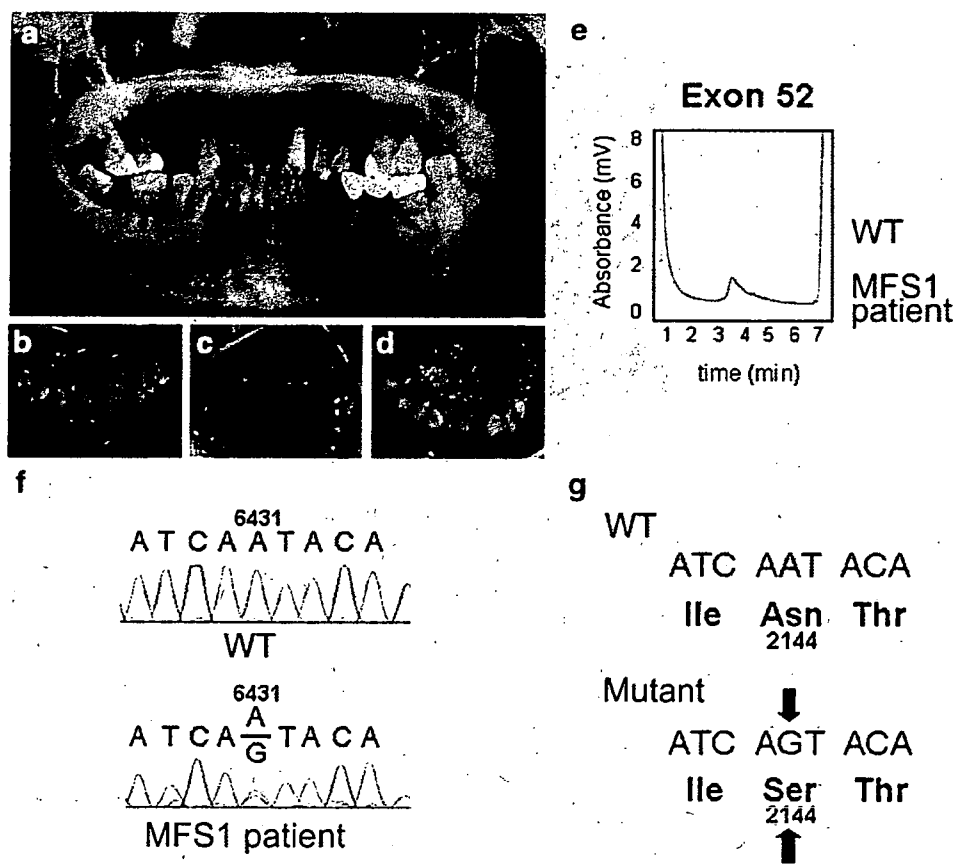

Fig. 1 Severe periodontitis and mutational analysis of the MFS1 patient. **a** In the panoramic X-ray, severe alveolar bone loss was observed around tooth roots. **b–d** Oral photographs showing the severe periodontitis of the patient. **e** DHPLC analysis of exon 52 in *FBN1*. Note the peak in the elution profile of the MFS1 patient shifted to the left compared with that of the wild-type (WT), demonstrating heteroduplex formation. **f** Nucleotide sequence of exon 52 in *FBN1*. **g** Amino acid sequence of fibrillin-1. The 6431A→G change resulted in the heterozygous missense mutation of Asn to Ser (N2144S)

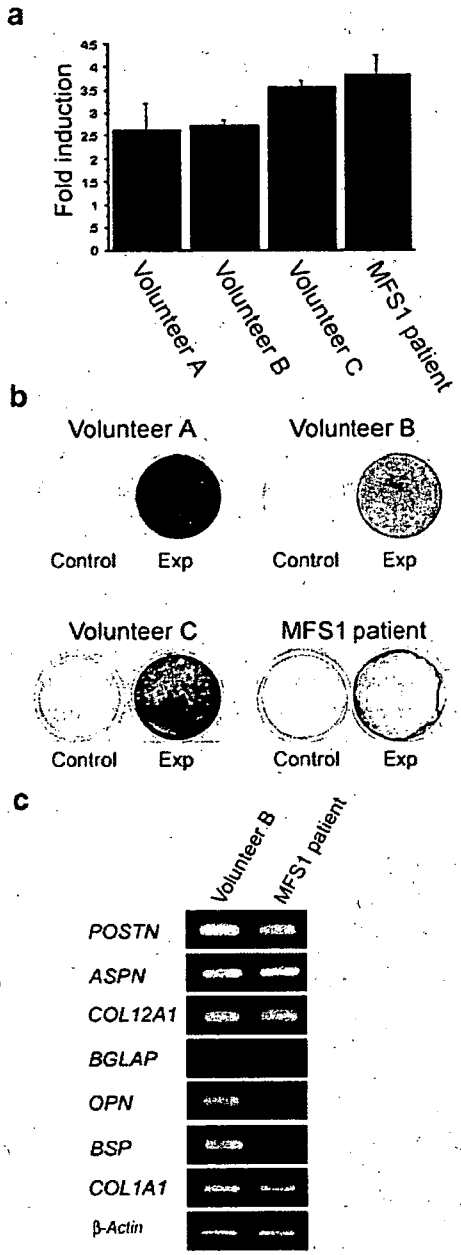

Fig. 2 **a** ALP activities of primary PDL cells isolated from three healthy volunteers (*volunteers A–C*) and the *MFS1 patient*. PDL cells were cultured in the cell differentiation medium containing ascorbic acid (50 μg/ml), β-glycerophosphate (10 mM), and dexamethasone (10 nM) for 3 weeks. Each ALP activity is represented as the ratio (*Fold induction*) to the value before culturing in the cell differentiation medium. PDL cells isolated from the MFS1 patient showed increased ALP activity as in healthy volunteers. **b** Mineralization of PDL cells cultured in the cell differentiation medium for 3 weeks. All PDL cells cultured in the cell differentiation medium were stained positively with Alizarin Red (*Exp*), but were negative when cultured in the medium containing 10% FBS solely (*Control*). **c** Expression of PDL-related genes, such as *POSTN* encoding periostin, *ASPN* encoding asporin, *COL12A1* encoding collagen type XII, *BGLAP* encoding osteocalcin, *OPN* encoding osteopontin, *BSP* encoding bone sialoprotein, *COL1A1* encoding collagen type I, and a human gene encoding β-actin, in cells from volunteer B and the MFS1 patient; reverse transcription/polymerase chain reaction (RT-PCR)

β-glycerophosphate, dexamethasone and ascorbic acid, these cells showed increased ALP activity, as did PDL cells of healthy volunteers (volunteers A–C) after a 3-week culture (Fig. 2a). The PDL cells from the MFS1 patient and healthy volunteers showed mineralization in the cell differentiation medium, but not in the medium only containing 10% FBS (Fig. 2b). The levels of the mineralization varied among cultures. Based on the similarities in the level of mineralization, PDL cells from volunteer B and MFS1 patient were selected for use in the further experiments.

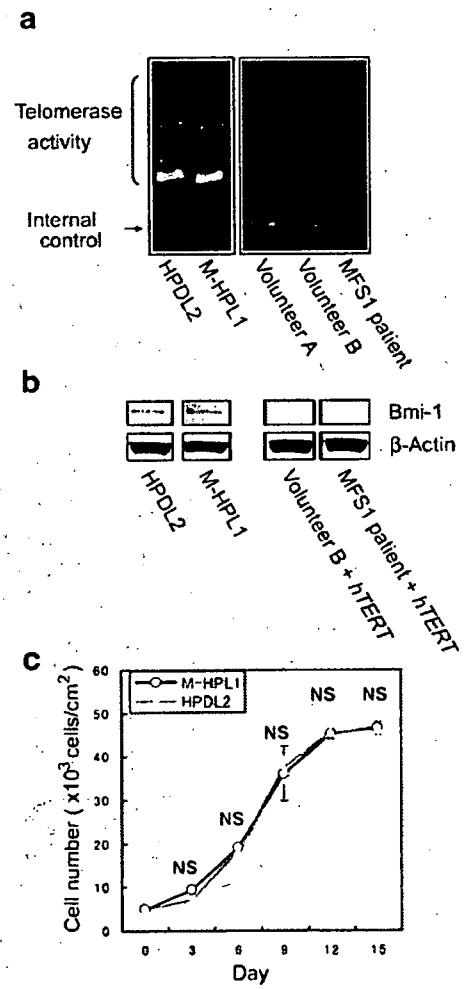

Fig. 3 **a**, **b** Telomerase activity and Western blot analysis of Bmi-1, respectively, in *hTERT*- and *Bmi-1*-transfected HPDL2 (originally from healthy volunteer B) and M-HPL1 (originally from the MFS1 patient). Note the characteristic ladder formation showing telomerase activity in HPDL2 and M-HPL1, but not in untransfected cells (volunteers A or B or MFS1 patient). Western blot analysis showing the expression of Bmi-1 in HPDL2 and M-HPL1, but not in cells transfected solely with *hTERT* (*Volunteer B + hTERT*, *MFS1 patient + hTERT*). **c** Proliferation of HPDL2 and M-HPL1 in culture. No significant difference occurs in the growth of the two types of cells at days 3, 6, 9, 12, 15 (*NS* not significant). Data represent means ± SD (n=3)

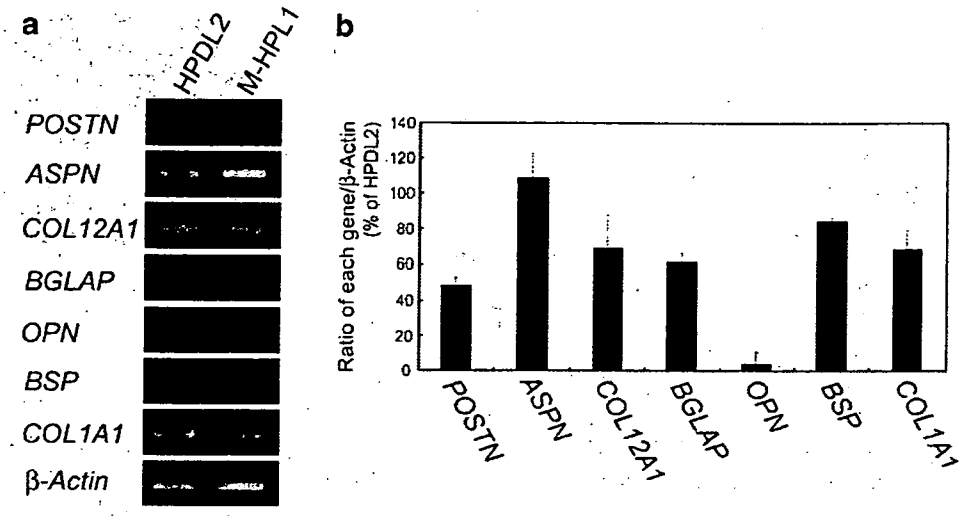

Fig. 4 **a** Expression of *POSTN* encoding periostin, *ASPN* encoding asporin, *COL12A1* encoding collagen type XII, *BGLAP* encoding osteocalcin, *OPN* encoding osteopontin, *BSP* encoding bone sialoprotein, *COL1A1* encoding collagen type I, and a human gene encoding β-actin in immortalized HPDL2 and M-HPL1; RT-PCR. **b** Densitometric data were normalized to β-actin in both types of cells. The bar graph represents the ratios of the expression of each gene in M-HPL1 (% of HPDL2). Data represent means ± SD (n=3)

The expression of various PDL-related genes was examined in cells from volunteer B and MFS1 patient by RT-PCR (Fig. 2c). Both types of PDL cells expressed *POSTN*, *ASPN*, *COL12A1*, *BGLAP*, *BSP*, and *COL1A1*. PDL cells from volunteer B expressed *OPN*. These results demonstrated that, induced by the culture conditions, the isolated PDL cells could differentiate into an osteoblastic phenotype.

Immortalization of isolated PDL cells

Since human cells have a limited life span (Sherr and DePinho 2000), and since the present PDL cells from the MFS1 patient with N2144S are valuable from a research viewpoint, the cultured cells (primary cells from volunteer B and MFS1 patient) were immortalized by retrovirus-mediated transduction. Non-transduced PDL cells and PDL cells transduced solely with *hTERT* showed senescence by passage 15. In contrast, PDL cells transduced with *Bmi-1* and *hTERT* did not show cellular senescence up to passage 20 as indicated by their cell morphology. Thus, we decided to transduce cells with both *Bmi-1* and *hTERT*. The transduced PDL cells from healthy volunteer B and the MFS1 patient were termed HPDL2 and M-HPL1, respectively.

Activation of telomerase by the transduction of *hTERT* was confirmed by the telomerase repeat amplification assay (Fig. 3a). Overexpression of Bmi-1 was confirmed by Western blot analysis (Fig. 3b). Bmi-1 was easily detected in M-HPL1 and HPDL2, but not in cells transduced solely with *hTERT*. No significant difference was seen in the cell growth between HPDL2 and M-HPL1 cultured in medium with 10% FBS up to day 15 (Fig. 3c). After day 12, the cell numbers of neither HPDL2 nor M-HPL1 increased extensively, suggesting that both types of cells had limited proliferation.

Phenotype of HPDL2 and M-HPL1

To characterize the phenotype of the immortalized PDL cells, expression of the reported PDL-related genes and β-actin was examined by semi-quantitative RT-PCR (Fig. 4a). HPDL2 and M-HPL1 both expressed *POSTN*, *ASPN*, *COL12A1*, *BGLAP*, *BSP*, and *COL1A1*. The relative expression of *POSTN*, *COL12A1*, *BGLAP*, and *COL1A1* was lower in M-HPL1 than in HPDL2 (Fig. 4b). *OPN* was expressed in HPDL2, but this was scarcely expressed in M-HPL1 (Fig. 4a, b). Immunohistochemistry with antibodies

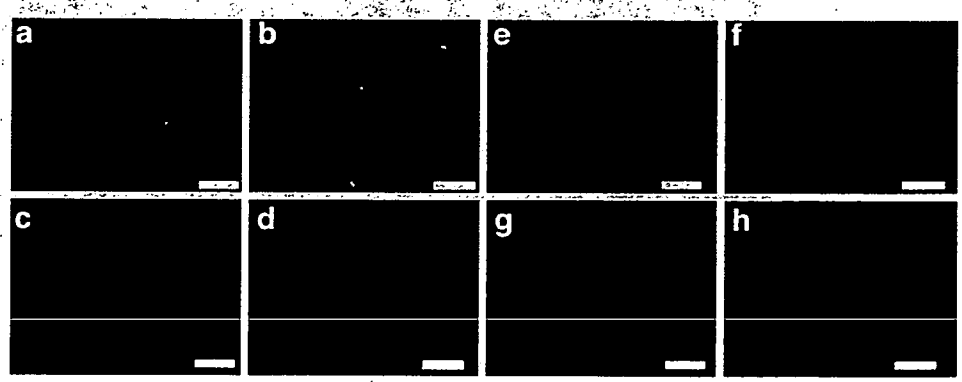

Fig. 5 Positive immunohistochemical localization of collagen type XII in cultured HPDL2 (**a**) and M-HPL1 (**b**) and of periostin in cultured HPDL2 (**e**) and M-HPL1 (**f**). Primary antibodies were replaced with mouse IgG (**c** HPDL2, **d** M-HPL1) or normal rabbit serum (**g** HPDL2, **h** M-HPL1) for negative controls. *Bars*. 100 μm

against collagen type XII (Fig. 5a, b) and periostin (Fig. 5e, f) showed numerous immunostained cells in cultures of HPDL2 and M-HDL1. Staining was scarcely seen in negative controls in which primary antibodies had been replaced with mouse IgG (Fig. 5c, d) or normal rabbit serum (Fig. 5g, h).

In the lung (Neptune et al. 2003) and cardiovascular (Ng et al. 2004) systems, gene mutation in *FBN1* has been suggested to be involved in the activation of TGF-β. To examine whether this is the case in M-HPL1, immunostaining with LC1-30, which only recognizes the active form of TGF-β, was performed. M-HPL1 showed more intense staining than HPDL2 (Fig. 6e, f), although a comparable reaction was seen in HPDL2 and M-HPL1 to the antibody against LAP-β1, which also forms complexes with TGF-β (Fig. 6a, b; Miyazono et al. 1993). Staining was scarcely seen in negative controls, in which primary antibodies were replaced with normal goat serum (Fig. 6c, d) or normal rabbit serum (Fig. 6g, h).

Cell and fiber alignments in tissues transplanted with M-HPL1

Ectopic fiber formation by M-HPL1 and HPDL2 in the subcutaneous tissues of SCID mice was examined by transplantation of these cells with hydroxyapatite particles. In this experiment, hydroxyapatite was chosen because it is the major inorganic component of teeth and bones (Ten Cate 1998). HPDL2 (Fig. 7a) and M-HPL1 (Fig. 7b) both attached to the hydroxyapatite particles 18 h after being mixed with the particles.

Four weeks after the transplantation of the cells with hydroxyapatite particles into SCID mice, sections of the cells were immunostained with anti-vimentin antibody recognizing only human but not mouse cells. HPDL2 aligned in parallel between the particles (Fig. 8a, c). In contrast, M-HDL1 were

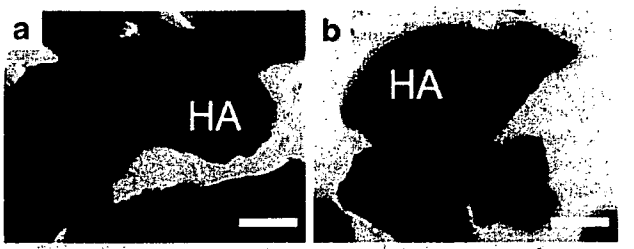

Fig. 7 Attachment of HPDL2 (**a**) and M-HPL1 (**b**) to hydroxyapatite particles (*HA*) after 18-h culture. *Bars* 100 μm

mainly located around the particles and were aligned irregularly (Fig. 8b, d). Similar observations were also seen in eight other transplants from a total of three SCID mice.

Transplanted tissues were immunostained with anti-human fibrillin-1 antibody. In contrast to the elaborate network of immunoreactive fibrillin-1 in HPDL2, M-HPL1 showed disorganized microfibril assembly (Fig. 9a, b). Staining was scarcely seen in the tissues in which the hydroxyapatite particles without cells were transplanted into SCID mice (Fig. 9c), demonstrating that the antibody only recognized human cells but not mouse cells.

Discussion

The present Japanese female patient had profound skeletal and cardiovascular symptoms including tall stature, arachnodactyly, aortic dissection, mitral valve prolapse, and severe periodontitis. Two types of Marfan syndrome (type I, MIM #154700; type II, MIM #154705) have been described so far. A large French family has been reported to exhibit the skeletal and cardiovascular features of Marfan syndrome in an autosomal dominant manner (Boileau et al. 1993). No mutation in *FBN1* has been seen in this family, and they have been classified as MFS2. Recently, *TGFBR2*

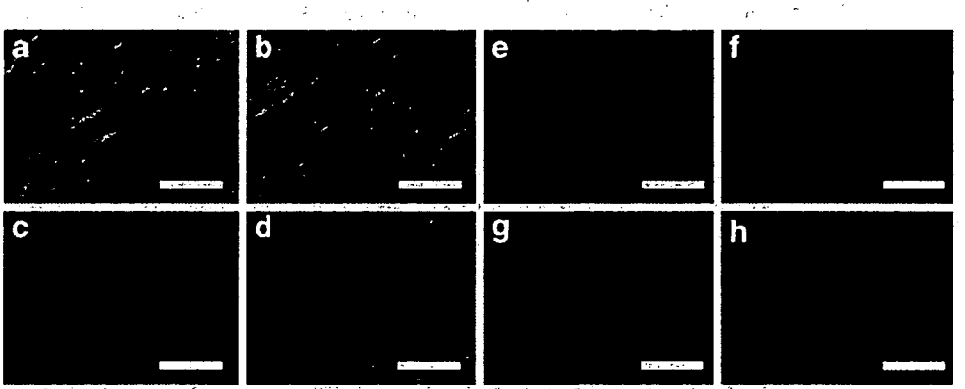

Fig. 6 Immunohistochemical localization of LAP-β1 in cultured HPDL2 (**a**) and M-HPL1 (**b**) and of LCI-30 in cultured HPDL2 (**c**) and M-HPL1 (**f**). Note that LCI-30, which recognizes only the active form of TGF-β, immunoreacts more abundantly in M-HPL1 than in HPDL2, whereas the level of LAP-β1 was comparable in the both types of cells. Primary antibodies were replaced with normal goat serum (**c** HPDL2, **d** M-HPL1) or normal rabbit serum (**g** HPDL2, **h** M-HPL1) for negative controls. *Bars* 100 μm