Piebald Trait: Implication of *kit* Mutation on *In Vitro* Melanocyte Survival and on the Clinical Application of Cultured Epidermal Autografts

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Piebald trait leukoderma results from "loss-of-function" mutations in the *kit* gene. Correlations between mutation type and clinical phenotype have been reported. However, mutation classification has been mainly based on the clinical features of patients. The aim of this study was to get a better understanding of the pathogenesis of human piebaldism by establishing whether the *kit* mutation type may affect the *in vitro* survival/proliferation of patient melanocytes. Overall, the research was finalized to implement the clinical application of the autologous cultured epidermis in the treatment of piebald patients. Seven patients, who were transplanted with autologous *in vitro* reconstituted epidermis, showed an average percentage of repigmentation of 90.7. Six novel and one previously reported mutations were found and their postulated effects discussed in relation to the clinical phenotype and *in vitro* behavior of epidermal cells. Although mutation type did not impair repigmentation given by autotransplantation, it was shown to influence the survival/proliferation of co-cultured melanocytes and keratinocytes. In particular, tyrosine kinase domain mutations were found with melanocyte loss and keratinocyte senescence during expansion of epidermal cultures. Results indicate that the clinical application of cultured epidermis in piebald patients may be optimized by investigating mutation functional effects before planning surgical operations.

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INTRODUCTION

Piebald trait (MIM no. 172800) is an autosomal dominantly inherited leukoderma typically affecting the forehead, anterior trunk, and central extremities.

Human piebaldism results from constitutional heterozygous ''loss-of-function'' mutations in the *kit* gene, which encodes the cell surface transmembrane tyrosine kinase (TK) receptor for KIT ligand (stem cell growth factor (SCF), mast cell growth factor, or steel factor) (Spritz, 1997, 2006; Murakami *et al.*, 2004, 2005). In the skin, SCF is produced by fibroblasts and keratinocytes (Brenner *et al.*, 2005; Okazaki *et al.*, 2005) and stimulates the KIT receptor expressed on melanoblasts/melanocytes and mast cells (Longley *et al.*, 1993; Grichnik *et al.*, 1998; Ito *et al.*, 1999; Botchkareva *et al.*, 2001; Kunisada *et al.*, 1998, 2001).

Deficient KIT-dependent signaling results in a primary defect of migration of neural crest melanoblasts to the skin during embryogenesis and interferes with local migration of skin melanoblasts and melanocytes during postnatal life (Alexeev and Yoon, 2006; Grichnik, 2006; Spritz, 2006). KIT-dependent signaling also seems to be required for melanoblast/melanocyte survival and proliferation (Botchkareva *et al.*, 2001; Kunisada *et al.*, 2001; Alexeev and Yoon, 2006; Grichnik, 2006; Spritz, 2006).

Heterozygous mutations in the *kit* gene have been demonstrated in about 75% of patients with piebaldism (Ezoe *et al.*, 1995; Murakami *et al.*, 2005), with a fairly good correlation between mutation site and type and severity of the resulting clinical phenotype (Spritz, 1997).

Medical therapies of piebaldism cannot be successful due to the lack of epidermal/hair follicle melanocytes within the achromic areas, where melanocytes have to be introduced by surgical techniques (Falabella *et al.*, 1995; Njoo *et al.*, 1998; Olsson and Juhlin, 2002). Emerging technologies based on autologous cell therapy are important alternatives to conventional surgical methods in the treatment of this mendelian disease (Falabella *et al.*, 1995; Kumagai and Uchikoshi, 1997; Olsson and Juhlin, 2002; Guerra *et al.*, 2004).

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Abbreviations: CFE, colony forming efficiency; M/K ratio, melanocyte to keratinocyte ratio; SCF, stem cell factor; TK, tyrosine kinase

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We have recently reported that the autologous cultured epidermis bearing melanocytes induces high levels of repigmentation in piebaldism, comparable to the highest success rate achieved by split-thickness skin grafts or epidermal blister grafts (Guerra *et al.*, 2004). Moreover, we have shown that the melanocyte to keratinocyte (M/K) ratio can be monitored in the *in vitro* reconstituted epidermis (Guerra *et al.*, 2000, 2003, 2004), and that an almost complete/fully complete repigmentation may be assured by a M/K ratio *in vitro* ranging from 1/40 to 1/400 (Guerra *et al.*, 2000, 2003, 2004).

Several considerations prompted us to choose the *in vitro* reconstituted epidermis bearing melanocytes for the treatment of some pigmentary disorders, such as vitiligo and piebaldism. Indeed, in the cultured epidermal sheet, keratinocytes regulate melanocyte growth and differentiation (De Luca *et al.*, 1994; Vancoillie *et al.*, 1999), and the proper M/K ratio (De Luca *et al.*, 1988, 1994; Guerra *et al.*, 2000, 2003, 2004). Melanocytes home into the basal layer of the cultured epidermis, develop dendrite arborization with melanosome-containing processes and transfer melanosomes into basal keratinocytes (Seiberg, 2001), being hence able to maintain their physiological characteristics when co-cultured with keratinocytes.

It has been also demonstrated that, if keratinocytes are subcultured 1–2 days after they reach confluence and seeded at high cell density, the M/K ratio is several fold higher than under conditions that privilege colony forming ability and stem cell maintenance (i.e. passaged during the exponential phase of growth) (De Luca *et al.*, 1988; Guerra *et al.*, 2000). In particular, the M/K ratio observed in secondary cultures, that is, in cultures eventually destined to transplantation, is particularly favorable (Guerra *et al.*, 2000, 2003, 2004) and can be preserved during repeated subcultivation (De Luca *et al.*, 1988; Guerra *et al.*, 2000).

Based on this experience, we planned to carry out further operations in piebald trait patients, by subcultivating previously frozen cells, in order to greatly amplify the epidermal cell population isolated from the original skin biopsy. Here, we report that we detected a progressive loss of melanocytes in three out of five tertiary cultures destined to the second transplantation, a finding that made it necessary to perform an additional skin biopsy in the three above-mentioned patients. In order to be able to evaluate different results achieved from different cases, we (i) investigated factors influencing the melanocyte survival in cultures destined to transplantations and (ii) performed long-term follow-up visits in seven piebald trait patients, who underwent one to two surgical operations for the treatment of their disease. In parallel, we tested all the patients for *kit* gene mutations and investigated the *in vitro* behavior of their epidermal cells (melanocytes and keratinocytes) by culturing them till senescence. Results obtained point to a correlation between the ''grade'' of *kit* mutation, as inferred by both intragenic location/type, and effect on the overall presentation of the patient and on the feasibility of the clinical application of the *in vitro* reconstituted epidermis in the surgical treatment of the pathology.

RESULTS

Transplant outcome in the investigated patients

Patients' clinical and biological data are shown in Table 1.

P129, P154, and P185 patients, who were treated twice, underwent two skin biopsies, because we did not manage to maintain melanocytes in tertiary cultures in order to treat a larger area.

On the contrary, we successfully treated P169 (twice) and P160 (three times) patients in subsequent transplantation procedures, starting from frozen cells that had been isolated from the original skin biopsy. P161 patient did not need further operations. All the remaining patients are awaiting further operations.

As shown in Table 1, the average percentage of repigmentation in our patients was 90.7 (4,867.7 repigmented cm^2/5,364.4 transplanted cm^2). Figure 1 shows transplanted areas in P169 (Figure 1a–d) and P185 (Figure 1e and f) patients, 12 months after autotransplantation.

In abdominal areas of P160 and P154 patients, the take of autografts was not complete, owing to the difficulty for patients to maintain an adequate immobilization in bed.

Variation of the M/K ratio in cultured grafts within the 1/36–1/417 range (Table 1) was not correlated to the percentage of final repigmentation, nor with the intensity of repigmentation. Repigmentation was stable within 18–60 months, as indicated by the follow-up column in Table 1.

Different *in vitro* lifespan of melanocytes/keratinocytes from the investigated patients

In order to investigate the melanocyte survival in the *in vitro* reconstituted epidermis from piebaldism patients, we performed serial expansion of epidermal cells until they reached senescence.

As shown in Figure 2a, the melanocyte survival/proliferation was not affected in the serially expanded *in vitro* reconstituted epidermis from P161 and P160 piebald patients, as indicated by M/K ratios equal to 1/168 and 1/95, respectively, at the end of cell lifespan. The M/K ratio in the cultured epidermis from P169 and P181 patients was also preserved during *in vitro* passages, even if at lower levels (1/421 and 1/311, respectively). On the contrary, a rapidly decreasing M/K ratio starting from the third passage (1/2.000–1/48.000) was seen in cultures from P129, P154, and P185 patients (Figure 2a). An average M/K ratio of 1/59 was shown at the end of the lifespan in control cultures (Figure 2b).

Unexpectedly, an impaired behavior of keratinocytes from piebald trait patients was detected during their *in vitro* lifespan. Piebaldism keratinocytes in confluent culture conditions underwent an average value of 38.84 ± 6.14 cell doublings (Figure 2c), without particular differences among mild, moderate, and severe patients, whereas control keratinocytes performed an average value of 54.4 ± 7.91 cell doublings (Figure 2d). It is not surprising that keratinocytes cultivated under conditions that privilege melanocyte growth (keratinocytes sub-cultivated at least 1 day after confluence) possess a lower cell doubling number than keratinocytes

Table 1. Clinical and biological data of 7 piebald trait patients, treated by means of the autologous *in vitro* reconstituted epidermis

Patient	Phototype/ age (years)/ sex, phenotype	Affected body sites	Affected family members	Number of skin biopsies/ number of operations	Melanocyte/ keratino-cyte ratio in auto-grafts	Treated body areas	cm^2 of treated areas (total treated areas)	cm^2 of repigmented areas (total repigmented areas)	Percentage of repigmen-tation (average percentage)	Total cm^2 of recipient area/total cm^2 of donor area (amplifica-tion folds)	Last follow-up visit (months)
1 P161	IV/16/F, mild	WF, left leg	WF: mother; sister, brother	1/1	1:68	Left leg (front) no need of further grafts	107.78	107.78	100%	107.7/1.5 (71.8)	48
2 P169	II/20/M, moderate	WF, forehead, knees, and legs	P: maternal grandfather WF: mother, maternal great-grandmother	1/2	1:55 1:136	Left knee and leg (front) Legs (back)	466.37 394.93 (861.3)	433.894 387.03 (820.924)	93% 98% (95.3%)	861.3/2 (430.6)	48 36
3 P181	II/32/M, moderate	WF, forehead, knees, and legs	P: paternal grandmother, father, paternal aunt	1/1	1:36	Knees and legs (front)	413	392.35	95%	413/3 (137.6)	18
4 P160	II/12/M, moderate	WF, forehead, abdomen, midthighs to midcalves	NI	1/3	1:43 1:48 1:126	Legs (front) Legs (back) Abdomen	320 380 435.53 (1135.53)	320 368.4 326.64 (1011.44)	100% 96% 75% (89.07%)	1135.5/1.5 (757)	60 36 24
5 P129	III/21/F, severe	WF, forehead, chest, abdomen, mid-upper arms to wrists, midthighs to midcalves	P: father	2/2	1:417 1:169	Right leg (front) Forehead Chest	193.35 40 670 (903.35)	183.68 39.2 636.5 (859.38)	95% 98% 95% (95.13%)	193.3/1.2 (161) 710/3 (236.6)	60 48
6 P154	III/27/M, severe	WF, forehead, chest, abdomen, mid-upper arms to wrists, midthighs to midcalves	P: father	2/2	1:342 1:195	Chest Abdomen Left forearm	563.24 213.85 187.15 (964.24)	506.916 53.46 159.07 (719.446)	90% 25% 85% (74.61%)	563.2/2.2 (256) 401/3.3 (121.5)	60 48
7 P185	II/29/M, severe	WF, forehead, chest, abdomen, mid-upper arms to wrists, midthighs to midcalves	P: paternal grandmother, father, sister, first cousin	2/2	1:169 1:228	Chest Abdomen	636.34 342.94 (979.28)	623.613 329.222 (952.835)	98% 96% (97.29%)	636.3/3.4 (187) 342.9/3 (114.3)	30 18
						Total values	5364.4	4867.7	90.7%		

F, female; M, male; NI, not identified; P, piebaldism; WF, white forelock.

cultivated under conditions that privilege stem cell maintenance (keratinocytes passaged during the exponential phase of growth) (Pellegrini *et al.*, 1999), because the latter culture system is aimed at permanent epidermal regeneration in massive full-thickness burns (Pellegrini *et al.*, 1999; Ronfard *et al.*, 2000). Indeed, piebald keratinocytes in subconfluent culture conditions underwent an average value of 69.97 ± 16.35 cell doublings, which however remains

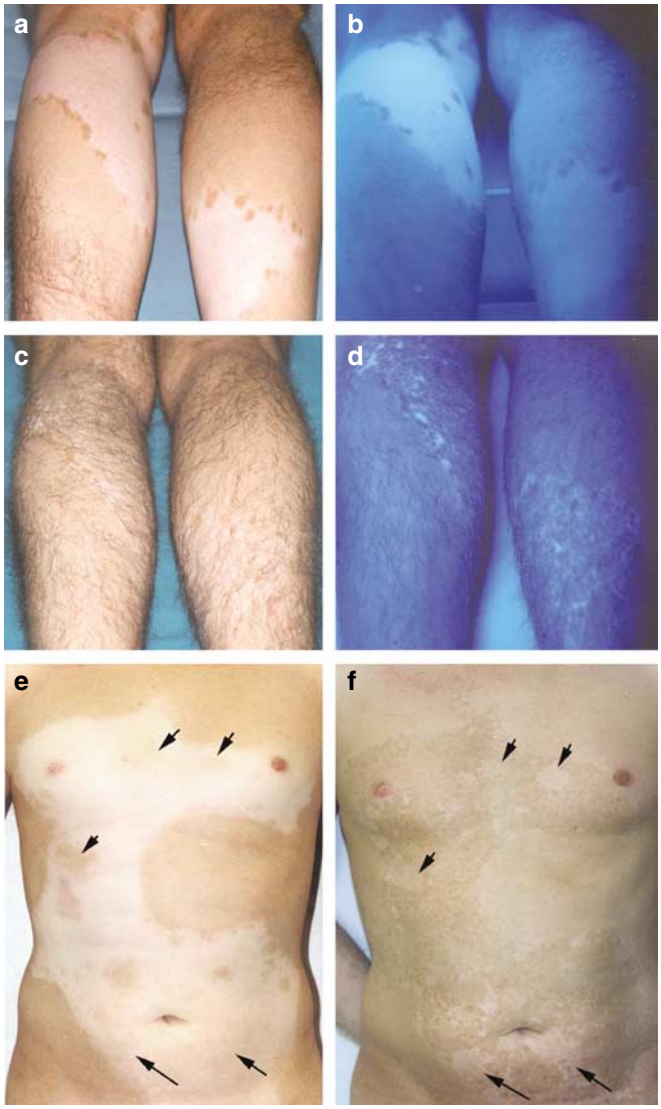

Figure 1. Results of cultured epidermal cell autotransplantation in two patients. (**a**) and (**b**) Depigmented areas of back of legs in P169 patient in normal (**a**) and Wood (**b**) light. (**c** and **d**) A 98% repigmentation was evaluated at the 12-month follow-up visit. The esthetic appearance of the almost completely repigmented area was improved by dyeing the leg hair, which the patient began to do 1 year after the transplantation. (**e**) Large leukodermic area of the chest and abdomen in P185 patient. (**f**) A 97% repigmentation was shown at the 12-month follow-up visit. Hyperpigmentation explains the non-homogeneous appearance of the treated areas: areas of pre-existing normally pigmented skin (arrows) are present within the darker transplanted regions. Our experience, however, suggests that this effect is transitory and does not require medical therapies.

significantly lower than that of 120±20 cell doublings observed in control keratinocytes in comparable culture conditions (data not shown) (Pellegrini *et al.*, 1999; Maurelli *et al.*, 2006).

Furthermore, all piebaldism keratinocyte cultures showed a rapid decrease of their colony forming efficiency (CFE) (Figure 2e) and a similarly rapid increase of the percentage of aborted colonies (Figure 2g). In contrast, both the naturally occurring decrease of the CFE and increase of the percentage of aborted colonies were more gradual in control keratinocyte cultures (Figure 2f and 2h).

Proliferation, differentiation, and senescence markers in cultured epidermal cells

The impaired behavior of cultured keratinocytes from piebald trait patients, prompted us to analyze proliferation, differentiation, and senescence marker expression in the *in vitro* reconstituted epidermis by Western blot.

As shown in Figure 3a, SCF expression was slightly upregulated in cultures from piebald trait patients. The expression level of p63, a marker of proliferative potential in epithelial cells, was comparable between control keratinocytes and piebald trait keratinocytes, except for P160 cultures, whose p63 expression was lower.

As previously described (Dellambra *et al.*, 2000), the senescence marker p16 was undetectable in control and piebald keratinocytes, except for P160 cultures, where p16 expression was higher. Lower levels of the tumor suppressor gene p53 and its effector p21 were detected in severe piebald trait patients. Differentiation markers such as involucrin and 14-3-3σ (Figure 3b) were similarly expressed in piebaldism patients and control cultures.

Mutational analysis

Direct sequencing of the proto-oncogene *c-kit* in seven piebald patients allowed to identify the pathogenetic mutations in all of them: they include six novel mutations and one (M541L in P160 patient) previously reported (Murakami *et al.*, 2004). Figure 4 shows an overview of the mutations that are mapped on the *kit*-cDNA and the predicted outcomes at the KIT receptor. The consequences of the mutations on KIT functioning are also indicated and inserted in the literature context. Last, the phenotype of the carrier patients, which is considered to be the ultimate effect of the mutation, is reported.

In detail, P161 and P169 patients were found to carry two different frameshift mutations: P161 at the 5′-terminus (30dupT) in exon 1, which leads 45 codons downstream (exon 2) to a translational truncation; P169 in exon 16 encoding the second TK domain ((2334_2336)delG), which leads 34 codons downstream (exon 17) to a translational truncation. Both mutations could lead to haploinsufficiency by nonsense-mediated decay of the *kit* transcripts containing a premature stop codon. Should the nonsense-mediated decay be inefficient, the 30dupT mutation is predicted to give rise to a defective KIT polypeptide truncated at the ligand-binding domain, determining in any case a 50% loss of function, in accordance with the mild phenotype of P161 patient. Conversely, the distal frameshift mutation (2334_2336)delG should result in termination of translation in TK2 domain, which leads to a combination of loss of function and dominant-negative effects (depending on the stability of the truncated transcripts and polypeptides). This results in a more complex phenotype, as in the case of P169 patient. Consistent with this prediction, a similar distal frameshift mutation affecting exon 16 was previously

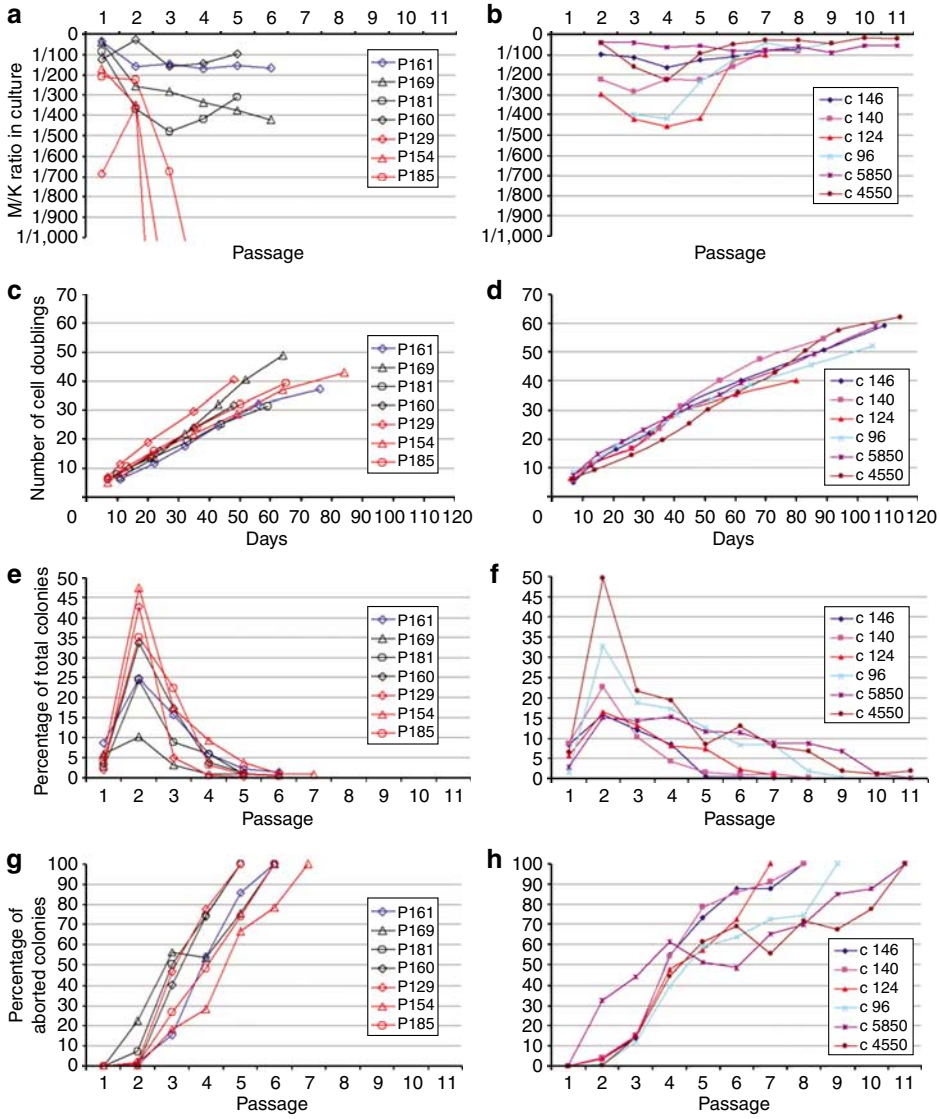

Figure 2. Biological data from serially expanded epidermal cells. (**a**, **c**, **e**, **g**) Cultures from piebald trait patients. (**b**, **d**, **f**, **h**) Cultures from controls. The blue, black, and red lines indicate mild, moderate, and severe piebald trait patients, respectively. (**a**, **b**) M/K ratio in cultured epidermal cells. (**c** and **d**) Cell doubling performed by cultured cells during serial amplification. (**e**, **f**) Percentage of total colonies (calculated on total plated keratinocytes). (**g**, **h**) Percentage of aborted colonies (calculated on total colonies).

described in association with moderate phenotype (Murakami *et al.*, 2004).

P181 patient, who displayed a moderate phenotype, was found to carry a new terminal frameshift mutation in exon 21 (2921_2922delAC), which shifts the stop-codon 71 residues downstream, leading to an elongated protein. We might hypothesize that the modified protein could enter the membrane bilayer and interfere with the KIT receptor function by changing, following frameshift, the specific terminal amino-acid motif HDDDV, and possibly altering its 3D structure. Interestingly, this KIT receptor terminal motif provides a binding site for the 10th PDZ (Postsynaptic density protein, Disk large, Zonula occludens) domain of MUPP-1 protein, which has been thought to enhance SCF-mediated signaling cascades (Mancini *et al.*, 2000).

In P160 patient, who also showed a moderate phenotype, we found a missense mutation in exon 10 (M541L), which has been reported previously (Murakami *et al.*, 2004). As proposed by the authors, this mutation possibly impairs insertion of the KIT receptor in the cell membrane or may affect dimerization of the receptor polypeptide.

Moving to mutations correlated to severe phenotype, we identified the A621S mutation in P154 patient. The same codon was altered by the A621T change in a Bangladeshi patient, who also showed a severe piebaldism (Ezoe *et al.*, 1995). A621S and A621T exert a similar effect at protein level, both replacing a nonpolar highly conserved amino acid (Ala) with an uncharged polar amino acid of hydroxyl group (Ser/Thr).

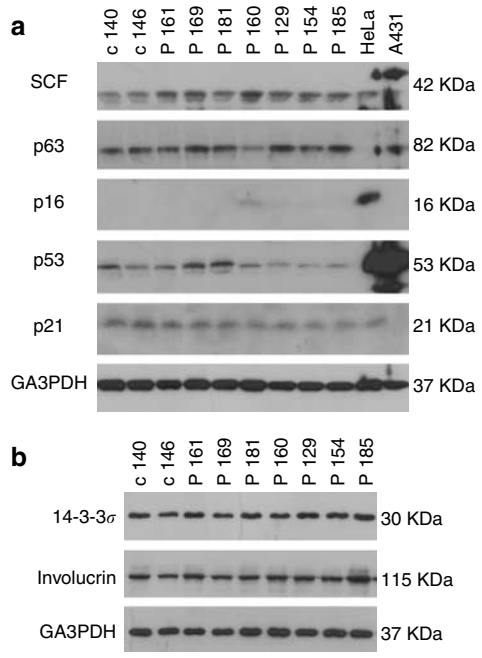

Figure 3. Western blot analysis of cultured epidermal cells. Confluent epidermal cultures from controls (c140, c146) and piebald trait patients (P161, P169, P181, P160, P129, P154, P185) have been analyzed. (**a**) SCF, proliferation (p63) and senescence (p16, p53, p21) markers were analyzed. GA3PDH was our loading control. (**b**) Differentiative (involucrin, 14-3-3 σ) markers were analyzed. GA3PDH was our loading control.

P129 and P185 patients were heterozygous for two novel mutations affecting splice-site junctions, represented by one base insertion at consensus sequence near IVS12 donor site (IVS12 + 3insT), and by a transition at the IVS 12 acceptor site (IVS12-1G > A), respectively.

Splicing effects of IVS12 + 3insT were evaluated by applying three different splicing prediction programs on the sequence surrounding the IVS12 splice donor (from exon 11 to exon 13): Neural Network (http://www.fruitfly.org/seq_tools/splice.html), Splice View (http://125.itba.mi.cnr.it/~webgene/wwwspliceview.html), and NatGene2 (http://www.cbs.dtu.dk/services/NetGene2). All three computer-assisted splice-site predictions identify the legitimate 5′ IVS12 splice site of wild-type sequence with a score exceeding the respective thresholds, whereas they fail to detect any splice donor for the mutant sequence.

We assume that the effects of IVS12 + 3T mutation are assimilable to those predicted by Spritz for the IVS12 + 1G > A mutation, which was identified in a proband with severe piebaldism (Spritz *et al.*, 1992). The author ascribes to this mutation a very complex combination of loss of function and dominant-negative effects leading to a 50–75% reduction of Kit function, responsible for the highly variable phenotypes observed in affected family members. The same interpretation holds for IVS12-1G > A mutation, detected in the severely affected P185 patient, where the lack of IVS12 acceptor splice could cause missplicing through (i) IVS12 retention, leading to a premature truncated KIT polypeptide, in case the transcript is not subjected to nonsense-mediated decay, and/or (ii) exon 13 skipping (111 bp), giving rise to an aberrant polypeptide with an in frame deletion of 37 amino acids in the first TK domain.

The apparent correlation between mutations and overall clinical phenotype also applies to the *in vitro* melanocyte lifespan. If we examine Figure 2a, it results that melanocyte survival/proliferation was not particularly affected in the serially expanded *in vitro* reconstituted epidermis from patients showing a mild-moderate phenotype (P161, P160, P169, P181), who were found to carry mutations with a prevalent loss-of-function effect. In contrast, the severely affected patients (P154, P129, P185), showing mutations that were predicted to have prevalent dominant-negative effects, are associated with a lack of melanocytes at the end of the *in vitro* lifespan of their cultured epidermis.

DISCUSSION

The identification of pathogenetic mutations of the *kit* gene in about 75% of piebald trait patients clearly indicated that KIT-dependent signaling plays key roles in melanoblast/melanocyte migration, maintenance and survival in both prenatal and postnatal life (Spritz, 2006). This is also attested by studies of *in vitro* proliferation of melanocytes carrying loss-of-function mutations (Spritz *et al.*, 1994).

The KIT protein, a member of the TK family of transmembrane receptors, undergoes dimerization within the cell membrane upon SCF binding to its extracellular domain, thus activating its intracellular TK (Spritz, 1997).

Loss-of-function mutations result in haploinsufficiency or dominant-negative effects, at levels depending on the nature of the mutation and location at different receptor domains. The following hierarchical ranking of the mutations has been proposed.

Missense mutations in the intracellular TK1 domain correlate with severe piebald phenotypes, because of full dominant-negative inhibition of the KIT receptor via formation of impaired receptor heterodimers between a normal and a mutant KIT monomer, and a 75% decrease of KIT-dependent signal transduction (Spritz, 1997).

Proximal frameshifts and missense mutations are by contrast associated with a mild piebald phenotype, the result of pure haploinsufficiency owing to a 50% decrease of KIT-dependent signal transduction (Spritz, 1997).

Within these two ends of the spectrum fall the distal frameshifts and splice junction mutations located near the intracellular TK1 domain. These are mainly associated with variable phenotypes, as the distally truncated polypeptides, via incorporation into non-functional receptor heterodimers, would decrease KIT-dependent signal transduction by 50–75%. Indeed, although these mutations abolish expression of fully functional KIT receptor, some truncated KIT receptors can still bind SCF and even form dimers. These inhibit function of the product of the normal allele by a dominant negative effect. However, as both the incompletely translated KIT mRNA and the truncated KIT polypeptide are probably unstable, they are likely to be present at less than a full dose (partial dominant-negative effect) (Spritz, 1997).

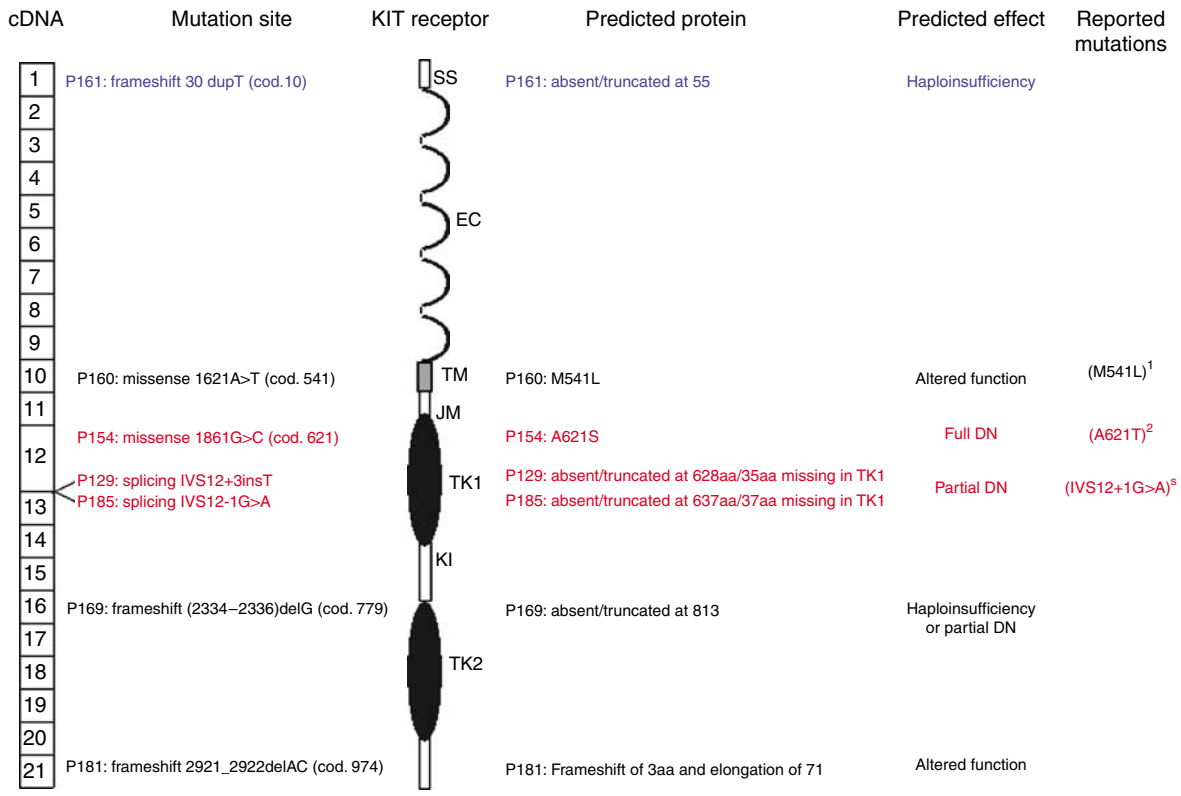

Figure 4. Mapping to *kit* cDNA and KIT receptor of the seven identified mutations. Three frameshift, two missense, and two splicing mutations have been identified. The blue, black, and red colors indicate mild, moderate, and severe phenotype of the carrier patients, respectively. The envisaged effects of the mutations and literature for identical or similar mutations are also provided (1: Murakami *et al.*, 2004; 2: Ezoe *et al.*, 1995; 3: Spritz *et al.*, 1992). SS: Signal sequence (aa 1–22), EC: Extracellular domain (aa 23–520), TM: Transmembrane domain (aa 521–543), JM: juxtamembrane domain (aa 544–581), TK1: TK domain 1 (aa 582–684), KI: Kinase insert domain (aa 685–761), TK2: TK domain 2 (aa 762–937). DN: Dominant-negative effect.

Such mutation classification has been mainly based on the clinical phenotype of the patients, as proliferation studies of defective melanocytes have only been performed sporadically, whereas functional KIT-dependent transduction studies have only been designed for a few activating *c-kit* mutations.

This study provides new hints on the biological rationale of autotransplantation in piebald patients carrying different c-*kit* mutation. First, the success rate with transplantation procedures is shown to be very high, approaching 90%, regardless of the overall phenotype, the severity of mutation and the survival/proliferation of co-cultured melanocytes and keratinocytes. The satisfactory repigmentation rate was confirmed to be stable until 5 years after transplantation. The appropriate and complex cytokine environment that exists *in vivo* is most probably the critical factor for melanocyte proliferation and survival after autografting (Grichnik, 2006).

In addition, the results achieved confirm that the type of *kit* mutation fairly correlates with the clinical phenotype of the patients and also may be interpreted in its functional effect by the pattern of proliferation and survival of patients' melanocytes *in vitro*. Analyses focused on transcripts and the activation status of KIT-dependent signaling translation should be designed to validate the above statement. However, the proliferation studies of KIT defective melanocytes have represented a complementary valuable approach.

Unexpectedly, keratinocyte cultures from piebald trait patients showed a shorter lifespan than those from healthy controls, the former having performed a lower number of total cell doublings. Obviously, a more rapid clonal evolution (i.e. the passage from stem cells to transient amplifying cells, which gradually occurs during natural aging, wound healing and keratinocyte subcultivation) was indicated by the quick decrease of total colonies and increase of aborted ones. Furthermore, p53 and p21 downregulation in cultured keratinocytes from severe piebald trait patients suggests an accelerated cell senescence (Munro *et al.*, 1999; Rheinwald *et al.*, 2002; Maurelli *et al.*, 2006). These data are consistent with an impaired behavior of cultured keratinocytes too, even if these cells are not primarily affected by the KIT dysfunction.

Melanocytes and mast cells are thought to be the only cutaneous cells, which express c-KIT. However, intriguing recent studies in mice suggest the existence of a population of KIT-positive hair matrix keratinocytes (Peters *et al.*, 2003), pinpointing a possible new function of KIT signaling in epithelial cell biology. The authors hypothesize that KIT-positive cells seen in the most proximal anagen hair bulb might represent a particularly rapidly proliferating

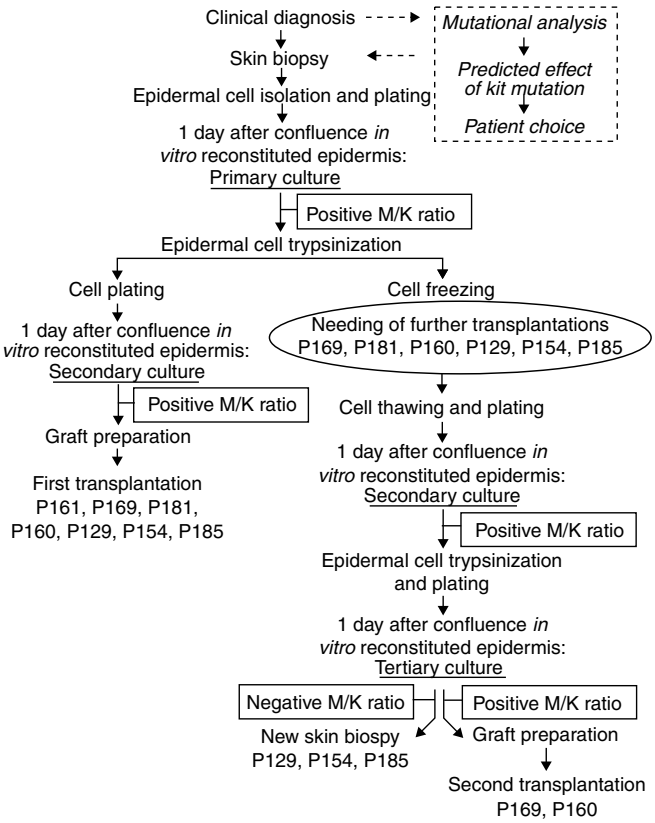

Figure 5. Flow chart showing the cell culture process. The broken line box indicates the additional procedures, which will be adopted to improve the entire technology.

sub-population of follicle transient amplifying keratinocytes, which depend on SCF/KIT signaling for proliferation and undergo terminal differentiation or apoptosis when signaling is downregulated during hair follicle regression (Peters *et al.*, 2003). Thus, SCF, which is also expressed by these KIT positive keratinocytes, becomes an important paracrine and autocrine regulatory factor for selected epithelial cells.

We speculate that keratinocyte contiguity to KIT-expressing melanocytes in the skin compartment might induce keratinocyte suffering in piebald trait patients, via an impaired SCF/KIT interaction. The slight SCF expression upregulation we detected in cultured keratinocytes from these patients might represent the keratinocyte response in restoring the normal SCF/KIT signaling control of melanocyte homeostasis. However, hair follicles may be present in the original donor specimen, as we perform full thickness skin biopsies (down to the fat layer), in order to be sure not to damage the epidermal basal cells. Consequently, we may hypothesize that keratinocyte suffering in piebald trait patients could be also related to hair follicle keratinocytes expressing mutated KIT receptors.

Evidence emerging from this study represents a first step in the transfer of experimental results into the clinical set.

Patients suffering from piebaldism experience increased sensitivity to sun exposure in depigmented areas. Moreover, the cosmetic disfigurement caused by piebaldism, particularly in severe cases, and the absence so far of treatments, has profound psychological effects on patients and gives rise to serious emotional stress.

Although topical or systemic medical treatments for piebaldism are usually unsuccessful, melanocytes taken from unaffected areas may be introduced by autotransplantation. The autologous cultured epidermis allows to produce a large amount of epithelial sheets starting from a small biopsy and also makes it possible to cover a large body surface in a single operation (Kumagai and Uchikoshi, 1997; Guerra *et al.*, 2004). However, the therapeutic outcome of any technology in the treatment of piebald trait patients is related not only to the successful result in repigmenting even a large area but also to the general strategy for restoring pigmentation in the largest possible number of depigmented areas. Patients suffering from piebaldism have very extensive depigmented areas and ask us to treat the entire affected body surface. Although completely repigmented in the transplanted area, some patients tell us that their quality of life is not changed, because they still have white areas.

Mutational analysis of *kit* gene in the proband is shown to be predictive regarding the *in vitro* survival of patient melanocytes. Indeed, in the case of mutations that correlate with mild-moderate phenotype, we have treated recipient areas even 750-fold larger than donor ones, by using cells both in secondary and tertiary cultures. Moreover, the patient's frozen cell bank will allow us to perform further transplantations. Conversely, mutations that correlate with a severe phenotype affect melanocyte survival. In these patients, the greatest recipient sites, which we have treated were only 250-fold larger than donor ones, and further operations have required additional donor sites.

In conclusion, the correlation of severity of *kit* mutations and melanocyte behavior in culture allows to plan a personalized transplantation procedure for each patient. Communication of timing and extent of treatment possibilities is important for patient compliance and avoids future disappointment.

MATERIALS AND METHODS

Patients

Seven patients suffering from piebaldism were enrolled in this study from March 1998 to January 2005 (Table 1). Preliminary data regarding patients 1, 2, 4, 5, and 6 had been already reported (Guerra *et al.*, 2004).

Written informed consent was obtained from all patients. Procedures were in accordance with the ethical standards of the Committees on Human Experimentation of IDI Institution. The study was conducted according to Declaration of Helsinki principles.

Cell cultures

Figure 5 summarizes the whole cell culturing process. Full-thickness skin biopsies (1.2–3.4 cm^2) were taken from unaffected body sites (pubis or buttock), under local anesthesia.

Epidermal cells were cultured on a feeder-layer of lethally irradiated 3T3-J2 as described (Guerra *et al.*, 2000, 2003, 2004). Briefly, biopsies were minced and trypsinized (0.05% trypsin/0.01% EDTA) at 37°C for 3 h. Cells were collected every 30 min, plated (4×10^4/cm^2) on lethally irradiated 3T3-J2 cells (2.4×10^4/cm^2) and cultured in 5% CO_2 and humidified atmosphere in keratinocyte growth medium: DMEM and Ham's F12 media (2:1 mixture) containing fetal calf serum (10%), insulin (5 μg/ml), adenine (0.18 mM), hydrocortisone (0.4 μg/ml), cholera toxin (0.1 nM), triiodothyronine (2 nM), epidermal growth factor (10 ng/ml), and glutamine (4 mM).

Quality and safety of culture media and additives were guaranteed. For bovine serum, the healthy status of the donor herd was well documented, and freedom at source from bovine spongiform encephalopathy (BSE) was ensured.

One day after confluence primary cultures were trypsinized, plated on 75–175 cm^2 flasks at a density of 4×10^4/cm^2, in the presence of lethally irradiated 3T3-J2 cells (2.4×10^4/cm^2), and cultivated as above. Grafts destined to the first transplantation were prepared from secondary cultures 1 day after confluence, as described (Guerra *et al.*, 2004).

Remaining epidermal cells from primary cultures were frozen in fetal calf serum containing 10% DMSO. Briefly, cells were centrifuged into a pellet; the pellet was resuspended in 1 ml/5×10^6 cells/vial of undiluted fetal calf serum. One milliliter/vial of fetal calf serum containing 20% DMSO was then added drop by drop. The vial was put on ice for 10 min to allow the DMSO to penetrate the cells, and then placed in a −80°C freezer overnight. Finally, cells were put into the liquid nitrogen container. At least 1 year after the first surgical operation, cells were defrosted and plated in secondary cultures, at the same cell density as above and on the feeder-layer of 3T3-J2 fibroblasts. In order to obtain a sufficient number of cells, secondary cultures were amplified in tertiary cultures. One day after confluence, tertiary cultures were utilized to prepare grafts for further operations in P169 and P160 patients. The M/K ratio and the keratinocyte CFE were determined at each passage of cell amplification (see below). A M/K ratio till 1/500 was considered favorable for transplantation procedures.

Surgery

A pretreatment with EMLA® cream (2.5% lidocaine–2.5% prilocaine, AstraZeneca S.p.A., Basiglio, MI) under 2-hour occlusion was performed. Achromic epidermis was removed by using the pulsed Er:YAG laser (Laser Smart 2940, DEKA M.E.L.A. s.r.l., Calenzano, Firenze, Italy) with a 2-mm spot-sized hand piece at a setting of 200–500 mJ of energy (Guerra *et al.*, 2003, 2004).

After disepithelialization, the receiving bed was covered with autologous cultured epidermal sheets. Grafts were secured and immobilized only by dressings. After 7 days, bandages were removed. Follow-ups were carried out every 3 months and 18, 24, 36, 48, and 60 months after transplantation.

Achromic and repigmented area sizes were calculated as described previously (Guerra *et al.*, 2003, 2004).

Serial amplifications of epidermal cells (lifespan cultures)

For serial propagation, epidermal cells from unaffected skin of piebald trait patients were serially passaged, 1 day after confluence, at a cell density of 4×10^4 cells/cm^2 and on the feeder-layer of 3T3-J2 fibroblasts, until they reached senescence. Frozen cells of piebald patients and of six unrelated controls who did not show pigmentary disorders (c146, c140, c124, c96, c5850, and c4550) were utilized in the same culture conditions. Among controls, in three patients, skin specimens were obtained by mammoplastic (c146) and abdominoplastic (c5850, c4550) surgery. The remaining three patients were non-massively (less than 20% of the body surface) second-degree burnt patients, in whom epidermal cells were isolated from unaffected skin, in order to treat them using the autologous *in vitro* reconstituted epidermis. The mean age of piebaldism patients and control donors was 22.4 and 28 years, respectively.

Staining with 3,4-dihydroxyphenylalanine was always performed on parallel cultures seeded in 24-multiwell plates, 1 day after cells reached confluence as described (Guerra *et al.*, 2003). The ratio between melanocytes and total keratinocytes (which include basal and sovrabasal cells) was evaluated under the phase-contrast microscope as follows. Under the culture conditions indicated above, cell density is of approximately 2,000 total keratinocytes/mm^2 (Barrandon and Green, 1987). Thus, to evaluate the M/K ratio, the number of keratinocytes was divided by the average number of melanocytes calculated in 10 randomly chosen 2.4-mm^2 areas (approximately the area seen with a magnification ×10) of confluent cultured epidermis (a_m:$a_k = 1_m$:x_k, where a_m is the calculated average number of total melanocytes in 2.4 mm^2; a_k is the theoretical number of total keratinocytes in 2.4 mm^2; m = melanocyte; k = keratinocytes).

CFE of keratinocytes at each culture amplification was determined by plating 1,000 cells on parallel 100-mm dishes, fixing colonies with 3.7% formaldehyde 12 days later, and staining them with 1% rhodamine B. Total colonies were calculated as a percentage of total plated cells; aborted colonies (made by large and flattened, terminally differentiated cells) were calculated as a percentage of total colonies.

The number of cell generations performed by cultured keratinocytes was then calculated using the following formula: $x = 3.322 \log N/No$, where N equals the total number of cells obtained at each passage and No equals the number of clonogenic cells plated (Rochat *et al.*, 1994). Clonogenic cells were calculated from the CFE data (total colonies), serially determined at each time of culture amplifications. In those cultures where melanocytes were maintained, the theoretical number of melanocytes, calculated on the basis of the M/K ratio, was subtracted from the total number of keratinocytes obtained and plated.

Western blot analysis of cultured epidermal cells

For immunoblots, confluent epidermal cells in secondary cultures were lysed for 30 minutes on ice in RIPA buffer, equal amounts of samples were electrophoresed on 12.5% SDS-polyacrylamide gels, and Western blots were performed as described (Dellambra *et al.*, 2000). The following antibodies were utilized: anti-p16^{INK4a} (N20), anti-p53 (DO-1), anti-p63 (4A4), anti-14-3-3 sigma (N14), anti-SCF (G-3), all from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); anti-involucrin from Sigma-Aldrich (St Louis, MO); anti-p21^{Waf1} was a kind gift from K Helin (IEO, Milan, Italy).

Mutational analysis

Genomic DNA was prepared from melanocyte/keratinocyte secondary cultures from the seven piebaldism patients. All 21 exons and flanking intron of the *kit* gene were amplified by PCR as described (Giebel *et al*., 1992). PCR fragments were subjected to direct DNA sequencing, using the Big dye terminator cycle sequencing ready reaction kit and an ABI automated sequencing system (Applied Biosystems, Foster City).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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