Proteomic comparison of human embryonic stem cells with their differentiated fibroblasts: Identification of 206 genes targeted by hES cell-specific microRNAs

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Abstract

Human embryonic stem (hES)-T3 (T3ES) cells were spontaneously differentiated into autogenic fibroblast-like T3DF cells, as feeder cells with the capacity to support the growth of undifferentiated hES cells. The proteomes of undifferentiated T3ES cells and their differentiated T3DF fibroblasts were quantitatively compared. Several heterogeneous nuclear ribonucleoproteins and glycolytic enzymes, including L-lactate dehydrogenase A (M), were found to be abundantly and differentially expressed in T3ES cells and T3DF fibroblasts, respectively. Both miRNA and mRNA profiles from the undifferentiated T3ES cells and their differentiated T3DF fibroblasts had been previously determined. In this investigation, 206 genes were found to be targets of the four hES cell-specific miRNAs of miR-302d, miR-372, miR-200c, and/or miR-367 by using two-fold differential expression and inverse expression levels (highly negative correlations) of miRNAs to their target mRNAs. That YWHAZ (14-3-3 zeta) is a target of miR-302d and miR-372 was further confirmed by proteomic comparison between T3ES cells and their differentiated T3DF fibroblasts. According to GeneOntology analyses, almost 50% of these 206 target proteins are nuclear and are involved in gene transcription. Identifying the target mRNAs of hES cell-specific miRNAs will provide a better understanding of the complex regulatory networks in hES cells. Furthermore, these miRNA-targeted proteins play important roles in differentiation of hES cells and during embryo development.

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Introduction

Human embryonic stem (hES) cell lines have been derived from the inner cell mass of preimplantation embryos donated at in vitro fertilization clinics, and the undifferentiated growth of hES cells has been traditionally maintained on inactivated mouse embryonic fibroblasts (MEF) [1,2]. These hES cell lines possess the remarkable abilities of both unlimited self-renewal and pluripotency to generate any cell type differentiated from the three germ layers (the ectoderm, mesoderm, and endoderm) [3]. Thus, these hES cell lines have a great potential for use in cell therapy for regenerative medicine, as experimental models for drug discovery and toxicity testing, and for studying the basics of stem cell biology and molecular embryogenesis [4]. To reduce the risks of cross-transfer of pathogens from xenogeneic feeders, an autogeneic feeder cell system, comprising fibroblast-like cells differentiated from hES cells, was developed to grow undifferentiated and pluripotent hES cells for various medical applications [5].

The proteome of the hES autogeneic differentiated fibroblasts has not yet been investigated, although the proteome of undifferentiated hES cells has previously been reported [6,7]. Proteomics has emerged as a robust method for performing large-scale studies of proteins to complement high-throughput gene expression analysis at the mRNA level. Two-dimensional gel electrophoresis (2-DE) is widely used in comparative studies of protein expression patterns between different cells to identify differentially expressed and/or modified proteins. The combination of 2-DE and nanoflow liquid chromatography-tandem mass spectrometry (LC-MS/MS) is broadly applied to proteomic analyses of stem cells, including hES cells [8–10].

MicroRNAs (miRNAs) are noncoding RNAs of approximately 22 nucleotides in length that play important roles in mammalian embryo development and cell differentiation [11–15]. In humans and mice, several embryonic stem cell-specific miRNAs have been reported [16–18]. These miRNAs were shown to be rapidly downregulated during differentiation, whereas miRNAs reported from other cell types were poorly expressed in embryonic stem cells. Identifying the target miRNAs of hES cell-specific miRNAs is an important prerequisite for understanding the complex and interesting networks of regulation in hES cells.

Our laboratory had previously derived five hES cell lines [19], and autogeneic differentiated fibroblast-like cells were established as feeders with the capacity to support the growth of undifferentiated hES cells [20]. In this investigation, the proteomes of undifferentiated hES-T3 (T3ES) cells and their differentiated fibroblasts (T3DF) were quantitatively compared. Several heterogeneous nuclear ribonucleoproteins (hnRNPs) and glycolytic enzymes, including L-lactate dehydrogenase A (M), were quantitatively compared. Several heterogeneous nuclear ribonucleoproteins (hnRNPs) and glycolytic enzymes, including L-lactate dehydrogenase A (M), were shown to be rapidly downregulated during differentiation [20]. Several heterogeneous nuclear ribonucleoproteins (hnRNPs) and glycolytic enzymes, including L-lactate dehydrogenase A (M), were shown to be rapidly downregulated during differentiation [20]. Several heterogeneous nuclear ribonucleoproteins (hnRNPs) and glycolytic enzymes, including L-lactate dehydrogenase A (M), were shown to be rapidly downregulated during differentiation [20]. Several heterogeneous nuclear ribonucleoproteins (hnRNPs) and glycolytic enzymes, including L-lactate dehydrogenase A (M), were shown to be rapidly downregulated during differentiation [20]. Several heterogeneous nuclear ribonucleoproteins (hnRNPs) and glycolytic enzymes, including L-lactate dehydrogenase A (M), were shown to be rapidly downregulated during differentiation [20]. Several heterogeneous nuclear ribonucleoproteins (hnRNPs) and glycolytic enzymes, including L-lactate dehydrogenase A (M), were shown to be rapidly downregulated during differentiation [20].

Materials and methods

hES cell culture

The hES cell line hES-T3, one of the five hES cell lines derived with institutional review board approval from preimplantation embryos donated at in vitro fertilization clinics in Taiwan, exhibits a normal female karyotype (46, XX). This cell line was continuously cultured on a mitotically inactivated MEF feeder layer in hES medium under 5% CO₂ at 37°C and subjected to freezing/thawing processes as previously described [20]. The undifferentiated state of hES-T3 cells (Passage 36) grown on MEF feeder layer was indicated by expression of OCT4 and NANOG, and these undifferentiated cells were designated as T3ES.

Establishment of autogeneic fibroblast-like T3DF cells

Autogeneic feeder cells with the ability to support the growth of undifferentiated T3ES cells were established according to the previously published procedure [5,20]. The hES-T3 (Passage 19) cells were transferred into feeder-free and noncoated plates (10 cm) in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum (GIBCO, Carlsbad, CA, USA) under 5% CO₂ at 37°C. After 10 days, cells appeared as fibroblast-like morphology, and these differentiated fibroblast-like cells were designated as T3DF. These T3DF cells were passaged using trypsin (0.05%, GIBCO) every 4 days.

Two-dimensional differential in-gel electrophoresis of proteins

Protein samples from approximately 1 × 10⁶ cells of T3ES and T3DF on each 10 cm plate were precipitated with 11% wt/vol trichloroacetic acid per 1 mL ice-cold acetone at −20°C for 30 minutes. The pellets were washed twice with ice-cold acetone and resuspended in a solubilizing buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, and 30 mM Tris. Protein concentration was measured by the Bradford method. CyDye labeling was carried out according to the manufacturer’s protocol (GE Healthcare, Little Chalfont, UK). In brief, 50 µg of proteins from T3ES and T3DF cells were labeled with Cy3 and Cy5 fluorescence dye, respectively, in the dark for 30 minutes. The sample labeling was stopped by the addition of 10 mM lysine for 10 minutes on ice in the dark. The two samples were then pooled and mixed with an equal volume of 2× sample buffer (7 M urea, 2 M thiourea, 2% wt/vol CHAPS, 1% immobilized pH gradient (IPG) buffer pH 3–10 nonlinear, 100 mM D-DeStreak reagent, and 0.002% bromophenol blue). Immobiline DryStrips (13 cm; pH 3–10 nonlinear; Amersham Biosciences, Uppsala, Sweden) were rehydrated for 10 hours in 250 µL rehydration solution [7 M urea, 2 M thiourea, 2% wt/vol CHAPS, 20 mM dithiothreitol (DTT), 0.5% IPG buffer, and 0.002% bromophenol blue]. Protein samples were loaded onto the strips using Ettan IPGphor cup-loading (Amersham Biosciences) according to the manufacturer’s protocol. Isoelectric focusing was carried out by an IPGphor
apparatus (GE Healthcare) as follows: 350 V, step and hold, 3 hours; 650 V, gradient, 1 hour; 1,100 V, gradient, 1 hour; 8,000 V, gradient, 1.5 hour; and 8,000 V, step and hold, 3 hours, giving a total of 16,000 Vh. After focusing, the strips were equilibrated by shaking for 15 minutes in 5 mL equilibration buffer (50 mM Tris-base, pH 8.8, 6 M urea, 30% vol/ vol glycerol, 0.2% wt/vol sodium dodecyl sulfate, and 1% wt/vol DTT), followed by 5 mL of the same solution but with 2.5% wt/vol iodoacetamine instead of DTT for 15 minutes. Separation of the proteins in the second dimension was performed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12% wt/vol separation gel with a constant voltage of 65 V for 0.5 hours followed by 120 V for 14 hours. The separated gel was visualized with the Typhoon 9410 scanner (Amersham Biosciences, Uppsala, Sweden), and the images of the protein spots were quantified and numbered using the DeCyder Differential Analysis Software, Version 5.0 (Amersham Biosciences). The protein abundance of each spot was expressed as a percentage of total volume on the 2-DE gel image. The proteins that were both abundantly (more than 0.1%) and differentially (more than three folds of changes) expressed were selected for LC-MS/MS analysis.

LC-MS/MS identification of proteins

The proteins of selected spots were digested in-gel overnight at 37°C using sequencing grade trypsin (20 μL of 10 μg/mL in 25 mM ammonium bicarbonate, pH 8.5). The supernatant was used for analysis by nanoflow LC and Micromass quadrupole-TOF (Micromass, Manchester, UK) Ultima Global mass spectrometer (Manchester, UK). Peptides were separated by a capillary LC system (Waters, Milford, CT), with a capillary analytical column (Symmetry C18, 75 μm ID, 100 mm; Waters), a desalting column (C18 PepMap, 300 μm ID, 5 mm; LC Packings, Sunnyvale, CA) and a switching valve. After on-line desalting with 0.1% formic acid for 3 minutes, the switching valve was auto switched to an analytical position. The tryptic peptides were then separated over a reversed-phase C18 column with a flow rate of 400 nL/min. Mobile phase A was 0.1% formic acid: acetonitrile = 95:5 (vol/vol) and mobile phase B was 0.1% formic acid: acetonitrile = 5:95 (vol/vol). The LC gradient conditions were as follows: base on time (t) set at the mobile phase: t = 0–3 minutes, hold% B = 10; t = 3–45 minutes, %B from 10 to 75; and t = 45–60 minutes, %B from 75 to 100.

Peptides separated from the capillary column were directed to the nanospray source by a 20 μm i.d. and 90 μm o.d. fused-silica capillary. A voltage of 3.2 kV was applied to the nanosource. The mass spectrometer was operated in positive ion mode with a cone voltage of 80 V and a source temperature of 80°C. The time-of-flight analyzer was set in the V-mode. MS/MS spectra were acquired in a data-dependent acquisition mode in which the two multiple-charged (+2 and +3) peaks with the three most abundant ions were selected for collision-induced dissociation. The parent ion was excluded if the same molecular weight ion was detected within 200 seconds. During the auto scans, collision energies were set at 10 V and 30 V, and argon was used as the collision gas. MS/MS spectra obtained for each of the parent ions were processed by MassLynx 4.0 software (Waters, Milford, MA) to get the corresponding peak lists. Proteins were identified by comparing experimental data with the NCBI nonredundant protein sequence database using the Mascot search engine (http://www.matrixscience.com). The mascot score was −10 × log(P), where P is the probability that the observed match is a random event, with a cutoff p value of 0.05.

miRNA and mRNA analyses

Total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA), and the same total RNA from each sample was used for both miRNA quantification and mRNA microarray analysis [20]. The expression levels of 250 human miRNAs were determined using the TagMan MicroRNA Assays (Applied Biosystems, Foster City, CA) as described previously [20–22]. Purified miRNAs were analyzed using Affymetrix Human Genome U133 plus 2.0 GeneChip (Santa Clara, CA) according to the manufacturer’s protocols. GeneChips from the hybridization experiments were read by the Affymetrix GeneChip scanner 3000, and raw data were processed using the GC-RMA algorithm. The raw data were also analyzed by GeneSpring GX software version 7.3.1 (Silicon Genetics, Redwood City, CA).

Target prediction of miRNAs

The miRNAs negatively regulate posttranscriptional expression by translational repression and/or destabilization of protein-coding mRNAs. Recent studies have revealed the impact that miRNAs can have on protein output. Although some targets were repressed without detectable changes in mRNA levels, those that were translationally repressed by more than a third additionally displayed detectable mRNA destabilization; and, for the more highly repressed targets, mRNA destabilization was usually the major component of repression [23,24]. The potential target genes of hES cell-specific miRNAs were predicted using the PicTar (four-way, http://pictar.bio.nyu.edu/) and TargetScanS (http://www.targetscan.org/) with a cutoff p value less than 0.05 [25]. The expression levels of the predicted target miRNAs were first analyzed by the Volcano plot using the parametric test and Benjamini-Hochberg false discovery rate for multiple testing correction. The abundantly and differentially expressed miRNAs were selected by more than a three-fold overall mean in T3DF cells and changes of more than two-fold (rather than three-fold) between T3DF and T3ES cells with a p value cutoff of 0.05. The negative correlation coefficients between miRNAs and their target mRNAs were calculated to demonstrate their inverse expression levels [20].

GeneOntology of hES cell-specific miRNA targets

The GeneOntology, including molecular functions and cellular localization, of differentially expressed, abundant targets of hES cell-specific miRNAs were analyzed using GeneSpring GX software version 7.3.1 GeneOntology Browser and MetaCore Analytical Suite (GeneGo Inc., St Joseph, MI). The current NetAffx annotation of transcript
26, dated July 8, 2008 was used for analyzing molecular functions of miRNA targets [20].

**Results**

Two-dimensional differential in-gel electrophoresis analysis and protein identification

Soluble proteins from T3ES and T3DF cells were separately labeled with Cy3 and Cy5, respectively, and then pooled together for comparison on a single two-dimensional differential in-gel electrophoresis (2D-DIGE). The fluorescently labeled proteins on 2D-DIGE were imaged using a Typhoon 9410 Variable Mode Imager (Fig. 1). The abundance of 884 protein spots was quantitated using DeCyder differential in-gel analysis software, and expressed as a percentage of total volume. The relative fold changes in protein expression levels were estimated by the fluorescent ratios of either Cy3%/Cy5% (T3ES/T3DF) or Cy5%/Cy3% (T3DF/T3ES). A total of 115 protein spots were selected for LC-MS/MS analyses, including 46 (green) and 66 (red) abundant (more than 0.1%) protein spots with more than three-fold changes, as well as three (yellow) protein spots of similarly abundant expression levels, from T3ES and T3DF cells. A sum of 64 protein spots, including 17 green, 44 red, and 3 yellow, were identified (Fig. 2, Supplementary Table S1). Interestingly, 13 of 17 differentially expressed abundant protein spots (green) in T3ES cells were found to be hnRNPs, and each of the 3 hnRNP proteins (A1, H1, and L) appeared to have two spots of different charges. Among the 44 differentially expressed abundant protein spots (red) identified in T3DF cells, cellular retinoic acid binding protein 1 (CRABP 1) and heat shock protein 90 (Hsp90) were the two most abundantly expressed proteins (1.84% and 1.53%, respectively), and several glycolytic enzymes, including aldolase, triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, enolase, pyruvate kinase, and l-lactate dehydrogenase A (M), were also found to be abundantly and differentially expressed. Actins and translation elongation factor 1 were abundantly expressed at similar levels between the T3ES and T3DF cells.

Target identification of miRNAs expressed highly in hES cells

The genome-wide mRNA expression in both undifferentiated hES cells hES-T3 (T3ES) and hES-T3 differentiated fibroblast-like cells (T3DF) was determined using the Affymetrix human genome U133 plus 2.0 GeneChip. The original data had been deposited in the NCBI database (GSE9440), and the results were described previously [20]. The expression levels of 250 human miRNAs in T3ES and T3DF cells were quantitated using TagMan MicroRNA Assays as described previously [21,22]. The four hES cell-specific miRNAs (miR-302d, miR-367, miR-372, and miR-200c) were found to be expressed abundantly (at levels higher than 20-fold that of U6 snRNA) in T3ES cells, but at low levels (0.03-fold of U6 snRNA) in T3DF cells [20]. The potential targets of these four hES cell-specific miRNAs were predicted using both the PicTar and the TargetScanS methods. The expression levels of their target mRNAs in T3ES and T3DF cells were analyzed by the Volcano plot, and 206 genes targeted by these four miRNAs were identified using two-fold differential expression and inverse expression levels (negative correlations) between miRNAs and their target mRNAs (Supplementary Table S2).

Proteomic confirmation of the miRNA target YWHAZ

The YWHAZ (14-3-3 zeta) protein targeted by miR-302d and miR-372 was found to be in common between the 44 abundantly and differentially expressed proteins in T3DF cells (Supplementary Table S1B) and the 206 targets of the 4 hES cell-specific miRNAs miR-302d, miR-372, miR200c, and/or miR-367 (Supplementary Table S2). The positions and abundance (measured as the volume) of this YWHAZ protein spot (756) on Cy3 and Cy5 images are indicated in Figure 1.

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**Figure 1.** Cy3 (green), Cy5 (red), and merged images. The proteins from human embryonic stem (hES)-T3 (T3ES) and T3 differentiated fibroblasts (T3DF) cells were labeled with (A) Cy3 (green) or (B) Cy5 (red), and the fluorescent images were obtained using a Typhoon 9410 Imager. (C) Cy3 and Cy5 images were merged, and yellow spots indicate the proteins with similar abundant expression levels in T3ES and T3DF cells.
Fig. 3. The results of LC-MS/MS identification and expression ratio of YWHAZ proteins, as well as its mRNAs, between T3DF and T3ES cells are given in Table 1. The expression ratio (T3DF/T3ES = 3.35) of YWHAZ protein was consistent with that (T3DF/T3ES = 2.49/1.06 = 2.35) of its mRNAs [20].

Molecular functions of hES cell-specific miRNA targets

The molecular functions of the 206 genes targeted by the four hES cell-specific miRNAs miR-302d, miR-372, miR-200c, and/or miR-367 were analyzed using the GeneSpring GeneOntology Browser and MetaCore Analytical Suite (Table 2). The top four molecular functions of the eight categories obtained by GeneSpring were the same as those obtained by MetaCore. All of the top four molecular functions are involved in gene transcription. As to their cellular locations, 102 targets (p value of 2.2E-07) of these 206 genes were localized in nucleus.

Discussion

Proteomics can provide a powerful approach for comparing the characteristics of hES cells with their autogenic differentiated fibroblasts. Thus, the proteomes of undifferentiated T3ES cells and their fully differentiated T3DF fibroblasts were quantitatively compared in this investigation. In the proteome of T3ES cells, hES cell-specific markers, such as OCT4, NANOG, and SOX2, were not detected among abundantly and differentially expressed proteins, whereas 13 of 17 abundantly and differentially expressed protein spots were found to be hnRNP proteins. The hnRNP proteins C and L were also reported to be enriched in undifferentiated hES cells but downregulated in differentiated embryoid bodies [26]. Our finding of abundant hnRNP proteins in undifferentiated hES cells is consistent with the high ratio of nucleus to cytoplasm in hES cells [1] because hnRNP proteins are among the most abundant proteins in the nucleus [27].

In humans, hnRNP proteins consist of a large group (more than 20) of nuclear RNA binding proteins, and they are given alphabetical names based on size from hnRNP A1 to hnRNP U. The hnRNP proteins A1 and M were found to be the two most abundantly expressed proteins (2.15% and 1.42%, respectively) in this investigation. The hnRNP proteins play important roles in alternative mRNA splicing and mRNA localization. Alternative mRNA splicing is a critical process that ensures the production of numerous proteins from a limited set of human genes [28]. Alternative splicing can also remove binding sites for posttranscriptional repression by miRNAs [29], and alternative splicing has recently been shown to regulate mouse embryonic stem cell pluripotency and differentiation [30]. It should further be noted that each of the three hnRNP proteins (A1, H1, and L) appeared to have two spots of different charges. The extremely abundant expression and multiple spots of hnRNP proteins indicate a very active pre-mRNA processing (i.e. alternative mRNA splicing) and the important role of posttranslational modifications on hnRNP protein functions in hES cells. The exact nature of posttranslational modifications (phosphorylation of Ser/Thr and/or methylation of Arg) and how these posttranslational
modifications affect the functions of hnRNP proteins in hES cells remain to be investigated.

Regarding the proteome of autogeneic fibroblast-like T3DF cells, the abundant expression of several glycolytic enzymes, including \(\text{L-lactate dehydrogenase A} \ (M)\), is consistent with the more anaerobic metabolism of fibroblasts. The multiple spots observed for annexin A2, enolase 1, galectin1, lamin A/C, and the eukaryotic translation elongation factor 2 may be because of different splicing isoforms and/or posttranslational modifications. Among the 44 abundantly and differentially expressed protein spots identified, CRABP 1, and Hsp90 were the two most abundantly expressed proteins. The CRABP 1 plays an important role in retinoic acid-mediated differentiation and proliferation processes. It is structurally similar to the cellular retinol-binding proteins but binds only retinoic acid at specific sites within the nucleus; this may contribute to vitamin A-directed differentiation in epithelial tissue. Hsp90 plays a well-known role of cytoplasmic-nuclear transport of steroid receptors as a molecular chaperone. Furthermore, incorporation of the Argonaute RNA-binding proteins into cytoplasmic P-bodies is mediated by Hsp90, and inhibition of Hsp90 interferes with posttranscriptional gene silencing by miRNAs [31].

The miRNA lin4 was first discovered to be essential for the normal temporal control of diverse postembryonic developmental events in nematodes by regulating lin14 mRNA translation via an antisense RNA-RNA interaction.

Table 1  Proteomic identification of miRNA target YWHAZ

<table>
<thead>
<tr>
<th>LC-MS/MS identification</th>
<th>Score, 195</th>
<th>5 Peptides matched</th>
</tr>
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<tbody>
<tr>
<td>aa</td>
<td>42–49</td>
<td>NLLSVAYK</td>
</tr>
<tr>
<td>aa</td>
<td>84–91</td>
<td>EKIETEIR</td>
</tr>
<tr>
<td>aa</td>
<td>104–115</td>
<td>FLIPNASAESK</td>
</tr>
<tr>
<td>aa</td>
<td>128–139</td>
<td>YLAEEAADKKK</td>
</tr>
<tr>
<td>aa</td>
<td>213–222</td>
<td>DSTLIMOLLR</td>
</tr>
</tbody>
</table>

Unigene: Hs.693311; Ratio of mRNAs (data from Li et al. [20]): T3DF/T3ES = 2.49/1.06 = 2.35.

Protein spot 756: fold changes of Cy5% /Cy3% on 2D-DIGE = 3.35.

LC-MS/MS = liquid chromatography-tandem mass spectrometry; T3DF = T3 differentiated fibroblasts; T3ES = human embryonic stem (hES)-T3; YWHAZ = 14-3-3 protein zeta = tyrosine 3/tryptophan 5-monoxygenase activation protein, zeta isoform.
A total of 759 probes for 206 genes. A comparison of the 206 human targets with the 253 mouse cells lacking Dicer of the miRNA processing enzyme [34]. were found by transcriptome analysis of embryonic stem 290 cluster (corresponding to the human miR-302 cluster) of p53 [33]. Recently, 253 target mRNAs of the mouse miR-302d and miR-372 was further confirmed by the proteomic analysis in this investigation. These results will provide a better understanding of the complex regulatory networks involved in hES cell differentiation. In summary, 206 genes targeted by four hES-specific miRNAs were identified, the proteins of which play important roles in hES cell differentiation and embryo development. YWHAZ, which belongs to the 14-3-3 family of conserved regulatory molecules expressed in all eukaryotic cells, is a target of miR-302d and miR-372, as confirmed by the proteomic analysis in this investigation. These results will provide a better understanding of the complex regulatory networks involved in hES cell differentiation.

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### Supplementary material

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### References


