Somatic chromosomal translocation between Ewsr1 and Fli1 loci leads to dilated cardiomypathy in a mouse model

Miwa Tanaka  
*Japanese Foundation for Cancer Research*

Shuichi Yamaguchi  
*Japanese Foundation for Cancer Research*

Yukari Yamazaki  
*Japanese Foundation for Cancer Research*

Hideyuki Kinoshita  
*Kyoto University Graduate School of Medicine*

Koichiro Kuwahara  
*Kyoto University Graduate School of Medicine*

See next page for additional authors

Follow this and additional works at: [https://digitalcommons.wustl.edu/open_access_pubs](https://digitalcommons.wustl.edu/open_access_pubs)

Please let us know how this document benefits you.

**Recommended Citation**
Tanaka, Miwa; Yamaguchi, Shuichi; Yamazaki, Yukari; Kinoshita, Hideyuki; Kuwahara, Koichiro; Nakao, Kazuwa; Jay, Patrick Y.; Noda, Tetsuo; and Nakamura, Takuro, "Somatic chromosomal translocation between Ewsr1 and Fli1 loci leads to dilated cardiomypathy in a mouse model." Scientific Reports. 5, 7826. (2015).  
[https://digitalcommons.wustl.edu/open_access_pubs/4286](https://digitalcommons.wustl.edu/open_access_pubs/4286)
Authors
Miwa Tanaka, Shuichi Yamaguchi, Yukari Yamazaki, Hideyuki Kinoshita, Koichiro Kuwahara, Kazuwa Nakao, Patrick Y. Jay, Tetsuo Noda, and Takuro Nakamura
Somatic chromosomal translocation between Ewsr1 and Fli1 loci leads to dilated cardiomyopathy in a mouse model

Miwa Tanaka1, Shuichi Yamaguchi1, Yukari Yamazaki1, Hideyuki Kinoshita2, Koichiro Kuwahara2, Kazuwa Nakao2, Patrick Y. Jay3, Tetsuo Noda4 & Takuro Nakamura1

1Division of Carcinogenesis, The Cancer Institute, Japanese Foundation for Cancer Research, 3-8-31 Ariake, Koto-ku, Tokyo 135-8550, Japan, 2Department of Cardiovascular Medicine, Kyoto University Graduate School of Medicine, 54 Kawara-cho Shogoin, Sakyo-ku, Kyoto 606-8507, Japan, 3Departments of Pediatrics and Genetics, Washington University School of Medicine, 660 S Euclid Avenue, St. Louis, MO 63110, U.S.A, 4Division of Cell Biology, The Cancer Institute, Japanese Foundation for Cancer Research, 3-8-31 Ariake, Koto-ku, Tokyo 135-8550, Japan.

A mouse model that recapitulates the human Ewing’s sarcoma-specific chromosomal translocation was generated utilizing the Cre/loxP-mediated recombination technique. A cross between Ewsr1-loxP and Fli1-loxP mice and expression of ubiquitous Cre recombinase induced a specific translocation between Ewsr1 and Fli1 loci in systemic organs of both adult mice and embryos. As a result Ewsr1-Fli1 fusion transcripts were expressed, suggesting a functional Ews-Fli1 protein might be synthesized in vivo. However, by two years of age, none of the Ewsr1-loxP/Fli1-loxP/CAG-Cre (EFCC) mice developed any malignancies, including Ewing-like small round cell sarcoma. Unexpectedly, all the EFCC mice suffered from dilated cardiomyopathy and died of chronic cardiac failure. Genetic recombination between Ewsr1 and Fli1 was confirmed in the myocardial tissue and apoptotic cell death of cardiac myocytes was observed at significantly higher frequency in EFCC mice. Moreover, expression of Ews-Fli1 in the cultured cardiac myocytes induced apoptosis. Collectively, these results indicated that ectopic expression of the Ews-Fli1 oncogene stimulated apoptotic signals, and suggested an important relationship between oncogenic signals and cellular context in the cell-of-origin of Ewing’s sarcoma.

Chromosomal translocation is a common feature of malignant neoplasms1. There is growing evidence that tumor-specific translocations and inversions commonly occur among hematopoietic, mesenchymal and epithelial tumors. An increasing number of gene fusions resulting from translocation have been observed as novel technological tools have been applied. Tumor-associated chromosomal translocations include two major molecular mechanisms. One is an oncogene juxtaposition to the enhancing elements of immunoglobulin or T-cell receptor associated with lymphoid neoplasms. As a result of the juxtaposition, constitutive expression of oncogenes such as c-MYC, BCL2 or CCND1 induces abnormal cellular functions, including cell cycle progression and apoptosis suppression1. Another important outcome of translocation in cancer is gene fusion or formation of chimeric genes. Two major functional aberrations of fusion gene products are constitutive activation of signal transduction and dysregulation of transcription. Most oncogenic gene fusions in human bone and soft tissue sarcomas belong to the latter group, and there is a specific relationship between tumor types and each gene fusion2.

To clarify the functional roles of sarcoma-specific chromosomal translocations and gene fusions, it would be ideal to induce chromosomal translocation in animal models in vivo. In contrast to transgenic expression of fusion genes, translocation-mediated gene fusion recapitulates gene expression levels equivalent to, and splice variants similar to those in human tumors. Inducible, site-specific chromosomal translocation has been achieved using Cre-loxP-mediated recombination in murine ES cells. Using this strategy, translocations between c-myc and immunoglobulin heavy chain loci, and between Dek and Can loci were successfully induced, though the efficiencies were not very high3,4. Indeed, a mouse model of Cre-loxP-mediated in vivo gene fusion between Mll and Af9 developed acute myeloid leukemia5. However, it is not known whether solid tumor-related translocation in vivo can induce malignancies of the anticipated phenotypes.
The ETS family of transcription factors includes FLI1 and ERG. They are major fusion partners for the EWSR1 gene in human Ewing’s sarcoma. EWS-FLI1 and EWS-ERG function as oncogenic transcription factors that dysregulate their downstream targets such as NKX2-2, NR0B1, and EZH2. It is, however, difficult to generate a good animal model by introduction of EWS-FLI1 or EWS-ERG into ES cells or mouse eggs. Moreover, conditional EWS-FLI1 expression in hematopoietic cells induced myeloid and erythroid leukemia in mice. Thus, it might be necessary to activate multiple target genes without activating pro-apoptosis signals for tumorigenic activity of EWS-ETS. We therefore hypothesized that EWS-ETS translocation is achieved by chance in human somatic cells of appropriate lineages and differentiation status, and such in vivo translocation could properly induce Ewing’s sarcoma.

In an effort to induce Ewing’s sarcoma in a mouse model, we have succeeded in promoting in vivo Cre-loxP-mediated translocation between Ewsr1 and FLI1 loci on chromosomes 11 and 9, respectively. Although the Ewsr1-FLI1 fusion was confirmed at both DNA and RNA levels, no neoplastic lesion was induced in the model. Unexpectedly, the mice with systemic translocation developed dilated cardiomyopathy due to degeneration and apoptotic cell death of cardiac myocytes. The result indicates that ectopic chromosomal translocation and gene fusion activates apoptotic signals, resulting in degenerative cardiac disease.

Results

Generation of a mouse model for somatic chromosomal translocation between Ewsr1 and FLI1. To induce locus-specific chromosomal translocation, loxP sequences were introduced into Ewsr1 intron 7 on mouse chromosome 11 and FLI1 intron 5 on chromosome 9 (Fig. 1A), since chromosomal breakpoints in human Ewing’s sarcoma are most frequently observed in these loci. Successful
knock-in of loxP sequences mediated by homologous recombination was confirmed for both loci in independent ES cells by Southern blotting (Fig. 1B). Both Ewsr1fl/fl and Fli1fl/fl mice appeared normal and healthy at birth. Germline transmission of the targeted alleles was confirmed. Ewsr1fl/fl and Fli1fl/fl mice were crossed to obtain mice having both mutations.

Genomic chromosomal translocation between chromosomes 9 and 11 in the Ewsr1fl/fl:Fli1fl/fl:CAG-Cre (EFCC) mice. The Ewsr1fl/fl and Fli1fl/fl mice were further crossed with CAG-Cre, Mx1-Cre or Rosa26-CreER mice to induce somatic chromosomal translocation between chromosomes 9 and 11 (Fig. 2A). Dual color fluorescence in situ hybridization (FISH) analysis of embryonic fibroblasts derived from the EFCC mice showed juxtaposition of the signal on der9 of BAC clone RPCI-23 64E17 from chromosome 11 and that of 218O21 from chromosome 9 (Fig. 2B). Reciprocal genomic translocations in systemic organs were examined by genomic PCR using Ewsr1- and Fli1-specific primers, and both Ewsr1-Fli1 and Fli1-Ewsr1 translocations were detected in tail skin of all the mice examined (n = 30). The translocations in systemic organs were examined in three mice, and both Ewsr1-Fli1 and Fli1-Ewsr1 translocations were detected in all the organs examined (Fig. 2C). The results indicated that loxP-mediated recombination was effective at inducing somatic translocation by ubiquitous Cre recombinase expression. The frequencies of the chromosomal translocations were 1.5 × 10⁻³ at the highest in heart and 1 × 10⁻³ in bone marrow as estimated by quantitative genomic PCR comparing Ewsr1-Fli1 and Trib1 signals (Fig. 2D). The estimated translocation frequencies in the model are higher than those observed in ES cells described in the previous report. When Cre recombinase was inducibly expressed by tamoxifen or polyIpolyC administration in a Rosa26-CreER or Mx1-Cre background, respectively, both Ewsr1-Fli1 and Fli1-Ewsr1 translocations were observed (four mice each) (Fig. 2E). However, the translocations...
were detected only by nested PCR in limited organs, indicating that recombination was less frequent in these Cre transgenes. In addition, inducible expression of Cre upon in the Mxi-Cre background resulted in translocations being limited to hematopoietic tissues.

**Detection of chimeric Ewsr1-Fli1 fusion transcripts in EFCC mice.** To confirm that gene fusion between Ewsr1 and Fli1 was accompanied by the anticipated transcription, RT-PCR was performed using RNA samples obtained from systemic organs of both adult and embryonic mice (three mice each) (Fig. 3A, 3B). The Ewsr1-Fli1 fusion was detected in all the embryonic organs examined, and the expression of the fusion gene was decreased in bone and liver of the adult mice. Diminished Ewsr1-Fli1 expression in adult bone and liver might be related to decreased proliferative activity of osteochondrogenic tissues and disappearance of embryonic hematopoietic cells, respectively. No reciprocal Fli1-Ewsr1 fusion transcript was detected in any of the organs examined (data not shown). The cDNA sequence of the Ewsr1-Fli1 fusion transcript was analyzed by sequencing, and in-frame fusion between Ewsr1 exon 7 and Fli1 exon 6 was confirmed (Fig. 3C). It is expected that the fusion product included both the EWS Q-rich repeats and the FLI1 ETS DNA binding domain. Thus, the data strongly suggested that a functional EWS-FLI1 protein was produced by somatic chromosomal translocation in the model.

**EFCC mice died of chronic cardiac failure due to dilated cardiomyopathy.** No malignant neoplasms, including Ewing’s sarcoma-like lesions, were observed in EFCC mice (n = 30) for a two year period after birth. Neither sarcomas nor benign neoplasms were detected by careful examination of mice irrespective of age. Instead, most of the EFCC mice showed growth retardation and decreased motility. All the EFCC mice died by 100 weeks of age with a mean survival time of only 40 weeks (Fig. 4A). The diseased mice were carefully examined at autopsy and they showed extensive dilatation of heart (Fig. 4B). The heart weight/body weight ratio as well as wall thickness of EFCC mice was significantly greater than that of control mice from 31 to 42 weeks (Fig. 4C, Table 1). Mice of the age were selected since the severity of cardiac lesions was significantly varied in younger EFCC mice. The pathological examination further revealed the cardiac lesions and subsequent systemic congestive changes. The hearts of EFCC mice showed extensive dilatation of both the ventricles and thin ventricular wall without any signs of cardiac hypertrophy (Fig. 4D). The earlier the mice became sick, the more severe the cardiac lesions were. High power views of cardiac sections indicated a disorganized arrangement of myocardial fibers with increased collagen fibers between the muscle bundles. The subendocardial area was severely affected and leukocytic infiltration was sometimes present. There was severe chronic congestion in systemic organs such as lung, liver or spleen accompanied by ischemic necrosis around the central vein of the liver (Fig. 4E).

Consistent with the pathological findings, echocardiographic analysis revealed reduced wall thickness, significant fractional shortening and decreased ejection fraction in EFCC mice (Fig. 5, Table 2). In contrast, there was no significant difference in blood pressure, heart rate or diastolic dimension between EFCC and wild-type mice (Table 2). Collectively, these findings are consistent with those of dilated cardiomyopathy.

**Ewsr1-Fli1 translocation and Ewsr1-Fli1 expression induced myocardial damage.** To obtain insights into the mechanisms of dilated cardiomyopathy in EFCC mice, the cardiac lesion was further investigated. Laser microdissection followed by genomic PCR to detect the Ewsr1-Fli1 translocation was carried out (Fig. 6A). Ewsr1-Fli1 was abundantly observed in the outer area of the ventricular wall, however, no signal was detected in the subendocardial area where the myocardial damage was more severe (Fig. 6A, 1 and 3). Severer damages in the subendocardial area were observed in most of mice, though the reason for such uneven distribution of cardiac lesions was unclear. The results suggested degeneration of cardiac myocytes with translocation and perhaps gradual loss due to the pathologic effects of Ewsr1-Fli1 expression. Indeed, a TUNEL assay using the cardiac sections showed significantly increased apoptosis in EFCC mice compared to wild-type (Fig. 6B).

The toxic effect of Ewsr1-Fli1 was directly evaluated by its exogenous expression in cultured cardiac myocytes. The murine neonatal cardiac myocytes were infected with Ewsr1-Fli1-lentivirus and the frequencies of apoptosis were evaluated (Fig. 6C). The TUNEL assay
showed that apoptosis of cardiac myocytes was significantly increased when Ewsr1-Fli1 was expressed in the cardiac myocytes. The Annexin V/PI flow cytometry analysis showed increases of both early and late apoptosis as well as necrosis in cardiac myocytes by Ewsr1-Fli1 expression (Fig. 6C). These results indicated that Ewsr1-Fli1 induced cellular apoptosis in the cardiac tissue, resulting in cellular damage and eventual dilated cardiomyopathy. In addition, Ewsr1-Fli1 expression in human cardiac fibroblasts induced increased expression of COL1A1 (Fig. 6D), suggesting that Ewsr1-Fli1 may also play some role in cardiac fibrosis.

A previous study indicated that the high level of expression of Cre recombinase itself showed cardiac toxicity. The expression level of the Cre protein in the hearts of the Efcc mouse was therefore compared with high-expressing Cre transgenic mice (Fig. 6E). Cre expression of Efcc mice was comparable to the low Cre transgenic mice that did not show cardiac lesions. The results indicated that the cardiac lesion was caused not by Cre expression but by Ewsr1-Fli1.

Discussion

Cre/loxP-mediated chromosomal translocations in mouse models have been reported. In those studies loxP sites were inserted into the introns of Mll or Af9 genes, and the mice carrying the mutations were crossed to place loxP sites in both genes. Both ubiquitous and hematopoietic-specific expression of Cre recombinase induced in vivo chromosomal translocation and the fusion of Mll and Af9, resulting in leukemia development. In contrast, leukemia was not observed in the mice bearing chromosomal translocation between AML1 and ETO in vivo using a similar protocol.

In the present study, Ewsr1-Fli1 fusion was successfully induced in various organs. Ewing’s sarcoma, however, did not develop in the mice, suggesting that the cell-of-origin of Ewing’s sarcoma might constitute a rare cellular population unlike hematopoietic neoplasms. Supporting this idea, we have recently succeeded in developing Ewing’s sarcoma-like small round cell tumors by introducing Ews-Fli1 or Ews-Erg into eSZ cells that are enriched in embryonic chondrogenic progenitors. Therefore, when chromosomal translocation between Ewsr1 and Fli1 is efficiently induced in eSZ cells, Ewing’s sarcoma can develop in a certain cohort using the current translocation model. It is likely that ubiquitous Cre expression affects most cell lineages both in developing and adult mouse tissues including the true cell-of-origin of Ewing’s sarcoma. However, the low frequency of chromosomal recombination could
Figure 5 | Echocardiographic analysis of 37-week-old EFCC and EF;wild-type mice. Analysis of cardiovascular function (top). DD, diastolic diameter of left ventricle; FS, fractional shortening; EF, ejection fraction. Representative echocardiogram for wild-type and EFCC mice (bottom). EDD, end-diastolic diameter; ESD, end-systolic diameter; IVS, interventricular septum; LV, left ventricle; PW, posterior wall.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Heart and body weight of EF;wt and EFCC mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EF;wt</td>
</tr>
<tr>
<td>Weeks</td>
<td>HW (mg)</td>
</tr>
<tr>
<td>31</td>
<td>180</td>
</tr>
<tr>
<td>36</td>
<td>140</td>
</tr>
<tr>
<td>38</td>
<td>210</td>
</tr>
<tr>
<td>38</td>
<td>180</td>
</tr>
<tr>
<td>40</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Weeks</td>
<td>HW (mg)</td>
</tr>
<tr>
<td>30</td>
<td>360</td>
</tr>
<tr>
<td>35</td>
<td>640</td>
</tr>
<tr>
<td>36</td>
<td>320</td>
</tr>
<tr>
<td>36</td>
<td>260</td>
</tr>
<tr>
<td>40</td>
<td>410</td>
</tr>
<tr>
<td>42</td>
<td>120</td>
</tr>
</tbody>
</table>

not induce detectable translocations in such a rare cell type. Perhaps eSZ cell-specific Cre expression may enable the induction of Ewing’s sarcoma by somatic Ewsr1 and Fli1 translocation, and efficient Cre expression in the specific spatiotemporal manner in the eSZ cell may be achieved using the promoter/enhancer elements of Gdf5 or Erg genes17,18. Expression of Ews-Fli1 in the majority of primary cells induced cellular apoptosis or senescence19–21. Activation of the Casp3 promoter by EWS-FLI1 was reported, and the activation of caspase 3-dependent signals may be responsible for apoptotic processes in mouse embryonic fibroblasts (MEFs) with ectopic Ews-Fli1 expression22. Indeed, Ews-Fli1 expression in cardiac myocytes induced apoptotic cell death, though activation of caspase 3 was not detected in cardiac myocytes unlike in MEFs (data not shown). Thus, the low capacity for cardiac myocyte regeneration after birth could not support cardiac homeostasis. This limitation, therefore, could result in gradual but irreversible cardiac damage. In support of this idea, the Ews-Fli1 fusion was not detected in the severely degenerated area but remained in relatively normal parts of the heart in EFCC mice. Moreover, introduction of Ews-Fli1 cDNA significantly induced apoptosis in primary cardiac myocytes, indicating the cardiac toxicity of the fusion gene. The cell type-specific epigenetic status may modulate growth inhibitory and tumorigenic activities of EWS-FLI1. Indeed, different chromatin modification was observed between Ewing’s sarcoma-sensitive eSZ and –resistant eGP cells22. It is noted that wild-type FLI1 protein represses Colla1 expression, inhibiting cardiac fibrosis23. Interestingly, EWS-FLI1 enhanced COLA1 expression in human cardiac fibroblasts, suggesting that it might accelerate fibrotic processes in cardiomyopathy.

A number of transcription factors are associated with the development and maintenance of cardiac myocytes, and mutations in these factors affect cardiac homeostasis, structure and functions24. Over-expression of E2F6 activates gene expression in myocardium and induces dilated cardiomyopathy in mice24. Moreover, mutations in Nkx2-5 and Pdmd16 were found associated with human congenital dilated cardiomyopathy25,26. It has been proposed that these proteins regulate genes involved in the ubiquitin proteasome system or proliferation of cardiomyocytes, suggesting different aspects of myocardial damage from the present model. Nevertheless, similar phenotypes shown in these models indicate the importance of cardiac-specific transcriptional regulation by transcription factors, given the low regenerative activity of adult cardiomyocytes.

Methods

Mice and gene targeting. The Ewr1 and Fli1 targeting vectors were assembled in a pBSKSTKLoxPNeoGFP plasmid containing appropriate loxP sites, a loxP-flanked thymidine kinase (Tk) promoter-driven neo gene and a Tk promoter-driven diphtheria toxin gene. A Gfp gene was inserted immediately downstream of the 3’ loxP site for the Ewsr1 vector. The homologous regions of the Ewr1 vector consisted of an 8.4 kb genomic fragment containing Ewr1 exons 5 to 7 and a 1.3 kb flanking exon (Fig. 1a). Similarly, the Fli1 vector included a 5.4 kb genomic fragment of Fli1 intron 5 and a 2.0 kb fragment flanking exon 6. A CMV promoter sequence was also inserted immediately upstream of the 5’ loxP site of the Fli1 vector. To establish mice carrying a single loxP allele of Ewr1 or Fli1 genes, the linearized targeting vectors were electroporated into C57/BL6 blastocysts and the resultant chimeric mice were bred to produce progeny having germ line transmission of the mutated allele. Mice harboring a targeted Ewr1 allele (Ewr1loxP) and a targeted Fli1 allele (Fli1loxP) were crossed to establish the mice that possessed loxP sites both in Ewr1 intron 7 and in Fli1 intron 5. The resultant

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Echocardiographic and hemodynamic analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>E/F; wt</td>
<td>EFCC</td>
</tr>
<tr>
<td>LVDd (mm)</td>
<td>3.01 ± 0.06</td>
</tr>
<tr>
<td>LSDs (mm)</td>
<td>1.43 ± 0.07</td>
</tr>
<tr>
<td>IVST (mm)</td>
<td>1.07 ± 0.03</td>
</tr>
<tr>
<td>LVPWT (mm)</td>
<td>1.06 ± 0.01</td>
</tr>
<tr>
<td>FS (%)</td>
<td>52.67 ± 2.67</td>
</tr>
<tr>
<td>EF (%)</td>
<td>89.67 ± 1.67</td>
</tr>
</tbody>
</table>

Hemodynamic data

| HR (bpm) | 580.7 ± 36.7 | 631.5 ± 11.9 |
| sBP (mm Hg) | 104.3 ± 3.8 | 105.5 ± 1.4 |
| dBP (mm Hg) | 57.7 ± 7.4 | 50.8 ± 4.5 |

Values are means ± SEM. LVDd, left ventricular end-diastolic dimension; LSDs, LV end-systolic dimension; IVST, interventricular septum thickness; LVPWT, left ventricular posterior wall thickness; FS, fractional shortening; EF, ejection fraction; HR, heart rate; sBP, systolic blood pressure; dBP, diastolic blood pressure; *p < 0.01, **p < 0.05.
Ewsr1fl/1 and Flilfl/1 mice were further crossed with CAG-Cre, Mx1-Cre or Rosa26-CreER mice. Genotyping of the mice was performed using primers described below. Animals were handled in accordance with the guidelines of the animal care committee at the Japanese Foundation for Cancer Research, which gave ethical approval for these studies.

Southern blotting. Southern blotting was carried out using standard procedures. Genomic DNA samples were digested with XbaI or SacI and probed with genomic DNA fragments derived from Ewsr1 or Flil loci. (Fig. 1a).

Fluorescence in situ hybridization (FISH). The BAC clones, RPCI-23 64E17 downstream from Ewsr1 on mouse chromosome 11 and RPCI-23 218O31 upstream from Flil on chromosome 9 were purchased from Invitrogen (Carlsbad, CA) for FISH analysis. The FISH analysis using metaphase spreads obtained from embryonic fibroblasts of the Ewsr1fl/1:Flilfl/1:CAG-Cre (EFCC) mouse was performed according to the methods previously described. Expression of EWS-FLI1 protein in cardiac myocytes was detected by Western blotting using anti-FLAG M2 antibody. (right). The expression of EWS-FLI1 protein was detected by Western blotting using anti-FLAG M2 antibody (right). (E) Expression of the Cre protein in the heart of EFCC mice and other Cre transgenic lines of variable expression levels.

Figure 6 | The cardiac lesion in the EFCC mouse and Ewsr1-Flil translocation. (A) Detection of Ewsr1-Flil translocation in the myocardium. The frozen section of the cardiac tissue from the EFCC mouse was laser microdissected for the indicated areas (1-4) (left). Genomic PCR using DNA samples obtained by laser microdissection (right). (B) A TUNEL assay showed a significantly greater increase of apoptotic cell death in the myocardium of the EFCC mouse than in that of the wild-type mouse (left). High power view of the apoptotic cell is shown in the magnified inset. Frequencies of TUNEL-positive cells per section are compared between wild type and EFCC mice (right). (C) Ewsr1-Flil cDNA expression induced apoptotic cell death of cardiac myocytes in vitro. The apoptotic cells were measured by positive signals in a TUNEL assay (left). Ewsr1-Flil-induced cell death was further analyzed by Annexin V/PI staining and FACS analysis. The lower right quadrant (Annexin V+/PI-) represents early apoptosis, while the upper right quadrant (Annexin V+/PI+) and the upper left quadrant (Annexin V-/PI-) represent late apoptosis and necrosis, respectively. Data are representatives of three independent experiments with similar results (center). The expression of EWS-FLI1 protein in cardiac myocytes was detected by Western blotting using anti-FLAG M2 antibody (right). (D) Quantitative real-time RT-PCR for COL1A1 in human cardiac fibroblasts with or without Ewsr1-Flil (left). Expression of EWS-FLI1 protein was detected by Western blotting using anti-FLAG M2 antibody (right). (E) Expression of the Cre protein in the heart of EFCC mice and other Cre transgenic lines of variable expression levels.
PCR primers to detect the Ewst-Fli1 translocation were as follows. For Ewst-Fli1, Ewst forward primer 5'-cctcgacagaccttgct-3' and Fli1 reverse primer 5'-tcctcttcacagccgac-3'. For Fli1-Ewst, Fli1 forward primer 5’-aggagaacatctcattgacg-3’ and Ewst reverse primer 5’-acgacatatcctgctagtacg-3’ were used. To detect the rare translocation in Ros36-CreER and Myo1-Cre transgenic mice, genomic DNA samples were pre-amplified using 35 cycles of PCR using the following primers. For Ewst1, the 5’ primer was 5’-caagttggtgtctcagtt-3’ and for Fli1, the 3’ primer was 5’-ggttatcttgagaggatac-3’. For Fli1-Ewst, the 5’ primer was 5’-ggctcctgcctaatagt-3’ and for Ewst1, the 3’ primer was 5’-gctgctgaaaaggacctct-3’. For Ewst1, the 3’ primer was 5’-gctgctgaaaaggacctct-3’.

Genetic PCR for the wild-type Ewst1 transgene, Cre recombinase or Tribi1 was performed using the following primers: Ewst1 forward, 5’-cctcgacagaccttgct-3’ and Fli1 reverse, 5’-extttcttggtcggtgggtgac-3’. For Fli1-Ewst, the 5’ primer was 5’-gcctgctgaaaaggacctct-3’ and for Ewst1, the 3’ primer was 5’-gctgctgaaaaggacctct-3’. The PCR products were analyzed by 2% agarose gel electrophoresis.

RT-PCR was carried out using cDNA generated from total RNA of systemic organs as previously described31. The Ewst1-Fli1 fusion transcript was amplified using Ewst1 exon 7 primer (5’-tctcttcacagccgac-3’) and Fli1 exon 6 primer (5’-ctgcagctgtgctgc-3’). The primers for Cre recombinase (forward, 5’-gcgctgctgaaaaggacctct-3’, reverse, 5’-gctgctgaaaaggacctct-3’) and Hprt (forward, 5’-gcgctgaaagacgcct-3’; reverse, 5’-cagacagtacggacg-3’) were also used. The PCR products were purified, subcloned into a plasmid and sequenced. Real-time quantitative RT-PCR was performed by using a Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The primers for Cre recombinase, 5’-tcctcttcacagccgac-3’ and Cre, reverse, 5’-ccatctctcctcctgctacttcg-3’, or Tribi1, forward, 5’-cctgctcctcctcctgctacttcg-3’ and Tribi1, reverse, 5’-gatttgctggtgatgtttgccc-3’ were used.

Echocardiography. Transthoracic echocardiography was performed on conscious, gently restrained mice using a 15-MHz linear probe (Power-Vision 8000, Toshiba, Japan), as described previously. M-mode recordings were obtained from short axis view at the level of the papillary muscles. Left ventricular internal diameter at end-diastole (LVEDV) and end-systole (LVDS) were measured from M-mode recordings. Fractional shortening (FS) was calculated as 100 × (LVEDV – LVDS/LVDS) (%). Ejection fraction (EF) was calculated using the Teichholz method.

Cell culture and recombinant lentivirus infection. Primary neonatal ICR mouse ventricular myocytes were purchased from Cosmo Bio (Tokyo, Japan). The cells were cultured with D-MEM/F12 medium supplemented 10% fetal bovine serum (HyClone, South Logan, UT). Human cardiac fibroblasts were purchased from Promocell (Heidelberg, Germany), and cells were cultured with Fibroblast Medium (ScienCell, Carlsbad, CA). The human EWSRT-FLI1 DNA (a kind gift from Susanne Baker) was FLAG-tagged and inserted into the pLSVN-CMV-neo plasmid (Takara Bio, Tokyo, Japan) and HEK 293 cells were transfected with the plasmid using Lipofectamine 2000 (Invitrogen). Cells were harvested 48 h after lentiviral infection and subjected to further analyses.

TUNEL assay and Annexin-V analysis. Formaldehyde-fixed and paraffin-embedded cardiac tissue sections or methanol-fixed murine primary cardiac myocytes were subjected to standard TUNEL assays using the DeadEnd Colorimetric TUNEL System (Promega, Madison, WI) according to the manufacturer’s protocol. For the Annexin V analysis cells were stained with Annexin V-FITC and propidium iodide (PromoCell (Heidelberg, Germany), and cells were cultured with Fibroblast Medium (ScienCell, Carlsbad, CA). The primers for Cre recombinase (forward, 5’-gcgctgctgaaaaggacctct-3’, reverse, 5’-gctgctgaaaaggacctct-3’) and GAPDH (forward, 5’-acgctgctgaaaaggacctct-3’, reverse, 5’-aaagggctggtgatgtttgccc-3’) were used.

Statistical analysis. Results are shown as means ± standard errors of the mean (SEM). Continuous distributions were compared with two-tailed Student’s t-tests. Survival analysis was performed using the Kaplan-Meier life table method, and the survival between groups was compared with the log-rank test. All P values were two-tailed, and a P value of less than 0.05 was considered significant.

Acknowledgments

We are grateful to Junichi Miyazaki for CAG-Cre, Klaus Rajewsky for MX1-Cre and Tyler Jacks for Ros36-Cre transgenic mice. We also thank Miki Yamazaki, Yohei Kanno, Hitomi Yamazaka and Tokushi Kawaguchi for technical assistance. This work was supported by...
the Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (23791672 and 26250029) to M.T. and T.Na.

**Author contributions**

M.T., T.No. and T.Na. designed the work. M.T., S.Y., Y.Y. and H.K. performed the experiments. M.T., K.K., K.N., P.Y.J., T.No. and T.Na. analyzed the data. M.T. and T.Na. wrote the paper. All co-authors contributed in the form of discussion and critical comments.

**Additional information**

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Tanaka, M. et al. Somatic chromosomal translocation between *Ewsr1* and *Fli1* loci leads to dilated cardiomyopathy in a mouse model. Sci. Rep. 5, 7826; DOI:10.1038/srep07826 (2015).

This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivs 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder in order to reproduce the material. To view a copy of this license, visit [http://creativecommons.org/licenses/by-nc-nd/4.0/](http://creativecommons.org/licenses/by-nc-nd/4.0/)