RUNX1 and its fusion oncoprotein derivative RUNX1-ETO induce senescence-like growth arrest independently of replicative stress

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Abstract

A role for the RUNX genes in cancer failsafe processes has been suggested by their induction of senescence-like growth arrest in primary murine fibroblasts and the failure of RAS-induced senescence in Runx2 deficient cells. We now show that RUNX1 induces senescence in human primary fibroblasts. High affinity DNA binding is necessary but not sufficient, as shown by the functional attenuation of the truncated RUNX1/AML1a isoform and the TEL-RUNX1 fusion oncoprotein. However, a similar phenotype was potently induced by the RUNX1-ETO (AML1-ETO) oncoprotein, despite its dominant negative potential. Detailed comparison of H-RAS\textsuperscript{V12}, RUNX1 and RUNX1-ETO senescent phenotypes showed that the RUNX effectors induce earlier growth stasis with only low levels of DNA damage signalling and a lack of chromatin condensation, a marker of irreversible growth arrest. In human fibroblasts, all effectors induced p53 in the absence of detectable p14\textsuperscript{ARF}, while only RUNX1-ETO induced senescence in p16\textsuperscript{INK4a} null cells. Correlation was noted between induction of p53, reactive oxygen species and phospho-p38, while p38\textsuperscript{MAPK} inhibition rescued cell growth markedly. These findings reveal a role for replication-independent pathways in RUNX and RUNX1-ETO senescence, and show that the context-specific oncogenic activity of RUNX1 fusion proteins are mirrored in their distinctive interactions with failsafe responses.

Keywords

RUNX1; RUNX1-ETO; premature senescence
INTRODUCTION

The phenomenon of oncogene-induced senescence was first described in primary cultured murine fibroblasts ectopically expressing mutant RAS alleles (Serrano et al., 1997). It is now generally recognised that this response, like apoptosis, represents an important failsafe process that can protect cells against malignant transformation, a view further supported by functional requirements for the p53 and pRb tumour suppressor pathways for its execution (Ferbeyre et al., 2002; Narita et al., 2003). Concerns that this may represent merely an aberrant response to non-physiological cell culture conditions in vitro (Parrinello et al., 2003) have been dispelled by observations that oncogene-expressing or premalignant cells can display a similar phenotypic response in vivo (reviewed in 5). Premature senescence induced by oncogene expression or other stress stimuli resembles the process of replicative senescence described in early studies of long term culture of primary cells in vitro (Hayflick, 1965) but differs in that it occurs in young cell cultures and does not reflect telomere attrition. While most studies of premature senescence have focused on primary fibroblasts, exploiting the advantages of genetic analyses, this phenomenon extends to cells from other lineages (Courtois-Cox et al., 2008).

The mechanism and downstream pathways involved in RAS-induced senescence have been subject to intense study. Recent studies implicate a DNA damage response secondary to sustained proliferative signalling as a crucial element of RAS-induced senescence (Di Micco et al., 2006; Bartkova et al., 2006). According to this model the aberrant expression of oncogenes such as activated RAS results in a transient round of cell proliferation followed by permanent withdrawal from the cell cycle and other phenotypic features indicative of cellular senescence. However, it is clear that the RAS-induced stress signaling response is complex and that cell types differ in the precise response and genetic requirement, as illustrated by the relative importance of p53 in murine embryonic fibroblasts, while p16\(^{Ink4a}/Rb\) pathways predominate in human skin fibroblasts (Brookes et al., 2002). The ability of the Runx gene family to induce senescence-like growth arrest in primary murine fibroblasts has been described previously and this property is shared by all three family members (Linggi et al., 2002; Wotton et al., 2004; Kilbey et al., 2007). Evidence that the endogenous Runx genes play an intrinsic role in oncogene-induced senescence was provided by the observation that primary MEFs lacking functional Runx2 fail to undergo senescence and are readily transformed by activated RAS\(^{V12}\) (Kilbey et al., 2007). Runx2 deficiency also leads to loss of senescence in the osteoblast lineage (Zaidi et al., 2007), while an analogous role for Runx1 in hemopoietic progenitors is suggested by the failure of N-RAS-induced growth suppression in cells lacking Runx1 (Motoda et al., 2007).

In light of the emerging role of Runx proteins in senescence-like growth arrest, we were interested in extending these observations to primary human diploid fibroblasts that have used extensively in the study of RAS-induced senescence. We also wished to explore the capacity of the common RUNX1 oncoprotein fusion derivatives to modulate this failsafe mechanism. The RUNX1-ETO fusion arises as a consequence of the t(8;21) chromosomal translocation and is a frequent feature of acute myeloid leukaemia (AML). RUNX1-ETO is a chimeric protein that lacks the transactivation domain of RUNX1, is fused to a transcriptional repressor, ETO, and has dominant negative activity with respect to wild type
RUNX1 in a variety of transcriptional contexts (reviewed in (Blyth et al., 2005)). Interestingly, RUNX1-ETO can repress p19\(^{Arf}\), an upstream effector of p53 which is required for RUNX1 induced senescence in murine embryonic fibroblasts (Linggi et al., 2002). TEL-RUNX1, which arises from the t(12;21) fusion in childhood pre-B acute leukaemias, is an N-terminal fusion that retains almost all of the RUNX1 reading frame fused to a truncated ETS family protein TEL(ETV6). It has also has dominant negative activity with regard to the wild-type RUNX1 gene on selected target promoters (Hiebert et al., 1996).

In this study we have found that RUNX1 is a potent inducer of senescence-like growth arrest in primary human fibroblasts, but more surprisingly the RUNX1 fusion proteins are divergent in this respect, having either gained (RUNX1-ETO) or lost potency (TEL-RUNX1) in relation to wild-type RUNX1. Moreover, premature senescence induced by the RUNX mediators differs markedly from RAS-induced senescence, with implications for its potency as a cancer fail-safe mechanism.

**RESULTS**

**RUNX1 induces senescence-like growth arrest, a property that is intensified in RUNX1-ETO but attenuated in TEL-RUNX1**

Ectopic expression of each of the three Runx genes in primary murine fibroblasts (MEFs) was previously shown to induce senescence-like growth arrest by a p53-dependent mechanism (Wotton et al., 2004; Kilbey et al., 2007). As this phenomenon had not been examined in human fibroblasts, which have distinct genetic requirements for RAS-induced senescence (Gil and Peters, 2006), we tested this further by expressing RUNX1 in human primary foreskin fibroblasts (Hs68) that have been used extensively to study the requirements for RAS senescence (Brookes et al., 2002). As shown in Figure 1 we found that ectopic RUNX1 expression in these cells induced growth arrest with early onset and a senescence-like morphology with a flatter appearance than seen with H-RAS\(^{V12}\), similar to previous observations in MEFs (Wotton et al., 2004). To determine the structural features of RUNX1 required for this activity, we examined several mutants disrupted in key functional domains. Mutants in DNA binding (K83N) or CBF\(^{\beta}\) cofactor interaction (T161A) (Cammenga et al., 2007) were unable to induce the senescent phenotype and transduced cells appeared virtually indistinguishable from controls. The short RUNX1 (AML1a, 1-250) isoform, which lacks the C-terminal transactivation and inhibitory domains of RUNX1 (Tanaka et al., 1995), was similarly impaired in senescence potential, indicating that RUNX1 has to bind DNA with high affinity and modulate gene expression to induce senescence-like growth arrest.

The activity of RUNX1 fusion oncoprotein derivatives was also of interest, as both RUNX1-ETO and TEL-RUNX1 have dominant negative activity with regard to wild-type RUNX (Lutterbach et al., 2000). We were surprised to find that RUNX1-ETO also induced growth arrest and senescence in Hs68 cells, with a flatter morphology and intense staining for senescence-associated \(\beta\)-galactosidase. A deletion mutant of RUNX1-ETO, which is essentially devoid of transcriptional repression activity (Lenny et al., 1995), was inactive.
further surprise was that the TEL-RUNX1 fusion protein, which includes an essentially full-length RUNX1 moiety, lacked the ability to induce senescence-like growth arrest.

As Runx senescence in murine fibroblasts entails potent induction of p53 and is p53-dependent for execution, we explored the possible similarity between these phenotypes and ectopic expression of p53 in Hs68 cells. While p53 also induced significant growth arrest, cells were less flat and enlarged, particularly in comparison with RUNX1-ETO, and the intensity of cytoplasmic SA-β-Gal staining was less pronounced (Figure 1). Essentially similar observations were made in an embryonic lung fibroblast line, TIG3 (Brookes et al., 2002), that displayed strong senescence-like growth arrest induced by both RUNX1 and RUNX1-ETO (not shown).

RUNX1 and RUNX1-ETO arrest cell growth more rapidly than H-RASV12 and display distinctive patterns of induction of senescence markers

The growth arrest induced by both RUNX1 and RUNX1-ETO occurred earlier and was more profound for H-RASV12, with RUNX1-ETO cells displaying the lowest uptake of BrdU (Figure 2A). RAS and RUNX1-arrested cells mainly in a 2N state, consistent with G1 arrest, while a greater proportion of RUNX1-ETO cells were arrested with 4N DNA content. This phenomenon correlated with a greater proportion of binucleate cells, suggesting a failure of cytokinesis. RUNX1 and RUNX1-ETO expressing cells lacked the initial growth phase seen in RAS cells during the first 3 days after selection (Figure 2B). Analysis of markers of senescence-associated growth inhibition (Figure 2C) showed that p53 was induced in all cases, though RAS and RUNX1-ETO cells displayed more potent induction. This was primarily a post-transcriptional response, as quantitative real-time PCR analysis showed a modest induction in Tip53 transcript levels for RUNX1-ETO but not for RASV12 or RUNX1 (Figure 2D). Examination of the levels of p21WAF1 showed an interesting phenomenon, as RUNX1 and RUNX1-ETO appeared, respectively, to increase or attenuate induction of this cdk inhibitor relative to its upstream regulator p53. This effect was highlighted by comparison with RASV12, which was, as expected, a strong inducer of p53 and p21WAF1 (Serrano et al., 1997; Ferbeyre et al., 2002). Induction of p16Inka was observed in all cases, but was much stronger in RASV12 cells and weakest in RUNX1-ETO cells. Basal expression of p14ARF was undetectable in Hs68 cells and was not induced by RAS as noted previously (Brookes et al., 2002). It was similarly undetectable in cells expressing RUNX1 or RUNX1-ETO (Figure 2C).

Since Runx2-/- MEFs display loss of RAS-induced senescence that is associated with failure to down-regulate G2/M cyclin expression (Kilbey et al., 2007; Kilbey et al., 2008), we considered the possibility that ectopic RUNX expression might induce growth arrest by repressing transcription of the same genes. As shown in Figure 2D, RAS expressing cells showed reduced expression of cyclin A2 and B1 while neither of these transcripts was significantly down-regulated in day 7 cultures of RUNX1 and RUNX1-ETO expressing cells.

Closer examination of the various senescent phenotypes also revealed a marked difference between RASV12 and the RUNX mediators, as only the RAS senescent cells displayed evidence of chromatin condensation or senescence-associated heterochromatin foci (SAHF).
(Zhang et al., 2007) as indicated in Figure 3 by DAPI staining (middle panel). The presence of SAHFs specifically in the RAS$^{V12}$ cells was also confirmed by immunostaining for K9 methylated histone H3 and heterochromatin protein 1γ (not shown). Moreover, only the RAS expressing cells showed organised foci of DNA damage as detected by immunostaining for phosphorylated histone H2AX, a sensitive marker of double-stranded DNA breaks (lower panel) (Di Micco et al., 2006). Indications of punctate staining above background levels were noted in some cells expressing RUNX1 or RUNX1-ETO, but these were clearly much less extensive than in the RAS$^{V12}$ cells. Cells ectopically expressing p53 did not display evidence of SAHF or double-strand breaks above background levels.

**RUNX1-ETO senescence in human fibroblasts is highly dependent on p53 but independent of p16$^{INK4a}$**

To explore the functional requirements for p53 in the various forms of senescence-like growth arrest, Hs68 cell were transduced with HPV16 E6 oncoprotein. E6 expressing wild-type cells showed an obvious growth advantage over cultures expressing the vector only (Figure 4A,B). After 4 days, these cells were super-infected with retroviruses encoding RUNX1, RUNX1-ETO, H-RAS$^{V12}$ or control vector (pBabe) and selected with puromycin. The ability of HPV16 E6 to down-regulate p53 was confirmed by western blot analysis (Figure 4C). Consistent with previous reports (Wei et al., 2001), p53 inactivation had only a modest effect on H-RAS$^{V12}$ growth arrest in human primary fibroblasts. RUNX1 growth arrest was partially rescued by E6 although this was statistically significant only at day 10. In contrast, proliferation of RUNX1-ETO expressing cells was substantially rescued by E6. β-gal staining of these cells revealed only a few blue cells, which may have escaped E6 transduction (not shown). These findings were also explored in primary MEFs, in which RUNX1 has previously been shown to induce senescence-like growth arrest in a p53-dependent manner (11). In p53 null MEFs, RUNX1-ETO did not induce senescence-like morphology or SA-β-gal staining (not shown), although a more marked growth delay was induced compared to RUNX1 (Figure 4D). These results suggest that both RUNX1 and RUNX1-ETO also have cell cycle inhibitory effects that are independent of p53, although it is clearly required for the full expression of the senescent phenotype.

The weak induction of p16$^{INK4a}$ by RUNX1-ETO and the lack of detectable expression of p14$^{ARF}$ in these cells suggested that this form of senescence-like growth arrest might be independent of INK4a (CDKN2A). We therefore examined the effects of RUNX1, RUNX1-ETO and RAS$^{V12}$ in the Leiden strain of human diploid fibroblasts which are homozygous for a germline deletion in the second exon of CDKN2A. These cells lack functional p16$^{INK4a}$ and have been shown to be resistant to H-RAS$^{V12}$ mediated senescence-like growth arrest (Brookes et al., 2002). Interestingly, Leiden cells were fully susceptible to RUNX1-ETO-induced senescence, with proliferation block, morphologic change and SA-β-Gal staining similar to normal fibroblasts. While RUNX1 and H-RAS$^{V12}$ caused moderate growth delay in these cells, they continued to increase from day 6 onwards and displayed no SA-β-Gal activity at day 8 (Figure 5A,B).
RUNX1-ETO is a potent inducer of reactive oxygen species and p38\(^{\text{MAPK}}\), a central mediator of senescence-like growth arrest

Since p53 was potently induced by RUNX1-ETO in cells lacking INK4a/ARF expression and without evidence of substantial DNA damage, we examined other stress-induced pathways that might account for its activation. Reactive oxygen species have been reported to be capable of inducing senescence-like growth arrest and to trigger p53 accumulation (Colavitti and Finkel, 2005). Levels of ROS in Hs68 cells were examined at day 7 post-selection by FACS-based detection of fluorescence emitted by the peroxide-sensitive fluorophore DCF. H-RAS\(^{V12}\) cells displayed high levels of intracellular ROS consistent with published data (Figure 6A,B) (Lee et al., 1999). Interestingly, both RUNX1 and RUNX1-ETO expressing cells were found to have elevated intracellular ROS levels compared to empty vector control cells. A small but statistically significant increase was seen in RUNX1 expressing cells, while RUNX1-ETO expressing cells showed a profound increase equal to or greater than seen in H-RAS\(^{V12}\) expressing cells.

It has been reported that ROS induction can activate p38\(^{\text{MAPK}}\) (Robinson et al., 1999). Furthermore, in a recent study it has been shown that p38\(^{\text{MAPK}}\) can act as a sensor of reactive oxygen species and thereby restrict H-RAS\(^{V12}\) induced tumorigenesis (Dolado et al., 2007). It was therefore interesting to test whether the p38\(^{\text{MAPK}}\) pathway also mediates RUNX1 and RUNX1-ETO induced senescence. The abilities of RUNX1 and RUNX1-ETO to activate p38\(^{\text{MAPK}}\) were examined using a specific antibody that recognizes two phosphorylation sites responsible for p38\(^{\text{MAPK}}\) activation (Thr180 and Tyr182). Indeed, western blot analysis on day 7 of culture period revealed that both RUNX1 and RUNX1-ETO induced elevated levels of phospho-p38 with RUNX1-ETO the more potent agonist, inducing phospho-p38 to a similar degree as H-RAS\(^{V12}\) (Figure 6C). Notably, the levels of total p38\(^{\text{MAPK}}\) remained unaffected (Figure 6C). Upregulation of phospho-p38 by RUNX1 and RUNX1-ETO correlated closely with the levels of induction of ROS and with the levels of p53 protein accumulation (Figure 2C) suggestive of a mechanistic link.

Inactivation of p38\(^{\text{MAPK}}\) by the inhibitor SB203580 at least partially rescues senescence induced by H-RAS\(^{V12}\) and oxidative stress induced senescence (Wang et al., 2002). The requirement for p38 MAPK in RUNX1 and RUNX1-ETO induced senescence was investigated using the p38 inhibitor (SB203580). Growth of cells containing vector was unaffected by the presence of 8μM SB203580. However, the p38\(^{\text{MAPK}}\) inhibitor caused a significant increase in the growth rate of RUNX1-ETO expressing cells (Figure 7A,B), and fewer cells had a flattened morphology or SA-β-Gal activity (Figure 7C). A similar effect was observed for H-RAS\(^{V12}\) expressing cells again consistent with previous reports (Wang et al., 2002). Treatment of RUNX1 expressing cells with SB203580 also resulted in a partial rescue of the growth arrest, however the effect was clearly less pronounced relative to RUNX1-ETO expressing cells, suggesting that RUNX1 may also activate growth arrest pathways independently of p38\(^{\text{MAPK}}\).

DISCUSSION

This study extends previous observations on the induction of senescence-like growth arrest by Runx proteins in primary MEFs (Linggi et al., 2002; Wotton et al., 2004; Kilbey et al.,...
2007), showing that human fibroblasts are strongly growth inhibited by ectopic RUNX1 expression. Moreover, the fusion oncprotein derivative RUNX1-ETO is even more potent in this regard, despite its dominant negative activity with regard to wild-type RUNX1 in other contexts (Meyers et al., 1995). Both RUNX1 and RUNX1-ETO differ from RAS\textsuperscript{V12} in the early induction of senescence-like growth arrest without an initial replicative phase and high levels of DNA damage. In this regard their effects resemble ectopic p53 expression, although both RUNX effectors induced a more marked flat phenotype and stronger SA-\beta-gal staining compared to p53 alone. Another common feature with ectopic p53 was the lack of induction of SAHF, a phenomenon that has been ascribed to p16\textsuperscript{INK4A} and a more stable form of growth arrest than that mediated by p53 and its downstream growth arrest mediator p21\textsuperscript{WAF1} (Beausejour et al., 2003). A diagram summarising the similarities and differences in the effects of RUNX1, RUNX1-ETO and H-RAS\textsuperscript{V12} on key senescence effectors and outcomes in Hs68 fibroblasts is presented in Figure 8.

While RUNX1 can both activate and repress transcription of target genes (Blyth et al., 2005), the fact that senescence-like growth arrest is also induced by RUNX1-ETO, in which the RUNX1 C-terminal domain is replaced by ETO, suggests that transcriptional repression may be a common denominator in their overlapping phenotypic effects. This suggestion is further indicated by the effects of a RUNX1-ETO deletion mutant lacking C-terminal ETO domains and transcriptional repression activity (Lenny et al., 1995). The activity loss of the truncated RUNX1/AML1a isoform was also consistent with this hypothesis and is noteworthy since this isoform can antagonise full-length RUNX1 and is often overexpressed in human leukemias (Tsuzuki et al., 2007). The relative inactivity of TEL-RUNX1 in this system was surprising, however, as this fusion derivative contains an essentially full-length RUNX1 protein and has repressor activity in other contexts. The possibility that engagement of this failsafe mechanism is selectively lost due to specific properties of the TEL moiety merits further investigation. While our findings would predict that loss of senescence is likely to be more strongly selected in RUNX1-ETO cancers, it is also possible that these results reflects cell type-specific responses and it is notable that TEL-RUNX1 has been reported to collaborate with loss of p16\textsuperscript{INK4A}/p19\textsuperscript{Arf} in murine leukemogenesis (Bernardin et al., 2002).

While RUNX and RUNX1-ETO senescence are phenotypically similar we noted some marked differences in the potency of activation of senescence effectors that correlated well with genetic requirements. RUNX1 displayed relatively modest induction of p53 compared to RUNX1-ETO, but markedly more expression of its downstream effector p21\textsuperscript{WAF1}. It is tempting to speculate that this is due to direct modulation of the p21\textsuperscript{WAF1} promoter which is an established RUNX target that can be regulated independently of p53 (Westendorf et al., 2002). However, it should be noted that despite the relatively lower level, RUNX1-ETO still led to p21\textsuperscript{WAF1} expression above background levels. The greater tendency of RUNX1 and RAS cells to accumulate in G1 was also consistent with this cdk inhibitor expression pattern, as was the lack of requirement for p16\textsuperscript{INK4A} function for RUNX1-ETO-induced senescence observed in Leiden cells. The fact that RUNX1-ETO cells are profoundly growth-arrested despite weak induction of the G1 cdk inhibitors p16\textsuperscript{INK4A} and p21\textsuperscript{WAF1} suggests that other inhibitory factors are also induced (shown as ? in Figure 8).
It is notable that RUNX1-ETO growth arrest is markedly dependent on p53 as revealed by interference with E6 and the resistance of p53 null MEFs to senescence induction. It is also notable that induction of p53 in human fibroblasts by all three effectors occurs without detectable expression of p14ARF, a canonical player in oncogene signalling to p53 in MEFs (Gil and Peters, 2006). Moreover, RUNX1 and RUNX1-ETO arrested cells lack the high level of double-strand breaks seen in RAS-expressing cells (Di Micco et al., 2006; Bartkova et al., 2006), pointing to another stress response pathway that does not require active DNA replication. The strong correlation between induction of p38MAPK phosphorylation and ROS by RUNX1-ETO is noteworthy in this respect. While ROS have the propensity to induce DNA damage, the rapid growth arrest and lack of ongoing DNA replication in Hs68 cells expressing RUNX1-ETO may have masked this potential. Strong induction of ROS, however, may activate p38MAPK independently through redox regulation of phosphatases (Robinson et al., 1999). Our results appear at first sight to contrast with observations in primary CD34 cells expressing RUNX1-ETO that were found to lack evidence of ROS but displayed DNA damage signals and down-regulation of a number of DNA repair enzymes (Krejci et al., 2008). However, as these cells were grown for five weeks in culture prior to analysis it is conceivable that these discordant observations are attributable to selection for survival rather than direct RUNX1-ETO regulation, and it is notable that DNA repair enzymes have not emerged as prominent direct targets for Runx regulation in general transcriptome analyses (Wotton et al., 2008). In future studies it will therefore be important to compare early responses to RUNX1-ETO in both cell types.

On the evidence presented here it may be predicted that the growth arrest induced by ectopic RUNX1 and RUNX1-ETO will be more readily reversible than RAS-induced senescence, with a greater propensity for cells to survive and acquire collaborating mutations. This prediction is consistent with the effects of ectopic Runx2 expression in murine thymus, which results in accumulation of a population of non-dividing cells (CD8ISP thymocytes) that are predisposed to malignant transformation, collaborating efficiently with loss of p53 or activated Myc (Blyth et al., 2006). It is also consistent with studies showing that a subset of human CD34+ hemopoietic precursor cells transduced with RUNX1-ETO can survive for extended periods with elevated expression of p53 and p21WAF1 expression, despite an increased rate of apoptosis (Krejci et al., 2008). Moreover, a recent study based on a murine model has advanced the interesting hypothesis that induction of p21WAF1 and cell cycle restriction by RUNX1-ETO may actually be required for leukemogenesis by preventing DNA damage and hence promoting leukemia stem cell survival (Viale et al., 2009). However, a caveat is that this study contradicts earlier observations that loss of p21WAF1 actually facilitates RUNX1-ETO leukemogenesis (Peterson et al., 2007).

A further conclusion from our findings in this study is that Runx functions impinge on cellular senescence in several different levels. We have previously reported that loss of RAS-induced senescence in Runx2/- MEFs operates at a late stage of the process, as these cells display a full cascade of growth inhibitors but are impaired in their ability to silence G2/M cyclin gene expression (Kilbey et al., 2007; Kilbey et al., 2008). It is clear that the induction of senescence-like growth arrest by ectopic Runx expression is not the simple mirror image of this process. While the Runx family are well known for their ability to activate or repress specific target genes (Swantek and Gergen, 2004), we suggest that their paradoxical
oncogenic and tumour suppressor potential may arise instead from their involvement in distinct steps in cellular growth regulation.

**Materials and Methods**

**Cells and retroviral constructs**

Hs68 neonatal foreskin fibroblasts and Leiden cells expressing ecotropic MLV receptor have been previously described (Brookes et al., 2002). Primary p53 null MEFs were prepared as described (11). The ecotropic virus packaging cell line Phoenix was obtained from G Nolan, Stanford University, Palo Alto, CA, USA. All cultures were maintained in DMEM (Invitrogen) plus 10% fetal calf serum (FCS), glutamine (2 mM), penicillin (200U/ml) and streptomycin (100μg/ml). For pharmacological treatment, SB203580 (Calbiochem) was dissolved in DMSO and added to cultures on the first day after selection (8μM). The medium with the drug was changed every day. The retroviral vectors used were pBabe-puro and pLXSN-neo. The pBabe puro constructs containing human RUNX1, RUNX1-ETO, RUNX1-ETOΔ469, TEL-RUNX1 were previously described and kindly supplied by S. Hiebert (Linggi et al., 2002). The pBabe–H-RASV12 construct has been previously described and kindly provided by S. Lowe (Serrano et al., 1997). pBabe puro containing p53 has also been described previously. The retroviral plasmids containing RUNX1 (1-250) and RUNX1 mutants : K83N, T161A were constructed by cloning of relevant cDNAs into pBabe puro vectors. pLXSN and pLXSN-E6 were a gift of S. Campo (University of Glasgow, UK). Retroviral mediated gene transfer was performed as described (Wotton et al., 2004). Hs68 cells were infected with retroviruses expressing E6 or empty vector (LXSN) and incubated in geneticin (Gibco, 300μg/ml). Infected cell populations were than superinfected with retroviruses expressing RUNX1, RUNX1-ETO, H-RASV12 or empty vector (pBabe) and selected in puromycin 2μg/ml for 4 days.

**Growth curves**

Cells were plated at 2.5 × 10^4/well in 12 well plates in selection medium containing 2μg/ml puromycin. Live cell counts were carried out in triplicate by haemocytometer and trypan blue vital staining. Media were changed out every 3-4 days. Graphs were plotted using Sigma plot. Error bars relate to standard deviations.

**Senescence staining**

Senescence staining was assayed using a solution of X-gal (Invitrogen) at pH6.0 to detect SA-β-galactosidase activity as described (Wotton et al., 2004).

**Immunofluorescence**

Cells were plated on poly-L-lysine-coated (13.3 μg/ml) glass chamber slides and fixed for 15 min with freshly prepared 4% paraformaldehyde (in PBS). After washing with PBS, cells were permeabilized for 15 min with PBST (PBS with 0.1% Triton X-100). Then cells were blocked with 10% FCS, 0.5% BSA in PBS for 30 min and incubated overnight in 4°C with primary antibody: mouse monoclonal γ-H2AX, (1:100, 05-636, Upstate) diluted in blocking buffer. After washing in 10% FCS, 0.5% BSA in PBS, cells were stained with FITC-conjugated secondary antibody (1:100, Jackson Immuno Research Laboratories) for 45 min.
at room temperature. Finally, cells were washed in PBS and coverslips were mounted in VectaShield containing DAPI (4', 6'-diamidino-2-phenylindole) (Vector Laboratories). Fluorescent images were captured using a confocal microscope (Leica DM IRE2).

**Cell cycle analysis**

The protocol for cell cycle analysis has been described (35). For simultaneous labelling with bromodeoxyuridine (BrdUrd), cells were incubated at 37°C for 3-hours with 10µM BrdU (Sigma) then rinsed three times in warmed PBS. After harvesting, cells (10⁶) were washed in 2ml cold PBS, resuspended in 0.2ml cold PBS and fixed for at least 30 minutes in 2mls 70% ethanol at 4°C. BrdU incorporation was identified using FITC-conjugated anti-BrdU antibody (Roche) according to manufacturers’ instructions. Samples were resuspended in PBS containing 10µg/ml PI and visualised on a Beckman Coulter Epics XL. The analysis was performed using EXPO32 software.

**Analysis of intracellular ROS**

To asses the generation of intracellular ROS levels, cells were incubated for 20 min in the dark at 37°C with 15µm DCF-DA (Calbiochem). DCF fluorescence was measured using a flow cytometer (Beckman Coulter Epics XL) with excitation at 488 nm and emission at 530 nm.

**Quantitative real-time PCR**

RNA extraction, cDNA preparation and amplification using primers for human Cyclin A2, Cyclin B1, p53 or endogenous control HPRT (Qiagen Quantitect Primer assays QT00014798, QT00006615, QT00060235 and QT00059066) were as described (Kilbey et al., 2007). Quantification was relative to pBabe puro.

**Western blotting and antibodies**

Cells were washed in cold PBS and whole-cell extracts were prepared in lysis buffer (20 mM HEPES, 5 mM EDTA, 10 mM EGTA, 5 mM NaF, 0.1 µg/ml okadaic acid, 10% glycerol, 1 mM DTT containing 0.4 M KCl, -0.4% Triton X-100 and protease inhibitors; 5 µg/ml leupeptin, 5 µg/ml aprotinin, 5 µg/ml pepstatin A, 1 mM benzamidine, 50 µg/m PMSF). Samples equivalent to 30 µg total protein (Biorad protein assay) were resolved on 8% or 15% SDS-polyacrylamide gels and transferred to Hybond-ECL nitrocellulose membranes (Amersham Biosciences). The following antibodies were used: H- RAS (Oncogene: Ab-1), p16INK4a, p21WAF1, p53, actin (Santa Cruz: sc-468, sc-397, sc-126, sc-1616), p14ARF (Neomarkers: Ab-2), p38, phospho-p38 (Cell signalling: 9212, 9211), AML1/Runx1 (Active Motif: 39300), RUNX1-ETO (antibody to ETO zinc finger motif, obtained from S.Hiebert). The positive control for p14ARF was purchased from Neomarkers (MS-850-PCL). Western blots were developed with ECL (Amersham Biosciences) according to the manufacturer's protocol.

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Figure 1.
(A) Comparison of senescence-like phenotypes in Hs68 human fibroblasts. Hs68 cells were transduced with retroviral vectors expressing RUNX1, RUNX1 (K83N), RUNX1 (T161A), RUNX1 (1-250; AML1a), TEL-RUNX1, RUNX1-ETO, RUNX1-ETO(Δ469), H-RASV12, p53 or control vector (pBabe), selected for 4 days in puromycin and stained for SA-β-Gal (day 7 post-selection). (B) Outline structure of RUNX1, RUNX1 mutants and RUNX1 fusion oncoproteins used in panel A. (C) Western blot analyses of ...
Figure 2.
Comparison of the effects of RUNX1, RUNX1-ETO and H-RASV12 on cell cycle status and expression of markers of proliferation and growth inhibition. Hs68 cells were transduced with retroviral vectors expressing RUNX1, RUNX1-ETO, H-RASV12 or control vector (pBabe) and selected for 4 days in puromycin. (A) Cell cycle-analysis of cell populations (day 7 post-selection) containing control vector (pBabe), RUNX1, RUNX1-ETO or H-RASV12 as determined by BrdU incorporation combined with propidium iodide staining (see Materials and Methods) The percentage of cells incorporating BrdU is indicated in the upper left of each box and percentage of cells in G1 (left corner) and G2/M (right corner) is also indicated. The number of 4N cells is significantly greater in RUNX1-ETO than in control (p=0.01) or in RAS senescent cells (p<0.05). (B) Representative growth curves showing viable cell numbers corresponding to cell populations containing control vector (pBabe), RUNX1, RUNX1-ETO or H-RASV12. Each curve was performed three times and each time point was determined in triplicate. (C) Percentage of cells expressing SA-β-gal. The differences between control and senescent cultures were all highly significant (p ≤0.005). (D) Western blot analysis of expression of p16, p21WAF1 and p53 and p14 in cells containing control vector (pB), RUNX1 (RX), RUNX1-ETO (RX-E) and or H-RASV12 (R) (day 7 post-selection). Human cells-13 lysate (MS-850-PCL, neomarkers) was used as a positive control for p14 antibody. (E) Expression analysis of Cyclin A2, Cyclin B1 and p53 transcripts by quantitative real-time PCR in cells containing control vector (pB), RUNX1 (RX), RUNX1-ETO (RX-E) and or H-RASV12 (R) (day 7 post-selection).
Figure 3.
Analysis of chromatin status and DNA damage in RUNX1 and RUNX1-ETO senescence-like growth arrest. Nuclear DNA was stained using DAPI (upper panel). γH2AX foci were detected by indirect immunofluorescence (lower panel) in Hs68 cells expressing control vector (pBabe), RUNX1, RUNX1-ETO, H-RAS\(^{\text{V12}}\) or p53 at day 7 post-selection. Only the H-RAS\(^{\text{V12}}\) expressing cells displayed clear chromatin condensation and expression of organised foci of DNA damage.
Figure 4.
Analysis of p53-dependence of senescent phenotypes by ectopic expression of HPV16 E6 in Hs68 human fibroblasts. Hs68 cells containing (A) control vector (LXSN) or (B) HPV16 E6 (E6) were co-transduced with retroviral vectors expressing RUNX1, RUNX1-ETO, H-RAS\textsuperscript{V12} or control vector (pBabe). A and B show growth curves. Similar results were observed in three independent experiments and each time point was determined in triplicate. (C) Western Blot analysis of p53 expression in the indicated populations of cells containing control vector (pB), RUNX1 (RX), RUNX1-ETO (RX-E), H-RAS\textsuperscript{V12} (RAS) or p53 on day 7 post-selection. (D) Representative growth curves of p53 null MEFs transduced with control vector (pBabe), RUNX1 and RUNX1-ETO. Statistical analyses of the experiment shown in panels A and B showed significant effects of E6 on cell numbers at day 10 for RUNX1-ETO (p \leq 0.005) and RUNX1 (p \leq 0.05) but not for RAS.
Figure 5.
RUNX1-ETO senescence is independent of p16\textsuperscript{INK4a}. Leiden cells (p16 mutant) were transduced with retroviral vectors expressing RUNX1, RUNX1-ETO, H-RAS\textsuperscript{V12} or control vector (pBabe) and selected for 4 days in puromycin. (A) Growth curves of cells containing control vector (pBabe), RUNX1, RUNX1-ETO or H-RAS\textsuperscript{V12}. Similar results were observed in three independent experiments and each time point was determined in triplicate. (B) SA-\textbeta-Gal staining of cells containing control vector (pBabe), RUNX1, RUNX1-ETO or H-RAS\textsuperscript{V12} (day 8 post-selection).
Figure 6.
ROS accumulation correlates with p38MAPK activation in senescent Hs68 human fibroblasts. Hs68 cells were infected with retrovirus encoding RUNX1, RUNX1-ETO, H-RASV12 or control retrovirus (pBabe) and analysed with DCF for intracellular ROS levels 7 days after puromycin selection. These results are presented as (A) FACS profiles and (B) histogram with error bars based on triplicate estimates. (C) Western blot analysis of phospho-p38 expression in cells containing control vector (pB), RUNX1 (RX), RUNX1-ETO (RX-E) or H-RASV12 (RAS). In panel B the differences between RUNX1 and control were significant (* p ≤0.05) while difference between control and RAS or RUNX1-ETO were highly significant (** p ≤0.005; p ≤0.0001)
Figure 7.
Requirement for p38MAPK function in senescence-like growth arrest phenotypes. Hs68 cells were transduced with retroviral vectors expressing RUNX1, RUNX1-ETO, H-RAS\textsuperscript{V12} or control vector (pBabe), selected in puromycin and then were fed daily with fresh medium containing (A) DMSO solvent alone or (B) 8μM SB203580 (SB) in DMSO. (C) SA-β-Gal staining of SB203580-treated (SB) and mock-treated (DMSO) RUNX1-ETO expressing cells on day 8 post-selection. Statistical analyses of the experiment shown in panels A and B
showed significant effects of SB203580 on cell numbers at day 12 for RUNX1 (p ≤0.05), RAS (p ≤0.01) and RUNX1-ETO (p ≤0.005) but not control cells.
Figure 8.
Alternative routes to senescence-like growth arrest in Hs68 fibroblasts. The principal findings in this study recapitulate previous observations on H-RAS induced senescence in human fibroblasts and indicate significant quantitative differences in activation of growth arrest effector pathways by RUNX1 and RUNX1-ETO. Arrow thickness indicate relative level of induction. DDR: DNA damage response, ROS: Reactive Oxygen Species, RAS: H-RASV12, RX: RUNX1, RX-E: RUNX1-ETO.