Epistatic interactions between modifier genes confer strain-specific redundancy for Tgfb1 in developmental angiogenesis

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Abstract

Tgfbm1 (chromosome 5, \( P = 8 \times 10^{-5} \)) and Tgfbm3 (chromosome 12, \( P = 6 \times 10^{-11} \)) were identified as loci that modify developmental angiogenesis of Tgfb1\(^{-/-}\) mice. Congenic mice validated these loci and demonstrated epistatic interaction between them. The novel locus, Tgfbm3, encompasses \(~22\) genes, colocalizes with both tumor susceptibility and atherosclerosis susceptibility loci, and is enriched in genes regulating cell growth and morphogenesis. The use of gene knockout and/or transgenic mice that predispose to a complex trait, such as vascular development/angiogenesis, facilitates the identification of modifiers by simplifying genetic analysis. Identification of genes that modify response to lack of transforming growth factor \( \beta \) (TGF\( \beta \)) will enhance the understanding of TGF\( \beta \) action in vivo and may help predict which patients would respond well to anti-TGF\( \beta \) therapy. Identification of angiogenesis-modifying genes may provide new targets for angiogenesis therapies and analysis of polymorphisms therein may contribute to assessment of risk for diseases involving angiogenesis.

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Keywords: Angiogenesis; Epistatic interaction; Genetic modifier; Mouse embryogenesis; Prenatal lethal; TGF\( \beta \); Yolk sac

Introduction

Deviations in normal embryonic and fetal development of the vascular system can contribute to adult pathologies with late age of onset, such as atherosclerosis and hypertension. Differential angiogenic capacity can also influence the ability to heal wounds and influence the progression of fibrotic diseases and cancer. The development of the vascular bed is regulated multifactorially with both strong environmental [1–4] and genetic influences [5–7]. Inbred mouse strains show innate variation in endogenous vascularity and in angiogenic capacity both during embryogenesis and postnatally [6–11], making it feasible to dissect the genetic components regulating angiogenesis [5]. However, because the genetics of regulation of angiogenesis is highly complex, it can be difficult to map those genes that act together to modify this process since each individual

Abbreviations: BC, backcross; HHT, hereditary hemorrhagic telangiectasia; QTL, quantitative trait locus; STB, survival to birth; TGF\( \beta \), transforming growth factor \( \beta \).

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modifier gene contributes only a small fraction of the genetic variance to the overall effect on angiogenesis, leading to weak linkage for any individual gene [6].

We have taken an approach that we believe simplifies the genetics of such modifier screens by potentiating mice toward defects in prenatal vascular development, in our case using the \( \text{Tgfb1}^{-/-} \) mouse [10–12]. Utilizing this approach, we expect that fewer loci would be identified, but that each locus would be more amenable to mapping at high resolution. The loci identified will certainly be important in developmental angiogenesis and may well also be important, either directly or indirectly, in the transforming growth factor \( \beta \) (TGF\( \beta \))^7 signaling pathway.

TGF\( \beta \)1 is a key regulator of normal angiogenesis and vascular integrity as demonstrated by the phenotypes of mice [13] and humans [14] with null mutations in components of the TGF\( \beta \)1 signaling pathway. Homozygosity for null mutations in Tgfb1, Tbr1, Tbr2, Eng, or Acvrl1 results in lethal embryonic vascular defects in mice [13]. Hemizygous mutation of TGFBR2 results in Marfan syndrome [15], and hemizygosity for ENG or ACVR1L results in hereditary hemorrhagic telangiectasia (HHT), a vascular dysplasia with late age of onset [14]. Genes encoding components of the TGF\( \beta \)1 signaling pathway, including TGFBL1 [16] and TBRL1 [17], have been shown to be functionally polymorphic in humans and confer susceptibility to atherosclerosis [16,18], hypertension [16,19], and other vascular diseases [20], as well as breast and prostate cancers [21,22]. TGF\( \beta \)1 is also important in pathological angiogenesis, such as tumor angiogenesis [13,23] and during wound healing [24].

Herein, we have used classical \( F_1 \) intercross mapping to validate the existence of a previously identified TGF\( \beta \)1 modifier locus on chromosome 5, in an independent cross between C57 and NIH mice (\( P = 8 \times 10^{-5} \)). This locus has now been named Tgfbm1. We have also identified a second strong modifier locus, Tgfbm3, on chromosome 12 (\( P = 6 \times 10^{-11} \)) that interacts with Tgfbm1 in a multiplicative fashion to determine the developmental response to Tgfb1 nullizygosity. The validity of these loci was confirmed using mice congenic for the respective modifier regions.

The identification of genes that modify the response to lack of TGF\( \beta \)1 should enhance our understanding of the mode of action of TGF\( \beta \)1 in vivo. This is particularly important now that small-molecule inhibitors that act on the TGF\( \beta \)1 signaling pathway are being developed to treat fibrosis and cancer [25]. Knowledge of the genetic basis for variation in response to attenuation of TGF\( \beta \) may eventually be useful in identifying patients that may or may not respond well to anti-TGF\( \beta \) therapy. Identification of angiogenesis modifying genes may provide new targets for angiogenesis therapies and analysis of polymorphisms therein may contribute to assessment of risk for diseases involving angiogenesis.

Results

Multifactorial nature of Tgfb1\(-/-\) genetic modifiers

Tgfb1\(-/-\) mice die at either 10.5 days \emph{postcoitum} (dpc) from defects in development of the yolk sac [10–12] or 3 weeks \emph{postpartum} from multifocal inflammation [26]. Yolk sac defects include both abnormal vascular development and anemia [10], but it has been suggested that the primitive hematopoietic defects are secondary to defects in vascular development [27,28]. On mixed genetic backgrounds, there have been reports of Tgfb1\(-/-\) prenatal loss before 8.5 dpc [11,29,30]; however, on relatively pure C57, NIH, or 129 genetic backgrounds, the fraction of 9.5 dpc Tgfb1\(-/-\) with yolk sac defects is consistent with this being the major cause of prenatal loss in each of these strains [11,12,31].

We previously identified a polymorphic genetic modifier locus, Tgfbm1, on chromosome 5 that accounts for a significant fraction of the genetic variance responsible for determining embryonic fate of Tgfb1\(-/-\) mice in a NIHCS7F1 intercross [11]. The phenotype scored was survival to birth (STB) of Tgfb1\(-/-\) mice compared to wildtype littersmates in a Tgfb1\(+/-\) \( F_1 \) intercross. Since then, the C57 and NIH strains harboring the Tgfb1 null allele were bred through further backcross generations onto the relevant strain, in order to purify the genetic backgrounds. At backcross generation 5 (BC5) mice were moved from a conventional facility in the United Kingdom to a barrier facility in the United States and re-derived by superovulation and embryo transfer. For each strain, between one and five Tgfb1\(+/-\) male mice were utilized for this re-derivation step. All breeding in the United States (post-BC6) was performed in a barrier facility.

The STB rate of Tgfb1\(-/-\) mice on the C57 background was zero in both the Glasgow- and U.S.-bred mice. However, it was notable that in all the crosses, the NIH STB rate in the U.S.-bred mice was roughly half that of the Glasgow-bred mice (Fig. 1), suggesting that the penetrance of the NIH modifier allele(s) was reduced ~40% following transfer to the United States. Nevertheless, within genetically similar populations bred within the same animal house the variance in STB was small. The incidence of NIH Tgfb1\(-/-\) neonates per litter was evenly distributed between the >30 litters of Glasgow-bred mice and >25 litters of U.S.-bred mice. Moreover, the different batches of Glasgow-bred F1 intercross mice showed little variance in STB rates (189 Tgfb1\(+/-\); 373 Tgfb1\(+/-\); 70 Tgfb1\(-/-\) versus 49 Tgfb1\(+/-\); 97 Tgfb1\(+/-\); 24 Tgfb1\(-/-\); \( P = 0.55 \)). All prenatal death of both the NIH and the C57 Tgfb1\(-/-\) embryos was due to yolk sac insufficiency [12], since STB rates corresponded directly to the incidence of vascular/ hematopoietic abnormalities. The reduced STB rate in U.S.-versus Glasgow-bred Tgfb1\(-/-\) mice may be explained by genetic and/or environmental differences (see Discussion), but clearly illustrates the multifactorial nature of the Tgfb1 genetic modifiers.
Fine-mapping of Tgfbm1

To confirm our earlier linkage association for Tgfbm1 [11] and to evaluate the impact on this linkage of reduced penetrance of the NIH modifier(s) in the U.S.-bred mice, our data set was increased from an original 73 NIHC57F1 intercross Tgfb1+/C0 intercrosses were set up on the indicated genetic backgrounds. According to standard convention, the maternal strain is shown on the left of the cross and the paternal strain is shown on the right. Tgfb1−/− STB rate was estimated as 100 × (number of Tgfb1−/− births/number Tgfb1+/+ births). Open columns are data gathered from U.S.-bred mice at BC6–7; solid columns are data collected in Glasgow, United Kingdom, at BC4. Data shows that in all the crosses Tgfb1−/− STB in U.S.-bred mice is consistently half that in Glasgow-bred mice.

Further 38 U.S.-bred Tgfb1−/− neonates. The additional Tgfb1−/− F2 intercross mice were genotyped using some of the same markers from our original study [11] together with 20 additional informative SSR makers across chromosome 5 (Fig. 2). LODmax = 4.6 was localized at D5Mit106.

An additional 181 U.S.-bred Tgfb1−/− mice were later collected and genotyped at D5Mit106. Linkage in the U.S.-bred F2 mice appeared weaker (LOD = 1.1, n = 181) than in the Glasgow data set (LOD = 4, n = 119);
however, the distribution of alleles in the two sets (Glasgow, 49NN: 53CN: 17CC at D5Mit106; United States, 54: 94: 33 at D5Mit106) was not significantly different ($P = 0.12$, $\chi^2$ test). Moreover, both data sets showed an excess of NIH alleles at D5Mit106, suggesting that, although contributing a weaker effect, Tgfbm1 is still detectable in the U.S. data set. The broad shape of the curve suggests that there may be multiple modifier genes on chromosome 5, since the 90% support interval [32] spans from 34 to 50 Mb. There are approximately 88 Ensembl-designated genes located in this 16-Mb interval, of which 43 have a LocusLink ID and ~24 have been characterized in some way (Supplementary Table 1).

Tgfbm1 requires interaction with another gene(s) to rescue Tgfb1+/− mice from prenatal lethality

Our initial linkage analysis [11] suggested that Tgfbm1 accounted for over 75% of the genetic variance in the ability of NIH $\times$ C57 Tgfb1+/− embryos to develop to term normally. Consequently, we tested whether this locus alone could rescue Tgfb1+/− embryos from prenatal lethality. Congenic mice were generated containing the Tgfbm1 region from the NIH strain on an otherwise C57 genetic background (C57.Tgfbm1NIH/C0/C0). STB rates of Tgfb1+/− mice were determined in Tgfb1+/− intercrosses of homozygous congenic C57.Tgfbm1NIH mice (Table 1). Although there was some rescue of Tgfb1+/− embryo lethality (Table 1, row 3), this was not significantly different from the STB rate for pure C57 (Table 1, row 2). However, in the light of finding weaker linkage to Tgfbm1 in the U.S.-born F1 intercross (see above), this result was not surprising.

We hypothesized that Tgfbm1 exists as a genetic modifier of the Tgfb1+/− phenotype, but that unlinked interacting modifier genes, bred out during generation of the C57.Tgfbm1NIH congenic (and possibly diluted during re-derivation of the “pure” lines), are required for the full effect of Tgfbm1. Two genetic crosses gave support to this hypothesis. First, when a Tgfb1+/− intercross was undertaken between pure (BC8) NIH mice and homozygous C57.Tgfbm1NIH, the STB rate was significantly ($P = 0.012$) increased compared with an intercross between pure NIH (BC8) and C57 (BC8) Tgfb1+/− mice (Table 2). Second, Tgfb1+/− intercrosses of homozygous congenic mice containing Tgfbm1 from the C57 strain on an otherwise NIH genetic background (NIH.Tgfbm1C57) showed a significant reduction ($P = 0.018$) in STB rate compared to pure NIH (Table 1, row 4). Since the variance in STB rate within any one genetic background is small ($P$ values for the $\chi^2$ test in the range of 0.3–0.97; data not shown), these data demonstrate a statistically significant functional alteration of Tgfb1+/− STB rates by Tgfbm1.

Identification of Tgfbm3 on chromosome 12

The reduction in overall STB rate in the U.S.-bred mice (Fig. 1) together with the congenic data (Table 1) suggest that there are additional genetic modifiers between the NIH and C57 strains that act in concert with and/or independently of Tgfbm1 to affect Tgfb1+/− STB rate. A cursory F1 scan was thus undertaken to identify such linkage. One SSR marker per autosome was utilized to scan the genomic DNA of 60 U.S.-bred F2 Tgfb1+/− (United States Batch A). Nonparametric linkage analysis (Table 3) revealed distortion from the expected Mendelian distribution of alleles at D12Mit46 ($P = 0.01$) and D11Mit258 ($P = 0.008$). On expansion of the data for these two markers, linkage at D11Mit258 became insignificant (44NN: 75CN: 23CC; $P = 0.3$), whereas linkage on chromosome 12 was increasingly strengthened, reaching a lod score of over 10 (Fig. 3). This locus is designated Tgfbm3.

Fine-mapping of Tgfbm3

Fine-mapping of Tgfbm3 was undertaken by analysis of DNA from 230 U.S.-bred F2 Tgfb1+/− neonates together with that from over 100 Glasgow-bred Tgfb1+/− neonates (Fig. 3). Each DNA was genotyped with 11 SSR markers mapping between 12 and 50 Mb on chromosome 12 (http://www.ensembl.org). Unlike Tgfbm1, genetic linkage in the U.S.-bred mice was higher than in the Glasgow-bred mice. The peak LOD of over 10 for the combined data far exceeds the commonly adopted threshold for definitive linkage (LOD = 4.3) [33]. The 95% confidence interval (CI), defined by LOD$_{\text{max}}$−1 [32], spans 4 Mb between the 14.4- and 18.4-Mb position on chromosome 12. There are 22 Ensembl-designated genes in this region, 11 of which have LocusLink IDs (tabulated in Supplementary Table 2).

### Table 1

Validation of Tgfbm1 and Tgfbm3 using congenic mice

<table>
<thead>
<tr>
<th>Cross</th>
<th>Line</th>
<th>Donor</th>
<th>Recipient</th>
<th>BC generation</th>
<th>+/-</th>
<th>+/-</th>
<th>--/--</th>
<th>Total</th>
<th>STB rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NIH</td>
<td>NIH</td>
<td>NIH</td>
<td>5–6</td>
<td>71</td>
<td>123</td>
<td>36</td>
<td>250</td>
<td>51%</td>
</tr>
<tr>
<td>2</td>
<td>C57</td>
<td>C57</td>
<td>C57</td>
<td>6–7</td>
<td>98</td>
<td>198</td>
<td>0</td>
<td>296</td>
<td>0%</td>
</tr>
<tr>
<td>3</td>
<td>C57.Tgfbm1NIH</td>
<td>NIH</td>
<td>C57</td>
<td>5</td>
<td>94</td>
<td>90</td>
<td>1</td>
<td>290</td>
<td>1%</td>
</tr>
<tr>
<td>4</td>
<td>NIH.Tgfbm1C57</td>
<td>C57</td>
<td>NIH</td>
<td>4–5</td>
<td>157</td>
<td>301</td>
<td>44</td>
<td>502</td>
<td>28%</td>
</tr>
<tr>
<td>5</td>
<td>C57.Tgfbm3NIH</td>
<td>NIH</td>
<td>C57</td>
<td>4–6</td>
<td>86</td>
<td>204</td>
<td>1</td>
<td>291</td>
<td>1%</td>
</tr>
<tr>
<td>6</td>
<td>NIH.Tgfbm3C57</td>
<td>C57</td>
<td>NIH</td>
<td>4</td>
<td>71</td>
<td>100</td>
<td>20</td>
<td>191</td>
<td>28.0%</td>
</tr>
<tr>
<td>7</td>
<td>C57.Tgfbm1NIH/Tgfbm3NIH</td>
<td>NIH</td>
<td>C57</td>
<td>5</td>
<td>73</td>
<td>78</td>
<td>5</td>
<td>104</td>
<td>7%</td>
</tr>
</tbody>
</table>

*Note. Tgfb1+/− intercrosses were set up within the above mouse lines. Neonates were collected at birth and genotyped for Tgfb1.*
Table 2

<table>
<thead>
<tr>
<th>Cross</th>
<th>Tgb1+/+</th>
<th>Tgb1+-</th>
<th>Tgb1--</th>
<th>Total</th>
<th>STB rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C57-Tgbm3.NIH × NIH</td>
<td>169</td>
<td>401</td>
<td>52</td>
<td>622</td>
</tr>
<tr>
<td>2</td>
<td>C57 × NIH</td>
<td>228</td>
<td>404</td>
<td>39</td>
<td>671</td>
</tr>
</tbody>
</table>

Note. Tgb1+- intercrosses were set up within the above mouse lines. Neonates were collected at birth and genotyped for Tgb1.

Cursory scan of F2

Table 3

<table>
<thead>
<tr>
<th>Marker</th>
<th>Centimorgan</th>
<th>NN</th>
<th>CN</th>
<th>CC</th>
<th>Total</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1Mit403</td>
<td>100</td>
<td>12</td>
<td>20</td>
<td>7</td>
<td>39</td>
<td>0.520</td>
</tr>
<tr>
<td>D2Mit277</td>
<td>69</td>
<td>8</td>
<td>29</td>
<td>17</td>
<td>54</td>
<td>0.192</td>
</tr>
<tr>
<td>D3Mit6</td>
<td>23.3</td>
<td>13</td>
<td>27</td>
<td>7</td>
<td>47</td>
<td>0.276</td>
</tr>
<tr>
<td>D3Mit38</td>
<td>70.3</td>
<td>14</td>
<td>31</td>
<td>9</td>
<td>54</td>
<td>0.348</td>
</tr>
<tr>
<td>D4Mit178</td>
<td>35.5</td>
<td>11</td>
<td>27</td>
<td>8</td>
<td>46</td>
<td>0.410</td>
</tr>
<tr>
<td>D5Mit79</td>
<td>26</td>
<td>18</td>
<td>32</td>
<td>10</td>
<td>60</td>
<td>0.301</td>
</tr>
<tr>
<td>D6Mit19</td>
<td>33.5</td>
<td>14</td>
<td>26</td>
<td>13</td>
<td>53</td>
<td>0.972</td>
</tr>
<tr>
<td>D7Mit363</td>
<td>2.2</td>
<td>2</td>
<td>14</td>
<td>35</td>
<td>51</td>
<td>3.0 × 10–12</td>
</tr>
<tr>
<td>D8Mit190</td>
<td>21</td>
<td>22</td>
<td>23</td>
<td>14</td>
<td>59</td>
<td>0.081</td>
</tr>
<tr>
<td>D9Mit18</td>
<td>71</td>
<td>18</td>
<td>24</td>
<td>9</td>
<td>51</td>
<td>0.187</td>
</tr>
<tr>
<td>D10Mit95</td>
<td>51</td>
<td>14</td>
<td>31</td>
<td>9</td>
<td>54</td>
<td>0.348</td>
</tr>
<tr>
<td>D11Mit258</td>
<td>65</td>
<td>23</td>
<td>34</td>
<td>6</td>
<td>63</td>
<td>0.008</td>
</tr>
<tr>
<td>D11Mit151</td>
<td>13</td>
<td>3</td>
<td>19</td>
<td>40</td>
<td>62</td>
<td>2.5 × 10–12</td>
</tr>
<tr>
<td>D12Mit46</td>
<td>16</td>
<td>23</td>
<td>28</td>
<td>7</td>
<td>58</td>
<td>0.012</td>
</tr>
<tr>
<td>D13Mit3</td>
<td>10</td>
<td>15</td>
<td>32</td>
<td>16</td>
<td>63</td>
<td>0.976</td>
</tr>
<tr>
<td>D14Mit193</td>
<td>40</td>
<td>14</td>
<td>21</td>
<td>15</td>
<td>50</td>
<td>0.517</td>
</tr>
<tr>
<td>D16Mit114</td>
<td>41</td>
<td>20</td>
<td>24</td>
<td>16</td>
<td>60</td>
<td>0.231</td>
</tr>
<tr>
<td>D17Mit16</td>
<td>18.15</td>
<td>8</td>
<td>36</td>
<td>16</td>
<td>60</td>
<td>0.104</td>
</tr>
<tr>
<td>D18Mit242</td>
<td>24</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>48</td>
<td>0.069</td>
</tr>
<tr>
<td>D18Mit149</td>
<td>24</td>
<td>15</td>
<td>18</td>
<td>16</td>
<td>49</td>
<td>0.175</td>
</tr>
<tr>
<td>D19Mit103</td>
<td>52</td>
<td>30</td>
<td>52</td>
<td>29</td>
<td>111</td>
<td>0.795</td>
</tr>
</tbody>
</table>

Tgbm3 function confirmed in F2 intercross and congenic mice

Additional support for the presence of Tgbm3 comes from STB studies on an F2 Tgb1+/+ intercross, where the F2 mice were derived from an F1 × F1 cross. Tgb1+/ mice, homozygous for Tgbm3<sup>C57</sup>, were intercrossed to each other, as were Tgb1+/- mice, homozygous for Tgbm3<sup>NII</sup>. Whereas those Tgb1-- mice derived from the homozygous Tgbm3<sup>C57</sup> parents showed only 7.5% STB (159 Tgb1+/+: 293 Tgb1+/-: 12 Tgb1--), mice derived from the homozygous Tgbm3<sup>NII</sup> parents showed 50% STB (88 Tgb1+/+: 156 Tgb1+/-: 44 Tgb1--), a very significant difference (P = 9.3 × 10<sup>−10</sup>).

Congenic mice were generated containing the Tgbm3 region from the NIH strain on an otherwise C57 genetic background (C57.Tgbm3<sup>NII</sup>) and vice versa (NIH.Tgbm3<sup>C57</sup>). STB rates of Tgb1+/ mice were determined in intercrosses of homozygous congenic Tgbm3, heterozygous Tgb1+/ mice (Table 1). Despite the strong genetic linkage to this locus, the Tgbm3<sup>NII</sup> modifier in isolation was able to rescue Tgb1-- mice at only low efficiency (Table 1, row 5), demonstrating that, like Tgbm1, this locus requires interaction with other genetic loci for full effect. However, homozygosity for Tgbm3<sup>C57</sup> on an otherwise NIH genetic background decreased the STB rate compared to that of NIH (Table 1, row 6), validating the presence of Tgbm3 on chromosome 12.

Tgbm1 acts in a codominant/additive fashion, whereas the Tgbm3<sup>NII</sup> allele may have recessive properties

The distributions of genotypes at D5Mit106 and D12Mit170 are not significantly different from each other (Table 4, P = 0.2), suggesting that the two loci act in a similar fashion. However, by examining the estimated penetrance (STB) of each genotype, a more detailed assessment suggests that Tgbm3 may have recessive properties.

By definition, the penetrance of a modifier gene depends on the genotype at that locus. If there is no linkage, the penetrance of each genotypic combination will be identical and will be equal to the overall penetrance of the trait. This is clearly not the case for Tgbm1 or Tgbm3 (Table 4). The 1.45-fold higher penetrance of Tgbm1<sup>NN</sup> compared to Tgbm1<sup>CC</sup> (P = 0.02) is similar to the 1.4-fold higher penetrance of Tgbm1<sup>NN</sup> compared to Tgbm1<sup>NC</sup> (P = 0.008), suggesting that the NIH allele of Tgbm1 acts in a purely additive/codominant fashion (Table 4), as we previously reported [11]. For Tgbm3 there is a 1.3-fold difference in penetrance between Tgbm3<sup>NC</sup> and Tgbm3<sup>CC</sup>, but this is not quite statistically different from the ratio of 1 that would be expected if the Tgbm3<sup>NII</sup> allele was purely recessive (P = 0.07). However, the 2-fold difference in
The penetrance between \textit{Tgfbm3}_{NN} \textit{and Tgfbm3}_{NC} (\(P = 6 \times 10^{-8}\)) suggests that homozygosity for \textit{Tgfbm3}_{NN} significantly exceeds the effect of heterozygosity at this locus. Namely, the \textit{Tgfbm3}_{NIH} allele has some synergistic properties rather than being purely additive. Indeed, with the present data set, we cannot rule out the possibility that \textit{Tgfbm3}_{NIH} acts as a recessive allele.

\textit{Tgfbm1} and \textit{Tgfbm3} interact in a multiplicative fashion to rescue \textit{Tgfb1}_{/\textit{C0}}_{/\textit{C0}} from prenatal lethality

Since it is necessary for both \textit{Tgfbm1} and \textit{Tgfbm3} to interact with other genes within the genome in order to manifest the trait of efficient \textit{Tgfb1}_{/\textit{C0}}_{/\textit{C0}} STB (Table 2), we addressed the question of whether these two loci show genetic interaction with each other. The distribution of alleles between the two loci for 289 mice supports our findings from the congenic mouse studies (Table 2) that each locus does not act in isolation. If one genetic locus is genotypically fixed, there is a significant deviation from expected Mendelian ratios at the remaining locus in five of the six possible groups (Table 5 and Materials and Methods). Thus, the two loci are not mutually exclusive.

Data analysis suggests that the two loci interact epistatically, i.e., in a multiplicative (\(P = 0.07\)) rather than in an additive fashion (\(P = 3 \times 10^{-8}\); see Materials and Methods and [34]). The penetrance of the \textit{Tgfbm1}_{NIH/NIH; Tgfbm3}_{NIH/NIH} genotype in the context of an F1 intercross background is \(\sim 70\%\) (Table 5). This indicates that, on the F1 intercross background, homozygosity for NIH at both of these loci is sufficient to rescue \(70\%\) of \textit{Tgfb1}_{/\textit{C0}}_{/\textit{C0}} mice from prenatal lethality (very similar to the STB level for pure U.S.-bred NIH mice). However, there must be additional genetic modifiers in the F1 intercross, since 13/289 \textit{Tgfb1}_{/\textit{C0}}_{/\textit{C0}} mice (~4\%) are homozygous C57 at both loci (Table 5).

The interaction of the two loci was directly tested by generating C57 mice homozygous NIH at both \textit{Tgfbm1} and

### Table 4

<table>
<thead>
<tr>
<th></th>
<th>\textit{D5Mit106}</th>
<th>\textit{D12Mit170}</th>
<th>\textit{D12Mit283}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expected</td>
<td>Observed</td>
<td>Genotype-specific</td>
<td></td>
</tr>
<tr>
<td>\textit{Tgfb1}<em>{/\textit{C0}}</em>{/\textit{C0}}</td>
<td>\textit{Tgfb1}<em>{/\textit{C0}}</em>{/\textit{C0}}</td>
<td>penetrance</td>
<td></td>
</tr>
<tr>
<td>NN</td>
<td>272.5</td>
<td>103</td>
<td>38%</td>
</tr>
<tr>
<td>NC</td>
<td>545</td>
<td>147</td>
<td>27%</td>
</tr>
<tr>
<td>CC</td>
<td>272.5</td>
<td>50</td>
<td>18%</td>
</tr>
<tr>
<td>Total</td>
<td>1090*</td>
<td>300</td>
<td>27.5%*</td>
</tr>
</tbody>
</table>

* Expected \textit{Tgfb1}_{/\textit{C0}}_{/\textit{C0}} conceptions = number of observed total \textit{Tgfb1}_{/\textit{C0}}_{/\textit{C0}}STB rate; expected genotype-specific conceptions = Mendelian fractions of expected total \textit{Tgfb1}_{/\textit{C0}}_{/\textit{C0}} conceptions (0.25:0.5:0.25).

* Genotype-specific penetrance = observed \textit{Tgfb1}_{/\textit{C0}}_{/\textit{C0}} conceptions/expected \textit{Tgfb1}_{/\textit{C0}}_{/\textit{C0}} conceptions.

* Total \textit{Tgfb1}_{/\textit{C0}}_{/\textit{C0}} STB rate in F1 intercross (see Fig. 1).
Table 5
Genetic interaction of Tgfbm1 and Tgfbm3

<table>
<thead>
<tr>
<th>Tgfbm1&lt;sup&gt;NN&lt;/sup&gt;</th>
<th>Tgfbm3&lt;sup&gt;NC&lt;/sup&gt;</th>
<th>Tgfbm3&lt;sup&gt;CC&lt;/sup&gt;</th>
<th>Total Tgfbm3</th>
<th>P value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>45 (68%)</td>
<td>38 (21%)</td>
<td>17 (25%)</td>
<td>100</td>
<td>6.6 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>64 (48.4%)</td>
<td>65 (24.6%)</td>
<td>14 (10.6%)</td>
<td>143</td>
<td>0.026</td>
</tr>
<tr>
<td>15 (22.7%)</td>
<td>18 (13.7%)</td>
<td>13 (19.7%)</td>
<td>46</td>
<td>0.037</td>
</tr>
<tr>
<td>Total Tgfbm3</td>
<td>124</td>
<td>121</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>P value&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.6 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>0.026</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note. Genotype-specific penetrance, calculated as described under Materials and Methods and in the footnote to Table 4, is shown in parentheses. For instance, 289/0.273 total conceptions; ∂: expected NNNN = 289/(0.273 × 16) = 66.1 and penetrance<sub>NNNN</sub> = 68%.

<sup>a</sup> Probability that Tgfbm1 and Tgfbm3 act in a mutually exclusive fashion.

Tgfbm3. This led to a significant increase in STB (P < 0.05), from ~1% for each of the two single congenic lines (Table 1, rows 3 and 5) to ~7% for the double congenics (Table 1, row 7). Data from the double congenic test cross (Table 1) are consistent both with epistatic interaction between Tgfbm1 and Tgfbm3 and with a requirement for additional NIH loci to bring the STB rate to the 50–60% level seen in U.S.-bred rows 3 and 5) to ~7% for the double congenics (Table 1, row 7). Our cursory genome scan may well have missed these other loci. Alternatively, there may be additional genes on distal chromosome 5 that contribute to STB, some of which were not included within the Tgfbm1 congenic region. Importantly, the analysis of C57,Tgfbm1<sup>NNNI</sup>H/,Tgfbm3<sup>NNNI</sup>H/ congeneric mice provides evidence for the localization of a major component of Tgfbm1 distal to D5Mit352 at 33.9 Mb but proximal to D5Mit11 at 46.8 Mb (Fig. 2).

Discussion

Here we have identified two unlinked but epistatically interacting genetic loci, Tgfbm1 and Tgfbm3, on chromosomes 5 and 12, respectively, that influence the extent of embryonic angiogenesis in vivo. These two loci together account for the major fraction (between 55 and 80%) of the genetic variance between the NIH and C57 mouse strains in their ability to undergo normal vascular development in the absence of TGFβ1. Both loci were found by whole genomic scanning of Tgfb1<sup>−/−</sup> mice that survive embryogenesis and are developmentally normal at birth. The existence of each locus was validated using mice congenic for the respective modifier regions. Tgfb1<sup>−/−</sup> NIH mice that retain C57 genomic DNA encompassing either of the modifier regions show a significant decrease in STB, compared to those carrying the Tgfbm<sup>NNNI</sup>H alleles. Each locus in isolation leads to only a very small percentage of mice showing STB on the invariably prenatal lethal background of the C57 Tgfb1<sup>−/−</sup> mouse. However, the two loci interact multiplicatively to significantly increase this Tgfb1<sup>−/−</sup> STB rate, illustrating the importance of genetic interactions in this phenotype.

Tgfbm1 was previously identified in a smaller data set of F<sub>1</sub> intercross Tgfb1<sup>−/−</sup> mice bred in Glasgow and, in this same data set, Tgfbm3 was noticed as a weak, insignificant linkage [11]. The mice were subsequently re-derived and bred through further backcross generations in barrier facilities in the United States. After embryo re-derivation of the lines, there was a noticeable twofold reduction in Tgfb1<sup>−/−</sup> STB on an NIH background. Moreover linkage at Tgfbm1, although still present, was far weaker than in the Glasgow-bred mice. The Tgfbm3 modifier on chromosome 12 exhibited stronger linkage in the U.S.-bred mice. These differences may have resulted from environmental influences on the phenotype. However, it is also likely that the data could be explained by genetic drift occurring as the lines were further purified during and following their re-derivation.

The NIH and C57 Tgfb1<sup>+/+</sup> lines as used here are not purely inbred, since for reasons of practicality they were backcrossed between only 4 and 8 generations onto inbred mice. The Glasgow linkage data were collected at BC4 [11], at which point 6.25% of the genome would be residual DNA from the original mixed genetic background. In contrast, linkage analysis of the U.S.-bred mice was performed at BC5–8 (0.4–3.1% extraneous DNA). On retrospective analysis of the genome-wide scans, it became apparent that many of the Glasgow-bred NIH mice, but very few of the U.S.-bred mice, contained residual 129 genomic DNA around D1Mit362, a region known to harbor another strong modifier, Tgfbm2<sup>129</sup>, previously detected in a 129 × C57 F<sub>1</sub> intercross [12]. It is possible that Tgfbm1<sup>NNI</sup>H requires interaction with Tgfbm2<sup>129</sup> [12] for its full effect. In fact, when the Glasgow linkage data for Tgfbm1 were stratified according to Tgfbm2 genotype, it appears that there is genetic linkage in mice that are homozygous (P = 0.007, n = 28) or heterozygous (P = 0.003, n = 50) for Tgfbm2<sup>129</sup>, but no linkage in mice lacking a Tgfbm2<sup>129</sup> allele (P = 0.13, n = 36) (data not shown), suggesting dependence of Tgfbm1 linkage on the presence of at least one Tgfbm2<sup>129</sup> allele. Stratification of this already small data set makes this statistical analysis questionable. Nevertheless, functional interaction between Tgfbm2 and the other modifiers is a possibility that should be addressed further.

Tgfbm1 and Tgfbm3 interact in a multiplicative manner to rescue Tgfb1<sup>−/−</sup> mice from embryo lethality. Genetic interactions do not necessarily translate into biochemical interactions. Without identifying the encoded proteins, it is therefore impossible to determine whether the two genes are on the same biochemical pathway. An equally plausible
possibility is that these modifiers act independently on alternative pathways normally redundant to that of TGFβ1, such that mutation or polymorphism in any of these pathways contributes in a multiplicative fashion to the phenotype of vascular dysgenesis.

The existence of loci that modify postnatal angiogenesis in a VEGF-induced corneal angiogenesis assay was recently reported using BXD recombinant inbred lines [5]. This phenotype (angiogenesis) is genetically very complex, exemplified by the inability to detect any linkage by simple interval mapping techniques and the relatively large number of loci and low individual P values (P in the range of 0.005–0.01) for each locus detected using composite interval mapping [5]. Intriguingly, 1 of the 10 suggestive loci identified by Rogers et al. [5] colocalizes with Tgfbm1 on chromosome 5 (P = 0.025) and another locus detected in that study maps within 23 Mb of the Tgfb1 gene on chromosome 7, albeit with a single marker (P = 0.014). Bearing in mind the low resolution of their genetic mapping [5], the chromosome 5 and 7 loci identified by Rogers et al. [5] could be Tgfbm1 and Tgfb1, respectively.

The approach of using gene knockouts or transgenics to potentiate mice toward a particular pathological fate should theoretically reduce the number of genes involved in the “subtrait” and thus simplify the genetics of the system in a manner similar to stratification of biological components of a trait. Each individual gene will make a larger contribution to the overall genetic variance, thus giving a more significant P value, which in turn allows genetic mapping at a higher resolution. In the Tgfb1+/− mouse model, animals are already predisposed to death from angiogenesis due to defects in this one specific signaling pathway, thus biasing the study toward identification of modifiers associated with that pathway.

Tgfbm1 could be mapped only at low resolution (16 Mb), due to its reduced effect in the U.S.-bred mice. The peak of the lod score maps around a cluster of genes encoding hematopoietic cell markers, including CD38, Bst1, and AC133. Also, within the 95% CI are a number of other genes that may be relevant to TGFβ1 and angiogenesis, including Fgfbp1, Rab2, Lim domain binding 2 (Ldb2), and Msc. Slit2, 4 Mb from the peak of the lod score, encodes a ligand that interacts with Roundabout to induce angiogenesis [35]. Finally, the Ellis-van Creveld gene, which is responsible for an autosomal recessive skeletal dysplasia, together with congenital cardiac defects in 60% of affected humans, maps within the region syntenic to Tgfbm1. Since TGFβ is known to be important in skeletal [15,36] as well as vascular [15,37,38] dysplasias, Evc could well contribute to the modifying effect of Tgfbm1 on angiogenesis.

Tgfbm3 yielded a very high lod score, allowing us to map this locus at high resolution (~4 Mb, 95% CI). It is remarkable that this small region contains a cluster of functionally related genes known to regulate cellular proliferation and migration. Indeed, two genes are known to be involved in TGFβ signaling. Tieg2b/Tieg3, TGFβ immediate-early gene 2b, is a TGFβ-inducible transcriptional repressor with anti-proliferative and anti-apoptotic functions, consistent with a role in mediating TGFβ actions [39]. Idb2 encodes Id2 (inhibitor of differentiation), which is also involved in transcriptional inhibitory responses to TGFβ [40–42], in cell growth control and angiogenesis [40,41,43,44]. Other genes involved in cell proliferation include Ornithine decarboxylase (Odc), Ribonucleotide reductase 2 (Rrm2), and E2f6, and those involved in modifying cellular plasticity and migration encode Rho kinase (Rock2), Integrin β1-binding protein 1 (Igfb1bp1/LCAP1), and TNFα-converting enzyme (Adam17).

A number of mouse qualitative trait loci (QTL) localized within the same intervals as Tgfbm1 and Tgfbm3, including susceptibility loci for skin cancer (Skts3 and Skts5, respectively) [45,46], autoimmune disorders (Sle6 and Asbb2, respectively) [47], and atherosclerosis (Ath6) [48]. The human QTL Susceptibility to Hypertension 3 also maps to the syntenic region in humans at 2p24–p25 [49]. It may be significant that such QTLs tend to cluster in the genome, suggesting some commonality in molecular mechanisms of etiology for vascular diseases, cancer, and autoimmunity, all of which have a TGFβ1 involvement.

Tgfbm3 is a particularly interesting locus in relation to genetic interactions with the Tgfb1 gene. The skin cancer susceptibility locus Skts5 has been mapped to this region of chromosome 12 in a large interspecific Mus spretus × Mus musculus NIH F1 backcross [46]. This locus was detected by multiple stepwise regression methods designed to detect possible synergistic interactions between loci throughout the mouse genome [46]. This series of simulations showed that the strongest interaction in the genome was between Skts1 on chromosome 7 and Skts3 on chromosome 12. Intriguingly, the Tgfb1 gene is within the proximal Skts1 region, and the region of Skts5 with which it interacts corresponds exactly with a 7-Mb interval encompassing Tgfbm3 (J. Mao, A. Balmain, and R.J.A., unpublished results).

The Tgfbm3 region is also significant in human cancer. First, polymorphisms in ODC have been shown to be associated with risk for recurrence of colon adenoma [50]. Second, a recent study utilizing a bacterial artificial chromosome (BAC) comparative genomic hybridization (CGH) microarray to detect recurrent copy number aberrations in a cohort of 64 human prostate tumors obtained following radical prostatectomy revealed that 60% of tumors have amplification of a small region at 2p25.1, corresponding to the syntenic area of Tgfbm3 [51]. Due to the high resolution of their BAC CGH, Paris et al. [51] were able to define the minimal amplicon as a 2-Mb interval that includes ODC, TIEG2, RRMI2, and IDB2, i.e., aligning perfectly with Tgfbm3. These observations taken together are particularly exciting since polymorphisms in the TGFβ1 gene are known to alter risk of developing invasive prostate cancer [22].

In conclusion, we have identified two interacting loci that can modify developmental angiogenesis. These loci may
also be important in postnatal angiogenesis [5], atherosclerosis [48], immune disorders [52], and cancer [46,51]. The bias of our study was to identify Tgfb1-interacting genes. Interestingly, synergetic interaction between Tgfb1 and Tgfbm3 has been independently detected in a totally different model system, mouse skin carcinogenesis [46] (J. Mao, A. Balmain, and R.J.A., unpublished results), suggesting that this genetic interaction might be important in other biological systems. Polymorphisms in TGFBI in humans have already been shown to be associated with risk of developing various diseases [16,18–22,53–57]. However, the magnitude of the effect of this single-gene polymorphism on disease risk is very small, almost certainly due to the existence of TGFβ1 modifier genes that may enhance or mask genetic association. Combinatorial genetic association studies between TGFBI and its modifying genes should be more powerful in determining disease risks for pathologies in which a genetic association between TGFBI has already been established.

Materials and methods

Animals

Tgfb1+/− mice [26] were bred through four generations into either the inbred C57BL/6JOlaHsd (C57) or NIH/OlaHsd (NIH) genetic backgrounds in a conventional animal facility in Glasgow, United Kingdom, and some experiments were performed at this stage. Mice were then re-derived by embryo transfer and bred in a barrier facility in the United States. C57 was bred a further six generations to C57BL/6NTac, i.e., to backcross generation 10 (BC10), and NIH mice were bred a further six generations to NIH/OlaHsd.

Genomic DNA extraction and PCR analysis

Genomic DNA was extracted from tail biopsies using a DNEasy 96-well kit (Qiagen, Valencia, CA) or by automatic DNA isolation (Autogene, Framingham, MA). Tgfb1 genotyping was performed using a four-primer PCR. The wild-type allele generated a 402-nt band using primers B1 (TCACCCGCTGCTAATGGTGAGCCGC) and B2 (ACACCTTCATCTCTTGTGAAGG). The null mutant allele generated a 350-nt product using primers B3 (CATG-GAGCTGGTGAAACGGGC) and B4 (TCCATCTGGACAGGACTAGT). Dinucleotide repeat (SSR) primer sequences were obtained from http://www.jax.org or http://www.celera.com.

Genetic crosses and genome scan

C57/NIH.F1 mice were generated by intercrossing NIH and C57 Tgfb1+/− mice. Tgfb1+/− C57/NIH. F1 mice were then intercrossed and DNA was collected from all newborn F2 mice and genotyped for Tgfb1. All Tgfb1−/− neonates were then genotyped with the SSR markers specified under Results. Genetic linkage disequilibrium was assessed by nonparametric genetic analysis performed using a χ2 test with 2 df for an additive model. Where markers showed significant linkage disequilibrium, Tgfb1+/+ and/or Tgfb1+/− F2 littermates were typed with the same marker, to exclude nonrandom segregation distortion unrelated to the Tgfb1−/− genotype. Lod scores were calculated as −log10(P value) [58]. CIs were estimated as support intervals according to the calculations of Dupuis and Siegmund [32], whereby LODmax − 1 approximates a 90% CI at low marker density, but at high marker density LODmax − 1 ≥95% CI.

Estimation of STB and genotype-specific penetrance

It was assumed that there was no selective loss of Tgfb1+/− embryos in utero. Thus, the total number of Tgfb1−/− conceptions was estimated to be equal to the total Tgfb1−/− born. The frequency of STB for Tgfb1−/− mice was calculated as the number of Tgfb1−/− neonates/the number of Tgfb1+/−. The overall penetrance of STB (PSTB) for a particular cross is equal to the frequency of STB.

The genotype-specific penetrance (PNN, PNC, or PCC) = observed Tgfb1−/− neonates/expected Tgfb1−/− conceptions, where the number of Tgfb1−/− conceptions was estimated from PSTB, together with the expected Mendelian ratio (1:2:1) of an F1 intercross.

Congenic mice

Congenic mice were generated by backcrossing F2 mice from an F1 intercross, through several generations (>n = 4 up to n = 10) to recipient C57 or NIH mice. At each generation, selection was made for donor markers linked to the chromosomal region of interest. The minimal congenic regions for C57.Tgfbm1NIH mice included the NIH markers D5Mit387 (26.6 Mb)–D5Mit114 (46.8 Mb) or D5Mit348 (23.1 Mb–D5Mit106 (43.9 Mb); for NIH.Tgfbm1C57 this included the C57 markers D5Mit76 (34.1 Mb)–D5Mit197 (63.7 Mb). For C57.Tgfbm3NIH and NIH.Tgfbm3C57 the congenic region spanned from proximal to D12Mit56 (12.4 Mb) to distal to D12Mit105 (19.4 Mb). Congenic test crosses were set up by intercrossing Tgfb1+/− mice, each of which was homozygous for the congenic region of interest, and assessing the Tgfb1−/− STB rate.

Genetic interaction between Tgfbm1 and Tgfbm3

Several tests were used to investigate the nature of genetic interactions between the Tgfbm1 and Tgfbm3. (i) In the test for mutually exclusive action, for each of the six possible groups of mice (i.e., Tgfbm1mNIH/NIH, Tgfbm1mNIH/C57, Tgfbm1mC57/C57, Tgfbm3mNIH/NIH, Tgfbm3mNIH/C57, and Tgfbm3mC57/C57), the genotype of one genetic locus was fixed and the distribution of genotypes at the second locus was compared to the expected Mendelian ratio by χ2 test. (ii) In the test for additive interactions, a modification of the methods of Risch et al. [34] was used to estimate additive
versus multiplicative mode of action. Mathematically, the “additive” mode of interaction between two genes indicates that the genotype frequency of a combined genotype for two genes is the modified sum of the frequencies of the single genotype for each gene. The genotype frequency for a specific genotype ($f(n)$ denotes the penetrance ($P(n)$ of that genotype or the fraction of the total animals that survive to birth and have the “n” genotype. If the “individual frequency” for the three possible genotypes (p, q, and r) of “gene A” and “gene B” is designated $f(Ap)$, $f(Aq)$, $f(Ar)$, $f(Bp)$, $f(Bq)$, and $f(Br)$, then the frequency for a joint genotype, $f(AnBn) = 1/6 \times [f(An)+f(Bn)] (n = p, q, or r)$. (iii) In the test for multiplicative interactions, the multiplicative mode of interaction indicates that the frequency of a joint genotype of two genes is the product of the individual frequency of the two single genotypes, $f(AnBn) = f(An) \times f(Bn) (n = p, q, or r)$. 

Acknowledgments

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Appendix A. Supplementary data


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