Congenic Mapping and Allele-Specific Alteration Analysis of Stmm1 Locus Conferring Resistance to Early-Stage Chemically Induced Skin Papillomas

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Abstract

 Genome-wide association studies have revealed that many low-penetrance cancer susceptibility loci are located throughout the genome; however, a very limited number of genes have been identified so far. Using a forward genetics approach to map such loci in a mouse skin cancer model, we previously identified strong genetic loci conferring resistance to early-stage chemically induced skin papillomas on chromosome 7 with a large number of [(FVB/N×MSM/Ms)×FVB/N] F1 backcross mice. In this report, we describe a combination of congenic mapping and allele-specific alteration analysis of the loci on chromosome 7. We used linkage analysis and congenic mouse strains to refine the location of Stmm1 (Skin tumor modifier of MSM 1) locus within a genetic interval of about 3 cM on proximal chromosome 7. In addition, we used patterns of allele-specific imbalances in tumors from F1 backcross and N10 congenic mice to narrow down further the region of Stmm1 locus to a physical distance of about 5.4 Mb. To gain the insight into the function of Stmm1 locus, we carried out a long term BrdU labelling experiments with congenic mice containing Stmm1 locus. Interestingly, we observed a decrease of BrdU-LRCs (Label Retaining Cells) in a congenic strain heterozygous or homozygous for MSM allele of Stmm1. These results suggest that Stmm1 resposible genes may have an influence on papillomagenesis in the two-stage skin carcinogenesis by regulating epidermal quiescent stem cells.

Introduction

Identification of the specific genetic variants responsible for increased susceptibility to familial or sporadic cancers remains an important but challenging goal with major implications for the prediction of individual cancer risk, as well as for improved strategies for prevention or targeted therapy [1–4]. Present approaches to detect low-penetrance tumor-susceptibility alleles in humans involve association studies using DNA samples from hundreds or thousands of cancer patients, and an equal number of well-matched controls. Such studies are plagued by confounding factors such as population heterogeneity, weak effects, and genetic interactions, and require a very large number of cases and controls to reach statistical significance [5–7]. For many complex-trait diseases, including cancer, low-penetrance susceptibility alleles account for a very small proportion of the total cancer risk [8], leading to considerable discussion of the best approaches to discover the majority of disease-causing alleles in human populations.

For these reasons, complementary gene mapping and validation approaches including cross-species comparisons using animal models are required to identify genes that modify disease phenotypes, including the risk of developing cancer [9–11]. Exploiting the resistance of M. spretus to the two-stage skin carcinogenesis model involving 7,12-dimethylnaphth[a]anthracene (DMBA) initiation and subsequent promotion with 12-O-tetradecanoylphorbol-13-acetate (TPA), 15 skin tumor susceptibility loci, Skts1-15, were identified in a cross between the resistant Japanese M. spretus (MSM/Ms) and FVB/N [12]. Interestingly, we observed a decrease of BrdU-LRCs (Label Retaining Cells) in a congenic strain heterozygous or homozygous for MSM allele of Stmm1. These results suggest that Stmm1 responsible genes may have an influence on papillomagenesis in the two-stage skin carcinogenesis by regulating epidermal quiescent stem cells.
wild-derived inbred strain MSM/Ms and the susceptible strain FVB/N [16]. In the present study, we used interval-specific congenic mouse strains to refine the location of Stmm1 within a genetic interval spanning approximately 3 cM on proximal chromosome 7. In addition, we used patterns of allele-specific imbalances in tumors from F1 backcross and N10 congenic mice to further refine the location of Stmm1. High frequency of either MSM allele loss or FVB allele gain was detected in the region corresponding to physical distance of about 5.4 Mb. To gain the further insight into the function of Stmm1 locus, we carried out BrdU chase experiments with congenic lines containing Stmm1. As a result, we observed a decrease of BrdU-LRCs in congenic strains heterozygous or homozygous for the MSM allele of Stmm1 on proximal chromosome 7. These results suggest that gene(s) located within the Stmm1 locus may have an influence on papillomagenesis in the two-stage skin carcinogenesis by regulating epidermal quiescent stem cells.

**Results**

**A Strong Papilloma Resistance Locus, Stmm1 (Skin Tumor Modifier of MSM), on Proximal Chromosome 7**

Previous QTL analysis identified at least two independent skin tumor susceptibility loci (Skts1 and Skts2) on mouse chromosome 7 [12]. We have recently identified a series of skin papilloma resistance loci, Stmm (Skin tumor modifier of MSM) loci, using F1 backcross mice between a wild derived inbred mouse strain (MSM) and a susceptible inbred mouse strain (FVB/N). Two of these loci are located in the vicinity of Skts1 and 2 on chromosome 7: Stmm1 was mapped near the markers D7SNP507 and D7SNP513, and Stmm2 near the markers D7SNP6 and D7Mit10 [16]. (Figure 1A). To confirm the presence of low-penetrance susceptibility genes in these regions, we selected resistant F1 backcross mice for further backcrossing to FVB/N mice to generate two N10 congenic mouse lines that span either the Skts1 and Skts2 regions. These results clearly suggest that gene(s) located within the Skts1 region may have a strong suppressive effect on papillomagenesis.

**Table 1. Papilloma incidence in the congenic lines.**

| Congenic line Chromosomal region (cM) | 10 weeks | 20 weeks | P-value |
|--------------------------------------|----------|----------|---------|
| a 1.91–60.49                          | 3.6 (0–14; n = 15) | 20.5 (16–22; n = 14) | 3.2E-10 |
| b 68.16–88.85                         | 12.5 (4–21; n = 14) | 20.8 (12–22; n = 14) | 0.13 |
| c 20.65–60.49                         | 2.7 (0–13; n = 7) | 18.2 (7–27; n = 5) | 3.6E-07 |
| d 43.4–60.49                          | 3.7 (0–13; n = 5) | 20.3 (16–25; n = 5) | 0.31 |
| e 50.35–54.45                         | 3.6 (0–13; n = 5) | 19.8 (11–26; n = 5) | 0.31 |
| f 1.91–20.65                          | 20.2 (1–11; n = 5) | 19.8 (11–26; n = 5) | 0.49 |
| g 20.65–54.45                         | 20.8 (1–11; n = 5) | 19.8 (11–26; n = 5) | 0.31 |
| h 1.91–51.6                           | 3.6 (0–13; n = 5) | 19.8 (11–26; n = 5) | 0.31 |
| i 20.65–54.45                         | 20.8 (1–11; n = 5) | 19.8 (11–26; n = 5) | 0.31 |

Abbreviations: cM, centi-morgan from MGI; N.D., not determined; MF, MSM/FVB; FF, FVB/FVB. P-value is from standard t-test.

**Figure 1B, 1C and Table 1.** In contrast, heterozygous MSM/FVB (MF) mice of a congenic line (a) developed an average of 3.6 ± 3.8 papillomas/mouse at 10 weeks after initiation (n = 15; compared with control: P = 3.2E-10). (Figure 1B, 1C and Table 1), consistent with this region conferring resistance to skin papillomas. On the other hand, MF heterozygous mice of a congenic line (b) had little effect on papilloma development. The number was an average of 12.5 ± 5 papillomas/mouse at 10 weeks (compared with control: P = 0.13) and 38.3 ± 8.7 papillomas/mouse at 20 weeks after initiation (n = 10; compared with control: P = 0.07) (Figure 1B, 1C and Table 1). In summary, MF heterozygous mice of a line (a), corresponding to Stmm1, developed significantly fewer papillomas than FVB/N mice. On the other hand, MF heterozygous mice of a line (b), corresponding to Stmm2, developed about the same number of papillomas as control wild type FVB/N mice. These results clearly suggest Stmm1 has a strong suppressive effect on papilloma development.
Figure 1. Genetic linkage map and papilloma incidence in congenic lines (a) and (b) on mouse chromosome 7. (A) Two significant linkage peaks, Stmm1 and 2 were mapped in the previous report [16]. Two orange and white bars represents two congenic lines (a) and (b). The orange bars indicate the heterozygous MF region, while the white bars indicate the homozygous FF region. Several well-known genes located on chromosome 7 are indicated with other papilloma resistance loci, Skts1, 2, and genetic markers. Genetic positions shown are according to the Ensembl database (http://uswest.ensembl.org/index.html), the Mouse Genome Informatics Database (http://www.informatics.jax.org/). (B) Comparison of average papilloma numbers/mouse between a congenic line (a) and (b). The top panel shows papilloma incidence in a congenic line (a). The bottom panel shows papilloma incidence in a congenic line (b). The orange bars represent papilloma numbers of MF heterozygous congenic mice. The grey bars represent those of FF heterozygous congenic mice. (C) Photos of representative mice on TPA treatment. Dorsal back skin of MF heterozygous mice of congenic lines (a), (b) and a homozygous FF mouse of a congenic line (a) as a control at 10 weeks after initiation. doi:10.1371/journal.pone.0097201.g001
Narrowing down the Genetic Distance of *Stmm1* on Chromosome 7

On the basis of skin carcinogenesis experiments of lines (a) and (b), we focused on a line (a), which contains the *Stmm1* locus and showed a much stronger suppressive effect on papilloma development. A series of congenic lines (c–i) containing different overlapping regions were generated from mice of a line (a) (Figure 2A and Table 1). These sub-congenic lines were subjected to the DMBA/TPA chemical carcinogenesis protocol and their papilloma development was monitored for a period of 20 weeks (Table 1). For each congenic line, we documented the number of papillomas at 20 weeks after initiation.

Figure 2A and 2B show a genetic map and papilloma incidence of each congenic line. Of the seven sub-congenic lines (c–i) tested, four lines (c, d, e, and f) showed significant linkage with papilloma incidence (Figure 2B and Table1). By determining the minimum-overlapping region within the four positive congenic lines (c, d, e, and f) and by excluding the regions of the three congenic lines (g, h, and i) that did not show an effect (Figure 2B and Table1), we were able to narrow down the location of the *Stmm1* region to an interval of about 3 cM (indicated by a blue arrowed line) from 54.54 to 57.52 cM (Figure 2A and 2B). This region does not overlap with the previously reported *Skts1* locus. These data suggest that *Stmm1* is a novel skin tumor susceptibility locus on chromosome 7, conferring resistance to papilloma development.

Allelic Imbalances in Favor of the FVB/N Allele of the *Stmm1* Locus in Skin Tumors from F1 Backcross and Congenic Mice

We carried out a detailed investigation of allelic imbalance on chromosome 7 to determine whether somatic changes would provide a more specific localization of *Stmm1*. This allelic imbalance analysis was performed using twelve informative microsatellite markers (for detailed information, see Table 2). Figure 3A shows the frequency of allelic imbalances near the *Stmm1* locus in papillomas from F1 backcross (n = 30) and N10 congenic mice (n = 26). 5/30 and 5/26 papillomas from F1 backcross and N10 congenic mice showed losses of MSM alleles across the whole region, but others exhibited regional losses involving smaller chromosome fragments (Figure 3B). Papillomas from N10 congenic mice showed the higher frequency of allelic imbalances than those from F1 backcross mice (17/26 vs 12/30, \(P = 0.0005\)), probably because other MSM alleles retained in genomic DNA of F1 backcross mice had an influence on the frequency of allelic imbalances in *Stmm1*. Two allelic imbalance peaks were detected within the minimum-overlapping region of 3 cM identified with the multiple congenic lines of *Stmm1* (Figure 3A and 3B). One was localized within 3.4 Mb region between D7Mit96 (100.5 Mb) and D7Mit131 (103.67 Mb), the other was within 2 Mb region between D7Mit95 (105.75 Mb) and D7Mit124 (107.68 Mb). When these two regions are combined, the total physical size of the candidate region can be cut down to about 5.4 Mb. This interval still contains a large number of genes. Further congenic and somatic mapping will refine the candidate region to facilitate gene identification.

BrdU Chase Experiments using *Stmm1* Congenic Mice

In the original report, we classified papillomas into three categories based on their diameter, and carried out linkage analysis for each category. This analysis revealed strong linkage at *Stmm1* and *Stmm2* on chromosome 7 to the total papilloma number, the number of papillomas <2 mm, and the number of papillomas of 2–6 mm in diameter. However, these linkage peaks at *Stmm1* and *Stmm2* completely disappeared when the analysis was confined to papillomas >6 mm. These results suggested that *Stmm1* and 2 genes function only at an early papilloma stage, but are not involved at the later stage of papilloma progression [16]. To gain the insight into the function of *Stmm1* locus, we carried out BrdU-LRC analysis with congenic lines (Figure 4). Using a thymidine derivative, such as BrdU, DNA strands of adult stem cells can be labeled at the moment they are synthesized during development. Adult stem cells are quiescent and their DNA strands segregate non-randomly. As such, epidermal stem cells can retain the BrdU label indefinitely during adulthood and are accordingly referred to as label retaining cells (LRCs). The timetable of this BrdU chase experiment is shown in Figure 4A (for detailed information, see Material and Methods).

As a result, heterozygous MF mice of a congenic line (a) showed significantly reduced BrdU-LRCs in the bulge of hair follicles, compared with those of homozygous FF mice of a line (a), as a positive control (\(P = 0.98^{-3}\)) (Figure 4A, 4B, and 4F). In addition, the same BrdU chase experiment was carried out using heterozygous MF mice of a congenic line having the region of D4Mit17–D4Mit15 on chromosome 4, previously found to confer strong resistance to late stage papillomas (>6 mm in diameter) [16]. Heterozygous MF mice of a chromosome 4 congenic line did not exhibit a change in the number of BrdU-LRCs compared with that of their homozygous FF littermates (Figure 4C, 4D, and 4F). These results suggest that gene(s) located within the *Stmm1* locus may have an influence on papillomagenesis step in the two-stage skin carcinogenesis by regulating epidermal quiescent stem cells.

Discussion

In this study, the location of *Stmm1* was refined to a genetic interval of about 3 cM on proximal chromosome 7 by using multiple congenic lines. By taking advantage of allelic alterations in papillomas from F1 backcross and N10 congenic mice, we further narrowed down the physical interval to about 5.4 Mb. We carried out BrdU chase experiments with congenic lines containing *Stmm1*, and observed a decrease of BrdU-LRCs in a congenic strain that is M/F heterozygous or M/M homozygous for *Stmm1* on chromosome 7. These results suggest that *Stmm1* modifies papilloma development by regulating epidermal quiescent stem cells.

As previously shown, we screened stage-specific papilloma modifier loci on the basis of the size. *Stmm1* and *Stmm2* were identified on chromosome 7 as modifier loci conferring resistance to papillomas of smaller size [16]. They were mapped in the vicinity of *Skts1* and 2 loci, previously identified using a wild-derived inbred mouse strain, *M. spreus* [12]. *Skts1* was narrowed down to around 15 Mb region between D7Mit193 (31.4 cM) and D7Mit248 (35.2 cM), which is proximal to *Igf1r* by congenic analysis [17]. Carcinogenesis experiments using congenic mice presented in this report show that *Stmm1* locus is distal to *Skts1* (Figure 2A and 2B). In addition, mice of congenic lines (d, e, and f) having only *Skts1* region produced almost the same number of papillomas as mice of a congenic line (c) having both *Skts1* and *Stmm1* region did. Congenic lines (h and i) having only *Skts1* region didn’t show any effect (Figure 2A and 2B). However, the number of mice in congenic lines (g, h, and i) is relatively low (Table 1). We cannot completely exclude the possibility that the region of congenic lines (g, h, and i) has additional other *Stmm1* genes. Nevertheless, it seems likely that MSM mice do not have a modifier gene in *Skts1* region and *Stmm1* is independently capable
Figure 2. Summary of linkage analysis of sub-congenic lines for Stmm1 locus on proximal chromosome 7. (A) Each bar represents a different sub-congenic line. The location of the orange and white bars indicates the region on chromosome 7. The orange bars indicate the heterozygous MF region, while the white bars indicate the homozygous FF region. The blue arrowed line indicates the Stmm1 region narrowed down by congenic mapping. (B) Papilloma incidence of sub-congenic lines c (n = 7), d (n = 13), e (n = 12), f (n = 9), g (n = 5), h (n = 3), i (n = 5), and FVB (n = 36)
of reducing the risk of developing DMBA-TPA-induced papillomas. In contrast, mice of a congenic line (b) having Stmm2 showed much weaker effect on papilloma suppression (Figure 1). Stmm2 identified in the vicinity of Skts2 and Hras on chromosome 7 showed the same effect on allele specificity of Hras mutation as Skts2 did, suggesting that the same gene might be responsible for Skts2 and Stmm2 [16]. Although carcinogenesis experiments using congenic mice having Skts2 have not been reported yet, data presented in this report suggests that Stmm2 and Skts2 genes may have a small effect on papilloma multiplicity compared with Stmm1 (Figure 1A). However, a congenic line (b) does not fully cover Stmm2 region. The interval between D7Mit98 (60.49 cM) and

| Chromosome | Name   | cM   | bp                           |
|------------|--------|------|------------------------------|
| Chr7       | D7Mit21| 1.91 | 3266568–3266693              |
| Chr7       | D7Mit156| 20.65| 34801081–34801224            |
| Chr7       | D7Mit18 | -    | -                            |
| Chr7       | D7Mit276| 34.35| 62277634–62277744            |
| Chr7       | D7Nds1  | 43.46| 74676373–74676604            |
| Chr7       | D7Mit30 | 45.71| 81485676–81485905            |
| Chr7       | D7Mit319| 46.43| 82038932–82039044            |
| Chr7       | D7Mit350| 47.43| 83586089–83586207            |
| Chr7       | D7Mit62 | 48.36| 84640886–84641032            |
| Chr7       | D7Mit216| 49.01| 87447195–87447372            |
| Chr7       | D7Mit320| 49.64| 89436896–89436978            |
| Chr7       | D7Mit352| 50.64| 90599957–90600077            |
| Chr7       | D7Mit148| 51.6 | 92745007–92745138            |
| Chr7       | D7Mit184| 52.81| 96843943–96844092            |
| Chr7       | D7Mit351| 52.92| 96901062–96901168            |
| Chr7       | D7Mit354| 53.93| 98911768–98911890            |
| Chr7       | D7Mit96 | 54.36| 100505707–100505782          |
| Chr7       | D7Mit323| 54.45| 100875936–100876050          |
| Chr7       | D7Mit37 | 54.85| 103860643–103860803          |
| Chr7       | D7Mit131| 54.85| 103870606–103870726          |
| Chr7       | D7Mit95 | 55.98| 105751411–105751503          |
| Chr7       | D7Mit302| 55.98| 105794470–105794615          |
| Chr7       | D7Mit124| 57.21| 107683097–107683208          |
| Chr7       | D7Mit219| 57.21| 109096065–109096188          |
| Chr7       | D7Mit130| 57.52| 109788134–109788282          |
| Chr7       | D7Mit53 | 57.86| 110792641–110792834          |
| Chr7       | D7Mit356| 58.21| 111514329–111514441          |
| Chr7       | D7Mit281| 58.74| 112212330–112212440          |
| Chr7       | D7Mit222| 59.13| 112934466–112934612          |
| Chr7       | D7Mit98 | 60.49| 114917267–114917439          |
| Chr7       | D7Mit40 | 62.01| 116817442–116817642          |
| Chr7       | D7Mit330| 64.07| 119656690–119656814          |
| Chr7       | D7Mit255| 68.16| 124914439–124914573          |
| Chr7       | D7Mit104| 71.29| 129101439–129101574          |
| Chr7       | D7Mit165| 72.74| 129762283–129762408          |
| Chr7       | D7Mit43 | 73.19| 130321003–130321213          |
| Chr7       | D7Mit10 | 81.76| 136391134–136391318          |
| Chr7       | D7Mit259| 88.85| 144757504–144757647          |

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Amplifying cells are imbalance patterns around FVB/N allele loss or MSM allele gain. (B) Detailed analysis of bar represents MSM allele loss or FVB/N allele gain. The red bar microsatellite markers (for detailed information, see Figure 3). The blue bar represents no imbalance; closed circles represent imbalance in favor of FVB/N allele.

Figure 3. Allelic imbalance analysis of Stmm1 region on chromosome 7. (A) Frequency of allelic imbalance detected by SSLP analysis on proximal chromosome 7. Data are derived from papillomas of F1 backcross (N2) and N10 congenic mice (N2, n = 30; N10, n = 26). The percentages of chromosome imbalances are plotted for different microsatellite markers (for detailed information, see Table 2). The blue bar represents MSM allele loss or FVB/N allele gain. The red bar represents FVB/N allele loss or MSM allele gain. (B) Detailed analysis of imbalance patterns around Stmm1 region in papillomas. Open circles represent no imbalance; closed circles represent imbalance in favor of the FVB/N allele.

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D7Mit255 (68.16 cM) is missing in any congenic line tested (Figure 1A). Further congenic study will give us the answer for whether Stmm2 gene is present in this interval in the near future.

Koning et al. (2007) showed that low-penetrance susceptibility genes, even when present in a heterozygous state in congenic mice, can influence somatic genetic changes in tumors and that these alterations can be exploited for the rapid fine mapping of putative susceptibility loci [17]. To narrow down Stmm1 candidate region, we carried out allelic imbalance analysis of papillomas from [(FVB/N × MSM) × FVB/N] F1 backcross and MF heterozygous mice of N10 congenic line (a) [Figure 3]. As a result, we detected allelic imbalance showing the highest frequency in Stmm1 region, in spite of the fact that both parental chromosomes carried the same FVB Hras gene as well as other alleles on distal chromosome 7 (Figure 3). This clearly shows that one or more low-penetrance susceptibility genes near Stmm1 locus can affect somatic genetic change in tumors, independently of the effects of Hras. Allelic alterations with the highest rate in favor of FVB alleles were detected within the minimum-overlapping region of Stmm1 congenic lines using the marker D7Mit323 and D7Mit37, which are located at approximately 100.98 Mb and 103.8 Mb, respectively. The physical size of this region showing allelic alterations is about 5.4 Mb. However this region still contains a large number of genes, some of which are already reported to be related to cancer. Ral1 (Ras association domain family) gene encodes the regulatory subunit of ribonucleotide reductase, the molecular target of gemcitabine. A SNP in the promoter region of Ral1 gene was reported to be associated with progression-free survival in non-small-cell lung cancer patients treated with gemcitabine-based chemotherapy [18]. The NUP98 protein has several distinct roles within the nucleus of the normal cell. Fusion of NUP98 to a large number of partner genes leads to the generation of leukemogenic NUP98 fusion proteins. NUP98 fusions are associated with a wide spectrum of hematopoietic malignancies, such as AML [19]. RhoG is another important candidate gene in this region. Rho proteins belong to the Ras superfamily. They are small (21–25 kDa) molecules that share structural homology and become activated when bound to GTP. Rho GTPases have been reported to contribute to most steps of cancer initiation and progression including the acquisition of unlimited proliferation potential, survival and evasion from apoptosis, angiogenesis, and tissue invasion [20]. RhoG was recently reported to be highly expressed in human glioblastoma and mediate glioblastoma cell invasion [21]. The precise functions of these candidate genes in DMBA-TPA induced skin papilloma model remain to be elucidated yet. Further congenic and functional study will be required for gene identification in the near future.

Several lines of evidence come from studies on mouse skin tumor development where the concept that slowly dividing LRCs rather than rapidly proliferating TA (Transit Amplifying) cells are capable to expand during skin tumor promotion is long established [22]. Already in the 1980th it was shown that in mice with [3H] thymidine labeled epidermal LRCs these LRCs scarcely underwent mitosis and remained in the basal layer upon TPA treatment, whereas the proliferating cells dislocated rapidly from the basal layer undergoing terminal differentiation [23]. Furthermore, LRCs of hair follicles retained carcinogen-DNA adducts [24], and even after ablation of cycling cells in the epidermis with a chemotherapeutic drug prior to DMBA treatment, the rate of carcinoma formation was unchanged, indicating that tumor initiation occurred in quiescent (stem cells) rather than rapidly proliferating (TA) cells [25]. On the basis of these concepts, we carried out BrdU-LRC analysis using congenic mice containing Stmm1 region. Interestingly, mice of a congenic line (a) containing Stmm1 locus exhibited a significant reduction of BrdU-LRCs in the bulge of hair follicles (Figure 4). These results strongly suggest Stmm1 gene could suppress papilloma formation by altering the behavior of adult epidermal quiescent stem cells in hair follicles. Combination of BrdU labelling, DMBA-TPA carcinogenesis and allelic imbalance analysis using congenic mice may facilitate gene identification step and functional characterization of the gene responsible for Stmm1.

Materials and Methods

Mice and Tumor Induction

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Education, Culture, Sports, Science, and Technology of Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments of Chiba
Cancer Center (Permit Number: 13–18). All efforts were made to minimize suffering. FVB/N mice were purchased from Japan Clea. MSM/Ms mice have been maintained in the Experimental Animal Facility at Niigata University and Chiba Cancer Centre for more than 20 years. In a large F1 backcross study using [(FVB/N × MSM/Ms) × FVB/N], papilloma resistance loci were identified by QTL analysis [16]. Resistant F1 backcross mice were selected for further backcrossing to FVB/N mice over at least 10 generations, ultimately leading to congenic lines (a) and (b) containing MSM/Ms allele of *Stmm1* and 2 on the FVB/N background (Figure 1A). Multiple congenic lines containing different overlapping regions were generated from line (a) mice (Figure 2A). These congenic mice were treated following the same skin tumor induction protocol, as reported previously [16]. In short, the mice (8–12 weeks) received a single dose of DMBA (25 μg per mouse with 200 μl of acetone) and, starting 1 week after initiation, the animals were subjected to TPA (200 μl of 0.1 mM solution in acetone) twice weekly for 20 weeks. Papilloma numbers were counted every week until 20 weeks after initiation.

**Figure 4.** *Stmm1* congenic mice show a decrease of BrdU-LRCs. (A) The timetable of BrdU chase experiment. Multiple color bars indicate the mouse hair cycles (orange, anagen; red, catagen; and blue, telogen) and the bold black line below indicates the postnatal days of mice. (B) Representative double-immunostaining patterns of BrdU-LRCs (green) and Keratin 14 (K14) (red) in the skin from a FF homozygous of a congenic line (a) on chromosome 7, (C) a MF heterozygous mouse of a congenic line (a), (D) a FF homozygous mouse of a congenic line having the linkage region on chromosome 4, and (E) a MF heterozygous mouse of a congenic line having the same linkage region on chromosome 4. (F) The numbers of BrdU-LRCs per hair follicle in dorsal back skin sections are plotted. The blue bars represent the number of BrdU-LRCs in each congenic line, FF homozygous mice of a congenic line having the same linkage region on chromosome 4 (n = 24). The P-value was calculated for BrdU-LRC number by t-test. Error bar represents standard deviation (S.D.). Abbreviation: Bu, Bulge. Scale bars, 100 μm. doi:10.1371/journal.pone.0097201.g004
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