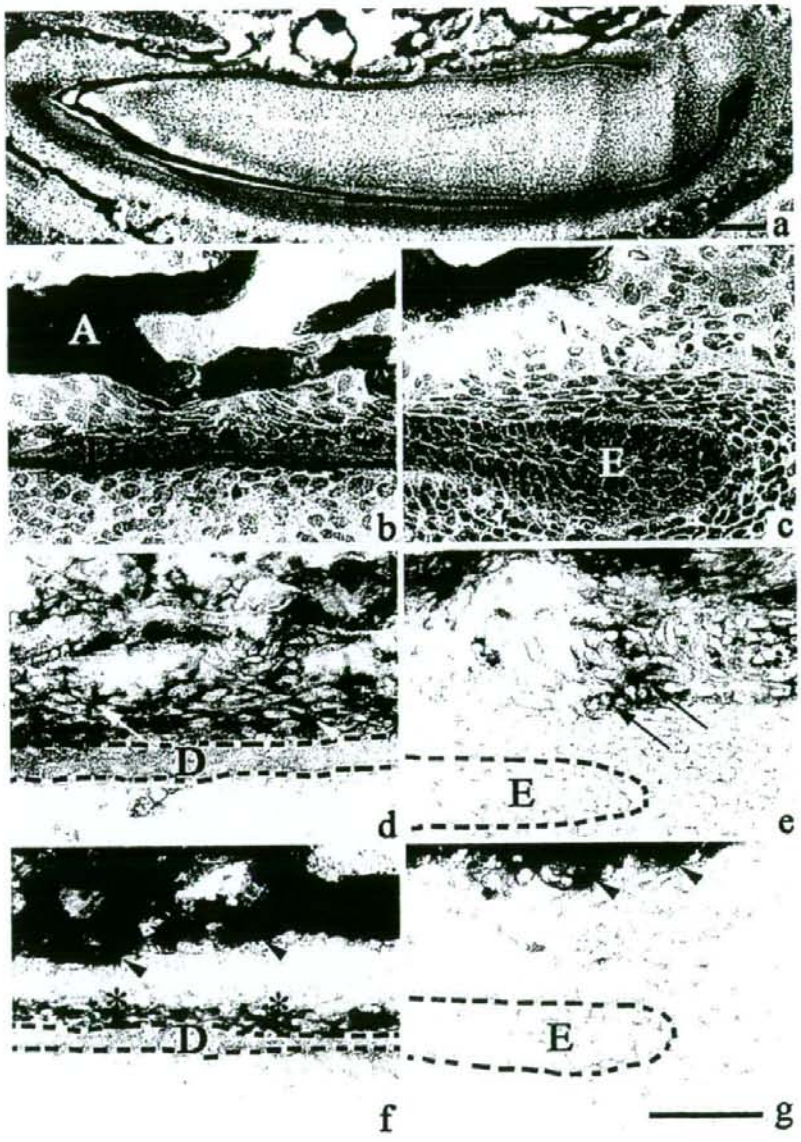

Fig. 1 In situ hybridization for *periostin* and *osteopontin* mRNA in newborn mice (*A* alveolar bone, *D* dentin, *E* dental epithelium). Sagittal sections of the incisor in the lower jaw of P1 mouse (**a**) were examined by hematoxylin and eosin staining (**a–c**) or in situ hybridization for *periostin* (**d, e**) or *osteopontin* (**f, g**). Higher magnifications of the anterior (**b, d, f**) and posterior (**c, e, g**) regions of the lingual side of the incisor are shown. Intense expression of *periostin* is seen in the developing PDL in the anterior region (**d**, *arrows*), with little expression in the posterior region (**e**, *arrows*). *Osteopontin* is expressed strongly throughout the alveolar bone (**f, g**, *arrowheads*) and in the differentiated cementoblasts in the anterior region (**f**, *asterisks*). No expression of *osteopontin* is observed in the dental follicle tissue in the posterior region (**g**). *Bars* 100 μm

histochemical analysis with immunohistochemical staining or in situ hybridization as described below. NIH3T3 cells were used for comparison.

Histochemical analysis

The transplants were fixed in 4% paraformaldehyde for 1 day, decalcified with 12.5% EDTA containing 2.5% paraformaldehyde for 3 days, and then embedded in OCT compound for the production of frozen sections. Subsequently, 30 serial sections of 5 μm in thickness were cut per implant and analyzed histochemically. Morphology was examined by hematoxylin and eosin staining. Observation by fluorescence microscopy (Axio imager, Carl Zeiss, Germany) was performed to distinguish between the cells of donor origin and host tissue. Expression of mRNA for *periosotin* and *Scx* was examined by in situ hybridization as described above. For immunohistocemical analysis, the

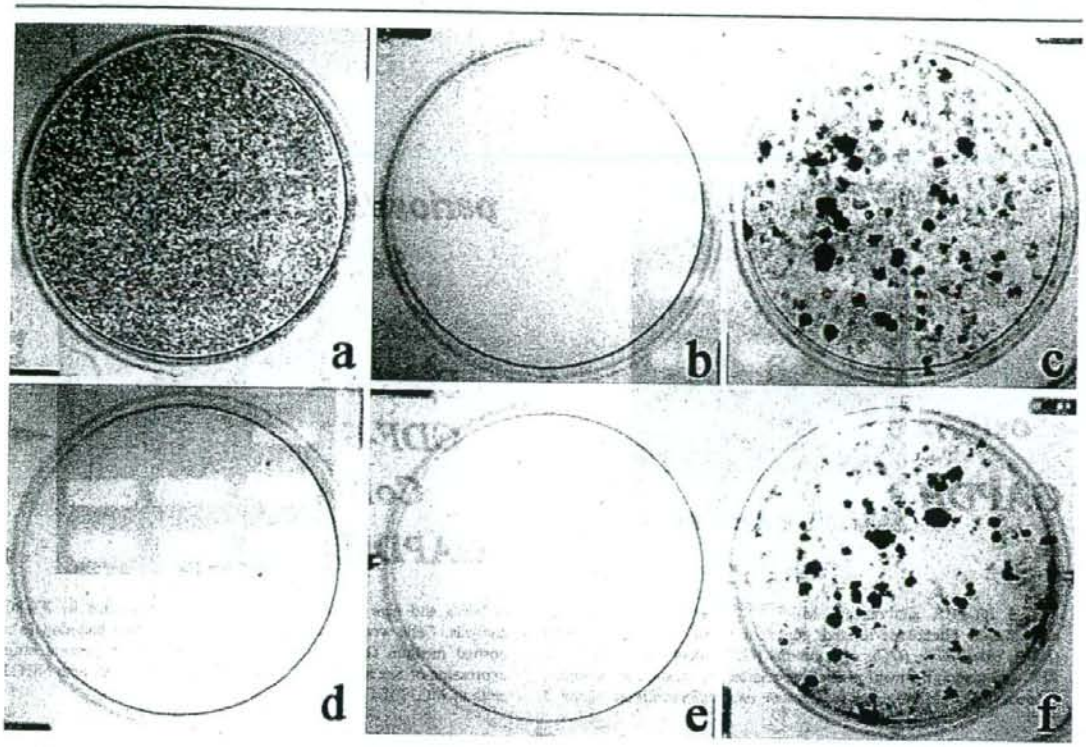

Fig. 2 ALP activity and mineralization potential of MDF$^{E6\text{-}EGFP}$ cells in vitro. MDF$^{E6\text{-}EGFP}$ cells were grown for 21 days in osteogenic differentiation medium in order to analyze ALP activity (**a–c**) and mineralization potential (**d–f**). High ALP activity by MDF$^{E6\text{-}EGFP}$ cells and MC3T3E1 (**a**, **c**) cells was apparent, whereas no activity was observed for NIH3T3 cells (**b**). Alizarin red staining showed no mineralized nodule formation for MDF$^{E6\text{-}EGFP}$ cells and NIH3T3 cells (**d**, **e**), whereas mineralized deposits were readily apparent for MC3T3E1 cells (**f**)

sections were blocked with 1% bovine serum albumin and probed with goat anti-type I collagen polyclonal antibody (Southern Biotech, Birmingham, Ala., USA) for 1 h. Sections were then probed with donkey anti-goat Alexa 555 (Invitrogen). After several washes, fluorescence in the sections was observed by fluorescence microscopy.

Results

Localization of periostin mRNA in the incisor tooth

Sequential developmental process of the dental follicle was observed in a sagittal section (in an anterior-posterior direction) of the P1 mice incisor (Fig. 1a). Immature cells were located posteriorly (Fig. 1c,e,g), and differentiation progressed toward the anterior region (Fig. 1b,d,f). To locate immature dental follicle cells, we first investigated the expression pattern of a PDL marker gene, *periostin*, and a cementoblast/osteoblast marker, *osteopontin*, in the lower incisor tooth germ of P1 mice. In a section stained with hematoxylin and eosin, dentin was seen in the anterior region (Fig. 1b), and invaginating dental epithelium was observed lingually in the posterior region (Fig. 1c). Intense expression of *periostin* was observed in the dental follicle cells close to the dentin layer in the anterior region (Fig. 1d, arrows). In contrast, only patchy expression of *periostin* was observed in the dental follicle cells in the posterior region (Fig. 1e, arrows). *Osteopontin* was expressed intensely throughout the alveolar bone (Fig. 1f,g, arrowheads). In accordance with the *periostin* expression pattern, *osteopontin* was expressed in the cementoblast adjacent to the dentin-forming layer in the anterior region (Fig. 1f, asterisks), but not in the posterior region (Fig. 1g). These data confirmed that the dental follicle cells in the posterior region were in an immature stage, whereas those in the anterior region were differentiated. We thus dissected dental follicle cells from the posterior region in order to establish a dental follicle progenitor cell culture system.

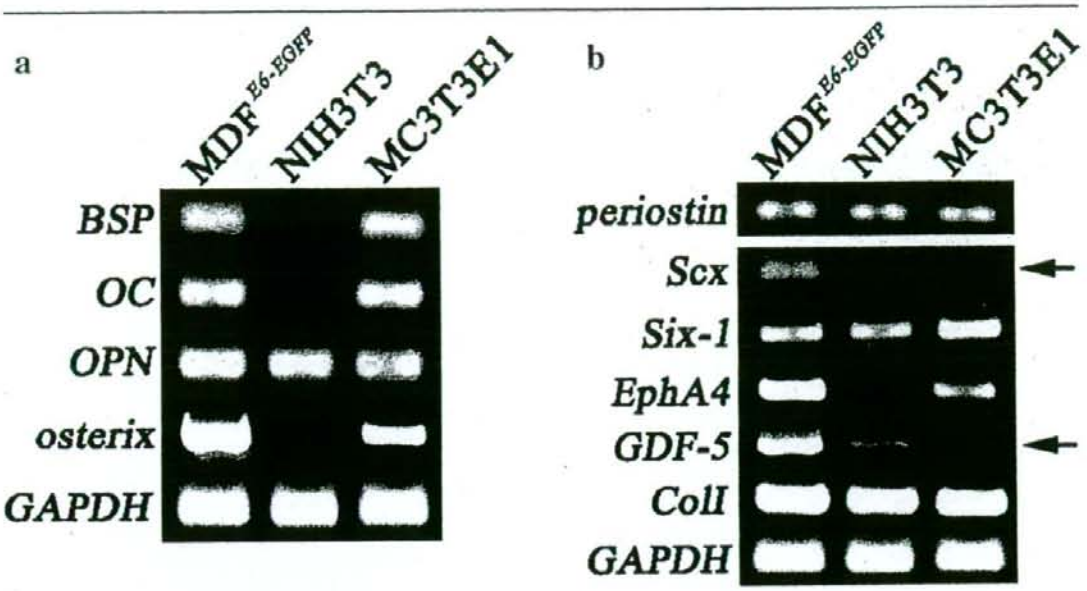

Fig. 3 RT-PCR analysis of $MDF^{E6\text{-}EGFP}$ cells. Expression of osteoblastic phenotype-related genes such as *bone sialoprotein* (*BSP*), *osteocalcin* (*OC*), *osteopontin* (*OPN*), and *osterix* (**a**), and PDL and tendon/ligament phenotype-related genes, such as *periostin*, *scleraxis* (*Scx*), *Six-1*, *EphA4*, *growth and differentiation factor 5* (*GDF5*), and *type I collagen* (*ColI*; **b**) were examined by RT-PCR analysis. Cells were cultured with osteoblastic differentiation (**a**) or normal medium (**b**), and total RNA was extracted (*arrows* strong expression of *Scx* and *GDF-5* in $MDF^{E6\text{-}EGFP}$ compared with NIH3T3 cells or MC3T3E1 cells)

Characterization of $MDF^{E6\text{-}EGFP}$ cells

MDF cells were isolated from the posterior region of the incisor tooth germ, and their life span was extended by using a retrovirus-expressing human papillomavirus type 16 (HPV16) *E6* gene lacking the PDZ-domain-binding motif ($E6^{\Delta 146\text{-}151}$), together with a lentivirus expressing *EGFP* for fluorescence detection. After viral infection, the expression of $E6^{\Delta 146\text{-}151}$ was confirmed by RT-PCR analysis (data not shown). $MDF^{E6\text{-}EGFP}$ cells maintained their original morphology and cell proliferation activity, even when the cells were cultured beyond population doubling (PD) 150, indicating that they had overcome replicative senescence. In contrast, normal MDF cells were only able to propagate until PD 10 (data not shown). We thus used $MDF^{E6\text{-}EGFP}$ cells for further analysis.

To assess the osteogenic potential of $MDF^{E6\text{-}EGFP}$ cells, the cells were treated with osteogenic differentiation medium supplemented with ascorbic acid, β-glycerophosphate, and dexamethasone for 21 days. ALP activity was observed in $MDF^{E6\text{-}EGFP}$ cells (Fig. 2a) and in osteoblast-like MC3T3E1 cells (Fig. 2c). Mineralized nodule formation by the $MDF^{E6\text{-}EGFP}$ cells was not observed (Fig. 2d), whereas MC3T3E1 cells were able to deposit mineralized nodules (Fig. 2f). Neither ALP activity nor mineral deposition was observed in the fibroblastic cells (NIH3T3 cells; Fig. 2b,e). RT-PCR analysis was performed to characterize the $MDF^{E6\text{-}EGFP}$ cells by using primers for osteoblast phenotype-related genes *BSP*, *OC*, and *osterix*. $MDF^{E6\text{-}EGFP}$ cells expressed all of these osteoblast marker genes, with a similar expression pattern to that of MC3T3E1 cells. In contrast, no expression of osteoblast phenotype-related genes was observed in NIH3T3 cells, except for *BSP*. From these data, we considered that osteoblastic populations were present in $MDF^{E6\text{-}EGFP}$ cells (Fig. 3a).

To characterize the PDL-forming properties of $MDF^{E6\text{-}EGFP}$ cells, gene expression of *periostin* and tendon/ligament phenotype-related genes encoding *Scx*, *EphA4*, *Six-1*, *GDF-5*, and *ColI* were examined by RT-PCR analysis. $MDF^{E6\text{-}EGFP}$ cells expressed all of these genes suggesting that they possessed PDL and tendon cell properties (Fig. 3b). Stronger expression of *Scx* and *GDF-5* was observed in $MDF^{E6\text{-}EGFP}$ cells than in NIH3T3 cells or MC3T3E1 cells (Fig. 3b, arrows). Expression patterns of *Scx* and *periostin* mRNA were examined in postnatal (P35) mouse molar PDL (Fig. 4a) by in situ hybridization. Intense expression of *periostin* was observed throughout the PDL (Fig. 4b, arrows), and *Scx* was also expressed in the PDL (Fig. 4c, arrowheads).

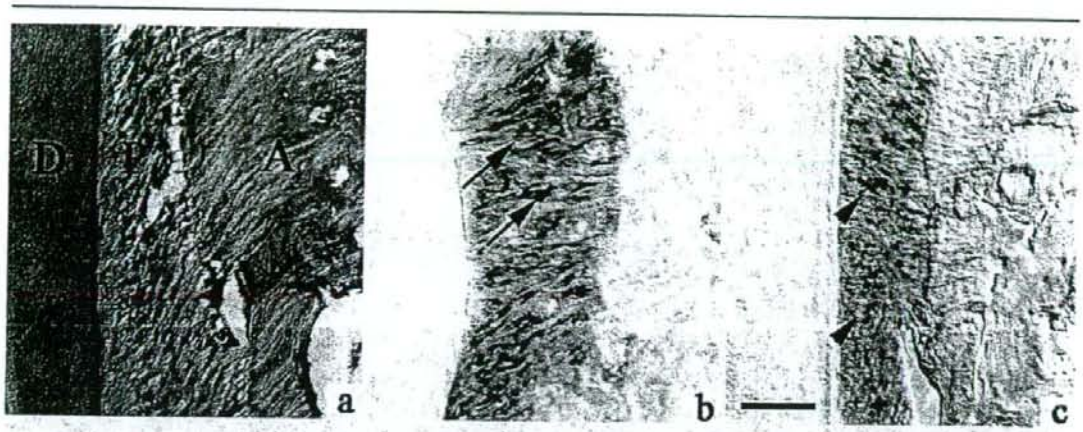

Fig. 4 In situ hybridization for *Scx* and *periostin* mRNA in postnatal PDL (*A* alveolar bone, *D* dentin, *P* PDL). Frontal sections of the molar tooth of P35 mouse (**a**) were examined by hematoxylin and eosin staining (**a**) or in situ hybridization with *periostin* (**b**) or *Scx* (**c**). Intense expression of *periostin* was seen within PDL (**b**, *arrows*). Expression of *Scx* can be seen throughout the PDL (**c**, *arrowheads*). *Bars* 100 μm

In vivo characterization of $MDF^{E6\text{-}EGFP}$ cells

To investigate the differentiation potential of $MDF^{E6\text{-}EGFP}$ cells, the cells were implanted into SCID mice. After 4 weeks, MDFE6-EGFP cell transplants formed PDL-like fibrous tissue (Fig. 5a-a) including scattered bone-like tissue formation (Fig. 5a-b). The PDL-like tissues resembled the structure of PDL with sheet-like cells (Fig. 5a-a, a-d, arrows). Cells within the PDL- and bone-like tissues were strongly positive for EGFP (Fig. 5a-d, a-e). In contrast, no such tissues were formed in the transplants without mouse cells (HAP transplants; Fig. 5a-f). Immunohistochemical staining revealed dense type I collagen fibril assembly in the PDL-like tissue (Fig. 5b-a), whereas type I collagen fibril assembly was not evident in the NIH3T3 cell or HAP transplants (Fig. 5b-b, b-c). To validate the capacity of $MDF^{E6\text{-}EGFP}$ cells to differentiate into PDL in vivo, the expression of *periostin* and *Scx* was examined in the transplants by in situ hybridization. Expression of *type XII collagen* has been shown in the PDL during tooth root formation; therefore, we also examined the expression of this gene in the transplants (MacNeil et al. 1998). As expected, the expression of *periostin*, *Scx*, and *type XII collagen* was observed in $MDF^{E6\text{-}EGFP}$ cells (Fig. 6a,d,g, arrows), whereas these genes were not expressed in the NIH3T3 cell transplants (Fig. 6b,e,h) or HAP transplants (Fig. 6c,f,i). All the transplants except HAP transplants were EGFP-positive, indicating that cells within the $MDF^{E6\text{-}EGFP}$ and NIH3T3 transplants had originated from the donor (Fig. 6j,k,l).

Discussion

We have immortalized dental follicle cells from cells isolated from mice incisor tooth germs. These cells express *Scx*, *GDF-5*, *EphA4*, *Six-1*, and *Coll*, which are expressed in a developing tendon (Bonnin et al. 2005; Brent et al. 2003; Luukko et al. 2005; Settle et al. 2003). Our findings also suggest that $MDF^{E6\text{-}EGFP}$ cells act as PDL progenitors since they form a PDL-like structure that expresses *periostin*, *Scx*, and *type XII collagen* and that is capable of producing dense collagen fibril assembly in vivo.

In the present study, $MDF^{E6\text{-}EGFP}$ cells have been isolated from the undifferentiated dental follicle region of the incisor in which there is minimal expression of *periostin* and no expression of *osteopontin*. Our findings indicate that $MDF^{E6\text{-}EGFP}$ cells act as PDL progenitors, as shown by their capacity to generate PDL-like tissue in vivo. $MDF^{E6\text{-}EGFP}$ cells are similar to tendon progenitors with respect to their expression of *Scx* and *GDF-5*, a marker for tendon/ligament. Since PDL is morphologically similar to the tendon/ligament in vivo, dental follicle cells may have a similar phenotype as tendon progenitors. The expression of *Scx* by mouse PDL has been seen in the present study, suggesting that the PDL has some characteristics similar to tendon. Salingcarnboriboon and coworkers (2003) have reported that a tendon-derived cell line isolated from mouse Achilles tissue shows expression of *Scx*, and that these cells are able to form tendon-like tissue when they are implanted into a mouse tendon-defect model. $MDF^{E6\text{-}EGFP}$ cells show a similar phenotype to these cells, and the transplants form PDL-like tissues comprising cells

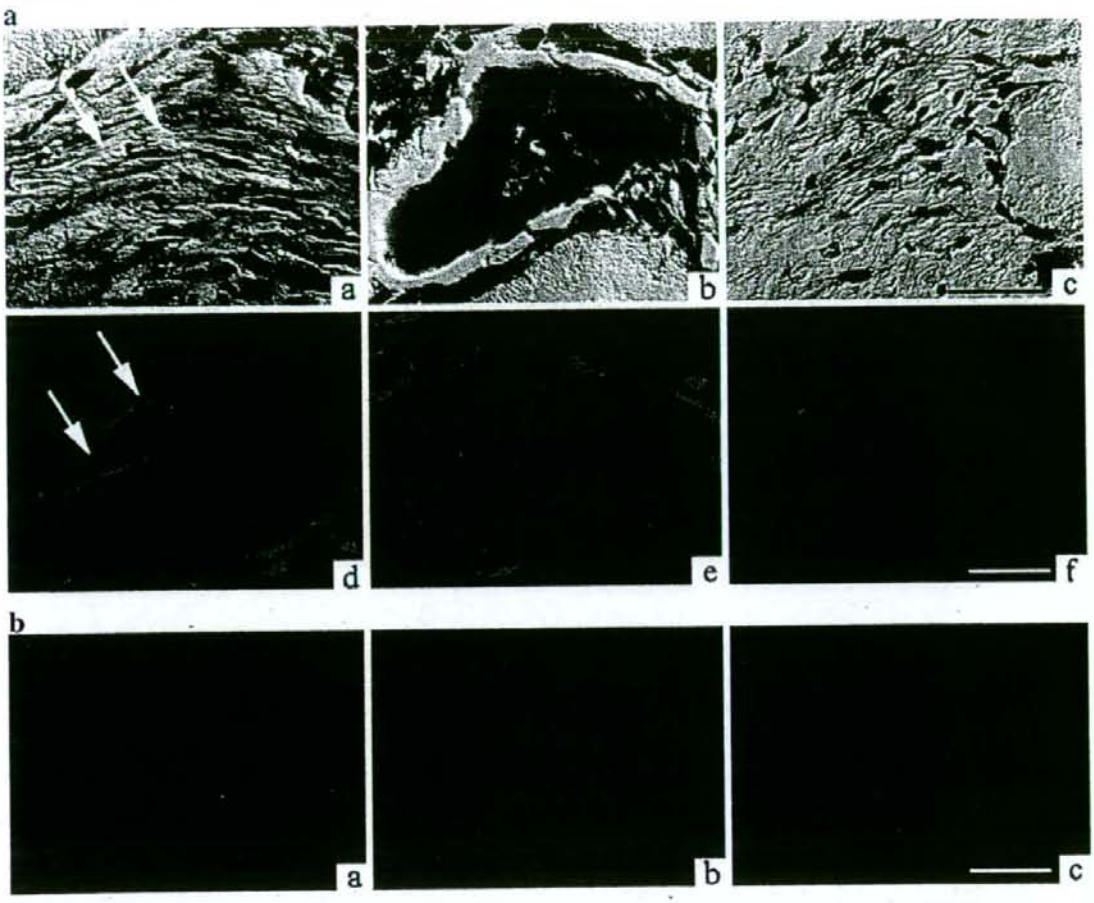

Fig. 5 Differentiation potential of MDF$^{E6\text{-}EGFP}$ cells in vivo. **a** Representative sections of MDF$^{E6\text{-}EGFP}$ cell transplants (**a-a**, **a-b**, **a-d**, and **a-e**) and transplants without mouse cells (HAP transplants; **a-c**, **a-f**). The transplant was stained with hematoxylin and eosin (**a–a** to **a-c**) or viewed for EGFP fluorescence (**a-d** to **a-f**). PDL-like fibrous tissues (**a-a**, **a-d**, *arrows*) and bone-like tissues (**a-e**) are visible in the MDF$^{E6\text{-}EGFP}$ transplants. Cells within the MDF$^{E6\text{-}EGFP}$ transplants are positive for EGFP (**a-d**, **a-e**). **b** Immunohistochemical staining with anti-type I collagen polyclonal antibody in the MDF$^{E6\text{-}EGFP}$ transplants (**b-a**), NIH3T3 cell transplants (**b-b**), or HAP transplants (**b-c**) are shown. Dense type I collagen fibril assemblies are seen in the MDF$^{E6\text{-}EGFP}$ transplants. *Bars* 50 μm

with sheet-like extensions surrounding a densely packed, collagen fibril assembly. Our data are also coincident with previous findings that human PDL cells are able to form PDL-like tissue upon implantation into immunodeficient mice for 4 weeks, suggesting that the differentiation potential of MDF$^{E6\text{-}EGFP}$ is comparable with these cells (Grzesik et al. 2000; Seo et al. 2004). In the present study, the PDL differentiation of MDF$^{E6\text{-}EGFP}$ cells has been assessed by the expression of *periostin*, *Scx*, and *type XII collagen* (Bohme et al. 1995; Karimbux and Nishimura 1995). The expression of *periostin* is observed in both alveolar bone cells and dental follicle cells on P1. However, previous findings and our data have shown that the expression of *periostin* is limited to the adult periodontal ligament, indicating that it could be used as a marker for differentiated PDL (Kruzynska-Frejtag et al. 2004). In the case of MDF$^{E6\text{-}EGFP}$ transplants, they form PDL-like tissue expressing *periostin*, *Scx*, and *type XII collagen*, thus indicating that the tissue is almost identical to PDL. From these findings, we suggest that the MDF$^{E6\text{-}EGFP}$ cells have PDL progenitors able to differentiate into PDL in vivo. Although the role of the tendon/ligament related genes in PDL development is not clear, these data strongly support our hypothesis that MDF$^{E6\text{-}EGFP}$ cells possess PDL progenitors that resemble tendon progenitors.

Progenitors for cementoblasts, PDL cells, and osteoblasts are generally believed to be present in dental follicle cells (Ten Cate 1994), and we have previously shown that

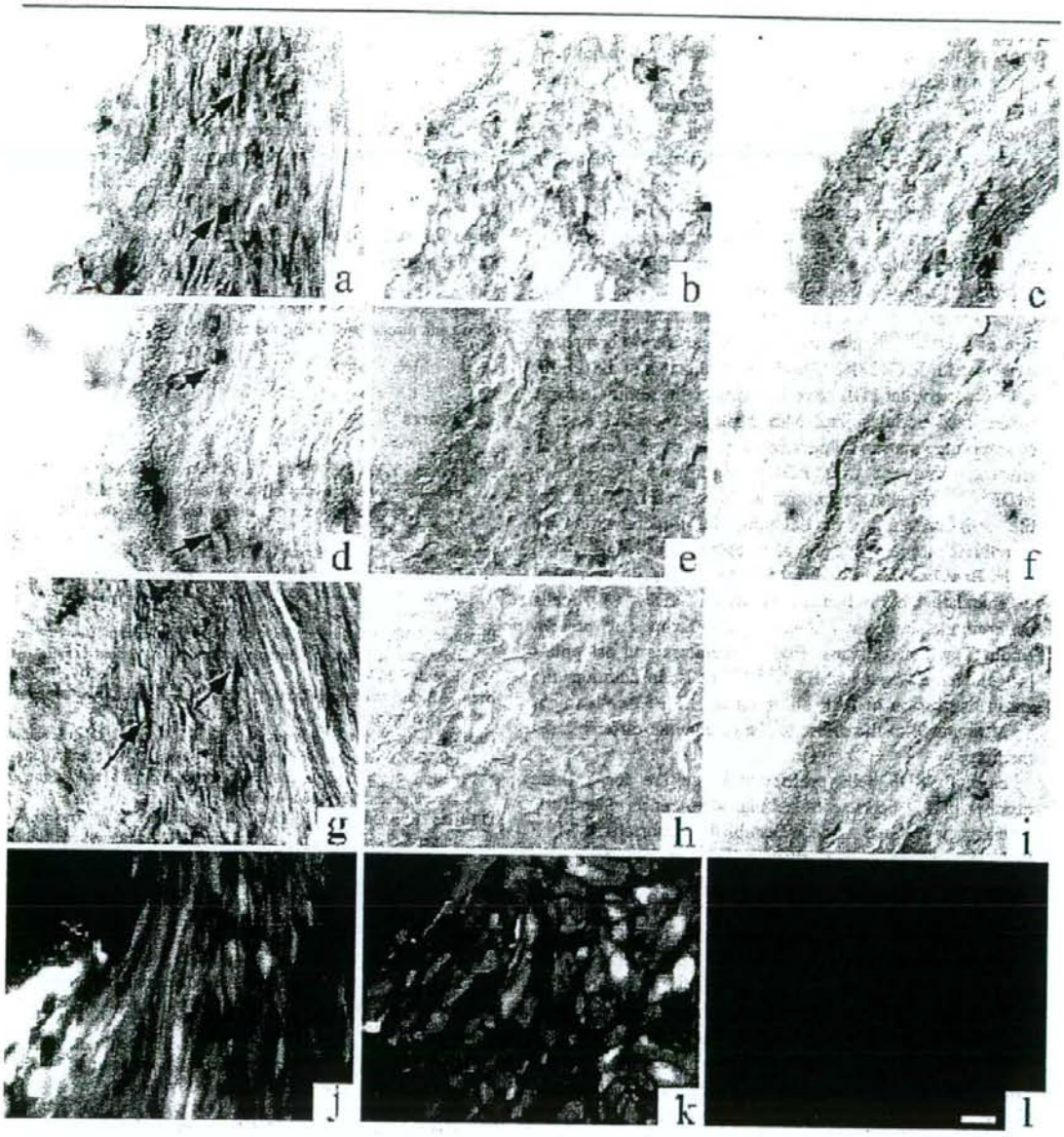

Fig. 6 In situ hybridization for *periostin*, *Scx*, and *type XII collagen* mRNA in $MDF^{E6\text{-}EGFP}$ cell transplants. Representative sections of $MDF^{E6\text{-}EGFP}$ cell transplants (**a**, **d**, **g**, **j**), NIH3T3 cell transplants (**b**, **e**, **h**, **k**), and transplants without mouse cells (HAP transplants; **c**, **f**, **i**, **l**) were analyzed by in situ hybridization for *periostin* (**a**–**c**), *Scx* (**d**–**f**), or *type XII collagen* (**g**–**i**), or viewed for EGFP fluorescence (**j**–**l**). Expression of *periostin*, *Scx*, or *type XII collagen* is seen in the $MDF^{E6\text{-}EGFP}$ transplants (**a**, **d**, **g**, *arrows*). Cells within the $MDF^{E6\text{-}EGFP}$ and NIH3T3 transplants are positive for EGFP (**j**, **k**). *Bar* 100 μm

cementoblast progenitors can be obtained from bovine dental follicle tissues (Handa et al. 2002). Cementoblast differentiation does not occur in $MDF^{E6\text{-}EGFP}$ cell transplants. This discrepancy may be explained by the isolation technique used for $MDF^{E6\text{-}EGFP}$ cells. The cells grown from mouse dental follicle tissues have been employed in this study, whereas in a previous study, we isolated bovine dental follicle cells by bacterial collagenase digestion; this

suggests that isolation of cementoblast progenitors requires enzyme digestion (Handa et al. 2002). We have also found that $MDF^{E6\text{-}EGFP}$ cells can act as osteoblast progenitors, since they form bone-like tissue in vivo. Although $MDF^{E6\text{-}EGFP}$ cells express an array of osteoblast marker genes, they cannot form mineralized nodules in vitro, as described previously for bovine dental follicle cells (Handa et al. 2002). This may be because the difference in time required for the calcification of MDF^{E6EFGP}. The timing and extent of mineralization varies substantially depending on the origin of the cells or experimental conditions. Mineralization of MDF^{E6EFGP} may require a longer period compared with that for MC3T3E1. Recently, Yoshizawa et al. (2004) have reported that PDL cells do not have the ability to form mineralized nodules, and Msx-2 plays a central role in suppressing matrix mineralization in these cells. Our findings suggest that PDL progenitors present in $MDF^{E6\text{-}EGFP}$ cells are similar to those in PDL, and that the cells preventing or delaying the mineralization of osteoblast progenitors are also present in $MDF^{E6\text{-}EGFP}$ cells. Based on these findings, future studies are necessary to elucidate the mechanism by which $MDF^{E6\text{-}EGFP}$ cells differentiate into osteoblastic cells. From our present findings, we suggest that PDL progenitors and osteoblast progenitors co-exist in $MDF^{E6\text{-}EGFP}$ cells. In addition, the slight expression of BSP observed in NIH3T3 cells might be attributable to the effect of the osteogenic differentiated medium.

Normal MDF cells proliferate in culture for a finite number of PD because of cellular senescence. Therefore, attempts have been made to establish immortalized MDF cells in order to analyze their differentiation potential. A deficiency in p53 is sufficient for the establishment of mouse clonal cell lines from various tissues (Hanazono et al. 1997). HPV16E6 has been shown to abrogate the function of p53 and has the ability to immortalize various cell types (Fehrmann and Laimins 2003; Kiyono et al. 1998). However, it also has other biological functions that depend on its C-terminal PDZ-domain-binding motif, such as cell transformation and skin hyperplasia in transgenic mice (Nguyen et al. 2003). For instance, mice expressing HPV-16 E6 in their epidermis develop epithelial hyperplasia and squamous carcinomas (Song et al. 1999). However, transgenic mice expressing HPV-16 E6 lacking the PDZ-binding motif fail to display epithelial hyperplasia but retain the ability to inactivate p53 (Nguyen et al. 2003). In the present study, we have used a mutant version of E6 that lacks the C-terminal PDZ-domain binding-motif to extend the life span of MDF cells and have succeeded in immortalizing MDF cells without affecting their differentiation potential. In addition, we have confirmed that no tumor formation occurs in $MDF^{E6\text{-}EGFP}$ cell transplants (data not shown). This suggests that the immortalization system used in this study might be useful for the immortalization of MDF cells.

In summary, we have established an immortalized mouse dental follicle cell culture system that possesses PDL progenitors. $MDF^{E6\text{-}EGFP}$ cells might provide new insights into the mechanisms of PDL formation, including those pertaining to PDL cell differentiation. They may also be a powerful tool in the development of therapeutic strategies for the treatment of periodontitis.

Acknowledgement We are grateful to Dr. Masato Yamauchi for his advice and discussions during the course of this work.

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Molecular mechanisms of cellular senescence and immortalization of human cells

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Cellular senescence was originally described as a phenomenon observed in cultured human cells. Accumulating lines of evidence now indicate that the same processes also take place *in vivo*, suggesting important implications for tumor development. Telomere shortening is the most well-established cause of cellular senescence that can be induced by many other intrinsic and extrinsic factors. The retinoblastoma susceptibility gene product is a convergent target that is downstream of these factors. p53, p38MAPK and cyclin-dependent kinase inhibitors p16INK4a (p16) and p21CIP1 (p21) are key mediators. As most stresses that induce cellular senescence are also known causes of cancer, a common strategy might be applied to the development of cancer chemopreventive agents and anti-ageing drugs.

Keywords: ageing, cyclin-dependent kinase, cyclin-dependent kinase inhibitor, immortalization, p16(INK4a), p21(WAF1/CIP1/SDI1), p38(MAPK), p53, polycomb group, reactive oxygen species, retinoblastoma protein, senescence, senescence-associated heterochromatic foci, telomerase reverse transcriptase, telomere

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1. Introduction

Most human cells undergo a limited number of divisions in culture and then enter a non-dividing state termed replicative senescence [1]. Unlike the case with quiescent cells, growth arrest of senescent cells is essentially irreversible and insensitive to growth factors. Cellular senescence refers to replicative senescence and replicative senescence-like states which can be induced by many stresses, such as genotoxic damage and active oncogenes. Activation of the retinoblastoma susceptibility gene product (pRb) is the most common downstream event of many senescing signals, though the other pRb family pocket proteins (p130 and p107) may substitute this function in the absence of pRb. pRb is inactivated by phosphorylation by cyclin-dependent kinases (CDKs) which are inactivated by CDK inhibitors. Induction of CDK inhibitors p16INK4a (p16) and p21CIP1 (p21), along with the activation of p53, are often associated with cellular senescence. By inhibiting CDKs, these inhibitors consequently activate pRb, which induces growth arrest at G1. Cdk4/Cdk6–cyclin D and Cdk2–cyclin E function as the major pRb kinases and mouse embryonic fibroblast (MEF) cells derived from double knockout of Cdk4 and Cdk2 feature early onset senescence [2], although a recent report indicated that Cdk1 can associate with all the cyclins and is the only essential Cdk for cell cycling ([3,4] for review). In contrast, overexpression of Cdk4/Cdk6 can delay cellular senescence [5]. In senescent cells, cell cycle arrest can be maintained by sustained growth arrest signaling and/or heterochromatinization of cell cycle-associated genes. There are numbers of intrinsic and extrinsic factors that induce cellular senescence; the same signaling pathway is often involved, although some of the underlying mechanisms are specific to the cell type and

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the inducer. In this review, processes contributing to cellular senescence induced by different factors and their significance for human health are discussed.

2. Telomere shortening and senescence

Telomere shortening is the most well-known cause of cellular senescence or replicative senescence. In the absence of telomerase activity, telomeres are continuously shortened following replication, due to the 'end-replication problem' [6]. The shortened telomeres are exposed as linear ends of double strand DNAs and are recognized as a type of DNA damage. Dysfunction of shelterin, a multimolecular complex that regulates telomere length and protects telomere ends, could also lead to cellular senescence ([7] for review). Based on the notion that most human somatic cells lack telomerase activity, telomere shortening is considered to be a molecular clock that determines cellular ageing. Although some human somatic stem cells express higher levels of telomerase, the activity is not sufficient to maintain telomere length. Therefore, the clock theoretically limits the maximum replicative capacity of human cells. Indeed, some human cell types can be immortalized by transduction of human telomerase reverse transcriptase (hTERT) alone [8].

However, the situation changes in cells with high telomerase activity. Mouse cells have relatively long telomeres and expression of telomerase is more ubiquitous. The observed 'replicative senescence' in MEF culture is not caused by telomere shortening and, therefore, is called premature senescence or culture shock [9].

3. Telomere-independent senescence

Telomere-independent senescence can be induced, not only in cell culture but also *in vivo*, due to many kinds of stresses and is known by different names in different situations, such as premature senescence, stress-induced premature senescence, culture shock and stress or aberrant signaling induced senescence to distinguish it from telomere-dependent senescence or replicative senescence [9,10]. However, telomere shortening or dysfunction itself is a stress to cells. The p53 and pRb pathways are common regulators that govern both telomere-dependent and -independent senescence. Furthermore, the term cellular senescence arose from the assumption that similar mechanisms of cellular ageing occur in both culture and *in vivo*, and the telomere-dependent and -independent forms of senescence could be equally important as biologic mechanisms to suppress tumor formation. Indeed, the observed cellular senescence is likely to be frequently induced by the sum of telomere-dependent and telomere-independent stresses. Therefore, the term replicative senescence in many papers refers to phenomena observed in culture, rather than being based on any mechanistic concept. To indicate mechanistic features of senescence, terms, such as telomere-dependent and oncogene-induced senescence are used in the present review.

4. DNA damage response and senescence

Many types of DNA damage have been identified as inducers of senescence, as well as apoptosis. In the case of telomere-dependent senescence, continuous growth arrest signals from shortened telomeres are causes. However, slight DNA damage induces transient cell cycle arrest followed by DNA repair, which then allows cells to re-enter the cell cycle. Therefore, relatively heavy or continuous DNA damage is generally chosen for experimental induction of senescence. Genotoxic stress due to reactive oxygen species (ROS), UV, γ-rays and DNA-damaging agents, including chemotherapeutic drugs, have been reported as inducing agents (**Figure 1**) ([11] for review). DNA damage results in activation of ataxia-telangiectasia mutation (ATM), p53 and subsequently, many p53 target genes, including *p21*. Depending on the cell type and the extent of DNA damage, activation of p53 induces either apoptosis or senescence. It has been suggested that *p21* plays a critical role in both inducing p53-dependent senescence and protecting against apoptosis [12-15]. Induction of p21 prevents cells from accumulating further damage by inducing growth arrest through an association with cyclin/CDKs and proliferating cell nuclear antigen, thereby providing time for repair to be accomplished.

5. Reactive oxygen species-induced senescence of mouse embryonic fibroblast cells

Replicative senescence is caused by shortened telomeres in human foreskin fibroblasts. However, primary MEF cells also enter senescence prematurely, independent of telomere length after several passages, as they have long telomeres and often express TERT. Cellular senescence of MEF was, thus, suggested to be caused by 'culture shock' [9] and later shown to be caused by ROS in the widely used carbon dioxide incubators, which contain a higher atmospheric oxygen concentration (20.8%) than that in the tissue environment [16]. As similar senescence of human foreskin fibroblasts can be induced by hydrogen peroxide, it is safe to say that the difference is based on relative sensitivity to ROS between human and mouse cells, and 'spontaneous' senescence of MEF cells should be interpreted as 'ROS-induced senescence'. Therefore, MEF cells can be essentially considered as immortal at lower oxygen concentrations (3%), and examples lacking p53, $p19^{Arf}$, inducer of acute promyelocytic leukemia (PML) or all the pRb family proteins (pRb, p107 and p130) are resistant to 'ROS-induced senescence' and Ras-induced senescence. However, it remains to be examined whether mouse cells derived from different tissues are also virtually immortal with low oxygen concentrations (3%) or in the presence of antioxidants. Similar effects of low oxygen conditions on human diploid cells were reported 30 years ago [17].

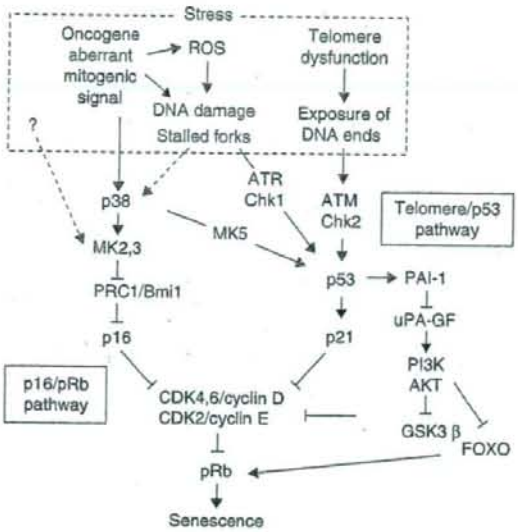

Figure 1. Major pathways to induce cellular senescence. Many stresses can induce cellular senescence. In this model, telomere dysfunction including telomere shortening, is also classified as a stress. The PI3K/Akt pathway can be suppressed by negative feedback signaling, originally triggered by activation of the Raf/MEK/ERK pathway.

ATM: Ataxia-telangiectasia mutation; ATR: Ataxia-telangiectagia and rad3-related; CDK: Cyclin-depedent kinase; Chk1: Cell cycle checkpoint kinase 1; FOXO: Forkhead transcription factor; GF: Growth factor; GSK: Glycogen synthase kinase; MK: MAPK activated protein kinase; PAI: Plasminogen activator inhibitor 1; PRC: Polycomb repressor complexes; pRb: Retinoblastoma protein; ROS: Reactive oxygen species; uPA: Urokinase-type plasminogen activator.

6. Oncogene-induced senescence

Oncogene-induced senescence (OIS) was first reported as an *in vitro* phenomenon in response to oncogenic Ras and Raf [10,18]. Both p53 activation and p16 elevation are induced in OIS, although changes in p16 appears to be less important in senescence of mouse cells [19,20]. Several common and specific mechanisms are proposed for OIS induced by different oncogenes. P14ARF is specifically activated by aberrant expression of E2F through a non-typical, E2F-responsive element in the promoter [21]. Independent of the E2F pathway, activation of Arf by Ras/Raf signaling is also indirectly mediated by Dmp1 through activation of Jun, explaining why Dmp1 null primary cells are highly susceptible to Ras-induced transformation [22]. p14ARF induces accumulation of p53 through inhibition of the human homolog of murine double minute 2 (HDM2), although p53 is activated by oncogenic Ras, independent of p14ARF in some human cells [23].

In animal models of OIS, most cells expressing oncogenic Kras neither form tumors nor enter senescence [24]. Similarly, endogenous oncogenic KrasG12D-expressing fibroblasts do not enter senescence, but demonstrate attenuation and altered regulation of canonical Ras effector signaling pathways [25]. Such attenuation is caused by a global negative feedback response induced by mutations affecting neurofibromin, a Ras GTPase activating protein (RasGAP), Raf and Ras [26]. This negative feedback program is regulated in part by Ras guanine-nucleotide exchange factors, sprouty proteins, RasGAPs and MAPK phosphatases also called dual specificity phosphatises (DUSPs). Their inhibitory signals can suppress the PI3K/AKT pathway, which can exert an impact on the senescence pathway through forkhead transcription factor and HDM2 (**Figure 1**) [26]. Expression of a constitutively activated mutant of forkhead transcription factor 1 (FOXO1) can rapidly induce cellular senescence with activation of pRb, but not p53 [26]. Inhibition of HDM2 results in activation of p53. As a p53 downstream target, PAI-1, can also inhibit the PI3K/Akt pathway and induce senescence in the absence of p53 [27], PAI-1 might be involved in negative feedback loops. Recently, it was suggested that OIS is also a DNA damage response triggered by DNA hyper-replication, associated with overexpression of CDC6 and an increased number of active replicons [28,29]. The DNA damage response requires DNA replication, as oncogenic ras expression does not induce DNA damage in quiescent cells. DNA damage in OIS could be induced by ROS. Activation of the p16–Rb pathway in cooperation with mitogenic signals causes elevated intracellular levels of ROS, thereby activating PKCδ in human senescent cells [30]. Importantly, once activated by ROS, PKCδ promotes further generation of ROS by activating NADPH oxidase, thus, establishing a positive feedback loop to sustain ROS–PKCδ signaling [30].

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

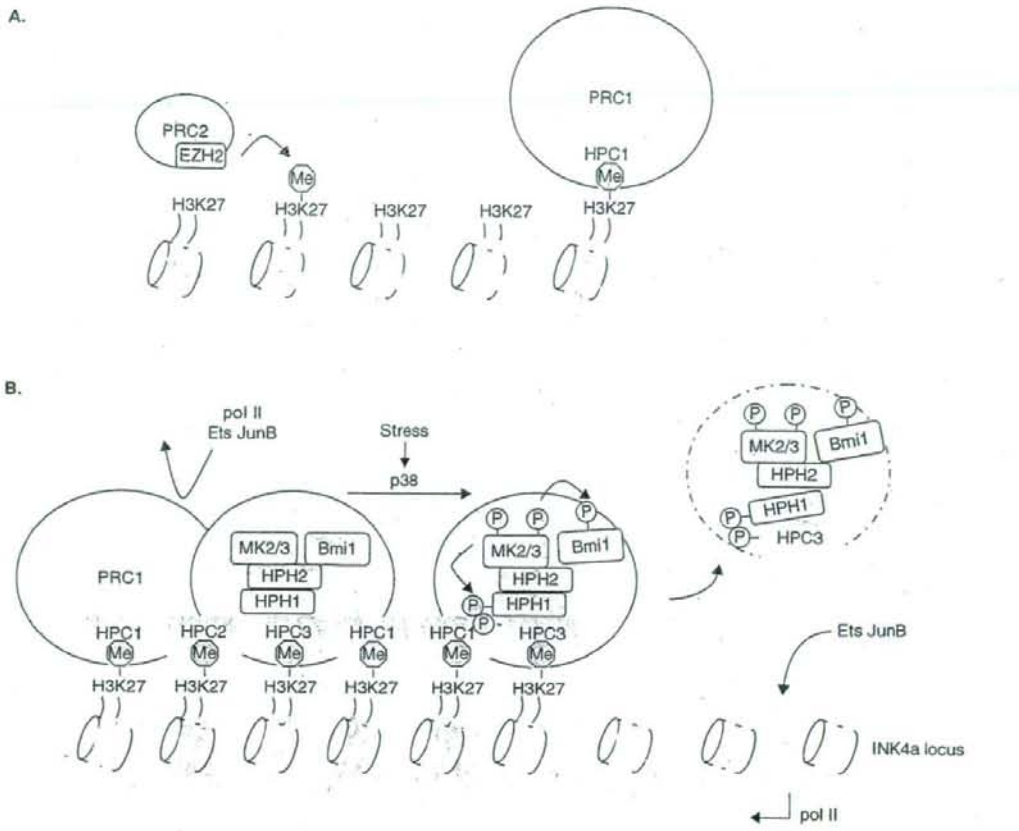

Figure 2. Polycomb repressor complexes-mediated repression and p38-mediated de-repression of the p16 promoter.
A. PRC2-mediated histone H3 methylation on lysine 27 initiates PcG-mediated gene silencing. A PRC2 component, EZH2, has methyl transferase catalytic activity. PRC1 recognizes the trimethylated 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

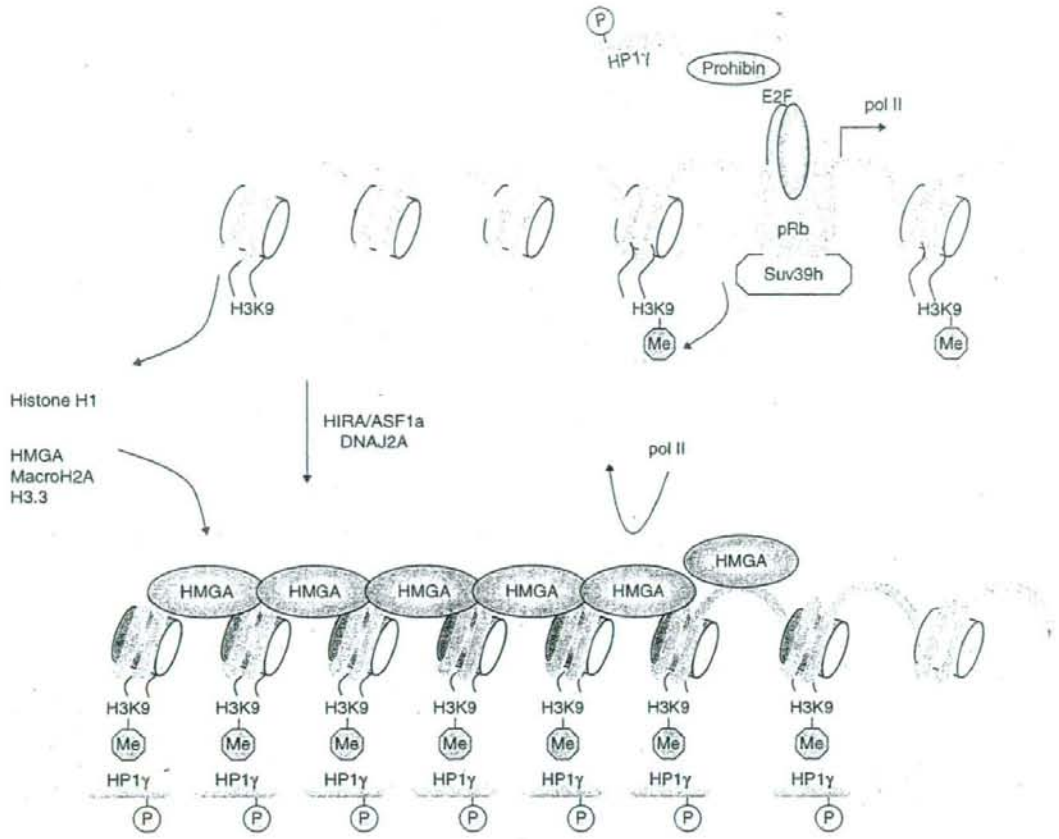

Figure 3. Molecular mechanisms of senescence-associated heterochromatic focus formation. E2F target genes are transcriptionally active in replicating cells. In senescent cells, repression of E2F-target genes is initiated by a multi-protein complex consisting of pRb, Suv39h, prohibin and HP1γ phosphorylated 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

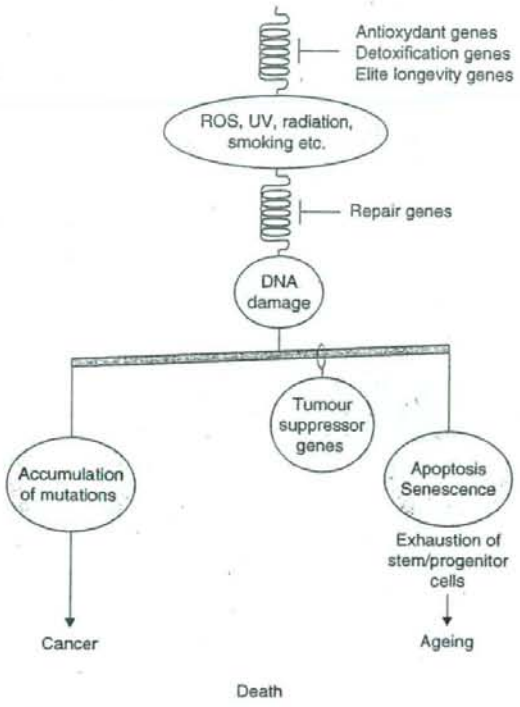

Figure 4. Genetic and environmental risk factors for cancer and ageing. Tumor suppressor genes evolved to balance risks of cancer and ageing. Although tumor suppressor genes are a burden to the balance, as some of the protective genes against DNA damage are regulated by a tumor suppressor gene p53, they 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

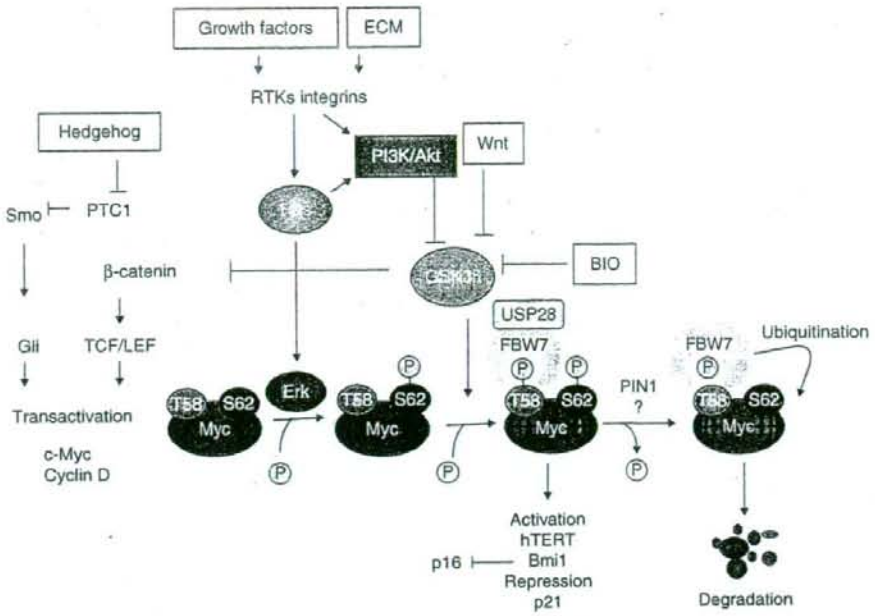

Figure 5. Regulation of Myc by various signaling pathways. Fine tuning of Myc activity by extracellular signaling molecules appears important for maintenance and expansion of stem and/or progenitor cells *in vitro*.

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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