Mouse keratinocyte stem cells originate from the bulge of hair follicle, and, according to definition, possess a clonogenic activity in vitro. We have investigated seven inbred (C57BL/6, C3H, DBA/2, BALB/c, FVB) and outbred (SENCAR, CD-1) mouse strains and found that three genetically distinct subsets of mouse strains differ significantly in the frequency of clonogenic activity in vitro. The analysis of keratinocyte colonies in two reciprocal backcross [(C57BL/6 × (BALB/c × C57BL/6)); BALB/c × (BALB/c × C57BL/6)] and intercross [(BALB/c × C57BL/6)] of BALB/c and C57BL/6 mice allowed us to identify two subpopulations of clonogenic keratinocytes able to produce small (less than 2 mm²) and large (more than 2 mm²) colonies. We conducted linkage analysis and found that small colonies associated with mouse chromosomes 1, 6, 7, 8, and 9; but large colonies—with the chromosome 4. We defined locus on the chromosome 9 that associated with small colonies as keratinocyte stem cell locus 1 (Ksc1), and locus on the mouse chromosome 4 associated with large colonies-keratinocyte stem cell locus 2 (Ksc2). Ksc1 and loci on chromosomes 6 and 7 are close if not equal to loci associated with sensitivity to skin carcinogenesis. We conclude that two subpopulations of stem cells able to produce small and large colonies regulated by different genes and genes regulating small colonies might be responsible for sensitivity to skin carcinogenesis.

Key words: clonogenic keratinocytes/genetic mapping/skin carcinogenesis/stem cells


The epidermis is a continuously renewing tissue in which keratinocytes are organized into multiple layers and undergo terminal differentiation as they move through the suprabasal layers toward the skin surface. The basal layer in adult mice consists primarily of keratinocytes, Langerhans cells, melanocytes, and Merkel cells (Leblond, 1964; Potten, 1983). Hair follicles are highly specialized cutaneous appendages contiguous with both the interfollicular epidermis and the sebaceous gland. A small population of self-renewing stem cells produces proliferative cells that undergo a series of amplification division prior to terminal differentiation. Stem cells are responsible for epidermal homeostasis and are required throughout life to maintain the self-renewing epithelium.

The traditional approach to the recognition of mouse keratinocyte stem cells has been the identification of slow-cycling label-retaining cells. Such cells in the bulge region of hair follicles have been demonstrated in adult mice after neonatal [³H]-thymidine injection (Cotsarelis et al, 1990; Morris and Potten, 1999). Hence, the hair follicle bulge region might function as a “niche” for most mouse keratinocyte stem cells.

Keratinocyte colony-forming cells are closely related or identical to multipotential stem cells. Most (95%) clonogenic keratinocytes originate from the bulge region of hair follicles (Kobayashi et al, 1993; Rochat et al, 1994; Oshima et al, 2001). Moreover, slow-cycling, label-retaining cells possess clonogenic activity in vitro (Morris and Potten, 1994). Thus, most visible mouse keratinocyte colonies are thought to originate from keratinocyte stem cells (Oshima et al, 2001), and clonogenic activity in vitro is one characteristic of mouse keratinocyte stem cells (Oshima et al, 2001).

Watt and colleagues distinguished human keratinocyte stem cells from transit-amplifying cells based on the type of colony they form (Zhu et al, 1999). Stem cells form self-renewing colonies (Zhu et al, 1999; Morris, unpublished), whereas transit-amplifying colonies containing fewer then 30–40 cells, all of which undergo terminal differentiation (Zhu et al, 1999), and can be identified by microscopy at low magnification. Therefore, most visible colonies probably originate from keratinocyte stem cells.

We have identified several factors that might influence the number of keratinocyte colonies. Although colony number remains virtually constant throughout most of adult life in mice (Morris et al, 1988), the frequency of colonies increases during tumor promotion of initiated skin (Morris et al, 1988). Thus, amplification of keratinocyte stem cells may play a role in skin carcinogenesis, and genes regulating keratinocyte colony number might be involved in regulating of skin carcinogenesis. The identification of regulatory...
genes involved in stem cell amplification would enhance the understanding of normal skin homeostasis and skin carcinogenesis. To address this issue, we carried out genetic analyses of keratinocyte colony formation in different mouse strains, and their genetic crosses.

**Results**

**Mouse keratinocyte colony number is a quantitative multigenic trait**

Recently we demonstrated that five inbred (C57BL/6, C3H, DBA/2, BALB/c, FVB) and two outbred (CD-1, SENCAR) mouse strains differ significantly in the number of keratinocyte colony forming cells per 1000 viable cells isolated from the back of mouse skin (Popova et al, 2002) (Fig 1). Keratinocyte colony frequency did not depend on numbers of hair follicles, basal or suprabasal cells, mitotic cells per cm² of skin, or percentage of viable cells per cm² of skin. Furthermore, there was no significant difference between high and low colony-expressing mice in the number of attached cells (Popova et al, 2002). This finding suggested that the different colony numbers reflected differences in the genetic background of these mouse strains.

We identified three subsets of mice that differed significantly in the frequency of clonogenic stem cells: C57BL/6 > SENCAR = C3H = DBA/2 = BALB/c > FVB = CD-1. C57BL/6 mice have high number of colonies per 1000 viable cells. SENCAR, C3H, DBA/2, BALB/c have intermediate colony numbers, whereas FVB, and CD-1 mice have low colony frequency (Fig 1). We found that the mouse strains from each subset had different genetic origins: based on the genealogies of mouse inbred strains (Beck et al, 2000). C57BL/6 mice are in category E; BALB/c, C3H, DBA/2 mice are in category B; and CD-1 and FVB mice are in category A (Fig 2). The finding of significant differences in keratinocyte colony numbers in three subsets of inbred, genetically distinct mouse strains further suggests that colony number is genetically regulated.

The biological significance of our observation that genetically distinct mouse strains have difference in the frequency of keratinocyte stem cells continue to be a mystery. But it also had been demonstrated for the blood (Haan et al, 1997) and liver stem cells (Kolesnichenko and Popova, 1979), and appears to be associated with the difference in life span of the investigated mouse strains. It is interesting that high keratinocyte colony number is associated with long-lived mouse strain (C57BL/6) (Storer, 1966; Goodrick, 1975), and all other investigated mouse strains have shorter life span (Festing and Blackmore, 1971). Therefore, we suggest that high frequency of clonogenic stem cells associated with long living mouse strains. Finally, our finding of the genetic regulation of keratinocyte colony frequency, and significant differences between different strains enabled us to map the gene(s) that regulate the high and low frequency of keratinocyte stem cells.

**Frequencies of stem cells able to produce small and large colonies are quantitative multigenic traits**

It is well documented that inheritance of quantitative characters is generally under the control of more than one gene (Falcone, 1963). Recently, we have demonstrated that clonogenic epidermal stem cells are a heterogeneous population (Popova et al, 2003). We have identified at least two sub-populations of clonogenic keratinocytes able to produce small (less than 2 mm²) and large (more than 2 mm²) colonies, and determined that they are regulated by the different genes (Popova et al, 2003). The frequencies of small and large colonies differed significantly between C57BL/6 and BALB/c mice (Fig 3). Moreover, they have a quantitative inheritance in these two strains of mice. Therefore, we concluded that the numbers of small and large colonies are new quantitative multigenic traits.

To determine the heritability of these two quantitative traits (frequency of small and large colonies), we investigated several genetic crosses between BALB/c and C57BL/6 mice (Table I, Fig 4). We determined that the ratio of small and large colonies per 1000 viable cells was 1:1 in C57BL/6 mice, whereas in BALB/c mice, this parameter was 9:1. The small and large colonies were inherited in a 3:1 ratio in the first hybrid (BALB/c × C57BL/6)F1, and this result was equivalent to the backcross (BALB/c × F1) mice (Table I, Fig 4). It is interesting that the ratio of the small and large colony frequency in the (C57BL/6 × F1) backcross was equivalent.
to parental strain C57BL/6 (1:1), and this trait in F2 generation was almost equivalent to the parental strain BALB/c (8.3:1.6). These results suggest that the frequency of small and large colonies have independent modes of inheritance, and genes regulating number of small colonies are differed from the genes regulating a number of large colonies.

Identification of keratinocyte stem cell regulatory loci
To map genes regulating numbers of both small and large colonies, we separated all large and small colonies from each individual dish, and monitored loci associated with numbers of small and large colonies in the two backcrosses and intercross mice using essentially as described by Angel et al, in 2000. Clonogenic activity of keratinocytes from 104 intercross mice was initially investigated (Popova et al, 2002) and the same protocol was used to determine the number of small and large colonies in intercross and backcross mice. Uses of 149 microsatellite polymorphic markers allowed us to generate a complete genome-wide scan at about 15 cM intervals. In regions suggestive of linkage small and large colony number, the density of markers was increased to obtain more accurate genetic dissection in the area. Overall, genomic coverage was about 95.3 percent across the 19 autosomal chromosomes. The keratinocyte colony number in mice homozygous and heterozygous for each locus was compared using the Qlink program based on the Wilcoxon rank test.

Recently, we have identified that the number of small colonies was linked to loci on mouse chromosomes 1, 6, 7, 8, 9, whereas the number of large colonies were linked to mouse chromosome 4 (Popova et al, 2003). Moreover, we have established that Ksc1 regulated the frequency of small colonies, whereas Ksc2—the frequency of the large colonies. Hence, different genes are involved in the regulation of the frequency of small and large colonies.

In order to identify gene(s) regulating frequency of small colonies in the Ksc1 locus we performed genetic mapping of the region on mouse chromosome 9 with higher resolution (Table II) and combined our data of backcross and intercross mice (Table III). Multilocus analysis of the chromosome 9 (Tables III) suggested that the most likely interval surrounding 41 and 49 cM in the mid-portion and distal region of the chromosome 9 contained the gene(s) involved in regulating the number of keratinocyte stem cells. Association of an increased number of small keratinocyte colonies with inheritance of the C57BL/6 allele was detected on the Wilcoxon rank test.

<table>
<thead>
<tr>
<th>Groups of mice</th>
<th>Number of mice</th>
<th>Average number of small colonies per %</th>
<th>Average number of large colonies per %</th>
<th>Ratio of the number of small and large colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>7</td>
<td>42 ± 13 (54%)</td>
<td>36 ± 12 (46%)</td>
<td>1:1</td>
</tr>
<tr>
<td>BALB/c</td>
<td>12</td>
<td>53 ± 19 (91%)</td>
<td>6 ± 3 (9%)</td>
<td>9:1</td>
</tr>
<tr>
<td>CBF1</td>
<td>10</td>
<td>41 ± 15 (71%)</td>
<td>17 ± 8 (29%)</td>
<td>3:1</td>
</tr>
<tr>
<td>C57BL/6 × F1</td>
<td>45</td>
<td>38 ± 24 (49%)</td>
<td>39 ± 23 (51%)</td>
<td>1:1</td>
</tr>
<tr>
<td>BALB/c × F1</td>
<td>44</td>
<td>31 ± 12 (73%)</td>
<td>12 ± 8 (27%)</td>
<td>3:1</td>
</tr>
<tr>
<td>F2</td>
<td>104</td>
<td>55 ± 21 (84%)</td>
<td>11 ± 7 (16%)</td>
<td>8.3:1.6</td>
</tr>
</tbody>
</table>

Table I. Independent inheritance of small and large colonies in several genetic crosses

*aAverage number of small colonies per 1000 viable cells ± SD.

*bAverage number of large colonies per 1000 viable cells ± SD.
for (BALB/c colonies were represented in ratio 1:1 for C57Bl/6; 9:1 for BALB/c; 3:1 for BALB/c/C2 segregating genetic crosses (BALB/c cultivation from parental strains C57BL/6 and BALB/c and their Small and large keratinocyte colony formation after 2 wk of Figure 4

expected to occur 0.007–0.006 times in a genome scan. We propose that genes regulating frequency of small colonies are located between 41 and 49 cM from centromere (Table III).

Suggestive evidence (Lander and Kruglyak, 1995) for linkage with the number of small colonies was found with markers on mouse chromosomes 1, 6, 7, 8 (Fig 5). On chromosome 1, the D1Mit178 locus had a LOD score of 1.65. Analysis of twelve markers on this chromosome suggests that a gene influencing the number of small colonies may reside in a 35-cM interval from D1Mit178 to D1Mit132. A third wide region showing an association with number of small colonies with C57BL/6 alleles was found on chromosome 6 between D6Mit93 and D6Mit323. A peak suggestive LOD score of 2.25 was obtained at D6Mit99. A fourth region with suggestive linkage to a high number of small colonies was identified between D7Mit66 and D7Mit259. A peak LOD score of 2.16 was obtained at D7Mit105 (63.5cM), and a peak LOD score of 2.7 (combined data of backcross and

Figure 4 Small and large keratinocyte colony formation after 2 wk of cultivation from parental strains C57BL/6 and BALB/c and their segregating genetic crosses (BALB/c × C57BL/6)F1, C57BL/6 × BALB/c, (F1 × F1) F2. The frequency of small and large colonies were represented in ratio 1:1 for C57Bl/6; 9:1 for BALB/c; 3:1 for BALB/c × C2 segregating genetic crosses (BALB/c cultivation from parental strains C57BL/6 and BALB/c and their Small and large keratinocyte colony formation after 2 wk of Figure 4

Table II. Loci associated with small keratinocyte colony number in intercross between BALB/c and C57BL/6 after 2 weeks of cultivation

<table>
<thead>
<tr>
<th>Marker</th>
<th>Map location (cM)</th>
<th>Colony number</th>
<th>p value</th>
<th>LOD score</th>
<th>Genome wide p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>aa</td>
<td>a/b</td>
<td>bb</td>
<td></td>
</tr>
<tr>
<td>D9Mit247</td>
<td>17</td>
<td>54</td>
<td>54</td>
<td>58</td>
<td>0.2</td>
</tr>
<tr>
<td>D9Mit191</td>
<td>26</td>
<td>53</td>
<td>53</td>
<td>60</td>
<td>0.09</td>
</tr>
<tr>
<td>D9Mit162</td>
<td>30</td>
<td>54</td>
<td>53</td>
<td>60</td>
<td>0.04</td>
</tr>
<tr>
<td>D9Mit303</td>
<td>35</td>
<td>55</td>
<td>50</td>
<td>63</td>
<td>0.004</td>
</tr>
<tr>
<td>D9Mit336</td>
<td>35</td>
<td>55</td>
<td>50</td>
<td>65</td>
<td>0.001</td>
</tr>
<tr>
<td>D9Mit104</td>
<td>35</td>
<td>55</td>
<td>50</td>
<td>65</td>
<td>0.001</td>
</tr>
<tr>
<td>D9Mit289</td>
<td>38</td>
<td>54</td>
<td>50</td>
<td>65</td>
<td>0.001</td>
</tr>
<tr>
<td>D9Mit166</td>
<td>41</td>
<td>51</td>
<td>51</td>
<td>66</td>
<td>0.0002</td>
</tr>
<tr>
<td>D9Mit262</td>
<td>41</td>
<td>52</td>
<td>48</td>
<td>69</td>
<td>0.0002</td>
</tr>
<tr>
<td>D9Mit8</td>
<td>42</td>
<td>51</td>
<td>51</td>
<td>66</td>
<td>0.0002</td>
</tr>
<tr>
<td>D9Mit156</td>
<td>42</td>
<td>51</td>
<td>51</td>
<td>66</td>
<td>0.0002</td>
</tr>
<tr>
<td>D9Mit236</td>
<td>43</td>
<td>53</td>
<td>47</td>
<td>69</td>
<td>0.0002</td>
</tr>
<tr>
<td>D9Mit133</td>
<td>43</td>
<td>55</td>
<td>49</td>
<td>66</td>
<td>0.0003</td>
</tr>
<tr>
<td>D9Mit269</td>
<td>43</td>
<td>55</td>
<td>49</td>
<td>65</td>
<td>0.0006</td>
</tr>
<tr>
<td>D9Mit196</td>
<td>48</td>
<td>56</td>
<td>49</td>
<td>65</td>
<td>0.0006</td>
</tr>
<tr>
<td>D9Mit273</td>
<td>49</td>
<td>56</td>
<td>49</td>
<td>65</td>
<td>0.0006</td>
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<tr>
<td>D9Mit182</td>
<td>55</td>
<td>55</td>
<td>50</td>
<td>63</td>
<td>0.0256</td>
</tr>
<tr>
<td>D9Mit51</td>
<td>61</td>
<td>54</td>
<td>52</td>
<td>62</td>
<td>0.2</td>
</tr>
</tbody>
</table>

*Microsatellites markers.
*Map location in cM from the centromere.
*Mean number of colonies ± SD.
*Single-point significance levels. Number of colonies was compared between genotypes using the Wilcoxon rank sum test (two sides). p Value was calculated using the program Qlink.
*LOD equivalent scores. The individual LOD equivalent score were calculated as described by Kruglyak and Lander.
*Genome wide p value < 0.05 significant for whole genome.
*a/a- homozygous genotype with two copies of the BALB/c allele.
*a/b- heterozygous genotype with one copy of the BALB/c allele and one copy of the C57BL/6 allele.
*b/b-homozygous genotype with two copies of the C57BL/6 alleles.
*Single-point combine p value of the colony number associated with one homozygous genotype (two C57BL/6 alleles or two BALB/c alleles) and corresponding heterozygous genotype (one C57BL/6 allele and one BALB/c allele) that have no significant differences (d.f. = 1).
intercross, Table III) was obtained at locus D8Mit58 near the centromere of Chromosome 8.

Suggestive linkage to large colony number was obtained for the markers on mouse chromosome 4 (Fig 5). D4Mit264 and D4Mit181 had a LOD score of 3.05. Analysis of eight markers on chromosome 4 suggested that the gene determining a high number of large colonies was situated between D4Mit264 (1.9 cM) and D4Mit181 (2.5 cM), and was associated with BALB/c alleles. Thus, gene(s) regulating the high number of large colonies reside in the D4Mit264 region. We designated this locus keratinocyte stem cell locus 2 (Ksc2).

**Discussion**

The keratinocyte stem cell regulatory mechanism and the involvement of stem cells in skin carcinogenesis continue to be a matter of intense investigation. Recently we have established that the frequency of clonogenic keratinocyte stem cells is a genetically defined and quantitative complex trait (Popova et al., 2002). Moreover, we demonstrated that clonogenic epidermal stem cells are a heterogeneous population, and mouse epidermis has at least two subpopulations of keratinocyte stem cells, which were regulated by the different genes (Popova et al., 2003). Hence, we suggested that different types of clonogenic keratinocytes could be involved in the regulation of different process in mouse epidermis.

We have determined that these two types of keratinocytes able to produce small (>2 mm²) and large (≤2 mm²) colonies in vitro have the independent modes of inheritance. We found that the ratio of small and large colony frequency is different in C57BL/6 and in BALB/c mice. Thus, the ratio of small and large colonies in C57BL/6 mice was 1:1, whereas in BALB/c mice this parameter was 9:1. Despite that the number of all colonies was significantly higher in C57BL/6 mice (Popova et al., 2002), the number of small colonies was higher BALB/c mice (p < 0.0001, Fig 3A), whereas the number of large colonies was higher in C57BL/6 mice (p < 0.0001, Fig 3B). Moreover, the frequency of small and large colonies inherited independently in different genetic crosses (Fig 4). This observation suggested that we could find the genes that regulating these two types of clonogenic stem cells.

The linkage analysis suggested that the gene(s) regulating the frequency of small colonies are situated on the mouse chromosome 1, 6, 7, 8, and 9 (Fig 5). Previously we have established that several factors, including in vivo treatment with the carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) and the promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) involved in two-stage skin carcinogenesis, might influence the frequency of clonogenic cells (Morris et al., 1988). This observation brought us to conclude that keratinocytes ability to produce colonies in vitro might be involved in skin carcinogenesis. Comparing of the loci regulating the frequency of small keratinocyte colonies with the loci published in association with resistance or susceptibility to two-stage skin carcinogenesis revealed

**Table III. Linkage of small colony frequency with mouse chromosomes 8 and 9**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Map location (cM)</th>
<th>Backcross C57BL/6 × F1</th>
<th>F2</th>
<th>Combined</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>p&lt;sup&gt;a&lt;/sup&gt;</td>
<td>LOD&lt;sub&gt;c&lt;/sub&gt;</td>
<td>p&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>D8Mit58</td>
<td>1</td>
<td>0.04</td>
<td>0.84</td>
<td>0.002</td>
</tr>
<tr>
<td>D8Mit94</td>
<td>13</td>
<td>0.02</td>
<td>1.06</td>
<td>0.03</td>
</tr>
<tr>
<td>D8Mit339</td>
<td>23</td>
<td>0.02</td>
<td>1.1</td>
<td>0.04</td>
</tr>
<tr>
<td>D9Mit191</td>
<td>26</td>
<td>0.4</td>
<td>0.1</td>
<td>0.09</td>
</tr>
<tr>
<td>D9Mit262</td>
<td>41</td>
<td>0.04</td>
<td>0.84</td>
<td>0.00002</td>
</tr>
<tr>
<td>D9Mit133</td>
<td>43</td>
<td>0.004</td>
<td>1.78</td>
<td>0.0003</td>
</tr>
<tr>
<td>D9Mit273</td>
<td>49</td>
<td>0.004</td>
<td>1.74</td>
<td>0.0006</td>
</tr>
<tr>
<td>D9Mit182</td>
<td>55</td>
<td>0.03</td>
<td>0.98</td>
<td>0.02</td>
</tr>
<tr>
<td>D9Mit51</td>
<td>61</td>
<td>0.05</td>
<td>0.8</td>
<td>0.2</td>
</tr>
</tbody>
</table>

<sup>a</sup>Single-point significant levels.
<sup>b</sup>Genome wide p value < 0.05 significant for whole genome.
<sup>c</sup>LOD equivalent scores. The individual equivalent scores were calculated as described by Kruglyak and Lander (1995).
<sup>d</sup>The combined LOD equivalent score, is the sum of the individual LOD equivalent scores, was calculating using the program Qlink.
overlap between a locus involved in skin tumor promotion, Pst1, previously mapped to a region of Chromosome 9 (Angel et al, 1997), and the Ksc1 locus (Popova et al, 2003). Moreover, Mock et al (1998) reported a higher than expected linkage score for the marker D9Mit271 (48 cM) in association with skin tumor susceptibility. Recently, Sks6, skin tumor susceptibility locus 6, have been mapped on the mouse chromosome 9 (49 cM) (Nagase et al, 1999). These loci (D9Mit271, and Sks6) are also closely related to our Ksc1 (Fig 5). Thus, gene(s) on chromosome 9 regulating the number of small keratinocyte colonies might also affect sensitivity/resistance to skin tumor promotion, or skin tumor development.

Nagase et al (1995) identified locus Sks2 linked to papilloma development on mouse chromosome 7. The Mus spretus allele of Sks2 (64 cM) in the distal region of chromosome 7 is associated with female-specific papilloma resistance, whereas the Mus musculus allele in this locus is associated with susceptibility to papilloma development. In our intercross mice, the BALB/c allele of D7Mit105 (63.5 cM) locus showed suggestive association with the high number of small colonies in almost the same region as Sks2. Thus, a gene(s) regulating small keratinocyte colony number might be involved in the regulation of susceptibility or resistance to papilloma development in the Sks2 locus.

Sks11 (36.5cM) is situated on mouse Chromosome 6, and linked with susceptibility or resistance to benign and malignant skin tumors (Nagase et al, 1995, 1999). The same region on mouse chromosome 6, D6Mit99 (36cM) with LOD score peak 2.3 linked with a high frequency of small colonies (Popova et al, 2003). Interestingly, the gene for transforming growth factor-α located on chromosome 6 at 35.8 cM from the centromere has been implicated in papilloma development (Vassar et al, 1992) and might also influence keratinocyte colony number (Morris, unpublished data). Together, the data suggest that a gene(s) linked with the number of small colonies on chromosome 6, also influences the development of benign and malignant skin tumors. Finally, the Ksc1 locus on Chromosome 9 and two minor suggestive loci on chromosomes 7 and 6 are surprisingly close to loci found to be associated with skin tumor promotion (Angel et al, 1997, 2001)/sensitivity/resistance to skin tumor development (Mock et al, 1998, Nagase et al, 1995, 1999). We hypothesize that the population of clonogenic keratinocytes able to produce small colonies represents a specific population of skin stem cells responsible for sensitivity or resistance to skin carcinogenesis. Identification of loci responsible for sensitivity to skin carcinogenesis awaits further studies of other genetic crosses with strains susceptible to skin carcinogenesis.

The Ksc1 region of mouse chromosome 9 shares linkage homology with human chromosomes 15q22.1 (MAP2K1), 15q21 (TCF12), 15q21-q22 (ADAM10), 6pter-qter (BMP5), 14pter-qter (COX7A3). The region of the Ksc1 locus contains a cluster of genes responsible for normal mouse development (Adam10, 4; Foxb1a; Bmp5; Hnf6; Tcf12; Oma2a, 2b) and possibly involved in regulation of keratinocyte stem cell number through their effects on growth and differentiation (Neurman et al, 1993; Sasaki and Hogan, 1993; West et al, 1996; Wolsberg and White, 1996; Furuta et al, 1997; Yavari et al, 1998; Jacquemin et al, 2000). Interestingly, that Adam10, plays a role in the Notch signaling pathway which has been implicated in keratinocyte growth arrest and entry into differentiation (Rangarajan et al, 2001), and may be expressed in the mouse hair follicle (Kopan and Weintraub, 1993). The involvement of that Delta-Notch signaling in stimulating human epidermal stem cell differentiation (Lowell et al, 2000), further suggests that Adam 10 as a candidate gene in the Ksc1 locus.

Another group of genes in the Ksc1 region includes those encoding mitogen-activated protein kinase 1 (Map2k1) (Alessandri et al, 1997) and Anxa2, a major substrate for growth factor receptor protein-tyrosine kinases and protein kinase C (Saris et al, 1987). Morris et al (1988) showed that the number of clonogenic keratinocytes increases in response to TPA treatment, possibly resulting in protein kinase signal transduction. Thus, Map2k1or/an Anxa2 might be involved in the regulation of the number of clonogenic keratinocytes.

The Ksc2 locus on mouse Chromosome 4 shares linkage homology with human chromosomes 8q13 (LYN, GWM), 8q11 (MOS), 8q13-q21 (GEM). Possible candidate genes for Ksc2 include Lyn, a member of the Src subfamily encoding protein tyrosine kinase and expressed in skin (Hibbs et al, 1995), and Mos, a post-transcriptionally regulated gene expressed during oocyte growth and maturation (Newman and Dai, 1996). Finally the mitogen-induced gene, Gem, encodes a GTP-binding protein that belongs to a new family within the Ras superfamily (Santoro et al, 1995); the regulated expression pattern of Gem suggests a role for this protein in cellular responses to growth stimulation and may be a candidate gene for the Ksc2 locus.

The application of different possible models of stem cells hierarchies to the distribution of small and large colonies in parental strains and different genetic crosses brought us to conclude that small colonies most likely are not the progeny of transit amplifying keratinocytes. The data issued from the laboratory of Barrandon (Kobayashi et al, 1993; Rochat et al, 1994; Oshima et al, 2001) suggested that 95% of all clonogenic cells were originated from the bulge of hair follicle. Let assumed that large colonies originate from stem cells. In this case, 85% of small colonies from BALB/c mice, and at least 45% of small colonies in C57BL/6 mice should originate from stem cells. The investigation of some other parameters in addition to the area of colonies will help to answer the question if these two types of clonogenic cells related to the same or different lineages.

Thus, our data demonstrate that the frequency of clonogenic keratinocyte stem cell is a genetically defined and quantitative complex trait. The clonogenic keratinocyte stem cells are a heterogeneous population. Two major subpopulations of clonogenic keratinocyte stem cells are regulated by different genes, with the locus responsible for small colony number on mouse chromosome 9 designated Ksc1, and the locus associated with large colonies on chromosome 4 designated Ksc2. Correlative data suggest that clonogenic keratinocytes producing small colonies might represent a specific population of keratinocytes stem cells responsible for sensitivity or resistance to skin carcinogenesis. Because these two subpopulations have independent inheritance and are regulated by different genes, we propose that they might be responsible for different processes in mouse epidermis.
The identification of genes regulating amplification of keratinocyte stem cell awaits the identification and eventual cloning of the Ksc1 and Ksc2 genes. Moreover, the mechanisms regulating the intrinsic number of keratinocyte stem cells, possibly part of a universal regulatory mechanism for maintaining resting numbers of stem cells or their amplification, undoubtedly underlie the cells responses to external manipulation. The ability to manipulate these genes in vivo raises the exciting possibility of a novel treatment for skin disease and cancer. Further studies are need to determine whether the stem cell regulatory genes might be targets in carcinogenesis caused by environmental chemicals or ultraviolet light.

Materials and Methods

Mice Six-week-old C57BL/6 [C57BL/6NcrIbr], DBA/2 [DBA/2NcrIbr], BALB/c [BALB/cAnNcrIbr], CD-1 [Crl:CD-1 (ICR)], C3H [C3H/HeNcrIbr], FVB [FVB/NcrIbr], (BALB/c × C57BL/6)F1, C57BL/6 × (BALB/c × C57BL/6)F1 and BALB/c × (BALB/c × C57BL/6)F2 female mice were obtained from Charles River Laboratories (Wilmington, Massachusetts), (www.criver.com). Six-week-old SENCAR female mice were obtained from NCI Frederick Cancer Research Center (Bethesda, Maryland). Groups of each mouse strains ranged between 24 and 57 animals; 30 F1, 44–45 backcrosses, and 104 F2 mice were used. A total of 496 mice were analyzed. All experiments were carried out with mice at 7 wk of age, when skin was still pink, indicating the resting stage of the hair follicle growth cycle.

Histology Skin of mice was fixed in 10% neutral buffered formalin, embedded in paraffin and prepared by serial sections (5 μm). Slides were stained with hematoxylin and eosin.

Phenotyping Keratinocytes harvested (Morris 1994) from individual mice were placed into four 60-mm plastic dishes (1000 viable cells per dish) on irradiated 3T3 (Swiss) mouse cells (American Type Culture Collection, Rockville, Maryland) and were grown for intervals of 2 and 4 wk. Dishes were fixed with neutral buffered formalin, and stained with rhodamine B. Keratinocyte colony size and colony size were analyzed with a Fluor-S MultilImager (BIO-RAD, Hercules, California) after 2 wk of cultivation. Colony size ranged from 0.1 to 12 mm². The number of keratinocyte colonies 0.1–2 mm² in area in duplicate dishes was designated as a “small” colony phenotype. The number of keratinocyte colonies greater than 2 mm² area was considered a “large” colony phenotype. Hence, we had two phenotypes. The first of our phenotypes was the number of small colonies from each individual animal of each segregating crosses; the second—large colonies, respectively.

Statistical analysis A balanced incomplete block design experiment (Box et al, 1978) was carried out with 12 replicates from each mouse strain and for the CB6F1 mice. Differences in the number of keratinocyte colonies between different mouse groups were analyzed using the Wilcoxon rank sum test and extension of the two-way ANOVA.

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