Gene expression response to EWS–FLI1 in mouse embryonic cartilage

Miwa Tanaka, Ken-ichi Aisaki, Satoshi Kitajima, Katsuhide Igarashi, Jun Kanno, Takuro Nakamura

A Division of Carcinogenesis, The Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan
B Division of Cellular and Molecular Toxicology, Biosafety Research Center, National Institute of Health Science, Tokyo, Japan

ABSTRACT

Ewing's sarcoma is a rare bone tumor that affects children and adolescents. We have recently succeeded to induce Ewing's sarcoma-like small round cell tumor in mice by expression of EWS–ETS fusion genes in murine embryonic osteochondrogenic progenitors. The Ewing's sarcoma precursors are enriched in embryonic superficial zone (eSZ) cells of long bone. To get insights into the mechanisms of Ewing's sarcoma development, gene expression profiles between EWS–FLI1-sensitive eSZ cells and EWS–FLI1-resistant embryonic growth plate (eGP) cells were compared using DNA microarrays. Gene expression of eSZ and eGP cells (total, 30 samples) was evaluated with or without EWS–FLI1 expression 0, 8 or 48 h after gene transduction. Our data provide useful information for gene expression responses to fusion oncogenes in human sarcoma.

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Direct link to deposited data

Deposited data can be found here: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE32618.

Experimental design, materials and methods

Preparation of mouse embryonic superficial zone (eSZ) and growth plate (eGP) cells

Femoral and humeral bones of BALB/c mouse embryos were removed aseptically on 18.5 dpc, and they were microdissected into eSZ and growth plate (eGP) under a stereomicroscope (Zeiss Stemi 2000-C, Carl Zeiss MicroImaging). Each region was minced and gently digested with 2 mg/mL of collagenase (Wako Pure Chemical) at 37 °C for 2 h. They were cultured in growth medium composed of Iscove’s Modified Dulbecco’s Medium (Invitrogen) supplemented with 15% fetal bovine serum, and subjected immediately to retroviral infection.

Retroviral infection

N-terminal FLAG-tagged EWS–FLI1 was introduced into the pMYs-IRES-GFP vector. The full length EWS–FLI1 cDNA was a kind gift from Dr. Susanne Baker. Retroviral infections of eSZ, eGP or shaft cells were performed as described [1]. Infection efficiency was examined using a FACSCalibur flow cytometer (Beckton Dickinson). Cells were harvested after forty-eight hours of infection.

RNA isolation and microarray

GeneChip analysis was conducted to determine gene expression profiles. The per cell normalization method (Percellome method) was applied to eSZ and eGP samples [2]. Briefly, cellular lysates were prepared with RLT buffer (QiAGEN). A 10 μL aliquot of each lysate was treated with DNase-free RNase A (Nippon Gene Inc., Japan) for 30 min at 37 °C, followed by Proteinase K (Roche Diagnostics GmbH., Germany) for 3 h at 55 °C. The aliquot was then transferred to a 96-well black plate. PicoGreen fluorescent dye (Molecular Probes Inc., USA) was added to each well, shaken for 10 s four times and then incubated for 2 min at 30 °C. DNA concentration was measured using a 96
well fluorescence plate reader with excitation at 485 nm and emission at 538 nm. λ phage DNA (PicoGreen Kit, Molecular Probes Inc., USA) was used as standard. As reported previously [2], the grade-dosed spike cocktails (GSCs) made of the *Bacillus subtilis* RNAs corresponding to the sequences in the Affymetrix GeneChip arrays (AFX-ThrX-3_at, AFX-LysX-3_at, AFX-PheX-3_at, AFX-DapX-3_at, and AFX-TrpnX-3_at) were prepared, and GSCs were added to the sample homogenates in proportion to their DNA concentrations. Total RNA was extracted using the RNeasy Mini Kit (QIAGEN). The GeneChip Mouse Genome 430 2.0 Array (Affymetrix) was hybridized with the cRNA generated from eSZ and eGP cells, and murine Ewing’s sarcoma tissue (Table 1). After staining with streptavidin–phycoerythrin conjugates, arrays were scanned using an Affymetrix GeneChip Scanner 3000 and analyzed using Affymetrix GeneChip Command Console Software (AGCC, Affymetrix) and GeneSpring GX 11.0.2 (Agilent Technologies) as described previously [3]. The expression data for eSZ and eGP cells were converted to Percellome data, i.e., absolute copy numbers of mRNA per one cell, by the homemade software SCal4 (Spike Calculation version 4). This software also graphically indicates the efficiency of in vitro transcription, the dose–response linearity of the five GSC spikes and the location of spike probe sets in the histogram of all probe sets (Fig. 1A). From the same treatment group (n = 3), all the pairs were plotted to a scatter graph as red (expression above detection level) or green dots (below detection level) with the data of five yellow spike probe sets (Fig. 1B). If any samples did not draw a symmetric scatter plot with yellow dot on the diagonal line, the sample were rejected for evaluation, and they were subjected to additional analyses.

**Data analysis**

Homemade software named RSort (Roughness Sort) [4] was used. This program sorts the probe sets as upward or downward peaks in a 3D isobologram (Fig. 2). To avoid biologically nonsense probe sets

### Table 1

| GEO accession no. | Cell types | Gene transfer | Time (h) |
|-------------------|------------|---------------|----------|
| GSM808581         | eSZ        | No            | 0        |
| GSM808582         | eSZ        | No            | 0        |
| GSM808583         | eSZ        | No            | 0        |
| GSM808584         | eGP        | No            | 0        |
| GSM808585         | eGP        | No            | 0        |
| GSM808586         | eGP        | No            | 0        |
| GSM808587         | eSZ        | Empty vector  | 8        |
| GSM808588         | eSZ        | Empty vector  | 8        |
| GSM808589         | eSZ        | Empty vector  | 8        |
| GSM808590         | eGP        | Empty vector  | 8        |
| GSM808591         | eGP        | Empty vector  | 8        |
| GSM808592         | eGP        | Empty vector  | 8        |
| GSM808593         | eSZ        | EWS–FLI1      | 8        |
| GSM808594         | eSZ        | EWS–FLI1      | 8        |
| GSM808595         | eSZ        | EWS–FLI1      | 8        |
| GSM808596         | eGP        | EWS–FLI1      | 8        |
| GSM808597         | eGP        | EWS–FLI1      | 8        |
| GSM808598         | eGP        | EWS–FLI1      | 8        |
| GSM808599         | eSZ        | Empty vector  | 48       |
| GSM808600         | eSZ        | Empty vector  | 48       |
| GSM808601         | eSZ        | Empty vector  | 48       |
| GSM808602         | eGP        | Empty vector  | 48       |
| GSM808603         | eGP        | Empty vector  | 48       |
| GSM808604         | eGP        | Empty vector  | 48       |
| GSM808605         | eSZ        | EWS–FLI1      | 48       |
| GSM808606         | eSZ        | EWS–FLI1      | 48       |
| GSM808607         | eSZ        | EWS–FLI1      | 48       |
| GSM808608         | eGP        | EWS–FLI1      | 48       |
| GSM808609         | eGP        | EWS–FLI1      | 48       |
| GSM808610         | eGP        | EWS–FLI1      | 48       |

eSZ, embryonic superficial zone; GP, growth plate.

![Fig. 1](image-url)
such as ones with expression below the detection level, the data were visually checked for their 3D isobologram shape.

**Discussion**

We describe a unique dataset of mouse embryonic cartilage with or without the Ewing’s sarcoma fusion oncogene, EWS–FLI1. Significantly different responses of gene expression between eSZ and eGP cells were observed. The dataset was used in the study published recently [5] and was informative to understand the tumorigenic mechanisms of Ewing’s sarcoma.

**References**

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