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Research paper

Selection of RNA aptamers against mouse embryonic stem cells

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ABSTRACT

Embryonic stem cells (ESCs) are capable of unlimited self-renewal and differentiation into multiple cell types. Recent large-scale analyses have identified various cell surface molecules on ESCs. Some of them are considered to be beneficial markers for characterization of cellular phenotypes and/or play an essential role for regulating the differentiation state. Thus, it is desired to efficiently produce affinity reagents specific to these molecules. In this study, to develop such reagents for mouse ESCs (mESCs), we selected RNA aptamers against intact, live mESCs using several selection strategies. The initial selection provided us with several anti-mESC aptamers of distinct sequences, which unexpectedly react with the same molecule on mESCs. Then, to isolate aptamers against different surface markers on mESCs, one of the selected aptamers was used as a competitor in the subsequent selections. In addition, one of the selections further employed negative selection against differentiated mouse cells. Consequently, we successfully isolated three classes of anti-mESC aptamers that do not compete with one another. The isolated aptamers were shown to distinguish mESCs from differentiated mouse cell lines and trace the differentiation process of mESCs. These aptamers could prove useful for developing molecular probes and manipulation tools for mESCs.

1. Introduction

Embryonic stem cells (ESCs), which are derived from the inner cell mass of blastocysts, can differentiate into all three germ layers [1–3]. Under optimized culture conditions, ESCs maintain the undifferentiated state and self-renew indefinitely [4,5]. Owing to pluripotency and growth ability, ESCs are valuable sources in the fields of regenerative medicine [6] and developmental biology [7]. Although extensive studies have been pursued to uncover the molecular mechanisms underlying the maintenance of the undifferentiated state and the regulation of differentiation [8,9], further studies are necessary for the comprehensive understanding of the molecular mechanisms, as well as the medical applications of ESCs.

Cell surface molecules can be ideal markers for the classification and isolation of desired cell populations, and play an essential role in cellular states by transferring extracellular signals to intracellular response systems [10,11]. Recent extensive, large-scale analyses of transcriptome, proteome, glycome, *etc*., have identified varieties of cell surface molecules on ESCs, including ESC-specific markers and signaling molecules [12,13]. Antibodies against such molecules are widely used for the characterization of cellular phenotypes associated with each marker and the isolation of target cell populations. However, there remains scope for developing new reagents to extend the range of markers and manipulation systems. In this study, we aimed to develop RNA aptamers as novel affinity reagents for mESCs.

Aptamers are short single-stranded nucleic acid molecules that are selected *in vitro* from a large random sequence library based on their high affinity to a target molecule by a process known as SELEX (Systematic Evolution of Ligands by EXponential enrichment) [14–16]. Aptamers can serve as attractive molecular probes in view of several advantages over canonical probes. First, aptamers exhibit high affinity against targets with dissociation constant in the nanomolar to picomolar range. Second, aptamers can achieve high specificity so as to distinguish subtle differences between target and non-targets. For example, RNA aptamers against the diphosphorylated form of extracellular regulated kinase 2 (ERK2) can distinguish one phosphate group and hardly bind to non-phosphorylated ERK2 [17]. Third, contrary to antibody, aptamers of constant quality are easily available due to a chemical production process. Finally, aptamers are relatively small molecules compared to antibody and accessible to targets masked by surrounding molecules, which are inaccessible to antibody [18].

In the literature, a wide variety of aptamers has been generated by SELEX, mostly targeting highly purified molecules [14–16].

Abbreviations: ESCs, embryonic stem cells; mESCs, mouse embryonic stem cells; SELEX, systematic evolution of ligands by exponential enrichment.

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However, recent development of cell-based SELEX procedures enabled us to isolate aptamers against cell surface molecules of unknown identity or proteins inappropriate for purification in fully active conformations [19–22]. Therefore, one could assume that cell-based SELEX might be appropriate for the selection of aptamers against cell surface markers of unknown identity on ESCs, by coupling with negative selection against differentiated cells.

In this study, we performed SELEX against live mESCs and isolated several RNA aptamers to mESCs. Unexpectedly, all the analyzed aptamers selected in the initial SELEX competed with one another for binding to mESCs, indicating that these aptamers recognize the same target. However, the subsequent selections employing one of the aptamers as a competitor gave rise to distinct anti-mESC aptamers that are non-competitive with one another. The aptamers bind to mESCs, but hardly to differentiated cells, suggesting the potential utility of these aptamers as molecular probes to detect or isolate mESCs.

2. Materials and methods

2.1. Cell culture

Mouse ESCs (cell line CCE) were cultured as previously described [23]. Induction of mESC differentiation was performed by treatment with 0.1 μM retinoic acid (RA; Sigma-Aldrich) in the medium without leukemia inhibitory factor (LIF; Chemicon/Millipore) for the indicated periods [24]. C2C12 (RCB0987), Hepa 1–6 (RCB1638) and NB2a (RCB2639) cells were provided by RIKEN BioResource Center (Japan) and cultured according to the supplier's recommendation. A-9 and NIH 3T3 were kind gifts from Dr. Tomoko Kozu (Saitama Cancer Center, Japan) and cultured with Dulbecco's modified Eagle's medium (Invitrogen/Life Technologies) containing 10% fetal bovine serum (Invitrogen), 20 U/ml penicillin and 20 μg/ml streptomycin (Invitrogen). All the cells were subcultured before confluence.

2.2. Preparation of random RNA pools

Template DNAs for transcription of random sequences were amplified from 8.3 pmol (5 x 10^{14} molecules) of synthetic DNAs (purchased from Operon Biotechnologies, Japan) carrying random sequences by the polymerase chain reaction (PCR) with *ExTaq* DNA polymerase (Takara Bio, Japan). The amplified sequences contained a 60- or 40-nucleotide random region (N60 or N40, respectively) flanked by two constant regions necessary for the reverse transcription (RT) and PCR amplification. Three lines of selections were carried out using distinct pool sequences: SELEX Line 1, 5′-TCTCGGATCC TCAGCGAGTC GTCTG-N60-CCGCATCGTC CTCCCTATAG TGAGTCGTAT TA-3′ (hereafter the T7 promoter region is underlined); SELEX Line 2, 5′-GCCTGTTGTG AGCCTCA-N40-CGTCCATTGT GTCCCTATAG TGAGTCGTAT TA-3′; SELEX Line 3, 5′-CTCTCATGTC GGCCGTTA-N40-TGGCATCCTT CAGCCCTATA GTGAGTCGTA TTA-3′. PCR primers were designed complementary to the constant regions in each selection line. The PCR products were transcribed in the presence of 2′-fluoro-CTP and 2′-fluoro-UTP (DuraScribe T7 transcription Kit, Epicentre Biotechnologies). After DNase I (Epicentre Biotechnologies) treatment, the RNAs were purified by polyacrylamide gel electrophoresis in the presence of 7 M urea.

2.3. SELEX against mESCs

Three lines of SELEX against mESCs were carried out under the conditions based on the reported procedure [20] (Fig. 1). Before the selection process, the purified RNAs were folded by denaturing at 95 °C for 5 min, followed by cooling to 4 °C and incubation in buffer A (20 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1.5 mM $CaCl_2$ and 0.5 mM $MgCl_2$) for 15 min at 37 °C. To reduce undesired, non-specific adsorption of RNAs to cells and culture dishes, 2 mg/ml of tRNA (Sigma-Aldrich) and 1 mg/ml of bovine serum albumin (Sigma-Aldrich) were added to the folded RNAs. As the negative selection in SELEX Line 3, the folded RNAs were transferred to 70%-confluent A-9 cells cultured on 60 mm-culture dishes followed by 5 min incubation at 37 °C. After the negative selection, the supernatant containing unbound RNAs was collected and used for the positive selection against mESCs. In the positive selection, the folded RNAs were transferred to 70%-confluent mESCs on culture dishes, followed by 10 min incubation at 37 °C. In SELEX Lines 2 and 3, an excess amount of a competitor aptamer, L1-65 (one of the anti-mESC aptamers selected in Line 1), and the pool RNAs were separately folded as described above and mixed just before the incubation with mESCs. After washing several times with buffer A, tightly bound RNAs were eluted from cells by adding EDTA-containing buffer (PBS-based cell dissociation buffer, Invitrogen), followed by the elimination of cell debris by centrifugation at

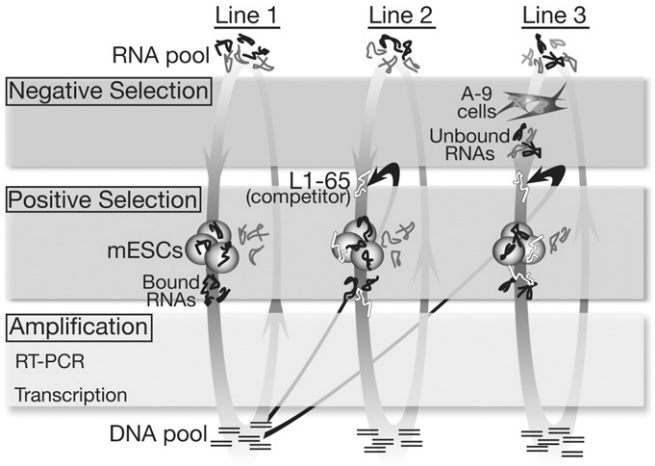

Fig. 1. Scheme of SELEX against live mESCs. In Line 1, unbiased positive selection against mESCs was carried out. In lines 2 and 3, the aptamer L1-65 selected in Line 1 was added as a competitor during the positive selection against mESCs. In Line 3, negative selection against A-9 cells (derived from mouse connective tissue) was further included before the positive selection.

Table 1
Conditions of SELEX against mESCs.

Round	Size of the culture dishes	Numbers of pool RNA molecules	Number of competitor RNA (L1-65) molecule	Numbers of washings[a]	Numbers of negative selection[b]
Line 1					
1	100 mm	1 x 10^{15}	–	–	–
2–3	60 mm	5 x 10^{14}	–	3	–
4–6	60 mm	5 x 10^{14}	–	5	–
7–10	60 mm	5 x 10^{14}	–	7	–
Line 2					
1	100 mm	1 x 10^{15}	–	3	–
2	60 mm	5 x 10^{14}	5 x 10^{14}	3	–
3–4	60 mm	5 x 10^{14}	5 x 10^{14}	4	–
5	60 mm	5 x 10^{14}	5 x 10^{14}	5	–
Line 3					
1	100 mm	1 x 10^{15}	–	3	–
2	60 mm	5 x 10^{14}	5 x 10^{14}	3	1
3	60 mm	5 x 10^{14}	5 x 10^{14}	4	1
4	60 mm	5 x 10^{14}	5 x 10^{14}	4	2
5	60 mm	5 x 10^{14}	5 x 10^{14}	5	2

[a] Numbers of washings after the incubation of the RNA pool with the target cells in the positive selection.
[b] Numbers of the negative selection against A-9 cells.

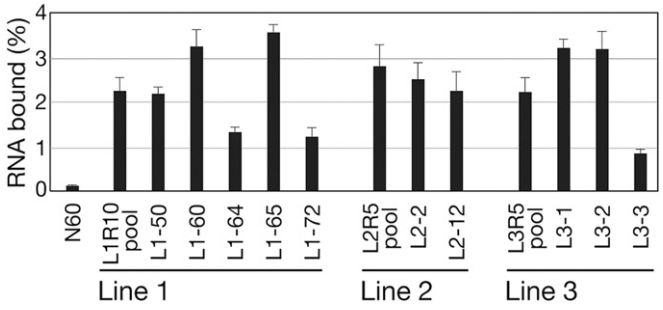

Fig. 2. Binding abilities of anti-mESC aptamers selected in biased and unbiased selection procedures. The binding abilities of the RNA pools (L1R10, L2R5, and L3R5) and individual selectants for mESCs were estimated using [^{32}P]-labeled RNAs. The random RNA pool for SELEX Line 1 (N60) was also analyzed as a control. The data represent the percentage of bound RNAs to input RNAs as the mean of three independent experiments and standard deviations with error bars.

200× g for 1 min. The recovered RNAs were precipitated with ethanol and reverse transcribed (ReverTra Ace, Toyobo, Japan) in the presence of ribonuclease inhibitor (Takara Bio). After 5–10 rounds of SELEX, variants in the pool DNAs were cloned into a TA cloning vector (pGEM-T Easy Vector system I, Promega). Detailed conditions of the SELEX are listed in Table 1.

2.4. Binding assay

Internally labeled RNAs were prepared by *in vitro* transcription in the presence of [α-^{32}P]-GTP or [α-^{32}P]-ATP (PerkinElmer). The RNAs were purified and folded as described above. The folded RNAs (~10,000 counts per minute, less than 200 fmol) were transferred to 70%-confluent cells cultured on 12-well multiwell plate (Sumitomo Bakelite, Japan). In the competitive binding assay, the separately folded labeled RNAs and unlabeled competitor RNAs were mixed just before transferring to the cells. After 10 min incubation at 37 °C, unbound RNAs were removed by washing three times with buffer A. The tightly bound RNAs were recovered as described above, and the radioactivity of the bound RNAs and the input RNAs were measured by the liquid scintillation counting (LS 3801, Beckman Coulter). Binding ratio was calculated as the proportion of bound RNAs to input RNAs. All assays were performed in triplicate and repeated at least twice.

2.5. Confocal microscopy analysis

Fluorescein was covalently attached to the 3′-end of the RNAs using fluorescein-5-thiosemicarbazide (Sigma-Aldrich) as described previously [25]. One day before confocal microscopy imaging, 10^5 cells were seeded on four-well Lab-Tek II CC2 chamber slides (Nalge Nunc/Thermo Fisher Scientific). Cells were washed with buffer A and incubated with 200 nM fluorescein-labeled RNAs in buffer A containing 2 mg/ml tRNA and 1 mg/ml BSA for 10 min at room temperature. After three washes, cells were stored in buffer A. In the case of staining with antibodies, cells were incubated with 10 μg/ml anti-stage-specific embryonic antigen-1 (SSEA-1) antibody (MC-480; Developmental Studies Hybridoma Bank, IA) or an IgM isotype control antibody (BioLegend) in buffer A containing 2 mg/ml tRNA and 1 mg/ml BSA for 20 min at room temperature, washed three times, and incubated with 0.67 μg/ml allophycocyanin (APC)-labeled anti-mouse IgM antibody (eBioscience) in buffer A for 20 min at room temperature. Differential interference contrast (DIC) and fluorescence images were obtained using a Nikon A1R laser scanning confocal microscope (Nikon, Japan).

3. Results

3.1. Selection of RNA aptamers against mESCs: SELEX Line 1

Aptamers were first selected against intact, live mESCs by SELEX in the absence of a competing aptamer or negative selection from an RNA pool randomized over 60 nucleotides (nts) with 2′-fluoro pyrimidine modifications to resist ribonucleases (SELEX Line 1) [26]. To select tightly binding aptamers, the number of washings after the incubation of the RNA pool with the target cells was progressively increased (Table 1). The bound RNAs were released from the cell surface by adding EDTA to chelate divalent cations. The formation of typical higher-order RNA structures often requires divalent cations and thus, its elimination with EDTA is expected to inactivate most of the bound aptamers [27,28]. It is noteworthy that dead and damaged cells tend to adsorb nucleic acids non-specifically [29] and the elimination of divalent cations rarely affects this non-specific adsorption. Therefore, EDTA-mediated recovery of aptamers is an effective process to distinguish specific binders from non-specific adsorbates (S.P.O., unpublished observations).

The affinity of the RNA pool to mESCs was monitored by [^{32}P]-labeled RNAs remaining bound to mESCs after repeated washings. After 10 rounds of SELEX, the RNA pool (L1R10 pool, standing for the 10th round pool of SELEX Line 1) showed apparent binding to mESCs (Fig. 2), and RNAs in the pool were cloned to analyze their sequences and binding activities. Of 20 clones randomly picked out, four sequences appeared multiple times: L1-50 (twice), L1-60 (twice), L1-64 (twice), and L1-72 (four times) (Fig. 3). The binding activities of four multi-clones and one single-clone L1-65 were analyzed, and all of them

```
Line 1 clones       10         20         30         40         50         60         70         80         90         100
L1-50 (x2) gggaggacga ugcggCAAGG GCCCCUCUGA UUAGACUAAA GACCGGUCCC ACGCACUUGG GCCCCCCUUA UUCC-cagac gacucgcuga ggauccgaga
L1-60 (x2) gggaggacga ugcggUCCCC AUGACUCCAU CAUUCGUAUC AGAGCCUAAC CGCCAUGCGG ACUCUGAACC AACCUcagac gacucgcuga ggauccgaga
L1-64 (x2) gggaggacga ugcggACGAA GAAUCCGGCU AAGGAAUGUU CAACGAUAUG CGCGUUCCCU GAACUCACCG CCUCUcacac gacucgcuga ggauccgaga
L1-65      gggaggacga ugcggAAAAU CCGGUUAAGC ACAAUUUAUG ACCUGUAUAA GUGUGCCUGA ACUCAUACGA CUCCCcagac gacucgcuga ggauccgaga
L1-72 (x4) gggaggacga ugcggGAUGU AAAAAUCCUG GAGGGUUAGU AAAUCGACUC UCAAAGAACA UCACCCACGG CAACCcagac gacucgcuga ggauccgaga

Line 2 clones       10         20         30         40         50         60         70
L2-2 (x12) gggacacaau ggacgCAAUC CAGCCAAGCC UCACCUCGGA UUAGACUAAC GACCCugagg cucacaacag gc
L2-12 (x2) gggacacaau ggacgCCGUC CCCCCCGGAG AAGACUAACG GCCCGGACCG CGUCUugagg cucacaacag gc

Line 3 clones       10         20         30         40         50         60         70
L3-1  (x3) gggcugaagg augccaAAGC CCAAACAGCG AAAACUCUCA CUCCCCCGGA GCAGACuaac ggccgacaug agag
L3-2       gggcugaagg augccaAAAC CUUCAACUCA CACAUACUUA UGAGCCCGGA GCAGACuaac ggccgacaug agag
L3-3  (x2) gggcugaagg augccaAAUC UCCAUGUAGA UAAACUUCCU UCACGGCCUC UAUGCCuaac ggccgacaug agag
L3-4       gggcugaagg augccaAUCA CGAAUCCUUC AACAAACUCA UGAGCCCGGA UUAGACuaac ggccgacaug agag
L3-5       gggcugaagg augccaACUA CUCCCGGCAA CCCGACAGCA GUCGCCCGGA UCAGACuaac ggccgacaug agag
L3-9       gggcugaagg augccaAAUC UAGCCCACAC AAAUCCCUCU GUCCCCCGGA GCGGACuaac ggccgacaug agag
```

Fig. 3. Sequences of anti-mESC aptamers isolated through three different selection procedures. Twenty, 23, and 20 clones were randomly chosen from the pools of SELEX Lines 1, 2, and 3, respectively. The numbers in parentheses indicate the frequency with which the sequence was selected. The primer and "stem-internal loop-stem-loop" motif regions are indicated in lower-case and bold letters, respectively.

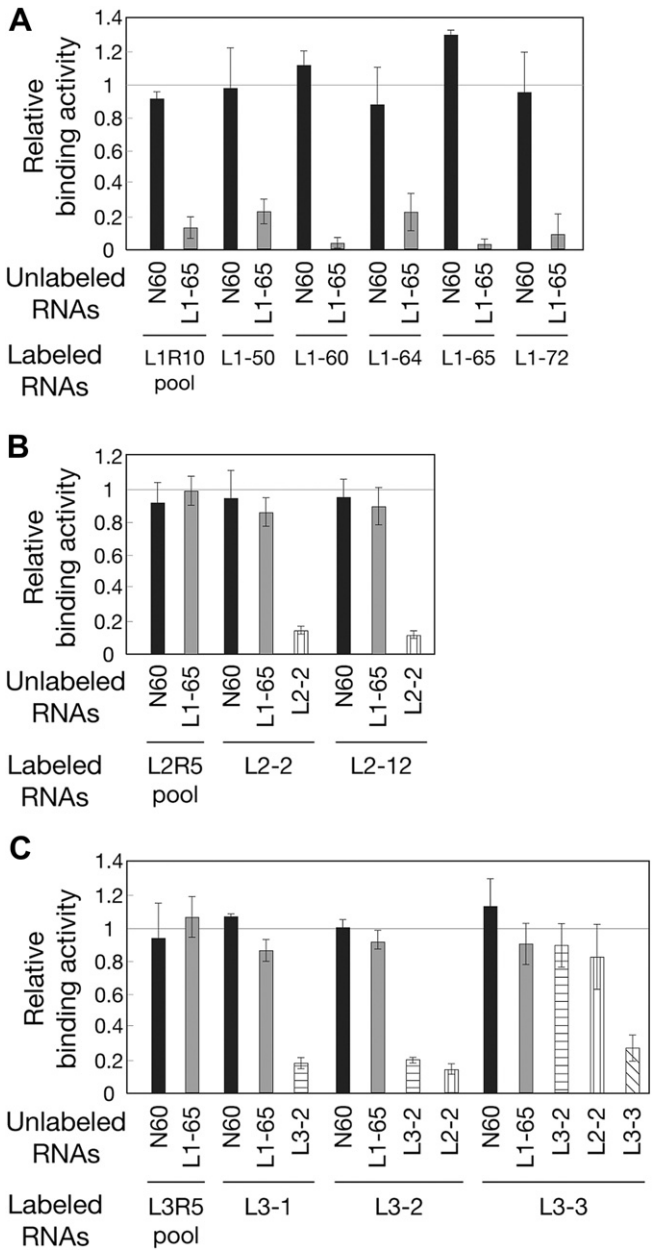

Fig. 4. Cross-competition between individual anti-mESC aptamers. The binding abilities of the indicated [^{32}P]-labeled aptamers were examined in the presence of more than 100 times molar excess of the indicated unlabeled competitor RNAs. The binding activity was represented as ratios relative to that without a competitor RNA. **(A)**, **(B)**, and **(C)** show the results of the clones isolated from SELEX Lines 1, 2, and 3, respectively. The mean and standard deviation of triplicates are shown.

exhibited significant binding to mESCs compared with the initial pool RNA (N60) (Fig. 2). Since the primary sequences, as well as the secondary structures predicted by Mfold [30], are completely different from each other (Fig. 3 and Supplemental Fig. S1), we first assumed that the targets of these anti-mESC aptamers might be different from one another. However, binding of not only [^{32}P]-labeled L1-65 but also [^{32}P]-labeled L1-50, L1-60, L1-64, and L1-72 to mESCs was hampered by an excess amount of unlabeled L1-65 (Fig. 4A). Furthermore, the competitor L1-65 also inhibited the binding of the L1R10 pool RNA (Fig. 4A), suggesting that the majority of anti-mESC aptamers in this pool bind to the same target on mESCs.

3.2. Selection of RNA aptamers for various cell surface molecules on mESCs: SELEX Lines 2 and 3

To isolate aptamers against mESC surface molecules other than the L1-65′s target, we carried out two lines of SELEX in which excess L1-65 was added as a competitor (Lines 2 and 3, Fig. 1). Previously, a similar approach has been made for yeast TATA-binding protein, and two classes of antagonistic aptamers with distinct inhibitory mechanisms were successfully isolated [31]. In addition to the involvement of L1-65 competitor, a negative selection process was also incorporated in SELEX Line 3 to remove RNAs binding to fully differentiated A-9 cells (derived from connective tissues of adult mouse). After five rounds of SELEX, both lines of the labeled RNA pools showed elevated binding activities to mESCs (L2R5 and L3R5 pools for Lines 2 and 3, respectively) (Fig. 2). Furthermore, the addition of excess competitor aptamer, L1-65, did not affect the binding (Fig. 4B and C), suggesting that these selected pools contained aptamers distinct from L1-65. Of 23 clones isolated from the L2R5 pool, L2-2 and L2-12 sequences appeared 12 and two times, respectively, whereas of 20 clones from the L3R5 pool, L3-1 and L3-3 sequences appeared three and two times, respectively (Fig. 3). All the clones except for L3-3 share highly similar sequences, which are predicted to form a characteristic “stem-internal loop-stem-loop” structure (Fig. 3 and Supplemental Fig. S1). The binding affinity of each clone was more or less comparable to that of the initial selectants (Fig. 2). As expected from the pool assay, L1-65 did not interfere with each selectant binding to mESCs (Fig. 4B and C).

Next, we examined cross-competition between these selected aptamers using [^{32}P]-labeled test aptamers and unlabeled excess competitors. The data clearly identified competing pairs, L2-2/L2-12, L3-2/L3-1, L3-2/L3-4, L3-2/L3-5, L3-2/L3-9, and L2-2/L3-2 (Fig. 4B and C, and Supplemental Fig. S2). Thus, all the analyzed aptamers, except for L3-3, isolated in modified selection lines compete with one another. Because these competing aptamers have the consensus motif as described above (Supplemental Fig. S1) and the motif region is sufficient for the binding activity (see below), they might bind to the same target. Through extensive selections of aptamers for mESC surface molecules, we acquired three classes of non-competitive anti-mESC aptamers. In the following studies, aptamers L1-65, L2-2, and L3-3, representing each class were further characterized.

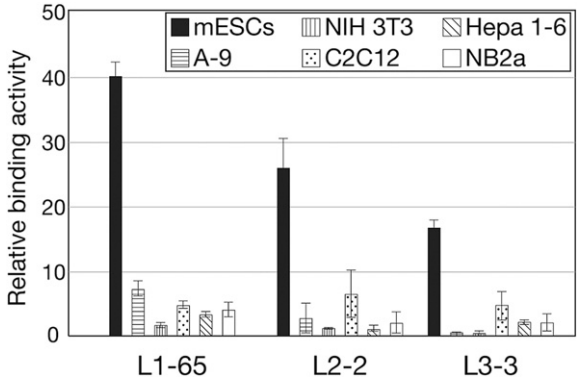

Fig. 5. Target cell specificity of anti-mESC aptamers. The binding abilities of aptamers L1-65, L2-2, and L3-3 for A-9, NIH 3T3, C2C12, Hepa 1–6 and NB2a cells were examined using [^{32}P]-labeled RNAs. The binding ratio normalized to that of the random RNA (N60) was shown as relative binding activity. The mean and standard deviation of triplicates are shown.

3.3. Binding specificity of anti-mESC aptamers

The binding specificity of aptamers L1-65, L2-2, and L3-3 was examined using mESCs and five differentiated mouse cell lines – connective tissue (A-9 cells), embryonic fibroblast (NIH 3T3 cells), muscle tissues (C2C12 cells), liver cancer (Hepa 1–6 cells), and neuroblastoma (NB2a cells). The data indicate that these three aptamers bound efficiently to mESCs but failed to bind, or only weakly bound if any, to differentiated mouse cell lines (Fig. 5). The loss of binding affinity of L3-3 and L2-2 (that belonged to the L3-1/L3-2 specificity group class) for A-9 cells is consistent with the negative selection process against A-9 (SELEX Line 3). It was rather surprising that unbiased selections, without any negative selection against non-mESCs, failed to produce affinity RNAs to non-mESCs under these experimental conditions. The reason is not immediately obvious but these findings might be interpreted as indicating that mESC-specific molecules, including L1-65, L2-2, and L3-3 targets, are composed of major surface molecules of mESCs.

3.4. Confocal microscopy imaging with fluorescein-labeled anti-mESC aptamers

To further confirm the binding specificity to mESC and to assess the utility of anti-mESC aptamers as molecular probes, confocal microscopy imaging was conducted using fluorescein-labeled aptamers. In the previous antibody-mediated imaging study, it has been shown that the known mESC markers are downregulated during the course of differentiation [32,33]. Hence, mESC-derived differentiated cells were prepared by treatment of mESCs with retinoic acid (RA) for 4, 8, and 14 days [24,34], and stained with the labeled aptamers. The same set of cells was also stained with an antibody against stage-specific embryonic antigen-1 (SSEA-1), a canonical mESC marker [35], as control. A set of fluorescence images demonstrated that aptamers L1-65, L2-2, and L3-3 as well as anti-SSEA-1 antibody bound to mESCs, while the N60 random RNA pool and the negative control antibody did not (Fig. 6). As reported previously, nearly the whole surface of mESCs was stained with the antibody but the staining intensity, or SSEA-1 expression level, differed among individual cells [35,36]. Similarly, the staining intensity with the aptamers varied from cell to cell. However, their staining patterns were completely different. The aptamers L1-65, L2-2, and L3-3 preferentially bound to dot-like spots on cell–cell contact regions rather than the whole cell surface (Fig. 6). These findings suggest that the targets for these aptamers are localized on some microdomain structures on the cell–cell contact regions, regardless of no cross-competition among L1-65, L2-2, and L3-3.

During the course of differentiation induced by RA, the staining intensities with anti-SSEA-1 antibody and aptamers L1-65, L2-2, and L3-3 exhibited different patterns and gradually decreased. Four days after RA addition, anti-SSEA-1 antibody signal was significantly weakened and completely disappeared after 8 days (Fig. 6). The SSEA-1 signal differed among individual cells, as described previously [32,37]. On the other hand, the reduction of staining with aptamers L1-65, L2-2, and L3-3 proceeded more slowly; a large proportion of the cells were stainable after 4 days and a half of the populations were stainable with the aptamers after 8 days. Even after 14 days, a few fractions were still stained with the aptamers. It is noteworthy that no apparent differences were observed in fluorescent images among aptamers L1-65, L2-2, and L3-3.

3.5. Exploration of the essential regions of anti-mESC aptamers

To assign nucleotide regions required for the activity of L1-65 and L3-3 aptamers, several deletion derivatives were prepared. Intriguingly, 3′ ten- or twenty-nt deletions of L1-65 and 3′ seven-nt deletion of L3-3 enhanced the binding activity (Fig. 7). Further 3′

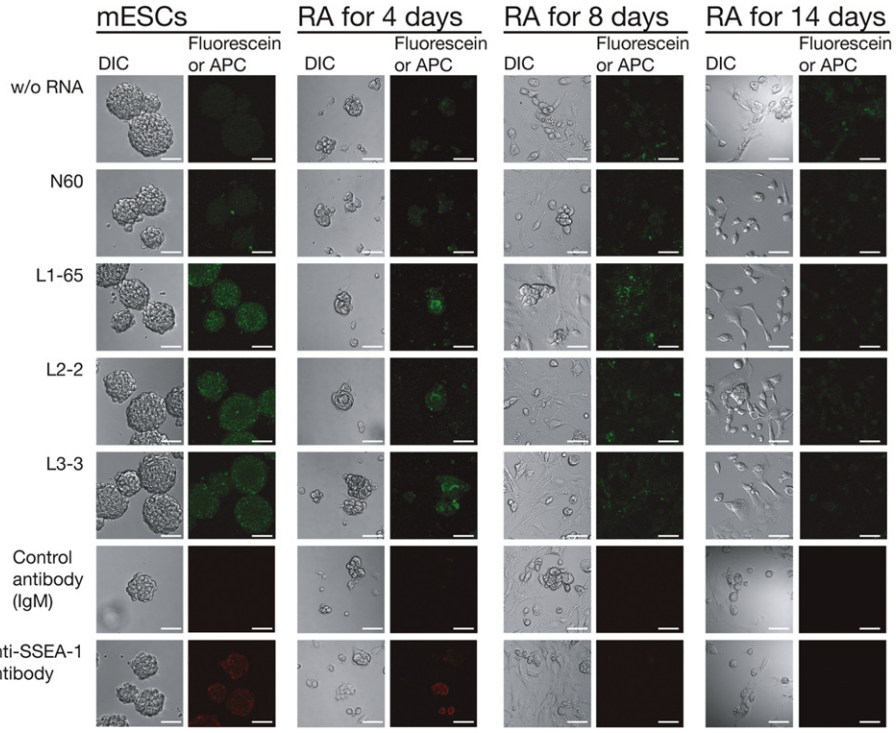

Fig. 6. The confocal fluorescence image with the fluorescein-labeled anti-mESC aptamers. Mouse ESCs before and after treatment with RA for 4, 8 or 14 days were stained with the indicated fluorescein-labeled aptamers and APC-labeled antibodies, and visualized by confocal microscopy. DIC and fluorescence images were shown. Scale bar, 50 μm.

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                  10         20         30         40         50         60         70         80         90         100
L1-65         gggaggacga ugcggAAAAU CCGGUUAAGC ACAAUUUAUG ACCUGUAUAA GUGUGCCUGA ACUCAUACGA CUCCCcagac gacucgcuga ggauccgaga    1.00(±0.16)
L1-65(1-90)   gggaggacga ugcggAAAAU CCGGUUAAGC ACAAUUUAUG ACCUGUAUAA GUGUGCCUGA ACUCAUACGA CUCCCcagac gacucgcuga               1.50(±0.33)
L1-65(1-80)   gggaggacga ugcggAAAAU CCGGUUAAGC ACAAUUUAUG ACCUGUAUAA GUGUGCCUGA ACUCAUACGA CUCCCcagac                        3.13(±0.32)
L1-65(1-70)   gggaggacga ugcggAAAAU CCGGUUAAGC ACAAUUUAUG ACCUGUAUAA GUGUGCCUGA ACUCAUACGA                                    0.17(±0.04)
L1-65(11-100)          g ugcggAAAAU CCGGUUAAGC ACAAUUUAUG ACCUGUAUAA GUGUGCCUGA ACUCAUACGA CUCCCcagac gacucgcuga ggauccgaga    0.10(±0.03)
L1-65(21-100)                    g CCGGUUAAGC ACAAUUUAUG ACCUGUAUAA GUGUGCCUGA ACUCAUACGA CUCCCcagac gacucgcuga ggauccgaga    0.01(±0.01)

                  10         20         30         40         50         60         70
L3-3          gggcugaagg augccaAAUC UCCAUGUAGA UAAACUUCCU UCACGGCCUC UAUGCCuaac ggccgacaug agag    1.00(±0.25)
L3-3(1-67)    gggcugaagg augccaAAUC UCCAUGUAGA UAAACUUCCU UCACGGCCUC UAUGCCuaac ggccgac            1.51(±0.10)
L3-3(1-60)    gggcugaagg augccaAAUC UCCAUGUAGA UAAACUUCCU UCACGGCCUC UAUGCCuaac                  0.19(±0.07)
L3-3(8-74)           gg augccaAAUC UCCAUGUAGA UAAACUUCCU UCACGGCCUC UAUGCCuaac ggccgacaug agag    0.32(±0.28)
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Fig. 7. Deletion analysis of aptamers L1-65 and L3-3. The sequences of deletion mutants and their binding activities (numbers in right column) are shown. The activity was examined using [^{32}P]-labeled RNA and normalized to that of the original, full-length aptamer. The mean and standard deviation of triplicates are shown. For the efficient transcription, one extra G residue was added to the 5′-ends of 5′-deletion mutants of L1-65 (shown in italics).

deletions and 5′ ten- or eight-nt deletions abolished the binding activity of L1-65 and L3-3 (Fig. 7). These findings are interpreted as indicating the non-essential 3′ extensions of L1-65 and L3-3 interfered with the correct folding of aptamers probably through forming multiple conformers [38–40].

All the other defined aptamers, L2-2, L2-12, L3-1 and L3-2, harbored the consensus motif (see Supplemental Fig. 1) and cross-competed with one another (see Fig. 4). When 5′ twenty seven-nts and 3′ eleven-nts were deleted from the parental L2-2, the resulting 34-nt RNA, referred to as L2-2c, conserved the consensus motif and bound mESCs (Fig. 8). These findings revealed that the consensus motif is sufficient for mESC binding and 5′ and/or 3′ extensions are somewhat inhibitory to functionality of L2-2c as shown with L1-65 and L3-3.

To further elucidate the functional significance of relevant nucleotides or each structural unit of the consensus motif, several base-substitution mutants of L2-2c were made and examined for the binding activity (Fig. 8B). The L2-2c-v1 mutant, in which stem 1 sequence was changed without affecting the base-pairing, retained the binding activity (Fig. 8B), suggesting that the secondary structure and not the primary sequence of stem 1 is crucial for the recognition of mESCs. On the other hand, similar base-pairing substitutions in stem 2 (L2-2c-v5, L2-2c-v6, and L2-2c-v7), except for one case (L2-2c-v4), abolished the aptamer activity (Fig. 8B and Supplemental Fig. 3). Because the primary sequences of stem 2 are highly conserved among all the consensus motifs isolated (Fig. 8A and Supplemental Fig. 1), the primary sequences likely contribute to the binding and/or tertiary structure formation. When single-stranded regions in the loops of L2-2c were mutated, the resulting L2-2c-v2 and L2-2c-v3 failed to bind mESCs (Fig. 8B), suggesting that, similarly to many other aptamers [41–44], the primary sequences of these single-stranded regions are important.

4. Discussion

In this study, we isolated three classes of anti-mESC aptamers, L1-65, L2-2, and L3-3, using three different selection schemes. L1-65 was isolated by the unbiased selection (Line 1), while L2-2 and L3-3 were isolated by the biased selections that employed L1-65 as a competitor (Line 2 and Line 3) as well as negative selection against differentiated A-9 cells (Line 3). As far as we know, this is the first report of the isolation of aptamers specific to ESCs. The selected aptamers, L1-65, L2-2, and L3-3, bound to mESCs, but hardly at all to differentiated mouse cell lines, A-9, NIH 3T3, C2C12, Hepa 1–6, and NB2a cells. It was rather surprising that three selection lines provided us with no aptamer against differentiated cells and no more than three distinct sets of aptamers to mESCs under the experimental conditions used. This is in sharp contrast to previous reports that a single selection line against live cells generates several aptamers that are likely to recognize distinct targets on the live cell surface [45,46]. The reason is not immediately obvious but we assume that the EDTA-mediated recovery step of bound RNAs might reflect the limitation in the variability of isolates. EDTA removes only divalent cations, which are often crucial for the tertiary structure formation of RNA, but not monovalent cations that are crucial for the guanine-quadruplex formation of RNA [47,48]. Therefore, the release of bound RNAs with EDTA might be a mild procedure, probably not effective for all the aptamers bound to mESCs, and are capable of distinguishing specifically bound certain sets of aptamers from non-specifically

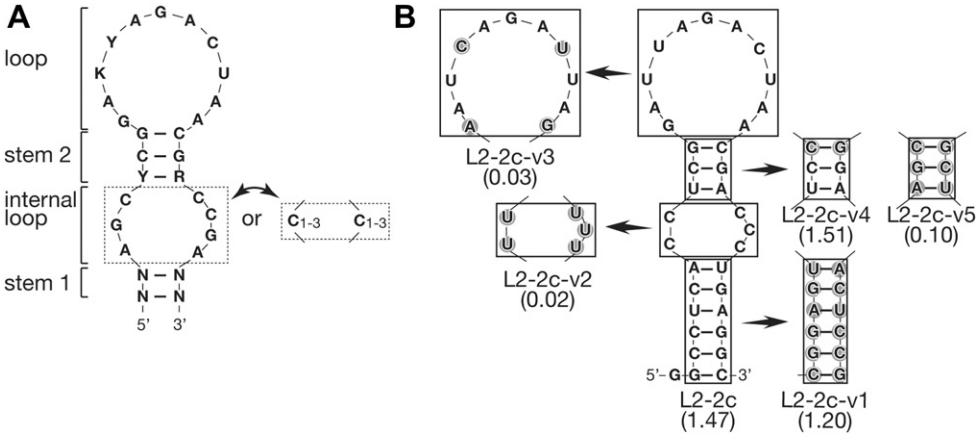

Fig. 8. Mutation analysis of the consensus motif of the aptamer L2-2. (A) The consensus motif conserved in the anti-mESC aptamers from SELEX Lines 2 and 3. N, K, Y, and R indicate any four, G or U, C or U, and A or G nucleotides, respectively. (B) the aptamer L2-2c derived from the consensus motif of the aptamer L2-2 and its derivatives. The altered residues of each derivative are indicated by gray shadows. Numbers in parentheses indicate relative binding activities that were normalized to the binding activity of the aptamer L2-2. The mean and standard deviation of triplicates are shown.

adsorbed RNAs. However, the procedure provided us with the aptamers that can be easily released from cell surfaces by the addition of EDTA. The aptamers with such features would be suitable for staining target cells repeatedly without damage to the cells (data not shown). In addition, possible problems caused by the existence of staining reagents on the cells would also be avoided by employing such aptamers.

The aptamers L1-65, L2-2, and L3-3 do not compete with one another. A most likely explanation might be the distinct target identity on the mESC surface. We investigated each target by the confocal microscopy imaging using fluorescein-labeled aptamers. The data revealed several interesting features. First, although anti-SSEA-1 antibody stained nearly the whole surface of mESCs, all the three aptamers stained similar dot-like spots on cell–cell contact regions (see Fig. 6). Therefore, it cannot be excluded at present that L1-65, L2-2, and L3-3 recognize different sites of the same molecule on the mESC surface. Second, upon induction of differentiation of mESCs with RA, the fluorescent signals of L1-65, L2-2, and L3-3 gradually, and similarly, reduced, much more slowly compared with anti-SSEA-1 antibody. Under these conditions, mESCs are predicted to differentiate into ectodermal or mesodermal cells [34]. Therefore, the aptamers' targets are likely expressed on the cells at early differentiation stages into ectodermal or mesodermal cells as well as mESCs. Because the aptamers hardly bound to the fully differentiated ectodermal or mesodermal cells (NB2a, A-9, and C2C12 cells; Fig. 5), these aptamers may be useful probes to monitor early ectodermal or mesodermal differentiation.

Given that anti-mESC aptamers could bind to artificially created, induced pluripotent stem cells (iPSCs) [49,50], these should provide an opportunity for the isolation and purification of iPSCs to evade tumor formation upon transplantation of iPSCs and iPSC-derived cells [51]. It has been demonstrated that cell-binding aptamers can be utilized not only as molecular probes but also as cell adhesion reagents to be plated on culture dishes, as drug (including short interfering RNA)-delivery systems, *etc.* [52–54]. Collectively, the anti-mESC aptamers isolated in this study might open the gate for diverse applications in the fields of regenerative medicine and developmental biology.

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Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.biochi.2011.10.017.

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