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RESEARCH ARTICLE

The role of oncogenic Ras in human skin tumorigenesis depends on the clonogenic potential of the founding keratinocytes

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ABSTRACT

The role of Ras in human skin tumorigenesis induction is still ambiguous. Overexpression of oncogenic Ras causes premature senescence in cultured human cells and hyperplasia in transgenic mice. Here, we investigated whether the oncogenic insult outcome might depend on the nature of the founding keratinocyte. We demonstrate that overexpression of the constitutively active Ras-V12 induces senescence in primary human keratinocyte cultures, but that some cells escape senescence and proliferate indefinitely. Ras overexpression in transient-amplifying- or stem-cell-enriched cultures shows that p16 (encoded by *CDKN2A*) levels are crucial for the final result. Indeed, transient-amplifying keratinocytes expressing high levels of p16 are sensitive to Ras-V12-induced senescence, whereas cells with high proliferative potential, but that do not display p16, are resistant. The subpopulation that sustains the indefinite culture growth exhibits stem cell features. Bypass of senescence correlates with inhibition of the pRb (also known as RB1) pathway and resumption of telomerase reverse transcriptase (TERT) activity. Immortalization is also sustained by activation of the ERK1 and ERK2 (ERK1/2, also known as MAPK3 and MAPK1) and Akt pathways. Moreover, only transduced cultures originating from cultures bearing stem cells induce tumors in nude mice. Our findings demonstrate that the Ras overexpression outcome depends on the clonogenic potential of the recipient keratinocyte and that only the stem cell compartment is competent to initiate tumorigenesis.

KEY WORDS: Primary human keratinocyte, Ras, Clonogenic potential, Tumorigenesis

INTRODUCTION

The epidermis relies on stem cells in the basal layer to self-renew. The transition from stem cells to transient-amplifying and, finally, to post-mitotic cells, named clonal evolution, is a continuous unidirectional regulated process that is instrumental in building up the epidermal structure (Barrandon and Green, 1987). As the epidermis protects the body from environmental insults, keratinocytes have a high risk of acquiring oncogenic mutations and undergoing uncontrolled proliferation, which is one of the hallmarks of cancer. Many epithelial cancers, including squamous cell carcinomas (SCCs), contain a minor population of tumor-initiating cells named cancer stem cells (CSCs) that can reconstitute and maintain the tumor (Adhikary et al., 2013; Patel et al., 2012; Thieu et al., 2013). CSCs share some characteristics of normal stem cells, give rise to heterogeneous tumors and are the source of metastatic outgrowths. The molecular mechanisms determining the development of these cells are not completely elucidated and are likely different among various cancer types. Indeed, CSCs might arise from normal stem cells in which accumulation of genetic and epigenetic modifications induces the deregulation of mechanisms governing self-renewal. However, some committed progenitors might transform into CSCs by acquiring stem-cell-like properties (Hanahan and Weinberg, 2011; Thieu et al., 2013). As epidermis is rapidly renewed, genetic hits necessary to initiate tumors might mainly accumulate in long-term resident stem cells rather than in transient-amplifying cells (Jonason et al., 1996; Thieu et al., 2013). The Ras pathway has been shown to be involved in the development of murine and human SCCs. Indeed, activating mutations in Ras genes have been found in SCCs and in experimentally induced SCCs in mice (Balmain and Yuspa, 2014). However, the role of Ras in the initiation of tumorigenesis is still ambiguous. Strong ectopic expression of oncogenic Ras causes premature senescence in primary human or murine cells through induction of p16 and/or $p14^{ARF}$ (also known as $p19^{ARF}$) (both encoded by *CDKN2A*), which in turn act on pRb (also known as RB1) and p53 (also known as TP53), respectively, to protect cells against Ras-mediated transformation (Serrano et al., 1997). In keratinocyte cultures, Ras overexpression arrests growth, even in the presence of p53 dysfunction. Thus, expression of Ras in primary cells does not promote transformation unless accompanied by cooperating oncogenes or coincident loss of functional p53 or pRb pathways (Dajee et al., 2003; Lazarov et al., 2002). However, the inability of Ras to transform primary cells in culture is in contradiction with clinical observations and data obtained from mouse models (Balmain and Yuspa, 2014). Indeed, transgenic mice tissues expressing activated *H-ras* and *K-ras* undergo hyperplastic growth or tumor development (Brown et al., 1998; Guerra et al., 2003; Jackson et al., 2001; Tuveson et al., 2004). However, in some animal models most of Ras-expressing cells do not display altered morphologies or hyperplastic properties, suggesting that tumor development is highly dependent on the cell type (Brown et al., 1998) and cell differentiation (Guerra et al., 2003; Lapouge et al., 2011; White et al., 2011). In addition, the role of p16 in Ras-induced senescence seems to rely on cell type (Bianchi-Smiraglia and Nikiforov, 2012). For instance, human fibroblasts isolated directly from skin are resistant to Ras-induced senescence and are capable of anchorage-independent growth, whereas p16-expressing cultured fibroblasts, are sensitive to Ras-induced senescence (Benanti and Galloway, 2004).

Primary human keratinocyte cultures bearing stem cells display undetectable levels of p16, but its levels progressively increase

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during the clonal conversion to transient-amplifying cells (Dellambra et al., 2000; Dickson et al., 2000; Maurelli et al., 2006). Thus, in this study, we overexpressed oncogenic Ras in stem-cell- or transient-amplifying-enriched primary cultures in order to define whether keratinocytes carrying a Ras mutation might have a different fate during tumorigenesis, depending on their clonogenic potential.

RESULTS

Ras-V12 overexpression and keratinocyte replicative senescence

Infections with a defective retrovirus carrying a full-length human Ras cDNA bearing an activating mutation at codon 12 (Ras-V12), were performed on three different strains (K53, K45 and K80) of primary human keratinocytes. Parallel infections were performed using empty vector as control (Fig. 1A). Cells were stably transduced with an efficiency near to 100%.

A total of 91±2.88% (mean±s.d., n=3 cultures) of the empty-vector-transduced cultures displayed regular keratinocyte colonies (Fig. 1B, Type A, Fig. 1C, V column), characterized by cells that tightly adhered to each other and that were able to stratify, with 9±2.88% of irregular colonies (Fig. 1B, Type B), characterized by enlarged and flattened cells, which are signs of keratinocyte senescence (Barrandon and Green, 1987). Although all Ras-V12-transduced cultures showed senescent features, three different colony types were identified after infection: 51.66±1.4% of colonies were Type B, 8.7±3.02% Type C and 39.6±1.73% Type D (Fig. 1B,C, Ras column). The Type C colonies were formed by small cells with a peculiar 'convex' surface (arrowheads) that adhered less tightly adherent to other cells and were surrounded by wide intercellular spaces. They displayed a stratification impairment resembling non-differentiating keratinocytes (Adams and Watt, 1988). The Type D colonies were composed of enlarged senescent cells with interspersed with a few small 'convex' cells (arrowheads). Of note, at least 30 min of trypsinization were needed to obtain empty-vector-transduced culture detachment from the flask, whereas Ras-V12-transduced cultures lost their adherence in about 15 min, underlining their adhesion impairment (data not shown).

Different percentages of proliferating keratinocytes per colony type were observed by analyzing the fluorescence labeling of 5-ethynyl-2′-deoxyuridine (EdU), a nucleoside analog of thymidine, which is incorporated during active DNA synthesis. As shown in Fig. 1D and Fig. S1A (2-h treatment panels), Type A colonies incorporated the EdU in 28.34±7.76% (mean±s.d., n=10 colonies) of cells, whereas only 1.16±1.13% of Type B colonies displayed EdU incorporation, but the half of cells (51.43±3.01%) of Type C colonies actively synthesized DNA; 24.27±6.88% of Type D colonies displayed proliferating cells and they were represented by 68.83±12.64% of small 'convex' cells. Also 24 h after the treatment, the Type B colonies displayed only 1.73±0.67% with incorporation, although the Type A and C increased their proliferation index to 77.98±8.65% and 92.82±5.88%, respectively. Of note, in Type D colonies the enlarged and flattened cells did not show any incorporation, whereas 98.07±3.47% of the small cells actively proliferated and came out from the colony (Fig. 1D, 24 h treatment; Fig. S1A).

Furthermore, a different percentage of resting keratinocytes per colony type was observed analyzing the expression of Ki-67 (also known as MKI67), a proliferation marker characterizing all active phases of cell cycle and downregulated during senescence (Tseng and Green, 1994). As indicated in Fig. 1E and Fig. S1B, Type A and C colonies displayed 7.8±3.8% and 3.42±2.85% Ki-67-negative cells, whereas in Type B colonies the 94.98±1.26% of the cells were resting. In Type D colonies, enlarged and flattened keratinocytes represented the Ki-67 negative resting cells, whereas small cells were actively cycling as the 98.78±2.2% of them was Ki-67 positive.

To investigate the proliferative capacity of Ras-V12-transduced keratinocytes, cells were serially cultured without drug selection. The three empty-vector-transduced cultures underwent 69.2, 89.7 and 137.6 cell generations, respectively (Fig. 2A). Their total cell outputs were 1×10^{15}, 1×10^{18} and 1×10^{26}, respectively (Fig. S1C). Unexpectedly, Ras-V12-transduced keratinocytes bypassed replicative senescence and continued to divide at a rate comparable to that of young keratinocytes without slowing down. Indeed, already after the first passage Type C proliferating colonies become predominant. Cultures were serially cultivated for 231, 243 and 253 days, respectively, before freezing them in liquid nitrogen, and then they underwent 283.9, 352.8 and 351.2 cell doublings, respectively (Fig. 2A). Their total cell outputs were greater than 1×10^{95} (Fig. S1C). Transgene and exogenous protein levels persisted during the entire lifespan of Ras-V12-transduced cultures (Fig. 2B,C). Thus, indefinitely growing cultures comprised Ras-V12-transduced cells.

Taken together, these findings demonstrate that Ras-V12-overexpression induces changes of cell morphology comparable to senescence in primary human keratinocytes. However, some cells are resistant to Ras-induced senescence, bypass replicative senescence and continue proliferating.

Comparison of p16- versus Ras-V12-induced senescence in primary human keratinocytes

A functional p16 and pRb pathway is required for Ras-induced senescence (Serrano et al., 1997). To understand whether K53, K45 and K80 strains were able to undergo a full premature senescence or exhibited an impaired pRb pathway, we transduced these cells with a defective retrovirus carrying a p16–EGFP cDNA (Fig. S2A,B, EGFP–p16, 5.55 doublings and p16-1, respectively). EGFP expression in cellular suspension confirmed a transduction efficiency of 90.02±0.35% (mean±s.d., data not shown). In contrast to Ras-V12-transduced cultures, p16-transduced cultures displayed only Type B colonies (Fig. 1C, p16 column). When cultures were serially propagated without drug selection, only p16-untransduced cells were able to grow, whereas p16-transduced cells become immediately senescent and did not proliferate (Fig. S2C). Indeed, EGFP-positive cells had already decreased to 0.2±0.1% after the first passage (data not shown), in parallel to the decrease in EGFP–p16 expression (Fig. S2A,B, compare lanes 5.55 vs. 18.3), and were lost thereafter.

To investigate these differences in p16- and Ras-V12-mediated senescence, key senescence pathways were analyzed 7 days after transduction, when senescent cells were still present in cultures. Upon Ras activation, ERK1 and ERK2 (ERK1/2, also known as MAPK3 and MAPK1, respectively) might play a key role in both cell proliferation and premature senescence given that the intensity or duration of its activity is a crucial determinant of cell fate. Sustained activation of ERK1/2 is required for continued expression of cyclin D1 in G1 phase (Balmanno and Cook, 1999) and facilitates the assembly and the stabilization of Cdk4–cyclin-D complex (Cheng et al., 1999). However, strong activation of ERK1/2 by oncogenic Ras causes cell cycle arrest by inducing the expression of the Cdk inhibitors. Both p16 and $p21^{Waf1}$ (also known as CDKN1A) are inhibitors of Cdk4–cyclin-D complex, and maintain

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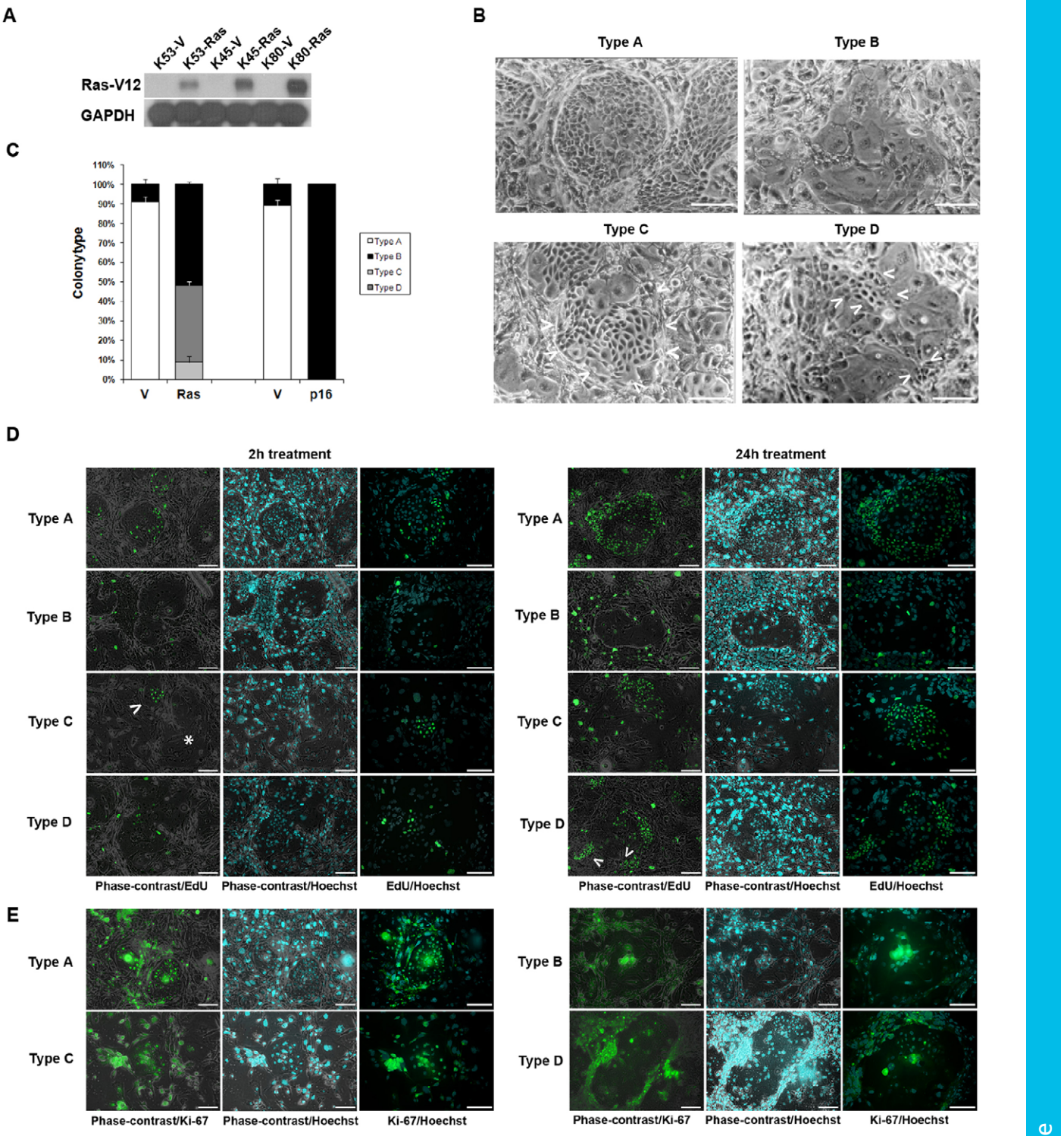

Fig. 1. Ras-V12 overexpression and early morphological changes in keratinocyte cultures. (A) Transcript expression was assessed by northern blotting using RNA obtained from Ras-V12-transduced keratinocytes (K53-Ras, K45-Ras and K80-Ras) 7 days after infection. Empty-vector-transduced cells (K53-V, K45-V and K80-V) were used as negative control. (B) Different colony types were identified in empty-vector- or Ras-V12-transduced cultures: Type A was a normal keratinocyte colony; Type B was a senescent colony; Type C was a colony formed by small cells with a 'convex' surface and surrounded by wide intercellular spaces (arrowheads); Type D was a colony composed of enlarged senescent cells with a few interspersed small 'convex' cells (arrowheads). (C) The percentage of the four colony types (Type A–D) was calculated for each culture 5 days after infection. V columns, empty-vector-transduced cells; Ras column, Ras-V12-transduced cells, p16 column, p16-transduced cultures. Results are mean±s.d. (n=3 cultures). (D) Cell proliferation was assessed by EdU incubation of transduced cultures for 2 h and 24 h and fluorescently labeling with Alexa Fluor 488. Merged phase contrast and EdU labeling, phase contrast and Hoechst 33342 staining, and EdU labeling and Hoechst 33342 staining is shown for each single colony type. Of note, in the 2-h Type C panel, both Type C and B (arrowhead and asterisk, respectively) colonies are present. In the 24-h Type D panel, small keratinocytes proliferating out of the colony rim are indicated by arrowheads. (E) The presence of resting cells was assessed by Ki-67 immunostaining. Merged phase contrast and Ki-67 staining, phase contrast and Hoechst 33342 staining, and Ki-67 staining and Hoechst staining is shown for each single colony. Scale bars: 300 µm.

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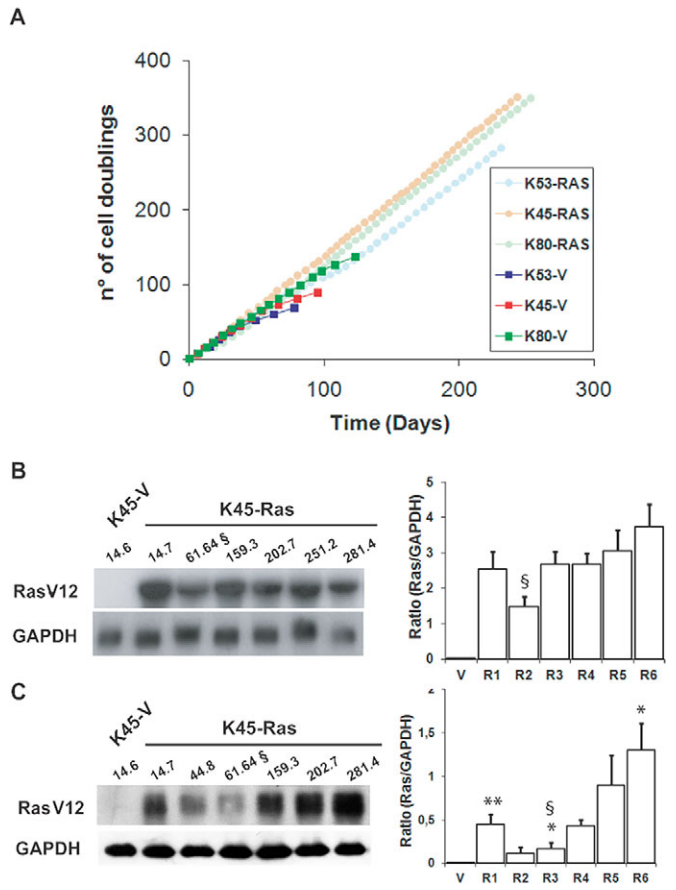

Fig. 2. Ras-V12 overexpression and keratinocyte replicative senescence. (A) Primary human keratinocytes (K53, K45 and K80) transduced with empty vector (blue, red and green squares) and Ras-V12 cDNA (light blue, orange and light green circles) were serially cultivated. The cumulative number of cell generations per passage was plotted against the total time in culture. The transgene persistence was assessed by northern (B) and western blotting (C) using RNA and cell extracts prepared from Ras-V12-transduced keratinocytes at different passages. Empty-vector-transduced cells (V) were used as negative control. Blots on the K45 strain are representative of experiments performed on all strains and passages are indicated by the numbers of cell doublings (§ indicates the passage approaching senescence bypass). Graphs show densitometric analysis of blots performed on three cell strains. As cell doublings at bypass of senescence are different for the three strains, the similar selected passages are generically named R1–R6, where R1 corresponds to low passages, R2 and R3 to senescence bypass (§), and R3–R6 to passages after senescence bypass. Results are mean±s.d. (n=3). *P<0.05, **P<0.01 for R1 versus V, and R2–R5 versus R1 (Student's t-test).

pRb in its hypophosphorylated active state that blocks the entry into S-phase (Lin et al., 1998).

pRb levels decreased following both Ras-V12 and p16 overexpression (Fig. 3A,B) suggesting that there is a block of the cell cycle. However, the hypophosphorylated form of pRb was predominant in p16-transduced cells, whereas both forms were equally expressed in Ras-V12-transduced cells suggesting that latter cells might restart cycling. Flow cytometry experiments underlined an increase in proportion of cells in G0/G1-phase and a decrease in S-phases with both Ras-V12- and p16-overexpression (Fig. 3C). Of note, the percentage of cells in S-phase was significantly reduced in p16-transduced cells compared to Ras-transduced ones, in agreement with EdU incorporation results (Fig. 3C,D).

The pRb pathway is activated by p16 overexpression itself in p16-transduced cells (Fig. 3A,B, p16 versus V lane). Cdk4 and cyclin D1 levels increased in these cultures. As p16 binds to Cdk4 and prevents its association with cyclin D, blocking formation of the active Cdk4–cyclin-D complex, the increased expression of these proteins was probably an attempt to counteract p16 overexpression. p53 expression and $p21^{Waf1}$ levels decreased, suggesting that p16 is the major factor leading this senescent phenotype. Of note, in p16-transduced cells the phosphorylated ERK1 isoform was barely detectable and the phosphorylated ERK2 isoform, the key isoform mediating the senescence of murine fibroblasts (Shin et al., 2013), was predominant. Thus, p16 overexpression is able to induce a full senescence suggesting that pRb the pathway was not impaired in these cultures.

p16 was undetectable and Cdk4 expression did not vary at 7 days after Ras-V12 transduction (Fig. 3A,B, Ras versus V lanes). These findings are in keeping with pRb hyperphosphorylation and suggest that the observed senescent phenotype in these cells might not be ascribed to this pathway. Enhanced expression of Ras-induced targets (phosphorylated ERK1/2 and cyclin D1), was observed. Specifically, both ERK isoform levels were increased compared to in the empty vector cells. The ratio of phosphorylated ERK (pERK) 2 to pERK1 was similar in Ras-V12- and empty-vector-transduced cells, whereas it increased in p16-transduced cells (Fig. S3A), strengthening the idea of a predominance of pERK2 isoform in inducing senescence in the latter cells. Of note, $p21^{Waf1}$ was significantly upregulated in Ras-V12-transduced cells, although p53 expression was significantly decreased.

Thus, our data suggest that, in this setting, cell cycle arrest in Ras-V12 cells might be due to p21 modulation.

Senescence by Ras-V12 in transient-amplifying- or stem-cell-enriched cultures

Human fibroblasts, isolated directly from the skin, are resistant to Ras-induced senescence and become susceptible to it only when p16 is accumulated during subcultivation. Thus, p16 levels seem to be crucial for cell fate after Ras-V12 transduction (Benanti and Galloway, 2004). Stem cells, which represent 5–10% of primary keratinocyte cultures (Barrandon and Green, 1987; Pellegrini et al., 1999), do not display p16 expression whereas the already-committed transient-amplifying-keratinocytes exhibit p16 upregulation (Cordisco et al., 2010).

Keratinocyte cultures approaching senescence (i.e. enriched in transient-amplifying-cells or depleted in stem cells) in which p16 is already expressed, were transduced with Ras-V12 and then serially cultured (Fig. 4A). Transient-amplifying cultures still displayed a good proliferative potential as empty-vector-transduced transient-amplifying cells underwent an additional 46 and 42 cell generations, corresponding to a total cell output of 5.81×10^{12} and 3.85×10^{12} (Fig. 4A; Fig. S1D). Furthermore, empty-vector-transduced transient-amplifying cultures showed 41±5.56% type A and 59±5.56% Type B colonies (mean±s.d., n=2 cultures) (Fig. 4B, TA-V column). Following Ras-V12-transduction, transient-amplifying cells immediately underwent senescence displaying only Type B colonies (Fig. 4B, TA-Ras column), did not reach subconfluence even after 15 culture days and were not able to grow when newly seeded (Fig. 4A).

pRb levels in transient-amplifying cultures were lower than those observed in first passage cultures (Fig. 4C,D, TA-V versus Fig. 3A,B, V-lanes), and further decreased after Ras-V12-transduction (Fig. 4C,D). p16 levels, which were higher in transient-amplifying cells than in primary culture (Fig. 4C,D, TA-V versus Fig. 3A,B, V lanes) became similar after Ras overexpression (Fig. 4C,D). Of note, Ras-V12 significantly suppressed the expression of CDK4

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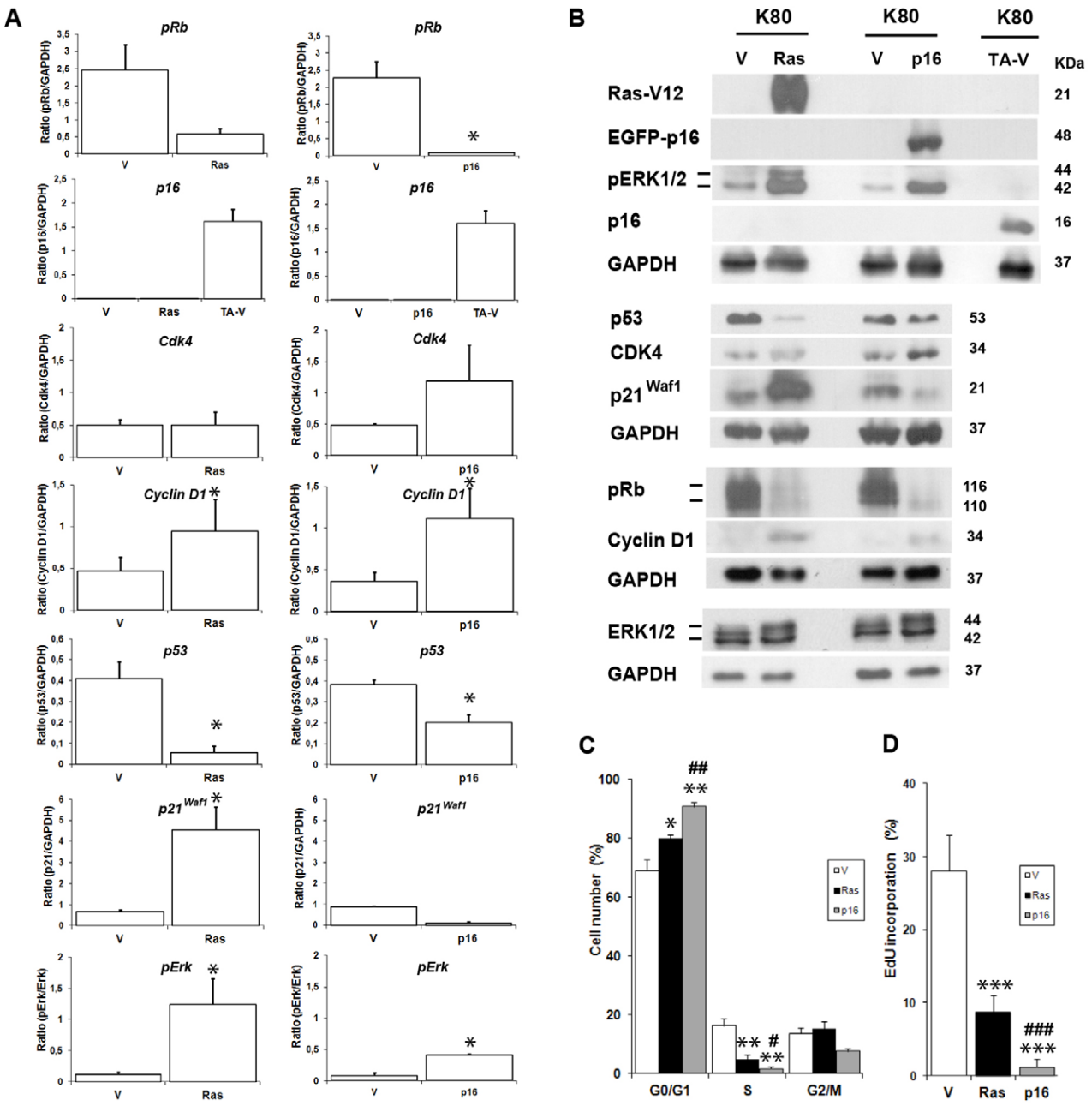

Fig. 3. Comparison of senescence induced by p16 versus Ras-V12 in primary human keratinocytes. (A,B) Ras-V12, EGFP–p16, pRB, p16, Cdk4, Cyclin D1, p53, p21^{Waf1}, pERK1/2 and total ERK1/2 expression was assessed by western blotting using cell extracts prepared from cells transduced with empty vector (V lanes), Ras-V12 (Ras lane) and p16 (p16 lane) at 7 days after infection. Here, transient-amplifying V cells were only used as a positive control of p16 expression. Note hypo- (lower line) and hyper-phosphorylated (upper line) forms of pRB, and the ratio of the pERK1 to total ERK1 (upper line) and pERK2 to total ERK2 (lower line). Graphs show densitometric analysis of blots performed on three cell strains. Results are mean±s.d. (n=3 cultures). *P<0.05 (Student's t-test). Blots on the K80 strain are representative of experiments performed on all strains. (C) Cell-cycle distribution of cells transduced with empty vector (V), Ras-V12 (Ras) and p16 as determined by flow cytometry analysis. The percentage of keratinocytes in G0/G1, S and G2/M phases are indicated in the graph. Results are mean±s.d. (n=3 cultures). *P<0.05, **P<0.01 for Ras or p16 versus V; $^{\#}P$<0.05, $^{\#\#}P$<0.01 for p16 versus Ras (Student's t-test). (D) The percentage (mean±s.d.) of proliferating (EdU-positive) keratinocytes in cells transduced with empty vector (V), Ras-V12 (Ras) and p16 was counted by analyzing the 2-h EdU nuclear fluorescence labeling normalized to total Hoechst 33342 counterstained nuclei. All nuclei of ten random fields (around 5000) were counted. ***P<0.001 for Ras or p16 versus V; $^{\#\#\#}P$<0.001 for p16 versus Ras (Student's t-test).

(Fig. 4C,D) as already observed in keratinocyte cultures (Lazarov et al., 2002). Cyclin D1 levels did not significantly vary, suggesting that CDK4 might be easily sequestered by the p16 that was already present at high levels. Both p53 and p21^{Waf1} expression decreased after Ras-V12 overexpression, strengthening the hypothesis that the irreversible arrest is mediated by p16. Phosphorylated ERK1/2 levels were barely detectable in empty-vector-transduced transient-amplifying cells and Ras-V12 overexpression significantly enhanced them. The ratio of pERK2 to pERK1 was similar to that observed in p16-transduced keratinocytes (Fig. 4C,D; Fig. S2A). These findings demonstrate that keratinocytes expressing high levels of p16 are more sensitive to Ras-V12-induced senescence and suggest that growth arrest might be due to Cdk4 decrease and/or pERK2 increase.

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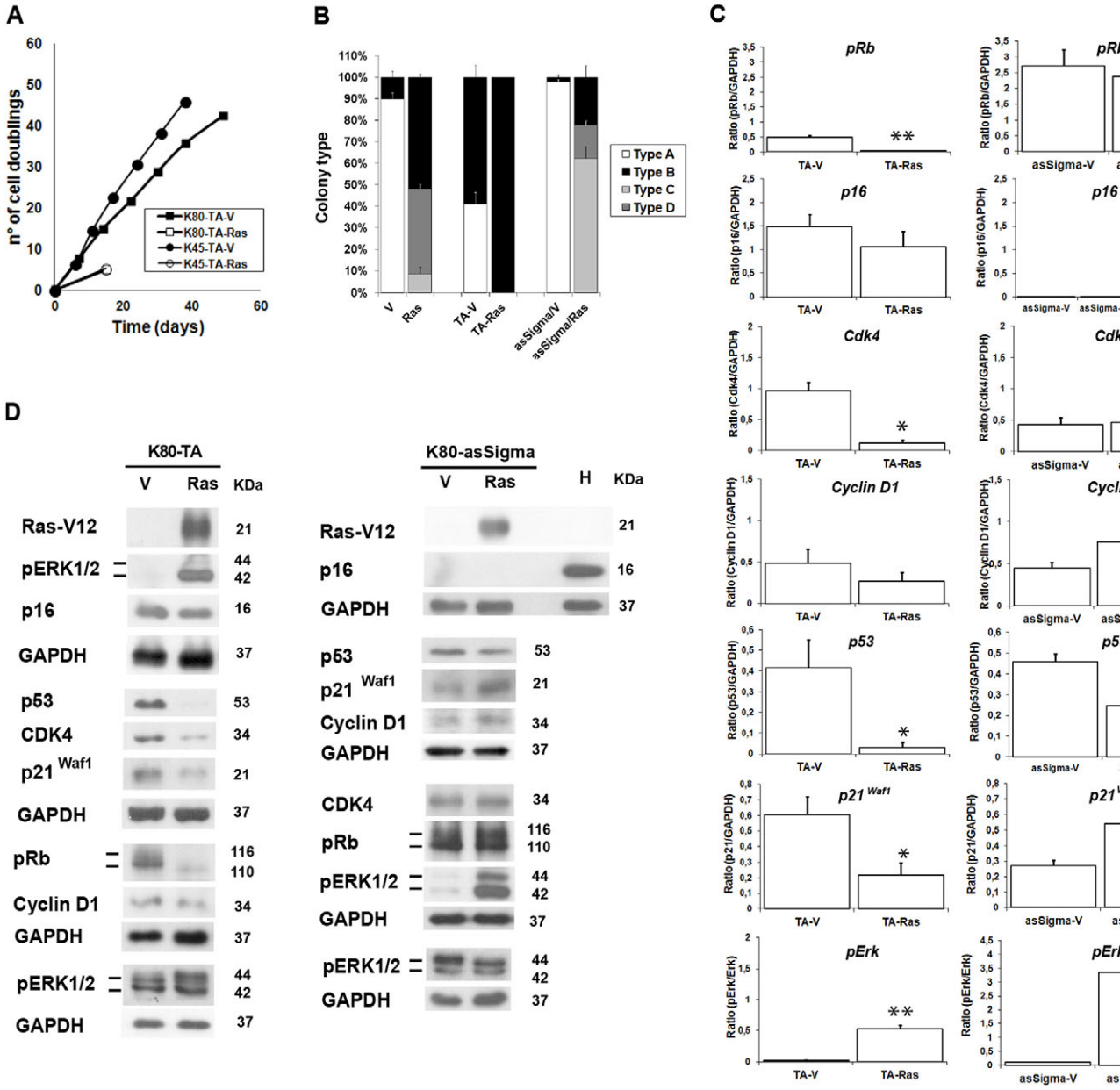

Fig. 4. Senescence induced by Ras-V12 in transient-amplifying- or stem-cell-enriched cultures. (A) Primary human keratinocytes (K80 and K45) were transduced with empty vector (closed squares or circles) or Ras-V12 (open squares or circles) in the half middle of their lifespan (80 and 50 cell generations, respectively), and then serially cultivated. Cumulative number of cell generations per passage was plotted against the total time in culture. (B) The percentage of the four colony types (Type A–D) was calculated for each culture: empty-vector-transduced cultures (V column), Ras-V12-transduced cultures (Ras column); empty-vector-transduced transient-amplifying cultures (TA-V column); Ras-V12-transduced transient-amplifying cultures (TA-Ras column); empty-vector-transduced asSigma cultures (asSigma/V column); Ras-V12-transduced asSigma cultures (asSigma/Ras column). Results are mean±s.d. (n=2–3 cultures). (C,D) Ras-V12, EGFP–p16, pRB, p16, Cdk4, Cyclin D1, p53, p21^{Waf1}, pERK1/2 and total ERK1/2 expression was assessed by western blotting using cell extracts prepared from cells transduced with empty vector (TA-V and asSigma-V lanes) and Ras-V12 (TA-Ras and asSigma-Ras lanes) 7 days after infection. HeLa cells (H lane) were used as positive control of p16 expression. Note hypo- (lower line) and hyper-phosphorylated (upper line) forms of pRB, and the ratio of the pERK1 to total ERK1 (upper line) and pERK2 to total ERK2 (lower line). Graphs show densitometric analysis of blots performed on all cell strains. Results are mean±s.d. (n=2–3 cultures). *P<0.05, **P<0.01 (Student's t-test). Blots on K80 strain are representative of experiments performed on all strains.

Human keratinocyte cultures, immortalized by 14-3-3σ downregulation (asSigma) (Dellambra et al., 2000; Pellegrini et al., 2001), show p16 inhibition and stem cell enrichment (50–60% of stem cells in these cultures versus 5–10% in primary cultures). asSigma cultures were transduced with Ras-V12. Empty-vector-transduced asSigma cultures displayed 98±1% Type A colonies and 2±1% Type B colonies (mean±s.d., n=3 cultures) (Fig. 4B, asSigma/V column). asSigma Ras-V12-transduced cultures showed 22.33±5.5% Type B, 62.33±5.5% Type C and 15.33±2.08% Type D colonies versus 51.66±1.4%, 8.7±3.02% and 39.6±1.73% for Ras-V12 cultures, respectively (Fig. 4B, asSigma/Ras column versus Ras column).

pRb levels were similar in both conditions (Fig. 4C,D, asSigma-Ras versus V lane), confirming that a higher percentage of cycling cells was present in these cultures (Type C, 62.33±5.5% in asSigma/Ras versus 8.7±3.02% in Ras cultures). p16 was undetectable in empty-

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vector-transduced asSigma cultures and did not increase after Ras-V12 overexpression. Cdk4 did not vary, whereas Cyclin D1 and p53 were significantly modulated in Ras-V12-transduced asSigma cells. $p21^{Waf1}$ and ERK1/2 were significantly upregulated, confirming the presence of senescent colonies (22.33±5.5% Type B and 15.33 ±2.08% Type D). As observed in Ras-V12-transduced cells, the ratio of pERK2 to pERK1 was similar in Ras-V12-transduced asSigma and empty vector cells (Fig. S3A), reinforcing the idea that the balance of the two isoforms could promote cell proliferation.

Taken together, these results show that only cells endowed with high proliferative potential are resistant to Ras-V12-induced senescence.

Bypass of senescence and stem cell features

In vivo findings have shown that the cell differentiation state plays a crucial role during oncogenic insults, as expression of K-Ras in stem cells but not in transient-amplifying-cells induces skin tumors (Lapouge et al., 2011; White et al., 2011). To investigate whether keratinocytes that had bypassed senescence exhibited stem cell features, we analyzed the ability of Ras-V12-transduced keratinocytes to form clones. As expected, empty-vector-transduced cells showed a progressive decrease in colony-forming efficiency (CFE) and increase in the number of paraclones during serial passages (Fig. 5A,B). In contrast, Ras-V12-transduced keratinocytes exhibited a progressive increase in CFE (Fig. 5A). The percentage of paraclones (a clonal evolution indicator) was already reduced at 7 days after transduction and decreased to 1–2% or below (as *in vitro* keratinocyte stem cells) during subcultivation (Fig. 5B).

Self-renewal is the hallmark of stem cells in normal and neoplastic tissues (Lee et al., 2014). Deregulation of signaling pathways, such as those involving Wnt/β-catenin, p63 and Bmi-1, can lead to transformation of stem cells into CSCs (Keyes et al., 2011; Lee et al., 2014; Lukacs et al., 2010). In addition, maintenance of telomerase reverse transcriptase (TERT) or resumption of its activity is crucial for self-renewal and cellular immortalization, a prerequisite for cell transformation (Hanahan and Weinberg, 2011). Here, the expression of clonal evolution regulators, such as p63 (also known as TP63) and Bmi-1, transiently decreased after Ras-V12 transduction and had already markedly increased before senescence bypass (Fig. 5C,D). The unphosphorylated form of β-catenin specifically mediates Wnt signals (Daugherty and Gottardi, 2007; Lee et al., 2014). Although β-catenin expression did not vary after Ras-V12 transduction, the phosphorylated form transiently increased after Ras-V12 transduction and had already markedly decreased before senescence bypass (Fig. 5C,D). TERT activity increased after Ras-V12-transduction and before bypass of senescence and further increased during subcultivation (Fig. 5E). Taken together, these findings indicate that the subpopulation resistant to Ras-V12-induced senescence possesses stem-cell-like properties, expresses stem cell markers and sustains the indefinite growth of cultures.

Growth suppression evasion and proliferative signaling maintenance

The p16, Cdk4, cyclin D1 and pRb, and $p14^{ARF}$, p53 and $p21^{Waf1}$ pathways are two key regulatory circuits governing the cell decision to proliferate or enter into senescence state (Hanahan and Weinberg, 2011). p16 was not upregulated during subcultivation of Ras-V12-transduced cells (Fig. 5F,G). Cdk4 expression had slightly increased whereas cyclin D1 had significantly decreased before bypass of senescence. After the first culture passage, expression of both pRb forms in Ras-V12-transduced cells returned to levels of the empty-vector-transduced cultures and had already strongly increased before senescence bypass. This pattern is compatible with increased cell proliferation by evading growth suppression in human keratinocytes (Alani et al., 1999, 2001; Dickson et al., 2000; Maurelli et al., 2006; Nickoloff et al., 2000; Takaoka et al., 2004).

In Ras-V12-transduced cells, $p21^{Waf1}$ returned to basal levels after the first subcultivation passage (Fig. 5F,G). $p21^{Waf1}$ and p53 expression significantly increased at the bypass of senescence. Ras-V12 activates the Raf–MEK–ERK and the PI3K–Akt pathways that are implicated in cell transformation *in vitro* and tumorigenesis (Kern et al., 2011). After first culture passage, expression of ERK1/2 in Ras-V12-transduced cells returned to basal levels and strongly increased at bypass of senescence (Fig. 5F,G). The ratio of pERK2 to pERK1 was similar in all passages for Ras-V12 and empty vector cells (Fig. S3B). Expression of phosphorylated Akt [pAkt; detected using an antibody recognizing phosphorylated Akt1 (Ser473), and Akt2 and Akt3 phosphorylated at corresponding residues] initially decreased and then increased during further subcultivations (Fig. 5F,G).

Taken together, these findings demonstrate that the RasV12-mediated bypass of senescence is coupled to inhibition of the pRb pathway and TERT resumption independently of modulation of the $p53–p21^{Waf1}$ pathway, and immortalization is also sustained by activation of the ERK1/2 and Akt pathways.

Immortality and cell transformation

To investigate whether immortalized cells display any gross chromosomal abnormalities, karyotyping was performed on Ras-V12-transduced keratinocytes after bypass of senescence. Metaphases analysis revealed that cells had the normal complement of 46 chromosomes (data not shown). Thus, no signs of aneuploidy were seen.

Epithelial–mesenchymal transition (EMT) might occur as an early step of cell transformation. Cells gain a fibroblast-like phenotype, become dissociated from each other and acquire the ability to migrate. Loss of E-cadherin is considered a fundamental event in EMT and is accompanied by upregulation of mesenchymal genes, such as vimentin (Sánchez-Tilló et al., 2011). Ras-V12-transduced-keratinocytes displayed a decreased E-cadherin and increased vimentin expression. Unexpectedly, E-cadherin and vimentin expression returned to levels comparable to those of control cells during subcultivation (Fig. 6A,B).

ZEB1 and ZEB2 are important regulators of EMT as they repress E-cadherin and activate vimentin, and are repressed by the non-coding microRNAs of the miR-200 family that induce the mesenchymal-to-epithelial transition (MET) maintaining an epithelial phenotype of tumor cells (Sánchez-Tilló et al., 2011). According to data shown in Fig. 6A,B, ZEB1 and ZEB2 expression increased following Ras-V12-transduction and decreased during subcultivation, whereas miR200a and miR200c expression showed the opposite behavior (Fig. 6C).

In agreement with these molecular data, cell and colony morphology changed during subcultivation of Ras-V12-transduced cells. Whereas empty-vector-transduced cells were able to form stratified colonies (Fig. 6D, 1) composed by cells tightly adherent to each other (Fig. 6D, 5), Ras-V12-transduced cells formed irregular colonies that showed a stratification impairment (Fig. 6D, 2, asterisks) and that were characterized by a fibroblast-like shape (Fig. 6D, 6) resembling non-differentiating keratinocytes (Adams and Watt, 1988). Following subcultivation, Ras-V12-transduced cultures were characterized by two colony types (Fig. 6D, 3,4): colonies (asterisks) with spreading cells

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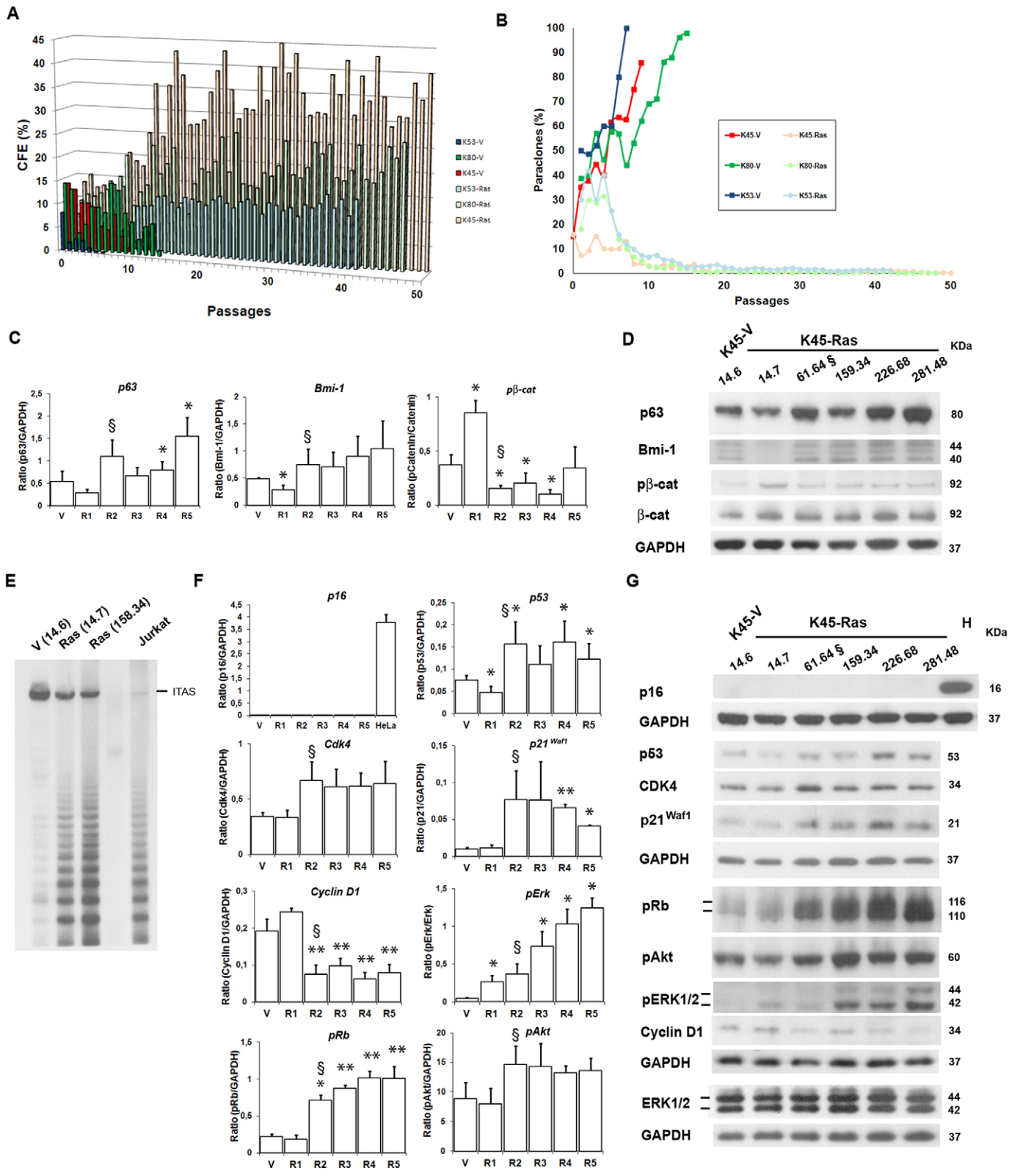

Fig. 5. See next page for legend.

displaying a non-differentiating-keratinocyte-like morphology that grew in monolayers (Fig. 6D, 7) and colonies with epithelial-like polygonal cells separated at cell–cell borders by wide intercellular spaces (Fig. 6D, 8), resembling SCC cells (Rheinwald and Beckett, 1981). The latter colony type became predominant during extensive subcultivation (compare asterisk numbers in Fig. 6D3 and D4). Although a slight decrease of involucrin expression was seen after Ras-V12-transduction, keratinocytes were able to synthesize this differentiation protein after bypass of senescence. However, its expression levels decreased during extensive subcultivation

Fig. 5. Bypass of senescence and stem cell features. (A) CFE assays were performed at each cell passage of keratinocytes (K53, K45 and K80) transduced with empty vector (blue, red and green columns) or Ras-V12 (light blue, orange and light green columns). CFE values were expressed as the ratio of the total number of colonies on the number of inoculated cells and plotted against the passages. (B) The paraclone percentage of cultures (K53, K45 and K80) transduced with empty vector (blue, red and green squares) or Ras-V12 (light blue, orange and light green circles) was expressed as the ratio of aborted colonies to the total number of colonies and plotted against the passages. (C,D,F,G) p63, Bmi-1, phosphorylated β-catenin (pβ-cat) and β-catenin (β-cat) (C,D), and p16, CDK4, Cyclin D1, pRb, p53, $p21^{Waf1}$, pERK1/2, total ERK1/2 and pAkt (F,G) expression was assessed by western blotting using cell extracts prepared from cells transduced with empty vector (V) and Ras-V12 (Ras lanes) at different passages. Blots on K45 strain are representative of experiments performed on all strains and passages are indicated by the numbers of cell doublings (§ indicates the passage approaching senescence bypass). Graphs show densitometric analysis of blots performed on three cell strains. As cell doublings are different for the three strains at bypass of senescence, the similar selected passages are generically named R1–R5, where R1 corresponded to low passages, R2 to senescence bypass (§), and R3–R5 to passages after senescence bypass. Results are mean±s.d. (n=3). *P<0.05, **P<0.01 for R1 versus V and R2–R5 versus R1 (Student's t-test). (E) TRAP assay was performed on cells transduced with empty vector (V) and Ras-V12 (Ras lanes) at different passages (indicated by number of cell generations); an internal TERT assay standard (ITAS) of 150 bp was used. Jurkat cells were used as positive control.

(Fig. 6E,F) as observed for SCC cells (data not shown). Ras-V12-transduced keratinocyte aberrant growth control was evaluated by an *in vitro* tumorigenicity assay. None of the cell strains formed colonies after 21 day culture in soft agar, as did the A431 cell line (positive control), indicating that Ras-V12-transduced keratinocyte growth is anchorage-dependent (Fig. S4A) as observed for *in vitro* subcultivation of some SSC (Rheinwald and Beckett, 1981). Thus, Ras-V12-transduced keratinocytes exhibit an immortalized phenotype and a peculiar morphology, but they do not display cell transformation features.

Tumorigenicity *in vivo*

To assess the *in vivo* tumorigenicity, Ras-V12-transduced keratinocytes were injected subcutaneously into nude mice. Ras-V12-transduced cells (K45-Ras or K80-Ras), after senescence bypass, induced tumors in 100% of cases by 10 days after injection (Table 1). The maximal mean tumor volume was 2693.46±972.95 mm^3 (mean±s.d., n=20) (Table 1, Fig. 7A). At 10 days after injection, the tumoral mass already had a mean volume of 572.66±156.59 mm^3 (Table 1; Fig. S4B). Interestingly, we also found tumors in 64% of cases after injection of Ras-V12-transduced keratinocytes at on day 7 after infection (K45-Ras-QS or K80-Ras-QS) (Table 1). In the latter, mice tumors were smaller and reached the maximal mean volume (518.09±181.18 mm^3) later (45.45±26.85 days after injection) (Table 1, Fig. 7A; Fig. S4C). Injection of Ras-V12-transduced transient-amplifying [K45(TA)-Ras or K80(TA)-Ras] and empty-vector-transduced cultures did not induce tumor formation (Table 1, Fig. 7A; Fig. S4C).

Histological examination of specimens by hematoxylin and eosin staining revealed a hyperplastic tissue with well differentiated 'horny pearls' in the dermis (Fig. 7B). These are typical SCC structures, which are composed of concentric layers of squamous cells (Fig. 7B, Ras 20×). Immunohistochemical analysis of specimens, using an antibody specific for human involucrin, showed that tumors were of human origin (Fig. 7C). Indeed, control mouse skin was unstained by this antibody.

Human cutaneous SCCs are characterized by inner cell masses of keratinocytes that do not proliferate and express differentiation markers (involucrin and 14-3-3σ). Proliferating keratinocytes are located on the outer edges (Lodygin et al., 2003). Here, involucrin and 14-3-3σ staining (Fig. 7C) showed that those tumors were well differentiated in the inner part. The proliferative marker PCNA was expressed in basal cells of control skin and in peripheral cells of tumor areas. Tumors were richly vascularized, as shown by human VEGF staining (Fig. 7D). Interestingly, human VEGF was found also in the region surrounding human tumor cells (compare involucrin staining versus human VEGF staining of the same specimen). Vascularization was mainly due to VEGF secreted by human cells as tissues were not stained by antibody specific for murine VEGF (Fig. 7E).

Thus, Ras-V12-transduced cultures show tumorigenic ability only when they include stem cells and when resident in the *in vivo* environment.

DISCUSSION

Ras and bypass of senescence

Overexpression of oncogenic Ras causes premature senescence in cultured primary cells and hyperplastic growth in transgenic mice (Balmain and Yuspa, 2014). Thus, the role of Ras in the induction of human skin tumorigenesis is still ambiguous. In the present study, we investigated whether the consequence of the oncogenic insult in human keratinocytes might depend on the nature of the founding cell.

Our results demonstrate that Ras-V12-overexpression induces senescence in primary human keratinocyte cultures, but some cells escape senescence and proliferate indefinitely. Ras-overexpression in transient-amplifying- or stem-cell-enriched primary cultures shows that: (1) transient-amplifying-keratinocytes expressing high levels of p16 are sensitive to Ras-V12-induced senescence; (2) cells with high proliferative potential, which do not display p16, are resistant to Ras-V12-induced senescence; and (3) $p21^{Waf1}$ levels modulate the cell behavior following Ras transduction when p16 is not still accumulated.

Following Ras-V12 transduction, expression of its targets (phosphorylated ERK1/2 and cyclin D1) and $p21^{Waf1}$ increases, although p53 is downregulated. Upregulation of ERK1/2 and $p21^{Waf1}$ is transient, as their expression returns to basal level after the first passage in culture. Taken together, these data are in line with findings indicating that ERK1/2 activation is sufficient to induce $p21^{Waf1}$ expression in a p53-independent manner (Bottazzi et al., 1999; Liu et al., 1996) and blocks proteasome-mediated $p21^{Waf1}$ degradation by cyclin D1 accumulation in fibroblasts (Coleman et al., 2003). Although the accumulation of $p21^{Waf1}$ has been associated with inhibition of Cdks and the block of cell cycle (Meloche and Pouysségur, 2007), the $p21^{Waf1}$-dependent arrest is reversible in cells expressing low levels of p16 and p53 (Beauséjour et al., 2003). In addition, a transient ERK1/2-mediated induction of $p21^{Waf1}$, concomitant with cyclin D1 accumulation, allows G1 progression (Meloche and Pouysségur, 2007). The $p21^{Waf1}$ pathway can either antagonize or synergize with p16 in senescence depending on the type and level of stress. Damaged cells might retain their proliferative potential, extending cell cycle duration to allow a compensatory repair mechanism, named 'assisted cell cycling'. To resume their proliferation, cells rely upon stress support pathways enabled by $p21^{Waf1}$ activation (van Deursen, 2014). Thus, in our setting, a $p21^{Waf1}$-mediated cell cycle arrest might induce senescence in the majority of the culture and allow a reversible block in a subset of keratinocytes expressing lower levels of p16. Indeed, the p16 basal level seems crucial for cell fate after Ras-V12 transduction (Benanti and Galloway, 2004). Primary human keratinocyte cultures comprise a heterogeneous cell population as

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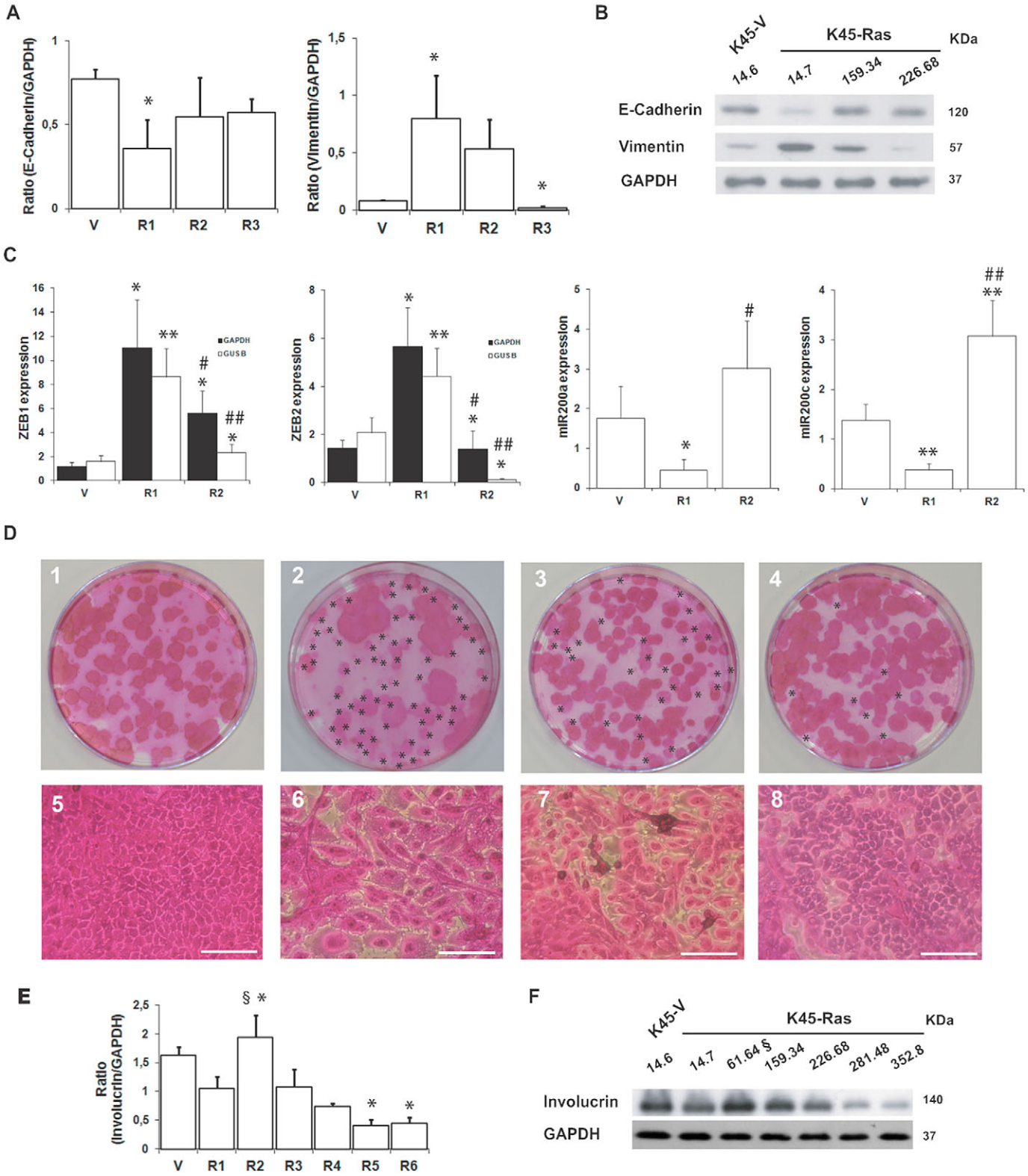

Fig. 6. See next page for legend.

clonogenic cells are endowed with different proliferative potential. Clonal analysis remains the most reliable means to discriminate stem cells from transient-amplifying cells. Indeed, none of the identified cell surface proteins is a keratinocyte stem-cell-specific marker, even though some of them are able to capture subpopulations that also contain stem cells (Barrandon et al., 2012). Keratinocytes can be classified as holoclones, meroclones and paraclones by clonal analysis (Barrandon and Green, 1987): the holoclone, generated from stem cell, has the highest proliferative capacity; meroclones and paraclones originate from young and old transient-amplifying cells, respectively. Specifically, paraclones derive from the largest colony-forming keratinocytes and generate

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Fig. 6. Growth suppression evasion and EMT. (A,B,E,F) E-cadherin, vimentin (A,B) and involucrin (E,F) expression was assessed by western blotting using cell extracts prepared from cells transduced with empty vector (V) and Ras-V12 (Ras) at different passages. Blots on K45 strain are representative of experiments performed on all strains and passages are indicated by the numbers of cell doublings. Graphs show densitometric analysis of blots performed on three cell strains. As cell doublings are different for the three strains, in A, the similar selected passages are generically named R1–R3, where R1 corresponds to low passages, and R2 and R3 to passages after senescence bypass. **P*<0.05 for R1 versus V, and R2–R3 versus R1 (Student's *t*-test). In E, the similar selected passages are generically named R1–R6, where R1 corresponds to low passages, R2 to senescence bypass (§), R3–R6 to passages after senescence bypass. **P*<0.05 for R1 versus V, and R2–R6 versus R1 (Student's *t*-test). Results are mean±s.d. (*n*=3 cultures). (C) ZEB1, ZEB2, miR200c and miR200a expression was evaluated by quantitative RT-PCR analysis on RNA extracted from cells transduced with empty vector (V) and Ras-V12 (Ras) at different passages. The RNA levels were normalized using the GAPDH or GUSB genes. Results are mean±s.d. (*n*=3 for each strain). As cell doublings are different for the three strains, the similar selected passages are generically named R1–R2, where R1 corresponds to low passages and R2 to passages after senescence bypass. **P*<0.05, ***P*<0.01 for R1–R2 versus V; $^{\#}P$<0.05, $^{\#\#}P$<0.01 for R2 versus R1 (Student's *t*-test). (D) Colony morphological changes were analyzed comparing the CFE of cultures transduced with empty vector (1) and Ras-V12 at selected passages indicated by the cell doublings number: 14.7 (2), 159.34 (3) and 281.48 (4). Modifications in cell shape and colony organization were analyzed. All empty-vector-transduced cells were tightly adherent to each other in the colony and were able to stratify (5), whereas most of the Ras-V12-transduced cells showed fibroblast-like shape and grew in a monolayer at early subcultivation passages (6). After bypass of senescence, Ras-V12-transduced cultures were composed by colonies with fibroblast-like shaped cells growing in a monolayer (7) and colonies with epithelial-like polygonal cells, surrounded by wider intercellular spaces (8). Asterisks indicate monolayer colonies in CFE dishes. Scale bars: 300 µm.

aborted colonies containing only terminally differentiated cells. Keratinocyte replicative senescence is accompanied by clonal evolution and is closely associated with increased expression of p16 (Dickson et al., 2000; Rheinwald et al., 2002). Thus, the progressive increase of paraclones during keratinocyte life-span is a direct measure of clonal evolution and in turn gradual stem cell depletion. Clonal evolution can be prevented by modulation of some specific regulators. For instance, 14-3-3σ downregulation (asSigma) in primary human keratinocytes induces stem cell enrichment by p16 inhibition (Dellambra et al., 2000; Pellegrini et al., 2001). We demonstrate that transient-amplifying-enriched cultures, in which p16 is already expressed, are susceptible to Ras-V12-induced senescence, suggesting that the arrest of the growth could be mainly ascribed to Cdk4 decrease and/or pERK2 increase. By contrast, cultures enriched in stem cells are also more resistant to Ras-induced senescence compared to primary keratinocytes. Indeed, we observed a higher percentage of cycling cells following Ras-V12 transduction, in keeping with the pRb levels observed. Although the presence of senescent colonies is confirmed by $p21^{Waf1}$ and ERK1/2 upregulation, this increase is lower than that observed in Ras-V12 primary cultures in which the senescent colony percentage is higher. These data strengthen the concept that $p21^{Waf1}$ levels modulate the cell behavior following Ras-V12 transduction when p16 is not still accumulated. Moreover, the ratio of pERK2 to pERK1 differs between cultures that are susceptible or resistant to Ras-V12. It increases with senescent phenotype in keeping with data obtained in fibroblasts (Shin et al., 2013). Taken together, these findings show that the consequence of the oncogenic insult also depends on the nature of the founding cell and basal p16 levels are crucial for the final outcome.

Ras and stemness

Only the stem cell compartments of the mouse skin are competent to initiate tumor formation (Lapouge et al., 2011; White et al., 2011), in keeping with our *in vitro* data. Keratinocytes resistant to Ras-V12-induced senescence bypass replicative senescence and their clonogenic ability increases during life-span. Of note, their paraclone percentage had already progressively decreased to 1–2% before senescence bypass. This finding suggests that cells bypassing senescence might have features of human keratinocyte stem cells, as holoclones display 0–5% aborted colonies (Barrandon and Green, 1987).

Deregulation of the machinery for self-renewal might induce the transformation of stem cells into CSCs. p63 and Bmi-1 levels are crucial for keratinocyte self-renewal (Cordisco et al., 2010; Pellegrini et al., 2001) and are enhanced in SCCs (Lukacs et al., 2010). They had already markedly increased during life-span of Ras-V12 transduced keratinocytes before the bypass of senescence. This is in agreement with findings indicating that overexpression of the ΔNp63a isoform blocks replicative senescence mediated by p16 and p19 inhibition and cooperates with Ras to maintain the stem cell population of mouse keratinocytes, promoting their malignant conversion (Ha et al., 2011; Keyes et al., 2011). Ras is also able to regulate Bmi-1 expression, and both proteins collaborate to induce an aggressive and metastatic phenotype (Hoenerhoff et al., 2009). TERT maintenance or resumption supports stemness, maintaining cellular immortalization (Pellegrini et al., 2004; Hanahan and Weinberg, 2011). TERT activity had already increased in Ras-V12-transduced keratinocytes before senescence bypass and further increased during cell passages. As both p63 and Bmi-1 are able to inhibit p16 expression in keratinocytes, in our setting they might foster cells in the stem cell compartment through TERT activation, in keeping with keratinocyte immortalization data obtained by the direct p16 inactivation (Maurelli et al., 2006). p63 also regulates the migration and invasion of esophageal squamous carcinoma cells through activation of β-catenin, a key downstream effector of the Wnt signaling pathway that maintains self-renewal in stem cells and CSCs (Lee et al., 2014). Activation of Wnt signaling leads to inactivation of glycogen synthase kinase-3β (GSK-3β), resulting in stabilization and accumulation of cytoplasmic β-catenin, which translocates to nucleus and induces target gene expression including c-Myc and VEGF. Indeed, the phosphorylation-dependent degradation of β-catenin by GSK-3β is a key step in turning off Wnt signals (Daugherty and Gottardi, 2007). In our setting, β-catenin is already mainly in the unphosphorylated form, which mediates Wnt signals, before senescence bypass. A feed-forward regulatory loop between TERT and Wnt/β-catenin pathways characterizes cancer development. Reactivated TERT modulates expression of Wnt target genes (such as TERT itself) that, in turn, exert tumor promoting functions (Li and Tergaonkar, 2014).

Thus, the indefinite growth of cultures is sustained by the subpopulation that is resistant Ras-V12-induced senescence, which possesses stem-cell-like properties and expresses stem cell markers.

Proliferative signaling maintenance, EMT and tumor formation

The subpopulation resistant to Ras-V12-induced senescence displays an enhanced ability to drive tumor formation.

The development of human tumors is a multistep process. Normal cells become progressively tumorigenic acquiring several hallmark capabilities, such as evading growth suppressors (e.g. p53 and pRb proteins), sustaining proliferative signaling, enabling

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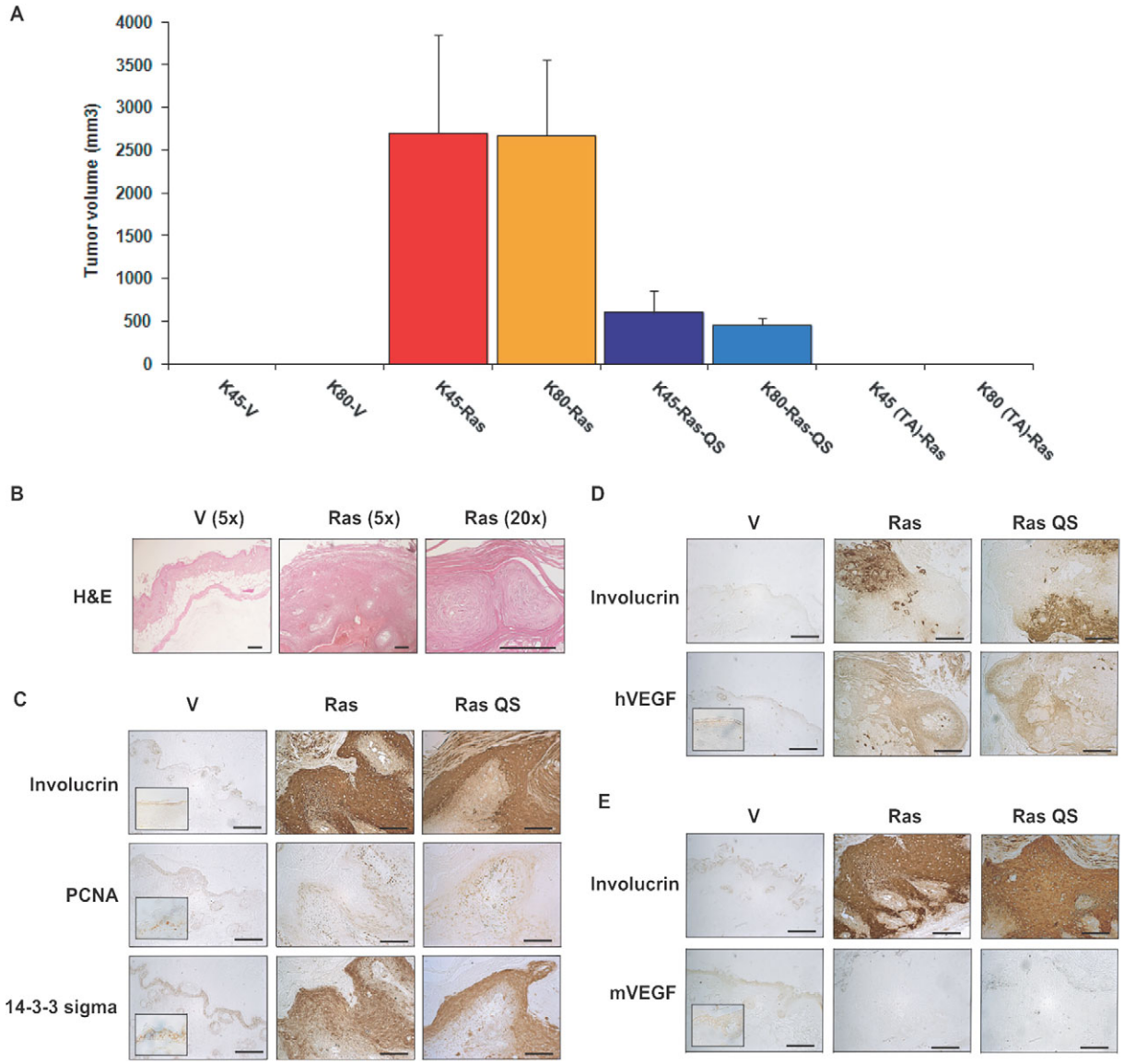

Fig. 7. Tumorigenicity *in vivo*. (A) Primary human keratinocytes overexpressing Ras-V12 were injected subcutaneously in nude mice. Specifically, they were from Ras cultures after bypass of senescence (K45-Ras or K80-Ras, see red and orange columns), at day 7 after infection (K45-Ras-QS or K80-Ras-QS, see blue and light blue columns), or Ras-V12-transduced-transient-amplifying cells at day 7 after infection [K45(TA)-Ras or K80(TA)-Ras]. Empty-vector-transduced cells (K80-V, K45-V) were used as control. Maximal tumor volumes are presented as the mean±s.d. tumor volume of each group from two independent experiments. Each culture was injected in five mice (for each experiment). (B) Paraffin-embedded specimens obtained from cells transduced with empty vector (K80-V) and Ras-V12 were stained with H&E. Scale bars: 100 µm. (C–E) Paraffin-embedded specimens obtained from cells transduced with empty vector (K80-V) and Ras-V12 (Ras and Ras QS) were immunostained with antibodies against involucrin, PCNA, 14-3-3σ, human (h)VEGF and mouse (m)VEGF. Insets display a high magnification view of empty vector staining. Scale bars: 100 µm.

replicative immortality, inducing angiogenesis, activating invasion by EMT and finally metastasis (Hanahan and Weinberg, 2011).

In parallel to TERT resumption, both forms of phosphorylated pRb had already strongly increased before bypass of senescence of Ras-V12-transduced cells, sustaining indefinite proliferation by evading growth suppression. Indeed, upregulation of p53 pathway at bypass of senescence does not appear to interfere with keratinocyte proliferation when the pRb pathway is strongly inactivated, as previously demonstrated (Maurelli et al., 2006). Immortalization is also sustained by activation of the ERK1/2 and Akt pathways. Indeed, these Ras targets, which are involved in cell transformation, are upregulated and progressively increase at the bypass of senescence. These data are in keeping with *in vivo* data demonstrating that both Erk and Akt pathways are activated during Ras-induced tumorigenesis from hair follicle stem cells (White et al., 2011). Activated PI3K–Akt signaling leads to a decrease of GSK-3β activity through phosphorylation. Phosphorylated GSK-3β dephosphorylates β-catenin and prevents its degradation (Daugherty and Gottardi, 2007). This is consistent with our data about the increase of the unphosphorylated β-catenin form.

pRb inactivation contributes to tumor progression not only through loss of cell cycle control but also through upregulation of ZEB factors, which are modulators of E-cadherin and vimentin, key players of EMT (Sánchez-Tilló et al., 2011). ZEBs are repressed by miR-200 family, which induce MET and have a key role in maintaining an epithelial phenotype in tumor cells. Indeed, the miR-200 family is considered a marker of well-differentiated epithelial cancers (Park et al., 2008). As miR-200 members are, in

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Table 1. Tumor incidence

	Tumor incidence	Tumor volume (mm^3)	Time (days)
V	0%	0	0
Ras-V12	100%	2693.46±972.95	10
Ras-V12-QS	64%	518.09±181.18	45.45±26.85
TA-Ras-V12	0%	0	0

The table shows the tumor incidence, the maximal mean tumor volume and the time of tumor development of transduced strains: empty-vector-transduced primary human keratinocyte cultures (V), RasV12-transduced primary human keratinocyte cultures (Ras-V12), RasV12-transduced primary human keratinocyte cultures at day 7 after infection (Ras-V12 QS) and RasV12-transduced transient-amplifying keratinocyte cultures (TA-Ras-V12). Results are mean±s.d. (*n*=20).

turn, transcriptionally repressed by ZEB1 and ZEB2, the unbalance of this loop might foster cells in an epithelial or mesenchymal state (Sánchez-Tilló et al., 2011). Interestingly, p63 negatively regulates ZEB1 and vimentin expression in keratinocytes (Tucci et al., 2012), whereas p53 and Akt pathways modulate miR-200 family in some cell types (Magenta et al., 2011).

At early passages Ras-V12-transduced cells show a fibroblast-like shape and a stratification impairment. These cultures display ZEB upregulation and miR-200 downregulation, with concomitant E-cadherin and vimentin modulation. These data are in keeping with pRb pathway resumption, and p63 and Akt downregulation, which might foster the ZEB and miR200 loop towards ZEB factor upregulation. Following extensive subcultivation, Ras-V12-transduced cultures are mainly comprised of colonies with epithelial-like polygonal cells resembling SCC cells. In these cultures, ZEB proteins are downregulated, whereas both miR200s are upregulated. Consequently, E-cadherin and vimentin levels return to the level of normal controls. These data are in line with p53 and Akt upregulation, which might foster the ZEB–miR200 loop towards miR-200 upregulation. The recruited keratinocytes display also high levels of p63 and Bmi-1. As p63 has already increased by senescence bypass, it might be the main protein responsible for miR-200 upregulation, acting by downregulating ZEB proteins. Bmi-1, which is a target of miR200, could contribute to the increase of both miRs as compensative effect.

Although Ras-V12-transduced keratinocyte cultures, originating from cultures bearing stem cells, do not display cell transformation features *in vitro*, they are able to induce tumors in nude mice. This suggests that microenvironmental factors are essential for tumorigenesis. As SCCs, these tumors are characterized by inner cell masses of differentiated keratinocytes and outer edges with proliferating keratinocytes. They are able to secrete human VEGF in keeping with the increase of the unphosphorylated β-catenin expression observed *in vitro*. Specifically, Ras-V12-transduced keratinocyte cultures at first passage, with a TERT expression increase and low levels of p63 and Bmi-1, formed tumors in 64% of cases with a major latency. By contrast, cultures after bypass of senescence, with high levels of TERT, p63 and Bmi-1, immediately formed tumors in 100% of cases with a greater maximal tumor volume. Ras-V12-transduced transient-amplifying keratinocyte cultures are not able to induce tumors in mice.

Taken together, our findings demonstrate that the consequence of Ras insult in primary human keratinocytes also depends on the clonogenic potential of the founding cell and that basal p16 and p63 levels are crucial for the final outcome, in keeping with data obtained in mouse models (Lapouge et al., 2011; White et al., 2011). Thus, these data might be predictive of what happens during tumorigenesis in humans. Activating mutations in Ras seem to be early events during the development of human cancer inducing a rapid hyperproliferation and an increased chance of acquiring additional mutations (Balmain and Yuspa, 2014). We provide evidence that the human epidermal stem cell compartment is competent to initiate tumorigenesis. Ras-V12 expression can drive cell immortalization and a second hit seems to be required for tumor development *in vivo*. Our data also highlight the inability of human epidermal transient-amplifying cells to respond to direct tumorigenic stimuli. However, we cannot exclude that transient-amplifying cells could generate tumors following additional genetic or environmental insults, such as inflammation generated by wounding.

MATERIALS AND METHODS

Cell culture, colony forming efficiency and cell generations

3T3-J2 cells (a gift from Howard Green, Harvard Medical School, Boston, MA), GP+E-86, and GP+*env* Am12 packaging cells were grown as described previously (Dellambra et al., 2000). Human keratinocytes were obtained from skin biopsies of healthy donors and cultivated on a feeder-layer of lethally irradiated 3T3-J2 cells (Rheinwald and Green, 1975). Procedures were performed in accordance with the ethical standards of the Committee on Human Experimentation of IDI-IRCCS. The study was conducted according to the principles of the Declaration of Helsinki. For serial propagation, subconfluent cells were passaged until they reached senescence (Dellambra et al., 2000). A CFE assay was performed and analyzed as described previously (Barrandon and Green, 1987; Dellambra et al., 2000).

Retroviral-mediated gene transfer

L(RasV12)SN was constructed by cloning RasV12 (a gift from Manuel Serrano, CNIO, Madrid, Spain) into the EcoRI site of LXSN retroviral vector. L(p16)SN construct and the amphotropic producer cell lines Am12/L(RasV12)SN and Am12/L(p16)SN were generated as described previously (Maurelli et al., 2006) with a viral titer of 1×10^6 CFU/ml. Keratinocyte infections were carried out as described previously (Dellambra et al., 2000). Subconfluent cultures were used for further analyses and serial cultivation.

Northern analysis and TRAP assay

RNA was extracted from cells using the TRIzol (Invitrogen), and northern blot analysis was performed as described previously (Dellambra et al., 2000). TERT activity was detected by a telomeric repeat amplification protocol (TRAP) assay using Telo TAGGG Telomerase PCR ELISA and Biotin Luminescent Detection kit (Roche).

Quantitative RT-PCR

RNA was extracted from cells using TRIzol (Invitrogen). For mRNA quantification, total RNA was reverse transcribed using an oligo(dT) primer (Invitrogen). mRNA levels were analyzed by a QuantiTect SYBR Green-PCR kit (QIAGEN) using an ABI PRISM 7000 (Applied Biosystems). mRNA levels were normalized using the GAPDH and GUSB genes as housekeeping genes. For miRNA quantification, total RNA was reverse transcribed using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Life Technologies). miRNA levels were analyzed with the TaqMan MicroRNA assays (Applied Biosystem, Life Technologies) using ABI PRISM 7000. miRNA expression was normalized using U6snRNA as a control. Relative expressions were calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$).

Immunohistochemistry

Immunohistochemistry was carried out as described previously (Dellambra et al., 2000), using the following antibodies: anti-involucrin (1:1000 dilution) from Sigma, anti-PCNA (PC10; 1:1000 dilution), anti-14-3-3σ (N14, 1:1000 dilution) from Santa Cruz Biotechnology, Inc.

Immunofluorescence and proliferation assay-

Keratinocytes were directly transduced onto 24-wells containing 1.4-cm^2 round glass coverslips and cultured as above.

Immunofluorescence was performed as previously described (Dellambra et al., 1998) using anti-Ki-67 antibody (NCL-Ki67p; 1:100 dilution) from Novocastra. The proliferation assay was performed using the Click-iT® EdU Alexa Fluor® 488 Imaging Kit (Thermo Fisher).

Western analysis

Subconfluent keratinocytes were extracted on ice with lysis RIPA buffer and equal amounts of samples (50 µg) were electrophoresed on 7.5–12.5% SDS-polyacrylamide gels. Western blotting was performed as described previously (Dellambra et al., 2000), using the following antibodies: anti-p16INK4a (N20; 1:500 dilution), anti-p53 (DO-1; 1:500 dilution), anti-p63 (4A4; 1:100 dilution), anti-Cyclin D1 (M20; 1:500 dilution), anti-E-Cadherin (H108; 1:2000 dilution), anti-β-Catenin (E5; 1:500 dilution) antibodies, all from Santa Cruz Biotechnology; anti-Cdk4 (06-139; 1:250 dilution) and anti-Bmi-1 (05-637; 1:100 dilution) antibodies from Upstate; anti-pRb (G3-245; 1:500 dilution), anti-h-Ras (18/Ras; 1:500 dilution) and anti-vimentin (RV202; 1:5000 dilution) from BD Pharmingen; anti-phosphorylated β-catenin (9561; 1:500 dilution), anti-phospho Akt (9271; 1:100 dilution), anti-phosphorylated p44-p42 MAPK (i.e. against pERK1/2; 9101; 1:100 dilution) and anti-p44-p42 MAPK (i.e. against ERK1/2; 9102; 1:500 dilution) from Cell Signaling; anti-$p21^{Waf1}$ antibody (1:3 dilution) was a kind gift from Kristian Helin (IEO, Milan, Italy). Protein levels were evaluated by densitometric analysis using a GS-710 scanner and Quantity One software (Bio-Rad) and normalized to GAPDH protein levels.

FACS analysis

Cell-cycle distribution analysis was performed on transduced keratinocyte cultures fixed in 70% ethanol, resuspended in PBS containing 1 mg/ml RNase and 50 µg/ml propidium iodide, and incubated in the dark for 30 min. Analyses of propidium-iodide-stained cells were carried out with a flow cytometer (Becton Dickinson, Mountain View, CA), and the data were analyzed using Multicycle software (Phoenix Flow Systems, San Diego, CA).

Soft agar assay

Single cells were mixed thoroughly in 0.3%-agar-containing complete medium [Agar Noble (BD Biosciences) dissolved in keratinocyte growth medium (Rheinwald and Green, 1975)] and seeded in 10 cm-diameter plates over a 0.5% agar layer. After 21 to 28 days, colonies were counted for each plate. Each soft agar assay was performed in triplicate.

Tumor growth assay

Balb/c–ByJ-Hfh11^{nu} athymic nude mice were purchased from Jackson Laboratory and housed at Istituto di Ricerche di Biologia Molecolare (IRBM), Pomezia (RM), to carry out experimental procedures. For tumor growth assay, 6- to 8-week-old mice were γ-irradiated (400 rads) and subcutaneously injected with a suspension of 1×10^6 cells in 100 µl of PBS and 100 µl of Matrigel (BD Biosciences) 4 h later (Elenbaas et al., 2001). Tumor size was measured at different times. Mice were killed when tumor grew to a volume of about 3000 mm^3 or after 12 weeks of monitoring. All experimental procedures complied with the Guidelines of the Italian National Institute of Health and EU Directive EC86/609 on the protection of animals used for experimental and other scientific purposes, which was ratified by Italian Legislation with DL no. 116/92.

Statistical analysis

Statistical analysis was performed using the Student's *t*-test. $P<0.05$ was considered statistically significant. Values are expressed as mean±s.d.

Competing interests

The authors declare no competing or financial interests.

Author contributions

R.M. performed experiments, analyzed data and prepared some figures. L.T., F.G., S.B., A.L.S., C.S. and C.A. performed experiments. G.M. performed *in vivo* experiments and analyzed data. L.G. analyzed data, participated in writing and reviewing the manuscript. M.C.C. critically revised the manuscript. E.D. conceived and designed the study, performed experiments, analyzed data, prepared the figures and wrote the manuscript.

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Supplementary information

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