RNA aptamers targeting cancer stem cell marker CD133

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The monoclonal antibody against the AC133 epitope of CD133 has been widely used as a cell surface marker of cancer stem cells in several different cancer types. Here, we describe the isolation and characterisation of two RNA aptamers, including the smallest described 15 nucleotide RNA aptamer, which specifically recognise the AC133 epitope and the CD133 protein with high sensitivity. As well, both these aptamers show superior tumour penetration and retention when compared to the AC133 antibody in a 3-D tumour sphere model. These novel CD133 aptamers will aid future development of cancer stem cell targeted therapeutics and molecular imaging.

1. Introduction

CD133, also known as Prominin-1, is a pentaspan, highly glycosylated, membrane glycoprotein that is associated with cholesterol in the plasma membrane [12]. Though this protein is known to define a broad population of cells, including somatic stem and progenitor cells, and is expressed in various developing epithelial and differentiated cells, its exact function is still being elucidated. It has, however, been linked to the Notch-signalling pathway which is critical for binary cell fate, differentiation of intestinal epithelium, and lymphopoiesis [3]. CD133 has gained its prominence in the cancer research field due to its reported role as a marker of cancer stem cells (CSCs) in glioblastomas [4]. Indeed, growing evidence has shown that CD133 is an important cell surface marker for CSCs in a variety of solid cancers, including those of the brain, prostate, pancreas, melanoma, colon, liver, lung and ovarian cancers [5] by virtue of the enhanced tumorigenic potential of CD133+ cells versus their negative counterparts in immunodeficient mice [6].

Most of the work in isolating CD133+ putative cancer stem cell subpopulation from the bulk cancer cells utilises one monoclonal antibody, AC133 [7]. However, there has been some controversy regarding the notion that CD133 can be used as a marker for CSCs, as investigators from independent laboratories showed that the CD133+ cells are also tumorigenic in immunocompromised mice [8–10]. This contention is further complicated by the near ubiquitous expression of CD133 on non-CSCs as well as CSCs, especially in tumours of the colon [11]. A recent study has shed some light on this, with a conformational change postulated to hide an epitope on the second extracellular membrane loop of CD133 during the differentiation process [5]. Kemper and co-workers suggested that the CD133 protein becomes differentially folded as a result of glycosylation, thus masking the AC133 epitope. These results were further supported by later studies [5,12] which suggested that the AC133 epitope, rather than the complete CD133 protein, is the marker for CSCs. As well, a recent report has shown that the AC133 epitope is lost upon cell differentiation, suggesting that this epitope is a marker of primitive cells [5]. While the AC133 epitope has been shown to be a marker for CSCs, not all cells positive for
the AC133 epitope are CSCs. In fact, the first description of this epitope was by Yin et al. in 1997, who described this as a marker of haematopoietic stem and progenitor cells [13]. However, the expression of AC133 on these cells is approximately 1000-fold lower than that observed in CSCs [14]. Despite the on-going debate of the utility of using AC133 to identify cancer stem cells, a retrospective study on colorectal patients showed that a high level of AC133 expression was associated with a poorer prognosis [15], though the sole use of the AC133 antibody is not recommended as it is thought to underestimate the level of CD133 expression [1,16].

AC133-positive cells have been shown to have an increased resistance to radiation therapy due to activation of the DNA damage checkpoint proteins, and an increased chemoresistance due to an increased Akt/PKB and Bcl-2 cell survival response [17]. These data suggest that a more targeted response is required to eradicate this population of cells, especially given the increasing evidence regarding the roles that CSCs play in the relapse of cancer after initial treatments. Immunotherapy has had a great impact on the treatment of cancer in recent years [18,19]. However, the use of antibodies, even humanised antibodies, can lead to adverse side effects with fatal consequences [20]. This has led to the search for ‘bigger and better’ options. There have been several attempts to use nucleic acids as therapeutics though these have met with disappointing results, not least because of the failure of these nucleic acids to enter the cell [21]. The reports in 1990 by two separate groups describing the generation of nucleic acids that can bind target molecules in the same manner as antibodies seemed to be the answer [22,23]. These chemical antibodies, termed aptamers, have been increasingly utilised for clinical applications recently. Indeed, one RNA aptamer has been approved by the FDA and several more are in clinical trials [21,24]. The increased interest in these aptamers is due to the fact that they exhibit no immunogenicity, little batch-to-batch variation due to being chemically synthesised, and are more stable than conventional antibodies. Due to their small size, aptamers also show superior tumour penetration. One important feature of these chemical antibodies is their versatility as they can be attached to nanoparticles, drugs, imaging agents or other nucleic acid therapeutics without loss-of-function [25,26]. This functionalisation is leading to new and more targeted therapies, with fewer side effects than current treatment modalities [25].

When compared to conventional treatment which is largely a passive process, targeted delivery systems are much more effective. For an aptamer to be an effective drug delivery agent, the aptamer must be efficiently internalised upon binding to its target on the cell surface [27].

In this study, we performed iterative rounds of an in vitro selection process, known as the systematic evolution of ligands by exponential enrichment (SELEX), to identify RNA aptamers that specifically bind to CD133. Further studies identified one aptamer, CD133-A15, specifically bound to the same epitope as the AC133 antibody, while the other aptamer, CD133-B19, bound to the extracellular domain of the CD133 protein. These aptamers were efficiently internalised into CD133-positive cancer cells and showed superior penetration of three-dimensional tumour sphere.

2. Materials and methods

2.1. Cell lines and cell culture

The cell lines of human origin used in this study were purchased from American type Culture Collection. They are human colorectal HT-29; human hepatocellular carcinoma HepG2; human glioblastoma multiform carcinoma T98C; human embryonic kidney cells HEK293T; human ductal breast carcinoma, T47D; human lung adenocarcinoma, A549; human ovarian teratocarcinoma, PA-1; human hepatoma, PLC/PRC/5; and human prostate carcinoma, DU145. Cells were grown and maintained in culture with Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen, Victoria, Australia) supplemented with 10% foetal calf serum (FCS) (HEK293T and HT-29), or Minimum Essential Medium (MEM) (Invitrogen) supplemented with 10% FCS (HepG2 and T98C). Cells were maintained at 37 °C in a 5% CO2 atmosphere.

2.2. Protein expression and cell SELEX

Human CD133 cDNA was purchased from Invitrogen and cloned into a mammalian expression vector, pcDNA 3.1/V5-HisTOPO (Invitrogen). The recombinant 6× His-tagged CD133 was transiently expressed in HEK293T cells. Briefly, HEK293T cells were seeded in 100 mm or 60 mm dishes to reach 70% confluency after 24 h incubation, and transfected with a total of 24 or 8 μg, respectively, of CD133 using Lipofectamine 2000 (Invitrogen Life Technologies) in antibiotic-free medium according to the manufacturer’s instructions. Following a 72 h incubation, the transfected cells were used as the target for cell SELEX. Successful transfection and expression of the recombinant CD133 were confirmed using flow cytometry and the AC133-APC antibody (Miltenyi Biotec) prior to each round of SELEX.

2.3. SELEX selection

A DNA library containing a central 40-nt randomised sequence (5′-TAA TAC GAC TCA TCA TAG GGA AAC AAT AAA GCC TCA A-N40-TTC GAC AGG AGG CCT ACA ACA A GC, with the T7 RNA polymerase promoter sequence underlined) was synthesised (GeneWorks, Australia). The double stranded DNA pool was generated from the original synthetic library via a large scale PCR using primers flanking the randomised sequence, 5′-TAA TAC GAC TCA TCA TAG GGA AAC AAT AAA AAA CGC TCA A-3′ and 5′-GCC TGT TGT GAG CCT CTC GCT GAA-3′. A portion of the large-scale PCR products (~1014 sequences) was used as a template for in vitro transcription to produce the initial 2′-fluoropyrimidine modified RNA pool using a Durascribe® T7 Transcription kit (EPICENTRE® Biotechnologies, USA). For SELEX, RNA, at a concentration of 5 μM for initial selection or 1 μM for each iterative rounds, was diluted in 100 μL of wash buffer (Dubecco’s phosphate buffered saline containing 2.5 mM MgCl2) and denatured at 85 °C for 5 min, allowed to cool to room temperature for 10 min, and annealed at 37 °C for 15 min, before incubation with the target protein expressed in HEK293T cells for 1 h at 4 °C. Following incubation and extensive washes, the bound RNA was reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen), followed by PCR amplification and in vitro transcription and used for the next round of SELEX. Counter-selection steps were included from round 4, using a His-tagged irrelevant protein expressed in HEK293T cells, to decrease the enrichment of species specifically recognising the His-tag, the HEK293T cells or the tissue culture plate. The number of PCR amplification cycles was also optimised to prevent over-amplification of non-specific “parasite” PCR products. In addition, the stringency of the selection process was enhanced to promote the selection of high-affinity aptamers through adjustments to aptamer concentration, incubation times, and the number of washes. To acquire aptamers of high specificity, the number of cells used was progressively decreased while the washing stringency increased during the progression of SELEX, with negative selections included from round four. Enrichment was monitored using restriction fragment length polymorphism (RFLP) and flow cytometry using live cells.

2.4. RFLP analysis

The enrichment of aptamer candidates during selection was determined by RFLP. Briefly, RFLP was performed as previously described [28,29], with minor modifications. Approximately 5 ng of cDNA from iterative cycles was amplified by PCR for eight cycles. The amplified DNA was digested with restriction enzymes, Afl I, Alu I, Hha I and Xsp I that recognise four nucleotides (frequent cutters) in Buffer T supplied by the manufacturer ( Takara) with 0.1% (w/v) bovine serum albumin at 37 °C overnight. Following the overnight digestion, the DNA was heated to 65 °C, cooled on ice, and separated via electrophoresis on a native 20% polyacrylamide gel in TBE buffer. The gel was then stained in GelStar and visualised using a standard gel imaging system.

2.5. Flow cytometry assays

Cells were harvested at 80% confluence with trypsin digestion and resuspended in washing buffer (DPBS with 2.5 mM MgCl2) and enumerated. Following centrifugation (1000g for 5 min), the cell pellet was resuspended in DMEM with 10% FCS and diluted to 1 × 107/mL. The cells were allowed to re-establish their cell surface markers for a period of 2 h prior to binding analysis. To confirm binding to the target protein, RNA from iterative rounds were labelled at the 3′-ends with fluoro-pyrimidine modified RNA pool using 50 L of binding buffer (DPBS without Ca2+ and Mg2+, 1014 sequences) was used as a template for in vitro transcription and used for the next round of SELEX. Counter-selection steps were included from round 4, using a His-tagged irrelevant protein expressed in HEK293T cells, to decrease the enrichment of species specifically recognising the His-tag, the HEK293T cells or the tissue culture plate. The number of PCR amplification cycles was also optimised to prevent over-amplification of non-specific “parasite” PCR products. In addition, the stringency of the selection process was enhanced to promote the selection of high-affinity aptamers through adjustments to aptamer concentration, incubation times, and the number of washes. To acquire aptamers of high specificity, the number of cells used was progressively decreased while the washing stringency increased during the progression of SELEX, with negative selections included from round four. Enrichment was monitored using restriction fragment length polymorphism (RFLP) and flow cytometry using live cells.
Fluorescent intensity was determined with a FACS Canto II flow cytometer (Becton Dickinson) by counting 50,000 events for each sample. The FITC-labelled RNA from the unselected library was used to determine non-specific binding. The binding for each round was calculated after subtracting the mean fluorescence intensity of the binding of round zero RNA to target cells as well as that for binding to negative control cells according to a method described by Ellington and colleagues [31].

2.6. Cloning, sequencing and structural analysis of selected aptamers

Following RFLP and flow cytometric analyses of iterative rounds, round six demonstrated a sufficient enrichment of RNA sequences that selectively recognised the target protein. This enriched pool was amplified by PCR for ten cycles and the PCR products were cloned into the plasmid pCR®-4-TOPO® (Invitrogen). Plasmid DNA from individual clones was prepared and their sequence determined using an automated DNA sequencing procedure. The aptamer sequences were analysed using ClustalX2 [32]. Secondary structures were predicted using the program RNAfold [33].

2.7. Determination of aptamer affinity

The dissociation constant ($K\text{d}$) of successful 2'-fluoropyrimidine RNA aptamer species to native CD133 expressed on the cell surface was determined using flow cytometry. HEK293T cells transfected with CD133 protein, or non-transfected HEK293T cells (5 × $10^6$) were first incubated with blocking buffer for 1 h on ice (binding buffer containing 0.2% (w/v) sodium azide) followed by two washes with binding buffer prior to incubation with serial concentrations (approximately 10-fold above and below the apparent $K\text{d}$) of FITC-labelled aptamer in a 100 μL volume of binding buffer for 1 h on ice. The cells were washed three times with binding buffer, resuspended in 150 μL binding buffer and subjected to flow cytometric analyses. The FITC-labelled unselected library was used as a negative control. The mean fluorescence intensity (MFI) of the unselected library was subtracted from that of the aptamer-target cell to generate the MFI of specific binding. The $K\text{d}$ for each aptamer was determined by Scatchard analysis according to the following equation:

$$\text{[Bound aptamer]/[aptamer]} = -1/[K\text{d}] \times \text{[Bound aptamer]} + (\text{MFI}_\text{un} / K\text{d})$$

where $\text{MFI}_\text{un}$ represents the total target concentration.

2.8. Aptamer truncation and determination of specificity

To generate the truncated aptamers, the sense and antisense DNA oligonucleotides of desired sequences were synthesised. CD133-AS8 (1st Truncation) was derived from a sense oligonucleotide, 5′-TAA TAC GAC TCA TAC ATA AGA CAA GAA TAA ACG CTC AAC CCA CCC TCC TAC ATA AGG ACC AGC CAT GTA CTA TAG CGT TCC TCT CTA CGT CAG TCT GTA GGA GGG TGG-3′ and antisense oligonucleotide, 5′-CTA TAG TAA CTA CTT CCT CCC TAT GTA GCA GGG TCG GTG GGT GCT TTA TTC TTG TCT C-3′. Transferrin (5 μg/mL) was added to the cells during the final 15 min of incubation. The aptamer solution was removed and the cells washed three times for 5 min each in binding buffer prior to visualisation using a Fluoview FV10i laser scanning confocal microscope (Olympus).

2.9. Confocal microscopy

Twenty-four hours prior to labelling, cells were seeded at a density of 75,000 cells per cm$^2$ in an 8-chamber slide (Lab-Tek II, Nunc). DY647-CD133-A15, DY647-CD133-B19 and the control aptamer were prepared in the same manner as for flow cytometry. Following removal of media, cells were incubated in blocking buffer containing 5% (w/v) foetal calf serum at 37 °C for 15 min, washed twice in binding buffer prior to incubation with 200 nM aptamer for 30 min at 37 °C. Bis-benzimide Hoechst 33342 (3 μg/mL) (Sigma) was added to the cells during the final 15 min of incubation. The aptamer solution was removed and the cells washed three times for 5 min each in binding buffer prior to visualisation using a Fluoview FV10i confocal microscope.

2.10. Inhibition of endocytosis

This was performed essentially as described for confocal microscopy with minor modifications. Briefly, cells were pre-treated with a potassium-depleted buffer (50 mM HEPES, 140 mM NaCl, 2.5 mM MgCl$_2$, and 1 mM CaCl$_2$) for 1 h at 37 °C prior to incubation with the aptamers. These buffers were also used in the incubation step with aptamers and all rinsing steps. The effectiveness of these treatments in inhibiting endocytosis was evaluated by qualitatively characterising the internalisation of human transferrin conjugated to Alexa Fluor 488 (Invitrogen Life Technologies). Transferin (5 μg/mL) was added to the cells following pre-treatment followed by a 30 min incubation at 37 °C. The cells were washed three times in their respective buffers and visualised using the Fluoview FV10i confocal microscope.

2.11. Tumour sphere preparation and incubation with aptamers and antibody

Two thousand HT29 and HEK293T cells were plated out in ultralow attachment wells and allowed to form spheres for 7 days in DMEM/F12 media (Invitrogen Life Technologies) containing 10 ng/mL epidermal growth factor, 10 ng/mL basic fibroblast growth factor, 50 μg/mL insulin and 100 units/mL E27. At 7 days, the spheres were washed three times in PBS containing 2.5 mM MgCl$_2$ and blocked for 20 min using binding buffer. The spheres were then incubated with 100 nM of aptamer or AC133 antibody (30 μg/mL Miltenyl Biotec) for 30 min, 60 min, 120 min, or 240 min. Following each time point, the spheres were washed three times with PBS prior to visualisation using the Fluoview FV10i confocal microscope. To determine the retention of aptamers within the tumour sphere, HT-29 tumour spheres were incubated with CD133 aptamers or CD133 antibody for a total of 4 h, washed three times in PBS, followed by incubation in sphere medium for a further 24 h before being imaged.

2.12. Differentiation assay with sodium butyrate

Twenty-four hours prior to differentiation, HT-29 cells were seeded at a density of 500,000 cells in a 12 well plate. Upon reaching 70% confluence, the cells were treated with 5 mM sodium butyrate for a total of 24 or 48 h and were then incubated with either the AC133 antibody, CD133-A15 or CD133-B19 aptamer for 30 min at 37 °C. The AC133 antibody was used at a concentration of 30 μg/mL as recommended by the manufacturer. After washing with PBS, the binding of AC133 antibody or aptamers was assessed using flow cytometric analysis.

2.13. Western blotting

HT-29 cells were treated with sodium butyrate as described in the previous section. The expression of CD133 protein in HT-29 cells was analysed by Western analysis as previously described [34]. Fifteen microlitres of each sample was loaded onto a 12% NuPAGE Bis-Tris mini gel (Invitrogen) along with a Precision Plus dual colour protein standard (BioRad). Following electrophoresis for 45 min at 200 V, the protein was transferred to a nitrocellulose membrane (Invitrogen) and blocked with 5% skimmed milk for 3 h at 25 °C, before being incubated with either anti-β-actin (Sigma) diluted 1:2000, or the anti-CD133 antibody, CD133/1 (clone W6B3C1, Miltenyl Biotec) diluted 1:200 in 1% skimmed milk, overnight at 4 °C. Chemiluminescence was detected using an ImageQuant™ LAS 4000 Biomolecular Imager (GE Healthcare).

3. Results

3.1. Cell SELEX facilitates the selection of aptamers against cell surface targets

CD133 is a complex pentaspan protein containing two extracellular loops. To effectively select aptamers against only the extracellular portion of the protein, it was necessary to devise a procedure that allowed us to express CD133 so as to preserve its native conformation. To this end, we sought to transiently express the protein...
on the surface of HEK293T cells. Using Lipofectamine 2000, the C-terminally His-tagged CD133 was transfected into HEK293T cells and allowed to express for 72 h prior to SELEX experiments, with expression confirmed by AC133 antibody staining. Similar to our previous SELEX experiments [26], a random RNA library of approximately $1 \times 10^{14}$ species containing 2'-fluoro-modified ribose on all pyrimidines was incubated with HEK293T cells expressing human CD133. Unbound RNA was removed via several washing steps prior to RT-PCR, and the process was repeated for a total of 12 rounds. Non-specific binding was eradicated through negative selection using an irrelevant His-tagged protein transfected into HEK293T cells. Non-radioactive RFLP was performed to confirm evolution of the species during iterative rounds and confirmation of enrichment was determined using flow cytometry using transfected and non-transfected HEK293T cells (Fig. 1A). As shown in Fig. 1B, round six showed a greater than 2.5-fold increase in binding to CD133-transfected HEK293T cells as compared to non-transfected HEK293T cells and that of the unselected library. Indeed, following round six, an almost complete loss of binding species was observed. This could be due to a proportion of the PCR amplicons being products of non-specific amplification which bound to the target via non-specific binding. These amplicons are preferentially amplified and possibly led to a decrease in the proportion of binding species in subsequent rounds [35]. Round six and round eleven were cloned and sequenced and a high proportion of the clones from round eleven displayed truncated sequences.

3.2. Post-SELEX engineering generated the smallest RNA aptamer

DNA from round six was cloned and sequenced and the clones were fluorescently tagged with FITC using an in-house method. The binding specificity of each clone was determined using CD133-negative HEK293T cells and HEK293T cells transfected with the expression construct of His-tagged CD133. The most encouraging results were shown with two aptamers, designated CD133-A and CD133-B (Fig. 2A(i) and B(i)). These two clones were sequentially truncated to determine the shortest number of bases required to maintain the structure of the binding region of the aptamer (Fig. 2 and Table 1). Clone CD133-A was truncated a total of four times to confirm the binding region of the aptamer (Fig. 2A and Table 1). This clone was successfully truncated to 15 nucleotides, making it the smallest published RNA aptamer against a cancer stem cell marker protein, and equivalent in size to the smallest published DNA aptamer directed against thrombin [36]. A second clone, CD133-B, was also investigated for its potential to bind with high affinity and specificity to CD133. This aptamer was truncated to 19 nucleotides (Fig. 2B(ii)), similar in size to our published aptamer targeting EpCAM [27]. In order for these CD133 aptamers to become effective cancer targeting agents, they must have negligible interactions with cells that do not express CD133. Therefore, both CD133-positive (HT-29 and Hep3B) and CD133-negative (T98G and HEK293T) cell lines of human origin were used to study the sensitivity and specificity of these two aptamers. As shown in Fig. 3A and B, determination of the equilibrium dissociation constant for both truncated aptamers showed a moderate binding affinity with CD133-positive cancer cells (33.85–145 nM) (Table 2), with the smallest aptamer, CD133-A15, having the better binding affinity. These results are consistent with results observed from previous experiments [27,37]. In addition, both aptamers did not bind to CD133-negative cells (Fig. 3C and D). The specific interaction between the CD133 aptamers was further verified using CD133-positive and -negative cell lines. The cell line HT-29 was used as a positive control to determine the specificity of our two aptamers. As shown in Fig. 4, the interaction of our aptamers with the different cell lines showed a similar staining pattern to that observed with the AC133 antibody, thus confirming the specificity of the aptamers.

3.3. CD133-specific aptamers are internalised via receptor-mediated endocytosis

For an aptamer to be developed into an effective cancer diagnostic, it must be efficiently internalised following binding to its target [27,38,39]. We studied whether the newly isolated CD133

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**Fig. 1.** Isolation of CD133 aptamers using systematic evolution of ligands by exponential enrichment (SELEX). (A) Flow cytometric binding analysis of FITC-labelled aptamers from iterative rounds of SELEX to CD133-transfected HEK293T cells. Fluorescein-labelled RNA from each round was incubated with target cells at 37 °C for 30 min, followed by flow cytometric analysis. (B) Comparison of the binding capacity of relative rounds of SELEX. The binding of each round was calculated after subtracting the mean fluorescent intensity of the binding of unselected library RNA to target cells, as well as that for binding to negative control cells. R, round in SELEX cycle; R0, unselected random library.
aptamers could internalise upon binding. To this end, we incubated both CD133-positive and CD133-negative cells with CD133 aptamers at 37°C for 30 min followed by confocal microscopy. As shown in Fig. 5A, CD133 aptamers were efficiently internalised upon binding. Such internalisation was specific as there was no fluorescent signal in CD133-negative cell lines. Furthermore, we confirmed that the mode of internalisation of CD133 aptamers was via receptor-mediated endocytosis as the aptamer fluorescence was found outside the cells and displayed a ring pattern along the plasma membrane upon the pre-treatment of endocytic blockers, such as potassium-depletion and hypertonic treatments (Fig. 5B). The effectiveness of these treatments in blocking receptor-mediated endocytosis has been previously confirmed using transferrin as a positive control [27].

3.4. CD133-specific aptamers are superior in penetrating tumour spheres than CD133 antibodies

To study the effectiveness of our aptamers as cancer theranostics, we investigated the potential of our aptamers to penetrate a tumour mass using an in vitro 3-dimensional culture, tumour sphere, as a model. We generated tumour sphere models of HT-29 and HEK293T cell lines under ultralow attachment condition. Upon reaching 200 μm in size, these spheres were incubated with 100 μM CD133-A15, CD133-B19 or 30 μM AC133 antibody. As presented in Fig. 6A, no tumour penetration was discernable with the AC133 antibody even after 4 h incubation and limited penetration was seen with our control RNA aptamer, which is of the same (19 nucleotide long) length as CD133-B19 but does not bind to CD133, after 4 h incubation. In sharp contrast, a superior penetration into the centre of the tumour sphere was seen with both our aptamers from 30 min up to 4 h during our assay period. A moderately enhanced signal was seen from the CD133-B19 aptamer as compared with CD133-A15. As well, only a weak signal was discernable with the HEK293T tumour spheroids which served as a negative control in this study (Fig. 6B). Importantly, 24 h after washing and further incubation in sphere medium, the fluorescence signals for CD133 aptamers in the tumour sphere were clearly detectable (Fig. 6C), demonstrating that the CD133 aptamers can not only penetrate into the core of the tumour sphere but can also be retained by the tumour cells in the centre of the sphere for at least 24 h.

Table 1

<table>
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<tr>
<th>Aptamer</th>
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<th>Base pairs</th>
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<tr>
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</tr>
<tr>
<td>CD133-A15</td>
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<tr>
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</tr>
<tr>
<td>CD133-B19</td>
<td>CAG AAC UUA UAC UAU G</td>
<td>19</td>
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Fig. 2. Post-selection engineering of CD133 aptamers. (A) CD133-A was serially truncated a total of four times (i: CD133-A; ii: CD133-A58; iii: CD133-A35, iv: CD133-A21, v: CD133-A15); (B) CD133-B (i: was truncated once, ii: CD133-B19).
3. CD133-specific aptamers recognise both the AC133 epitope and the CD133 protein

As discussed, the AC133 epitope, instead of the entire CD133 protein, has been used as a marker of CSCs. Treatment with sodium butyrate has been shown to differentiate colorectal cancer cells, thus decreasing the expression of the AC133 epitope [40, 41]. Using the colorectal cancer cell line HT-29 as a model system, we studied whether our CD133 aptamers could recognise both the AC133 epitope and the non-AC133 extracellular segment of CD133 displayed on the cancer cell surface. Following differentiation treatment with sodium butyrate for 24 h, HT-29 cells were analysed by flow cytometry to determine the binding epitope of our aptamers used at 100 nM concentration. The binding of the AC133 antibody at 300 nM to the treated cells was used as a benchmark and showed a 20% decrease in AC133-positive cells following treatment with sodium butyrate for 24 h (Fig. 7A). These results are consistent with previous reports showing a decrease in the AC133 epitope during differentiation [5, 7, 40–43]. In parallel, the binding of the CD133-A15 aptamer also showed a similar decrease in binding to the differentiated HT-29 cells (Fig. 7A). Given that the level of binding of both the CD133-A15 aptamer and AC133 antibody decreases with differentiation, it is plausible that this aptamer marks the same population of cells as the AC133 antibody, the CSCs. In contrast, the binding of the other aptamer, CD133-B19, to the differentiated HT-29 cells was increased, indicating that CD133-B19 binds to the extracellular domain of the CD133 protein, but not the AC133 epitope. Indeed, using another CD133 antibody which recognises a spatially distinct epitope to AC133 [44], we confirmed that there was no substantial change of the total CD133 protein level in the HT-29 cells before and after butyrate-induced differentiation in a separate Western analysis (Fig. 7B and C).

4. Discussion

Cancer stem cells (CSCs) are considered to be the root of cancer responsible for cancer recurrence. This model has gained acceptance because it explains radiation- and chemotherapy-resistance [45], and has led to numerous attempts to specifically target this population of cells within the tumour. While there is not one specific marker which defines all CSCs, a number of markers, including CD133, CD44, ALDH, EpCAM and ABCG2 [45, 46], have proven useful for defining the CSC population in solid tumours. CD133 has been implicated as a marker of the CSC population in brain, prostate, pancreas, melanoma, colon, liver, lung and ovarian cancers [5], and it has been suggested to be the most important marker of CSCs so far [47, 48]. While the function of CD133 is yet to be elucidated, this marker is upregulated in hypoxic conditions and has been associated with vasculogenic mimicry in triple negative breast cancer and prostate cancer [49, 50], indicating the importance of CD133 in tumour growth and metastasis. As well, given the conformational change that occurs during the differentiation process, the AC133 epitope represents a unique target for the treatment of various tumour types. Given how critical these CD133+ cells could be to the continuing spread of the tumour, we have isolated RNA aptamers against CD133 that are 25–32 times smaller in size than a monoclonal antibody.

We have previously described the success of our SELEX procedures to isolate aptamers targeting another CSC marker [27]. The isolation of aptamers targeting CD133 required a modification to our protocol due to the pentaspan nature of this protein and the necessity to use proteins in their physiological conformation for selection of aptamers. The success of our selection protocol was shown using flow cytometric binding assays, as well as RFLP analysis. Successful evolution was shown following the sixth SELEX cycle, and several aptamers were cloned. Two aptamers were chosen...
for further characterisation using both CD133-positive and negative cell lines. These aptamers were also truncated to determine the minimal size required to maintain binding affinity. One of our aptamers, CD133-A15 was truncated to a size of 15 bases. This truncation makes this the smallest RNA aptamer described so far. Both of these aptamers were shown to be sensitive and specific, and of more importance, these two aptamers were rapidly internalised by receptor-mediated endocytosis following binding to their target. This latter feature of the aptamers is a necessary requirement for these aptamers to be modified as theragnostic reagents.

Aptamers possess many benefits that make them ideal escort modalities for both treatment and imaging of tumour masses. Their

Fig. 4. Determination of specificity of CD133 aptamers. CD133-A15 or CD133-B19 were incubated at a concentration of 100 nM with CD133-positive or -negative cell lines for 30 min at 37 °C. Cells were also incubated with the AC133 antibody to confirm the presence of AC133 epitope. (A) HT-29 cells incubated with AC133-APC; (B) HT-29 cells incubated with CD133-A15 and CD133-B19; (C) PA-1 cells incubated with AC133-APC; (D) PA-1 cells incubated with CD133-A15 and CD133-B19; (E) PLC/PRF/5 cells incubated with AC133-APC; (F) PLC/PRF/5 cells incubated with CD133-A15 and CD133-B19; (G) A549 cells incubated with AC133-APC; (H) A549 cells incubated with CD133-A15 and CD133-B19; (I) DU145 cells incubated with AC133-APC; (J) DU145 cells incubated with CD133-A15 and CD133-B19; (K) T47D cells incubated with AC133-APC; (L) T47D cells incubated with CD133-A15 and CD133-B19. Black: Autofluorescence; Purple: AC133 antibody; Blue: CD133-A15 aptamer; Orange: CD133-B19 aptamer. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
small size means that they are capable of penetrating the tumour much more efficiently than conventional immunotherapy options, and these nucleic acids also lack immunogenicity, leading to far fewer side effects. One of the problems associated with the use of monoclonal antibodies for tumour therapy is the lack of penetration into the tumour mass. We have shown that our aptamers are

Fig. 5. CD133 aptamers are endocytosed via receptor mediated endocytosis following binding to CD133-positive cells but not to CD133-negative cells. (A) DY647-labelled CD133 aptamers were incubated with indicated cancer cells for 30 min at 37 °C, followed by imaging using laser scanning confocal microscopy. (B) CD133 positive cells were incubated with potassium depleted buffer to inhibit endocytosis. Transferrin or DY647-labelled CD133 aptamers were then incubated with HT-29 or Hep3B cells for 30 min at 37 °C, followed by imaging using laser scanning confocal microscopy. Transferrin was used as a positive control to confirm inhibition of endocytosis. (C) HT-29 or Hep3B cells were incubated with transferrin for 30 min at 37 °C without endocytic inhibitors. Data are representative of three independent experiments. For each pair of panels, fluorescent images are on the top, and optical (phase) images are on the bottom. Red: CD133 aptamer; blue: nuclear stain; green: transferrin. Scale bar = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
capable of not only penetrating a tumour sphere, but are also retained for a minimum of 24 h, suggesting that these aptamers would act as ideal drug delivery modalities. In contrast, the AC133 antibody was not capable of penetrating the tumour sphere core, even at a 300-fold higher concentration than that for the aptamers. This important property of the aptamers in this study highlights the invaluable attribute of aptamers for molecular imaging and targeted therapy. Not only are these aptamers internalised efficiently within a short amount of time by the tumour cells, they are also capable of penetrating the tumour mass, indicating their potential to target all of the cells in the tumour, including those that are usually poorly accessible due to their location in the tumour core when used as a molecular imaging probe. Through additional functionalisation, either by direct conjugation to a chemotherapeutic agent, e.g. doxorubicin or siRNA, or by surface conjugation onto nanoparticles containing a drug cargo, aptamers can function as very effective drug escorts [21]. While some aptamers can be effective solely by binding to their target, the majority of aptamers are much more successful as targeting ligands to improve the therapeutic index of cytotoxic agents. Therefore, by conjugating chemotherapeutic drugs directly to the CD133 aptamers, the drugs would not only be delivered to the tumour cells, but would also be retained for a sufficient amount of time within the tumour mass for the chemotherapeutic to kill the tumour cells. We are currently investigating the effectiveness of functionalising our aptamers for future theragnostic applications. Further studies will be required to confirm that these aptamer-nanoparticle and aptamer-drug constructs possess the same ability as aptamers to penetrate the tumour mass.

It is important to distinguish between the CD133 protein and the AC133 epitope when discussing the expression of either in relation to CSCs. AC133 is generally considered to be the marker of CSCs [51–53] though it should be pointed out that there is the same heterogeneity in CSCs as seen in normal cancer cells [17]. While AC133 may be a marker of CSCs, not all CSCs express AC133. There is also discordance over whether AC133 is truly a CSC marker as studies have shown that both CD133⁺ and CD133⁻ cells are capable of propagating tumours in mouse models [7–10]. There are a number of reasons for this: there is a heterogeneity in cell surface markers on CSCs [54,55]; the studies have used an
antibody directed against an epitope other than AC133 to determine CD133 expression [56]; and the AC133 antibody is unable to determine low expression or variants of the AC133 epitope so a ‘negative’ AC133 cell population does not necessarily mean this population of cells are not cancer stem cells [42,44,57]. It should be noted, however, that differentiation studies examining the expression of CD133 has consistently shown a down-regulation in the expression of the AC133 epitope as compared to CD133 [40,41,43,58]. This data indicates that the AC133 is an important marker of more primitive cells, such as CSCs. We examined if either of our CD133 aptamers were able to distinguish this epitope versus the CD133 protein using sodium butyrate differentiation treatment of HT-29 cells. The AC133 antibody was used as a positive control and showed a more than 20% decrease in expression levels following 24 h treatment with 5 mM sodium butyrate. Interestingly, aptamer CD133-A15 also showed a similar decrease in cell binding, indicating that this aptamer marks the same population of cells as identified by the AC133 epitope. An additional antibody used to confirm the expression of CD133 did not change following differentiation treatment. This antibody recognises the same epitope as AC133, and no significant decrease was observed following treatment as determined by Western blot analysis. This has previously been reported to show that the AC133 epitope is masked upon differentiation but is detectable by Western blot indicating the cells still retain their CD133 protein. As well, no decrease was seen in the percentage of positive cells recognised by aptamer CD133-B19, indicating that this aptamer not only recognises a different epitope to CD133-A15, but is also capable of identifying differentiated cancer cells. The use of both of these aptamers together have the potential to target the bulk cancer cells and CSCs in a tumour, thus reducing the bulk of the disease, as well as reducing the metastatic potential at the same time. Indeed, it is becoming well recognised that this is the only way we will successfully eradicate cancer in the future [59].

An important aspect of aptamers in cancer research is their lack of immunogenicity, a problem often encountered when antibodies are used for therapeutics or for flow cytometric cell sorting prior to xenotransplantation. A recent study demonstrated that the Fc fragment of antibodies can cause a profound inhibitory effect on engraftment of tumour cells in xenograft models due to antibody-mediated clearance [60]. This study on antibody-coated cells flow cytometrically sorted based on cell surface markers suggests that the proportion of CSCs could be highly under-estimated in xenotransplant studies using AC133 antibody-sorted cells. In addition, a further study has suggested that by stimulating the immune system, and thus targeting and attacking the bulk cancer cells, a reduction in the size of the tumour results in an increase in the proportion of CSCs [61]. These results taken together, along with prior knowledge of the effect that the majority of therapeutic antibodies have on the immune system, would indicate that modalities other than antibodies are required both for research into CSCs and also for the complete eradication of cancer in clinical studies. Aptamers, which are identical to monoclonal antibodies in their ability to bind to their targets with a high degree of selectivity, are superior to antibodies due to the lack of the Fc fragment and thus devoid of an immune response when injected into mammals. This attribute alone makes aptamers the ideal choice for cancer therapeutics, in addition to be a valuable tool in cell sorting for the isolation of CSCs. The ability to functionalise these nucleic acids without loss-of-function is an added benefit. As well, these aptamers do not suffer from the disadvantages associated with other nucleic acid species, such as RNAi or antisense oligonucleotides [21] due to their inability of entering the target cells, as we select aptamers that are internalised upon binding.

The targeting of CD133* cells in certain tumour types would seem to be a highly effective strategy to assist with the eradication of cancer [62]. As CD133* cells have been implicated in vasculogenic mimicry, this could explain the lack of efficacy of anti-angiogenic treatments, given that removing the oxygen supply from the tumour increases the number of CD133* cells. It is possible that by combining the targeting of CD133* cells continually alongside anti-angiogenic therapy may increase the efficacy of these treatments, though care must be taken that this does not increase the invasiveness of the tumour. Further studies are required to determine the effectiveness of targeting CD133 cells alone, or in combination with other therapeutic options using the novel CD133 RNA aptamers.

In summary, we have isolated the smallest RNA aptamer against CD133. Our AC133-targeting aptamers represent the ideal
molecules for the development of CSC targeted therapeutics without the side effects associated with immunogenicity.

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