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Differentiation stage-specific analysis of gene function with inducible short hair-pin RNA in differentiating embryonic stem cells

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Abstract

Cell differentiation is regulated by spatial and temporal coordination of gene expressions. Previously, we have established an embryonic stem (ES) cell differentiation system that can trace early cardiovascular developmental process in vitro. Here we show that tetracycline-induced short hair-pin RNA (shRNA) expression in differentiating ES cells successfully suppressed stage-specific genes for differentiation and modified cell fates. We established ES cell lines carrying shRNA gene driven by $tRNA^{val}$ promoter with tetracycline operator sequences (tet-ON system). When expression of vascular endothelial growth factor receptor-2 (VEGFR2) gene, a vascular progenitor and mesoderm marker and an essential gene for endothelial cell (EC) differentiation, was suppressed by shRNA in early ES cell differentiation, appearance of $VEGFR2^+$ mesoderm cells was substantially reduced. Suppression of VEGFR2 expression at mesoderm stage almost completely inhibited EC differentiation from $VEGFR2^+$ mesoderm cells. This novel experimental system, thus, can selectively determine stage-specific roles of genes in differentiation in vitro.
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Cell differentiation is a dynamic process regulated with fine tuning of gene expressions at the genetic and epigenetic levels. Various sets of genes are expressed in spatial and temporal regulation, and the same genes are often used repetitively with different roles in different developmental stages [1–3]. To precisely understand molecular mechanisms of cell differentiation, it is necessary to evaluate differentiation stage-specific roles of genes. Spatial and temporal gene modification using inducible gene regulation systems such as cre-loxP- or tetracycline (tet)-regulated system have been largely contributed to elucidate the developmental roles of target genes [4,5].

Embryonic stem (ES) cells are important materials for regenerative therapeutic approaches and developmental research. We have developed a novel ES cell differentiation system using 2-dimentional culture and fluorescence activated cell sorting (FACS) that can trace cell differentiation processes [6]. Vascular endothelial growth factor receptor-2 (VEGFR2) is the earliest differentiation marker for endothelial cells (ECs) and blood cells, and a marker for lateral plate mesoderm [7,8]. We induced $VEGFR2^+$ cells from ES cells, purified them by FACS, and re-cultured the purified cells [6]. We have succeeded in inducing vascular ECs and mural cells (pericytes and vascular smooth muscle cells) from common progenitor $VEGFR2^+$ cells [9], and have reproduced the early process of vascular development including arterial, venous, and lymphatic diversification in vitro [6,9–11]. This system is amenable to collect cells at various intermediate differentiation stages [12].

RNA interference is a nucleotide sequence-specific gene silencing phenomenon mediated by double-strand RNA

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[13,14]. Introduction of small interfering RNA such as short hair-pin RNA (shRNA) into living cells and/or individuals is now a potent tool for gene function analysis [14,15]. Recently, vector-based shRNA expression systems using pol III promoter, such as U6, H1, and tRNA promoter have been developed to efficiently express shRNA in mammalian cells [16–23]. Furthermore, various inducible gene expression systems, including tet- or cre-loxP-regulated ones, have been demonstrated to be applicable to pol III promoter-driven short RNA expression systems [24–28]. Continuous inhibition (or stimulation) of genes in ES cells should broadly affect ES cell derivation, proliferation, and differentiation in the early phase, hampering precise determination of stage-specific roles of target genes. Inducible gene manipulation system, thus, should be critical to investigate differentiation mechanisms using ES cells.

In the present study, to establish a novel experimental strategy to evaluate stage-specific gene functions for differentiation in vitro, and to elucidate cell differentiation mechanisms at the cellular level, we applied tet-inducible shRNA expression systems to our ES cell differentiation system, and demonstrated that controlled expression of shRNA during ES cell differentiation can successfully suppress target gene expression in a stage-specific manner and can modify differentiating cell fates by targeting essential genes for differentiation.

Materials and methods

Plasmids. Tet-inducible tRNA^{val} promoter was generated by inserting seven tetO sequences upstream of tRNA^{val} promoter in pPUR-tRNA plasmid (pPUR-tet-tRNA plasmid) [23]. Tet-inducible U6 promoter was established by inserting tetO sequence into the downstream of proximal promoter of U6. The tet-inducible U6 promoter showed detectable transcriptional activity within 4 h after tetracycline treatment [29]. Tet-inducible U6 promoter was then applied to hairpin-type siRNA expression vector using pU6i cassette vector (ptetU6icassette vector) [30]. CMV promoter-driven tTS plasmid and EGFP plasmid were purchased from Clontech Laboratories (Mountain View, CA).

shRNA construction. We constructed our own algorithm for the prediction of target sites of RNAi [31,32]. Nucleotide sequences of shRNA were selected using our algorithm and others, such as BLOCK-iT™ RNAi designer (invitrogen, Carlsbad, CA; https://rnaidesigner.invitrogen.com/rnaiexpress/), siDirect™ (Funakoshi Life Science, Tokyo, Japan) [33], and siRNA Design Support System (Takara Bio Inc., Ohtsu, Japan; http://www.takara-bio.co.jp/rnai/intro.htm/). For tRNA promoter system, single-strand DNAs of 5′-proximal region of tRNA^{val} promoter (CGAAA CCGGGCACTACAAAAACCAACTCC)-sense target sequence (19 nt)-loop (15 nt; ACGTGTGCTGTCCGT)-antisense target sequence (19 nt)-TTTTTT-GCATG-3′ and 5′-CAAAAAA-complementary antisense target sequence (19 nt)-complementary loop (15 nt; ACGGACAGCACACGT)-complementary sense target sequence (19 nt)-complementary proximal region of tRNA^{val} promoter (GGAGTTGGTTTTTGTAGTGCCCG GTTT)-3′ were synthesized, and annealed to form double-strand DNA. This double-strand DNA was subcloned between BstBI and SphI site of pPUR-tet-tRNA vector. CMV promoter-driven EGFP gene was subcloned upstream of shRNA expression gene, and CMV promoter-driven tTS gene and CMV promoter-driven puromycin-resistant gene were subcloned downstream of shRNA expression gene, respectively (GFP-tRNAtetshRNA-tTS vector) (Fig. 1A). For U6 promoter system, single-strand DNAs of 5′-CACC-sense target sequence-loop-antisense target sequence-TTTTT-3′ and 5′-GCAT-AAAAA-complementary antisense target sequence-complementary loop-complementary sense target sequence-3′ were synthesized (Invitrogen), and annealed to form double-strand DNA. The double-strand DNA was subcloned into BspMI site of ptetU6icassette vector (GFP-U6tetshRNA-tTS vector) (Fig. 1A).

shRNA sequences for VEGFR2 (shRNA-VEGFR2) were as shown in Fig. 1B. C → T or A → G mutations were introduced only in the sense strand of target sequences. The introduction of the multiple mutations in the sense strand could contribute to the stable maintenance in their bacterial host, and in some cases, could increase the silencing effect of the generated siRNA [30].

Cell culture. EB5, a subline derived from E14tg2a ES cell line, was a generous gift from Dr. H. Niwa (Center for Developmental Biology, RIKEN, Japan). EB5 cells were maintained as described [34]. Stable ES cell lines with tet-inducible shRNA were generated by introduction of GFP-tRNAtetshRNA-tTS vector to EB5 ES cells using mouse ES cells Nucleofector Kit (Amaxa Biosystems, Cologne, Germany) and selection by 1 μg/ml puromycin. Puromycin-resistant, GFP-expressing colonies were selected and subjected to further study.

To test the effects of shRNA-VEGFR2, CMV promoter-driven tTS gene was introduced into F2, a mouse endothelial cell line [35], to generate stable transformant of F2 cells expressing tTS gene (F2-tTS cells). GFP-tRNAtetshRNA(VEGFR2)-tTS vector was transfected into F2-tTS cells using FuGene6 (Roche Diagnostics, Basel, Switzerland), and 0–10 μg/mL of doxycycline (Dox) (BD Biosciences, Bedford, MA) was added 48 h after transfection. Forty-eight hours after Dox treatment, cells were harvested for Western blot analysis.

ES cell differentiation. Induction of VEGFR2^{+} cells and ECs was performed as described [6,9]. Undifferentiated ES cells were cultured in differentiation medium (alpha minimum essential medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) and 50 mmol/l 2-mercaptoethanol) on type-IV collagen-coated dishes (Becton–Dickinson Labware, Bedford, MA) to induce differentiation.

Antibodies and reagents. Monoclonal antibodies (MoAbs) for murine VEGFR2 (AVAS12) and vascular endothelial cadherin (VEcad) (VECD1)

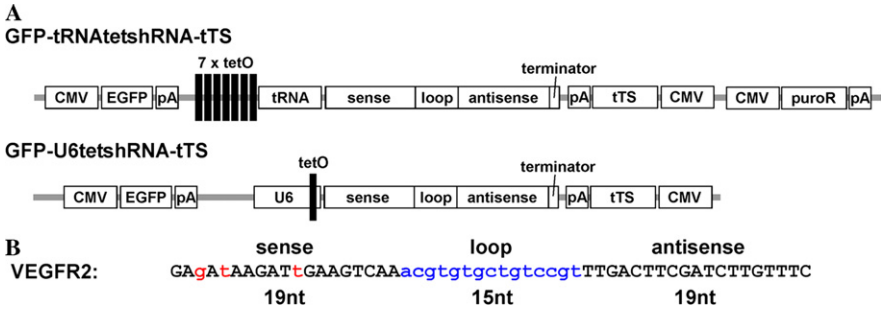

Fig. 1. Structure of shRNA vectors. (A) Schematic structure of tet-inducible tRNA^{val} promoter-driven or U6 promoter-driven shRNA expression vectors. (B) Nucleotide sequences of shRNA for VEGFR2. Loop sequences are indicated by small letters in blue. Small letters in red are C → T or A → G mutations introduced only in the sense strands.

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