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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 β1 (TGFβ1) will enhance the understanding of TGFβ1 action in vivo and may help predict which patients would respond well to anti-TGFβ 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.

Keywords: Angiogenesis; Epistatic interaction; Genetic modifier; Mouse embryogenesis; Prenatal lethal; TGFβ; 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; TGFβ, transforming growth factor β.

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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 *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 β (TGFβ)[7] signaling pathway.

TGFβ1 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 TGFβ1 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 *ACVRL1* results in hereditary hemorrhagic telangiectasia (HHT), a vascular dysplasia with late age of onset [14]. Genes encoding components of the TGFβ1 signaling pathway, including *TGFB1* [16] and *TBRI* [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]. TGFβ1 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 TGFβ1 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 TGFβ1 should enhance our understanding of the mode of action of TGFβ1 in vivo. This is particularly important now that small-molecule inhibitors that act on the TGFβ signaling pathway are being developed to treat fibrosis and cancer [25]. Knowledge of the genetic basis for variation in response to attenuation of TGFβ may eventually be useful in identifying patients that may or may not respond well to anti-TGFβ 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 *postcoitum* (dpc) from defects in development of the yolk sac [10–12] or 3 weeks *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 NIHC57F1 intercross [11]. The phenotype scored was survival to birth (STB) of *Tgfb1*−/− mice compared to wildtype littermates 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 F_1 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.

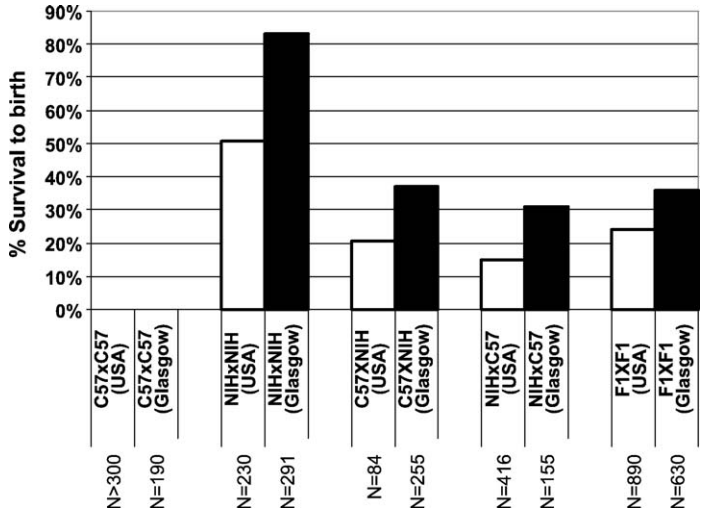

Fig. 1. The penetrance of *Tgfb1*−/− STB is reduced in US-bred mice. *Tgfb1*+/− intercrosses were set up on the indicated genetic backgrounds. According to standard convention, the maternal strain is shown on the left of the cross and the paternal strain is shown on the right. *Tgfb1*−/− STB rate was 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.

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*−/− mice [11] to 157. The U.S.-bred *Tgfb1*−/− neonates were collected in batches from F_1 parents of BC5 through BC8. Initially, 46 new Glasgow-bred *Tgfb1*−/− neonates were added, together with a further 38 U.S.-bred *Tgfb1*−/− neonates. The additional *Tgfb1*−/− F_2 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). LOD_{max} = 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 F_2 mice appeared weaker (LOD = 1.1, n = 181) than in the Glasgow data set (LOD = 4, n = 119);

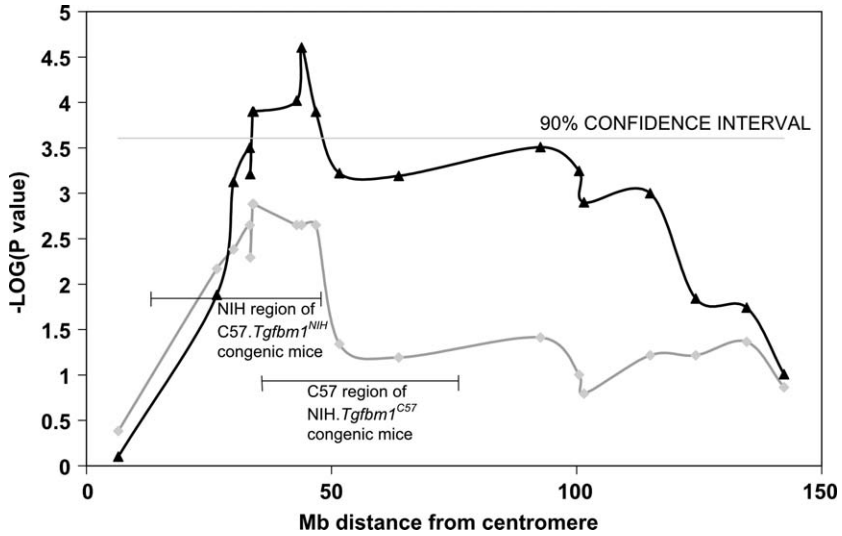

Fig. 2. High-resolution genetic linkage analysis of *Tgfbm1*. *Tgfb1*−/− neonates from *Tgfb1*+/− F_1/NIHC57F1 intercrosses were collected in batches according to country of birth (United States or Glasgow, UK). Animals were genotyped with over 20 SSR markers on chromosome 5, with the highest density around *D5Mit106*. The data were analyzed by χ^2 with 2 df for deviation from a simple additive genetic model (1NN: 2NC: 1CC). Lod score = $-\log_{10}(P \text{ value})$. The lower curve represents data from 73 of the original 80 Glasgow-bred mice reported in Bonyadi et al. [11]. The upper curve represents data from 157 mice, 119 Glasgow-bred together with 38 U.S.-bred. The 95% CI is shown. Markers mapped are as follows: *D5Mit145* (6.5 Mb), *D5Mit387* (26.6 Mb), *D5Mit228* (30 Mb), *D5Mit420* (33.3 Mb), *D5Mit268*, (33.4 Mb) *D5Mit352* (33.9 Mb), *D5Mit76* (34.1 Mb), *D5Mit79* (42.9 Mb), *D5Mit106* (43.9 Mb), *D5Mit11* (46.8 Mb), *D5Mit233* (51.6 Mb), *D5Mit197* (63.7 Mb), *D5Mit7* (102.6 Mb), *D5Mit23* (100.5 Mb), *D5Mit115* (101.5 Mb), *D5Mit188* (115 Mb), *D5Mit161* (124.3 Mb), *D5Mit168* (134.7 Mb), and *D5Mit191* (142.3 Mb). Megabase positions of markers are based on http://www.ensembl.org m32 assembly. The approximate locations of congenic regions of our homozygous congenic lines are indicated in the figure.

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 ($P = 0.12$, χ^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 × 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.$Tgfbm1^{NIH}$). STB rates of *Tgfb1−/−* mice were determined in *Tgfb1*+/− intercrosses of homozygous congenic C57.$Tgfbm1^{NIH}$ 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 F_1 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.$Tgfbm1^{NIH}$ congenic (and possibly diluted during rederivation 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.$Tgfbm1^{NIH}$, 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.$Tgfbm1^{C57}$) 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 F_1 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 F_2 *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 F_2 *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 $LOD_{max}-1$ [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

Cross	Line	Donor	Recipient	BC generation	+/+	+/−	−/−	Total	STB rate
1	NIH	NIH	NIH	5–6	71	123	36	230	51%
2	C57	C57	C57	6–7	98	198	0	296	0%
3	C57-$Tgfbm1^{NIH}$	NIH	C57	5	94	190	1	290	1%
4	NIH-$Tgfbm1^{C57}$	C57	NIH	4–5	157	301	44	502	28%
5	C57-$Tgfbm3^{NIH}$	NIH	C57	4–6	86	204	1	291	1%
6	NIH-$Tgfbm3^{C57}$	C57	NIH	4	71	100	20	191	28.0%
7	C57-$Tgfbm1^{NIH}$/$Tgfbm3^{NIH}$	NIH	C57	5	73	78	5	104	7%

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

Table 2
Tgfbm1 interacts with other modifier genes to rescue *Tgfb1*−/− mice from prenatal death

	Cross	*Tgfb1*+/+	*Tgfb1*+/−	*Tgfb1*−/−	Total	STB rate
1	C57-*Tgfbm1*.NIH × NIH[a]	169	401	52	622	30.8%
2	C57 × NIH[a]	228	404	39	671	17.1%

Note. *Tgfb1*+/− intercrosses were set up within the above mouse lines. Neonates were collected at birth and genotyped for *Tgfb1*.
[a] Reciprocal crosses showed no statistical differences and were therefore pooled.

Tgfbm3 function confirmed in F_2 intercross and congenic mice

Additional support for the presence of *Tgfbm3* comes from STB studies on an F_2 *Tgfb1*+/− intercross, where the F_2 mice were derived from an $F_1 \times F_1$ cross. *Tgfb1*+/− mice, homozygous for $Tgfbm3^{C57}$, were intercrossed to each other, as were *Tgfb1*+/− mice, homozygous for $Tgfbm3^{NIH}$. Whereas those *Tgfb1*−/− mice derived from the homozygous $Tgfbm3^{C57}$ parents showed only 7.5% STB (159 *Tgfb1*+/+: 293 *Tgfb1*+/−: 12 *Tgfb1*−/−), mice derived from the homozygous $Tgfbm3^{NIH}$ parents showed 50% STB (88 *Tgfb1*+/+: 156 *Tgfb1*+/−: 44 *Tgfb1*−/−), a very significant difference ($P = 9.3 \times 10^{-10}$).

Congenic mice were generated containing the *Tgfbm3* region from the NIH strain on an otherwise C57 genetic background (C57.$Tgfbm3^{NIH}$) and vice versa (NIH.$Tgfbm3^{C57}$). STB rates of *Tgfb1*−/− mice were determined in intercrosses of homozygous congenic *Tgfbm3*, heterozygous *Tgfb1*+/− mice (Table 1). Despite the strong genetic linkage to this locus, the $Tgfbm3^{NIH}$ modifier in isolation was able to rescue *Tgfb1*−/− mice at only low efficiency (Table 1, row 5), demonstrating that, like *Tgfbm1*, this locus requires interaction with other genetic loci for full effect. However, homozygosity for $Tgfbm3^{C57}$ on an otherwise NIH genetic background decreased the STB rate compared to that of NIH (Table 1, row 6), validating the presence of *Tgfbm3* on chromosome 12.

Tgfbm1 acts in a codominant/additive fashion, whereas the $Tgfbm3^{NIH}$ 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 *Tgfbm3* 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 *Tgfbm1* or *Tgfbm3* (Table 4). The 1.45-fold higher penetrance of $Tgfbm1^{NC}$ compared to $Tgfbm1^{CC}$ ($P = 0.02$) is similar to the 1.4-fold higher penetrance of $Tgfbm1^{NN}$ compared to $Tgfbm1^{NC}$ ($P = 0.008$), suggesting that the NIH allele of *Tgfbm1* acts in a purely additive/codominant fashion (Table 4), as we previously reported [11]. For *Tgfbm3* there is a 1.3-fold difference in penetrance between $Tgfbm3^{NC}$ and $Tgfbm3^{CC}$, but this is not quite statistically different from the ratio of 1 that would be expected if the $Tgfbm3^{NIH}$ allele was purely recessive ($P = 0.07$). However, the 2-fold difference in

Table 3
Cursory scan of F_2 *Tgfb1*−/− neonates from U.S.-bred *Tgfb1*+/− F_1 intercross

Marker	Centimorgan	NN	CN	CC	Total	*P* value
D1Mit403	100	12	20	7	39	0.520
D2Mit277	69	8	29	17	54	0.192
D3Mit6	23.3	13	27	7	47	0.276
D3Mit38	70.3	14	31	9	54	0.348
D4Mit178	35.5	11	27	8	46	0.410
D5Mit79	26	18	32	10	60	0.301
D6Mit19	33.5	14	26	13	53	0.972
D7Mit363	**2.2**	**2**	**14**	**35**	**51**	**3.0 × 10−12**
D8Mit190	21	22	23	14	59	0.081
D9Mit18	71	18	24	9	51	0.187
D10Mit95	51	14	31	9	54	0.348
D11Mit258	**65**	**23**	**34**	**6**	**63**	**0.008**
D11Mit151	13	3	19	40	62	2.5 × 10−12
D12Mit46	**16**	**23**	**28**	**7**	**58**	**0.012**
D13Mit3	10	15	32	16	63	0.976
D14Mit193	40	14	21	15	50	0.517
D16Mit114	41	20	24	16	60	0.231
D17Mit16	18.15	8	36	16	60	0.104
D18Mit243	24	16	16	16	48	0.069
D18Mit149	24	15	18	16	49	0.175
D19Mit103	52	30	52	29	111	0.795

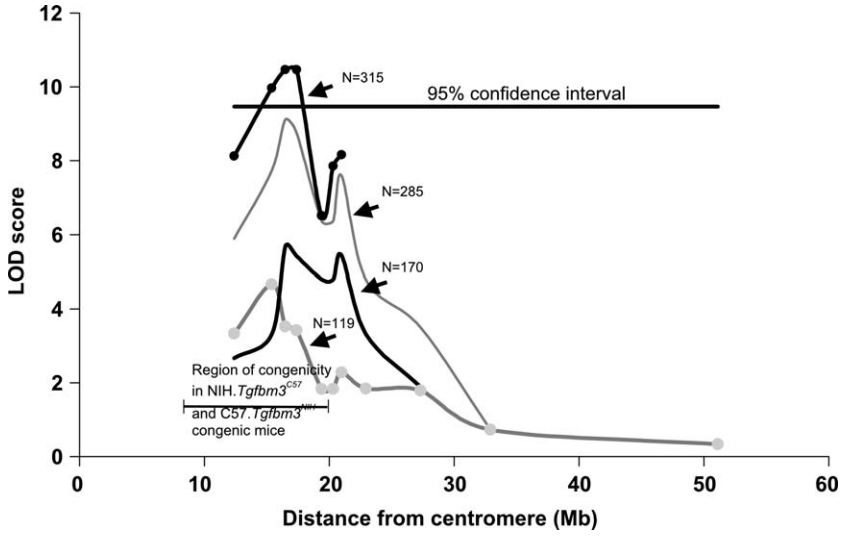

Fig. 3. High-resolution genetic linkage analysis of *Tgfbm3*. *Tgfb1*−/− neonates from *Tgfb1*+/− F_1/NIHC57F1 intercrosses were collected in batches according to country of birth (United States or United Kingdom) and backcross generation of parents. Animals were genotyped with 11 SSR markers on proximal chromosome 12 [*D12Mit56* (12.4 Mb), *D12Mit169* (15.4 Mb), *D12Mit170* (16.5 Mb), *D12Mit291* (17.4 Mb), *D12Mit105* (19.4 Mb), *D12Mit83* (20.3 Mb), *D12Mit283* (21 Mb), *D12Mit242* (22.9 Mb), *D12Mit46* (27.3 Mb), *D12Mit109* (32.9 Mb), and *D12Mit191* (51.1 Mb)]. The data were analyzed by χ^2 with 2 df for deviation from a simple additive genetic model (1NN: 2NC: 1CC). Lod score = $-\log_{10}(P$ value). The four curves, from lowest lod score to highest, represent the following: (a) $n = 119$ Glasgow-bred mice, (b) $n = 170$ U.S.-bred mice, (c) $n = 289$ data pooled from (a) and (b), (d) $n = 315$ for the seven most proximal markers. The 95% CI is shown. Megabase positions of markers are based on http://www.ensembl.org m32 assembly. The approximate locations of congenic regions of our homozygous congenic lines are indicated in the figure.

penetrance between $Tgfbm3^{NN}$ and $Tgfbm3^{NC}$ ($P = 6 \times 10^{-8}$) suggests that homozygosity for $Tgfbm3^{NN}$ significantly exceeds the effect of heterozygosity at this locus. Namely, the $Tgfbm3^{NIH}$ allele has some synergistic properties rather than being purely additive. Indeed, with the present data set, we cannot rule out the possibility that $Tgfbm3^{NIH}$ acts as a recessive allele.

Tgfbm1 and Tgfbm3 interact in a multiplicative fashion to rescue Tgfb1−/− from prenatal lethality

Since it is necessary for both *Tgfbm1* and *Tgfbm3* to interact with other genes within the genome in order to manifest the trait of efficient *Tgfb1*−/− 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 $Tgfbm1^{NIH/NIH}$; $Tgfbm3^{NIH/NIH}$ genotype in the context of an F_1 intercross background is ~70% (Table 5). This indicates that, on the F_1 intercross background, homozygosity for NIH at both of these loci is sufficient to rescue 70% of *Tgfb1*−/− 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 F_1 intercross, since 13/289 *Tgfb1*−/− 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 *Tgfbm1* and

Table 4
Relative penetrance of different genotypes of *Tgfbm1* and *Tgfbm3*

	D5Mit106			*D12Mit170*		
	Expected $Tgfb1^{-/-}$ conceptions[a]	Observed $Tgfb1^{-/-}$ neonates	Genotype-specific penetrance[b]	Expected $Tgfb1^{-/-}$ conceptions[a]	Observed $Tgfb1^{-/-}$ neonates	Genotype-specific penetrance[b]
NN	272.5	103	38%	284.5	129	45.3%
NC	545	147	27%	569	134	23.6%
CC	272.5	50	18%	284.5	50	17.6%
Total	1090[a]	300	27.5%[c]	1138[a]	313	27.5%[c]

[a] Expected total *Tgfb1*−/− conceptions = number of observed total *Tgfb1*−/− neonates/*Tgfb1*−/− STB rate; expected genotype-specific conceptions = Mendelian fractions of expected total *Tgfb1*−/− conceptions (0.25:0.5:0.25).
[b] Genotype-specific penetrance = observed *Tgfb1*−/−neonates/expected *Tgfb1*−/− conceptions.
[c] Total *Tgfb1*−/− STB rate in F_1 intercross (see Fig. 1).

Table 5
Genetic interaction of *Tgfbm1* and *Tgfbm3*

	$Tgfbm3^{NN}$	$Tgfbm3^{NC}$	$Tgfbm3^{CC}$	Total *Tgfbm1*	*P* value[a]
$Tgfbm1^{NN}$	45 (68%)	38 (21%)	17 (25%)	100	2.2×10^{-5}
$Tgfbm1^{NC}$	64 (48.4%)	65 (24.6%)	14 (10.6%)	143	1.4×10^{-8}
$Tgfbm1^{CC}$	15 (22.7%)	18 (13.7%)	13 (19.7%)	46	0.3
Total*Tgfbm3*	124	121	44	Grand total = 289	
P value[a]	6.6×10^{-4}	0.026	0.037		

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_{NNNN} = 68%.
[a] 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 NIH mice (Fig. 1 and Table 1, Row 1). 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 $C57.Tgfbm1^{NIH/NIH};Tgfbm3^{NIH/NIH}$ congenic 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*−/− 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*−/− NIH mice that retain C57 genomic DNA encompassing either of the modifier regions show a significant decrease in STB, compared to those carrying the $Tgfbm^{NIH}$ 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*−/− mouse. However, the two loci interact multiplicatively to significantly increase this *Tgfb1*−/− STB rate, illustrating the importance of genetic interactions in this phenotype.

Tgfbm1 was previously identified in a smaller data set of F_1 intercross *Tgfb1*−/− 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*−/− 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*+/− 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, $Tgfbm2^{129}$, previously detected in a 129 × C57 F_1 intercross [12]. It is possible that $Tgfbm1^{NIH}$ requires interaction with $Tgfbm2^{129}$ [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 $Tgfbm2^{129}$, but no linkage in mice lacking a $Tgfbm2^{129}$ allele ($P = 0.13$, $n = 36$) (data not shown), suggesting dependence of *Tgfbm1* linkage on the presence of at least one $Tgfbm2^{129}$ 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*−/− 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 *Msx. 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 (*Itgb1bp1/LCAP1*), and TNFα-converting enzyme (*Adam17*).

A number of mouse qualitative trait locus (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 F_1 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 *Skts5* on chromosome 12. Intriguingly, the *Tgfb1* gene is within the proximal *Skts1* region, and the region of *Stks5* 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*, *RRM2*, and *IDB2*, i.e., aligning perfectly with *Tgfbm3*. These observations taken together are particularly exciting since polymorphisms in the *TGFB1* 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, synergistic 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 *TGFB1* 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 *TGFB1* and its modifying genes should be more powerful in determining disease risks for pathologies in which a genetic association between *TGFB1* 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 (TCACCCGCGTGCTAATGGTGGACCGC) and B2 (ACACCTTCCATTCTCTTGAGCTGGG). The null mutant allele generated a 350-nt product using primers B3 (CATGGAGCTGGTGAAACGGAAGCGC) and B4 (TCCATCTGCACGAGACTAGT). Dinucleotide repeat (SSR) primer sequences were obtained from http://www.jax.org or designed in-house using Primer3 software (Massachusetts Institute of Technology, Cambridge, MA), based on genomic sequences available at http://www.celera.com.

Genetic crosses and genome scan

C57/NIH.F_1 mice were generated by intercrossing NIH and C57 *Tgfb1*+/− mice. *Tgfb1*+/− C57/NIH. F_1 mice were then intercrossed and DNA was collected from all newborn F_2 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 χ^2 test with 2 df for an additive model. Where markers showed significant linkage disequilibrium, *Tgfb1*+/+ and/or *Tgfb1*+/− F_2 littermates were typed with the same marker, to exclude nonrandom segregation distortion unrelated to the *Tgfb1*−/− genotype. Lod scores were calculated as $-\log_{10}(P$ value) [58]. CIs were estimated as support intervals according to the calculations of Dupuis and Siegmund [32], whereby $\mathrm{LOD}_{\max} - 1$ approximates a 90% CI at low marker density, but at high marker density $\mathrm{LOD}_{\max} - 1 \geq 95\%$ 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 (P_T) for a particular cross is equal to the frequency of STB.

The genotype-specific penetrance (P_{NN}, P_{NC}, or P_{CC}) = observed $Tgfb1^{-/-}$ neonates/expected $Tgfb1^{-/-}$ conceptions, where the number of $Tgfb1^{-/-}$ conceptions was estimated from P_T, together with the expected Mendelian ratio (1:2:1) of an F_1 intercross.

Congenic mice

Congenic mice were generated by backcrossing F_2 mice from an F_1 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.$Tgfbm1^{NIH}$ mice included the NIH markers *D5Mit387* (26.6 Mb)–*D5Mit11*(46.8 Mb) or *D5Mit348* (23.1 Mb–*D5Mit106* (43.9 Mb); for NIH.$Tgfbm1^{C57}$ this included the C57 markers *D5Mit76* (34.1 Mb)–*D5Mit197* (63.7 Mb). For C57.$Tgfbm3^{NIH}$ and NIH.$Tgfbm3^{C57}$ 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., $Tgfb1m^{NIH/NIH}$, $Tgfb1m^{NIH/C57}$, $Tgfb1m^{C57/C57}$, $Tgfb3m^{NIH/NIH}$, $Tgfb3m^{NIH/C57}$, and $Tgfb3m^{C57/C57}$), 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 $\chi 2$ 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 × [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) × f(Bn) (n = p, q, or r).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygeno.2004.09.003.

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