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Targeting Ephrin Receptor Tyrosine kinase A2 with a selective aptamer for glioblastoma stem cells

Alessandra Affinito\textsuperscript{1,2}, Cristina Quintavalle\textsuperscript{2,3,§}, Carla Lucia Esposito\textsuperscript{3}, Giuseppina Roscigno\textsuperscript{1}, Catello Giordano\textsuperscript{1}, Silvia Nuzzo\textsuperscript{4}, Lucia Ricci Vitiani\textsuperscript{5}, Iolanda Scognamiglio\textsuperscript{1}, Zoran Minic\textsuperscript{6}, Roberto Pallini\textsuperscript{2}, Maxim V. Berezovski\textsuperscript{6}, Vittorio de Francisis\textsuperscript{3}, and Gerolama Condorelli\textsuperscript{1,8,§}

\textsuperscript{1}Department of Molecular Medicine and Medical Biotechnology, “Federico II” University of Naples, V. Tommaso de Amicis 95, 80131 Naples, Italy. Tel: +39 0815452821; Fax: +39 0817704795;

\textsuperscript{2}Percuros B.V., Enschede, The Netherlands;

\textsuperscript{3}IEOS, CNR, Naples, Italy. V. Tommaso de Amicis 95, 80131 Naples, Italy. Tel: +390813722343, Fax: +39 0812296674;

\textsuperscript{4}IRCCS SDN, Naples Italy;

\textsuperscript{5}Department of Oncology and Molecular Medicine, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy. Tel: +39 06 4990 3673, Fax:+39 06 4990 2137;

\textsuperscript{6}Department of Chemistry and Biomolecular Sciences and John L. Holmes Mass Spectrometry Facility, University of Ottawa, Ottawa, Ontario K1N 6N5, Canada;

\textsuperscript{7}Institute of Neurosurgery, Università Cattolica del Sacro Cuore, Largo Agostino Gemelli 8, 00168 Rome, Italy. Tel: +39 06 8818881, Fax: +39 06.35510321;

\textsuperscript{8}IRCCS Neuromed – Istituto Neurologico Mediterraneo Pozzilli.

Correspondence should be addressed to Gerolama Condorelli and Cristina Quintavalle, Department of Molecular Medicine and Medical Biotechnology, “Federico II” University of Naples, V. Tommaso de Amicis 95, 80131 Naples, Italy. Tel: +39 0815452821; Fax: +39 0817704795; Email: gecondor@unina.it, cristina.quintavalle@gmail.com

Running title: A40s binds EphA2 and blocks glioblastoma stem cells
Abstract

Despite the benefit associated with radiotherapy and chemotherapy for glioblastoma treatment, the majority of the patients experience a relapse following initial therapy. Recurrent or progressive GBM usually do not respond anymore to standard therapy and this is associated to poor patient outcome. Glioblastoma stem cells (GSCs) are a subset of cells resistant to radio- and chemotherapy and play a role in tumor recurrence. The targeting of GSCs and the identification of novel markers are a crucial issue in the development of innovative strategy for GBM eradication. By differential cell SELEX we have recently described two RNA aptamers, 40L sequence and its truncated form A40s, able to bind human GSCs cell surface. Both aptamers were selective for stem-like growing GBM cells and are rapidly internalized into target cells. Here we demonstrate that their binding to cells is mediated by direct recognition of the Ephrin type-A receptor 2 (EphA2). Functionally, the two aptamers were able to inhibit cell growth, stemness, and migration of GSCs. Furthermore, A40s was able to cross the blood brain barrier (BBB) and was stable in serum in in vitro experiments. These results suggest that 40L and A40s represent innovative potential therapeutic tools for GBM.

Keywords: Glioblastoma, Cancer Stem Cell, Aptamer, EphA2

Introduction

Between the malignancies of the brain, glioblastoma (GBM) has the worst prognosis with a median survival of 15 months \(^1\). A disease relapse rate is very high. Very often the disease rapidly evolves and patients succumb in few months. The current state of care for patients
suffering of GBM is surgical resection, when possible, followed by temozolomide (TMZ) chemotherapy associated with radiotherapy. However in patients undergoing relapse the standard therapy has minimal benefits. The improvement in omics approaches of GBM has led to the identification of specific molecular markers able to stratify patient subgroups and to better improve GBM treatment and survival prediction. The genetic loss of chromosomes 1p/19q is predictive of an increase sensitivity to both radiotherapy and chemotherapy. The presence of mutations of IDH 1 or 2 genes are indicative of an evolution of the GBM from a low-grade glioma and usually their presence is associated with a more favorable prognosis while the overexpression of a EGFR variant III is usually associated with a worst prognosis. Methylation of the methyl-guanine-methyl transferase MGMT gene promoter is usually associated with a better response to TMZ treatment and it characterizes a sub-population with a better prognosis. Identification of new biomarkers are needed for a better sub stratification of patients and to adopt new therapeutic strategy.

GBM stem cells (GSCs) are a heterogenous subpopulation of cells characterized by an increased resistance to conventional GBM therapies and could cause tumor relapse. Therefore, GSCs identification and their eradication are an important challenge to treat GBM.

Within key molecules in GBM development are the Eph (erythropoietin-producing hepatocellular carcinoma) receptors family members. These receptors are mainly expressed in early development and crucial for embryonic development, regulating processes like cell migration and adhesion. Expression of Eph receptors that is very low in adult and differentiated tissues become up-regulated in a number of human malignancies such as melanoma, breast, lung and ovarian cancer as well as glioma. In these malignancies, contrary to Eph receptor, the endogenous ligand ephrin appears downregulated. In GBM, the Eph receptor A2, EphA2, has
been proposed as a novel molecular marker and therapeutic target since it is strongly overexpressed in GBM cells but not in normal brain\textsuperscript{10, 11}. The overexpression of EphA2 correlates with poor patient outcome and it is essential in the maintenance of the GSCs pool, promoting their invasiveness \textit{in vivo}\textsuperscript{12}. EphA2 overexpression promotes GSCs tumorigenesis in GBM and its blockage strongly induces a tumor suppressive phenotype\textsuperscript{13}. Moreover, EphA2 is co-expressed with other stem cell markers such as CD133 and integrin alpha\textsubscript{7}\textsuperscript{13, 14}.

Aptamers are short sequences of single stranded oligonucleotides generated by a SELEX approach; they bind to molecular targets in the same fashion as antibodies\textsuperscript{15}. Due to their intrinsic properties, such as selectivity, easy modification, low immunogenicity and high affinity for their targets, they appear as an ideal novel therapeutic agent for GBM treatment to improve the standard of care.

In this study we characterized biological functions of the 2’ fluoropyrimidine containing RNA aptamer 40L and its truncated form A40s, recently identified by our group\textsuperscript{16}. These two aptamers (long and short) are able to specifically recognize GSCs and to discriminate them from differentiated cells. Both aptamers appear to be functionally active on GSCs, inhibiting stem properties, growth and migration. We characterized 40L and A40s as a high affinity ligand for EphA2, that is overexpressed by a subset of GSCs grown as tumorspheres.

**RESULTS**

**Functional effects of aptamers**- To determine functional effects of aptamers on GSCs growth we used primary stem cells derived from GBM patients. We first performed limiting dilution assay (LDA) on GSC#83 upon treatment with the 40L aptamer. Data were analyzed using ELDA
Cells treated with 40L for 2 weeks showed approximately a 50% reduction in the estimated stem cell frequency compared to the starting pool of the aptamer selection G0, used as a control (Fig. 1A). Furthermore, as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT assay), 40L inhibited cell viability by about 50% after 6 days of treatment (Fig. 1B). We also assessed stem cell/differentiation marker expressions upon 40L incubation on GSC#83 and GSC#1. We found that 400nM of 40L induced downregulation of the stem cell-specific transcriptional factor Nanog and upregulation of the astrocyte differentiation marker, Glial fibrillary acidic protein, GFAP (Fig. 1C). We then compared the effects of 40L on GSC#1 and GSC#83 on cell migration. We found that 40L interfered with both cell lines’ ability to migrate (Fig. 1D).

Next, we tested whether the truncated aptamer preserves the functional properties of the long sequence, evaluating the efficacy of A40s to reduce colony formation with a LDA. We found that like the long aptamer, A40s reduced stem cell frequency of about 40-50% in GSCs of several patients (#1, #83, #74, and #163) (Fig. 2 A-B-C-D).

We also assessed stem cell/differentiation marker expression upon A40s incubation. A40s is able to affect stemness inducing downregulation of stem cell markers such as Nestin, Oct4, CD133, Nanog and Olig2 as detected by qRT-PCR, immunofluorescence or Western Blot analysis (Fig. 2 E-F-G-H). Furthermore, A40s treatment increases the differentiation marker GFAP (Fig 2 F-H) indicating that the active functional site of 40L is preserved in the shorter A40s aptamer.

Moreover, as for the long aptamer, A40s demonstrated to reduce both GSC viability and migration (Fig. S1 A-B). On the contrary, no functional effect was detected on differentiated
cells. In fact, as shown in fig.S1 C-D, no change was observed in viability and migratory ability upon A40s treatment in differentiated cells. Taken together, these results indicate that once bound to GSCs, 40L and A40s elicit an intrinsic biological activity which is expressed by a strongly reduction of stemness characteristics, suggesting that these aptamers could be used to target stem cell phenotypes.

**EphA2 is A40s and 40L target**- In order to identify 40L and A40s aptamer target, we firstly performed a receptor tyrosine Kinase (RTK) proteome profile Array. After the incubation of the GSC#1 with 40L or G0, the phosphorylation of EphA2 was mainly impaired in 40L treated cells compared to G0 (Fig. 3A). Then we performed a protein pull down experiment incubating the protein extract of GSC#1 and GSC#83 with biotinylated A40s and scrambled control. Eluted proteins were analysed by mass spectrometry. Among the identified proteins, in both groups we restricted the analysis for proteins whose expression was annotated for cellular component as “membrane” and “cell surface” with an FDR q-value <0.003. Eight proteins were shared between the two analysed samples (GSC#1 and GSC#83) and among them EphA2 was the only one with a clear cell component annotation of “cell surface” further indicating EphA2 as candidate target for A40s (Fig. 3B). Notably, we found that EphA2 expression levels correlate with A40s aptamer GSCs binding profile reported before. Indeed, EphA2 was overexpressed in GSC #1, #83, #74 and #163 spheres compared to the differentiated counterpart and was present at higher levels in the two cell lines used for the aptamer cell SELEX (Fig. 3C). Moreover, as shown in Fig. 3D, EphA2 is overexpressed in U251-derived stem-like cells compared to U87MG-derived stem-like cells in which EphA2 is absent. Indeed, A40s selectively
bind U251-derived stem-like cells but not U87MG-derived stem-like cells, as previously demonstrated\textsuperscript{16}.

To establish the specific interaction of A40s with EphA2, aptamer-pull down proteins were analysed by Western Blot with anti-EphA2 antibodies. Results confirmed A40s binding to EphA2 (\textbf{Fig. 4A}). Furthermore, overexpression of EphA2 made the differentiated cells U87MG able to be bound by A40s (\textbf{Fig. 4B}). By using Enzyme-Linked Oligonucleotide Assays (ELONA)\textsuperscript{19} with the recombinant protein we further proved the direct interaction between the A40s aptamer and the EphA2 as a specific target (\textbf{Fig 4 C}). Finally, by using ELONA we also determined the apparent dissociation constant of A40s for the recombinant EphA2 (Kd 0.76nM ±02641) (\textbf{Fig 4D}). All together this data indicates that A40s targets at high affinity and specificity the EphA2 expressed on GSCs cell surface.

\textbf{Serum stability and in vivo functional aspects of A40s}- An important feature for clinical translation of new therapeutics is \textit{in vivo} stability. Therefore, we evaluated the stability of A40s, incubating the aptamer in human serum for up to one week. Serum RNA aptamer samples were recovered at different time-points and analyzed through 15\% polyacrylamide/urea 7M denaturing gel (\textbf{Fig. 5A}). The aptamer was found to have good stability, it is gradually degraded but at least the 45\% of the sequence remains stable in 90\% serum for 8 hours.

Moreover, in order to evaluate the aptamer use \textit{in vivo} for future applicability of this molecule, we investigated A40s capability to cross healthy BBB after systemic injection. A40s proved to be able to reach brain and to be present until one hour upon the systemic injection (\textbf{Fig. 5B}). As a result, our \textit{in vivo} and \textit{in vitro} data altogether demonstrate that A40s is able to reach the tumor overcoming BBB when systemically injected.
Discussion

The presence of GSCs within GBM represents a major impairment for the treatment of this tumor. It is well established that GSCs are usually more resistant to conventional therapy and give rise to recurrence and more aggressive tumor\textsuperscript{4,5}. Therefore their targeting is an important goal for cancer therapy. The use of specific “bullets” targeting the GSCs in combination with conventional therapy for the differentiated population could represent a more effective approach to treat GBM, to ameliorate patients condition, and to prolong survival reducing tumor recurrences. Several proteins have been shown to be overexpressed in GBM and in particular in GSCs population. Between them, the EphA2 and EphA3 are the most investigated, showing a role in self renewal of GSCs compartment and blocking their differentiation\textsuperscript{20}.

Here we demonstrated that A40s targets specifically EphA2 both as a recombinant protein and when expressed on the cell surface of the stem-like population of GBM. Indeed, EphA2 is a transmembrane receptor tyrosine kinase overexpressed in stem like cells and required for self-renewal and GBM tumor propagation\textsuperscript{13}. We showed that EphA2 expression was restricted to GSCs indicating that EphA2 may represent a good candidate to discriminate between GSCs and differentiated cells. Other investigation\textsuperscript{14} has also reported this same observation on EphA2 expression and its inverse correlation with cell differentiation, supporting our idea that EphA2 is a marker for discriminating between GSCs and differentiated cells. Moreover, EphA2 knockdown has been demonstrated to suppress both self-renewal and tumorigenicity and several intracellular pathways such as AKT, JNK and mTORC1 have been reported to crosstalk with EphA2 signaling, regulating the GSCs proprieties\textsuperscript{7}. In its mechanisms of action, the A40s aptamer could induce an internalization of the EphA2 receptor decreasing the amount of the
receptor on cellular surface or could impair the activation of the intracellular crosstalk responsible of GSCs maintenance.

EphA2 has been reported as a promising therapeutical and molecular target for GBM diagnosis and therapy\textsuperscript{21, 22}. Indeed, we showed that upon binding to EphA2 the A40s aptamer downregulated self renewal and the expression of the stem-like phenotype reducing cell viability of GSCs. The ability of A40s to bind and specifically recognize EphA2 highlights this aptamer as a great candidate for selective inhibition and targeting of GSCs.

Our finding strongly enlarges the possibility of using the A40s as a novel targeting molecule for EphA2. To our knowledge our aptamer is the first example of a novel molecule able to recognize and to inhibit specifically EphA2 and toughts this, to impairs GSCs expansion in GBM tumor. Different strategies are under investigation in order to block its signaling\textsuperscript{13} or to develop novel immunotherapeutic vaccines\textsuperscript{23}. For example, GLPG1790 is a small inhibitor molecule shown to inhibit various Eph receptor kinases resulting in reducing tumor growth and stem cell population\textsuperscript{24}. In a preclinical model in fact it was shown to stop the EphA2 receptor signaling, miming the Ephrin A1 mediated phosphorylation\textsuperscript{25}. However, GLPG1790 lacks specificity, since it showes an inhibitory activity also on other members of Eph receptor family such as EphA3 and EphB4\textsuperscript{24}. Another pan inhibitor of Eph receptor family UniPR1331, also showed in a xenograft model to inhibit GBM angiogenesis and vasculogenesis toughts EphA2 blockage\textsuperscript{26}. A more specific approach targeting indirectly EphA2- overexpressing GSCs population was done by Arnold and colleagues who developed an antibody against CD44, conjugated with an antisense oligonucleotide against EphA2 mRNA\textsuperscript{27}, able to be internalized in GSCs population and to reduce EphA2 expression. Highlighting the importance of EphA2 targeting, several attempts have also be done in the reeducation of the immune system with a generation of panel of CAR-T
cells for EphA2 as a novel GBM immunotherapy strategy \(^2\). As we have previously reported, A40s is able to serve as a targeting moiety for therapeutic si/miRNAs\(^2\) thus combining the intrinsic inhibitory potential of EphA2 intracellular signaling to the selective silencing of GBM tumor targets as STAT3 and miR-10b\(^30, 31\).

An important limit for the development of therapeutic strategy for GBM is the passage of the molecules through the blood brain barrier (BBB). Even if only molecules smaller than 400 Da and lipophilic are considered able to cross BBB\(^32\), recent evidence supports the ability of aptamers to overcome the BBB\(^31, 33\); in fact receptor and/or channel mediated endocytosis, fluid-phase pinocytosis and transcytosis could be implicated in BBB permeability to aptamers\(^34\). Here, we demonstrated that A40s was able to reach the brain in healthy mice. Moreover, previously we have demonstrated that A40s and CD133 co-stained in sections from human brain tumor, indicating that A40s localizes in human brain cancer stem cells.

As a result, our \textit{in vivo} and \textit{in vitro} data indicate that not only A40s can be stable in human serum allowing systemic administration, but it is also able to reach the brain where it can potentially give its antitumoral effect targeting GSCs, halting tumor growth and reducing tumor relapse. Taken together this data suggest that A40s is a good candidate to selectively target EphA2 on GSCs and shows potential applicability as a therapeutic tool to block a GSCs population and thus GBM recurrence.

\textbf{Methods}

\textbf{Patient-derived tumor stem cells and continuous cell lines-} GBM tissue derived stem cells were obtained from the Institute of Neurosurgery\(^35, 36\), School of Medicine, Università Cattolica, Rome, Italy as indicated previously\(^16\). U251-MG and U87-MG cell lines were from ATCC and
were cultured in DMEM medium (Sigma Aldrich, Milan, Italy) supplemented with 10% FBS and 1% antibiotic and antimicotic (ThermoFisher Scientific, Milan, Italy)

**Western blot analysis**- Western blot analysis was performed as described previously\(^{37}\). Band intensity quantification was performed using ImageJ software. Primary antibodies were: anti-β3 tubulin, anti-Sox2, anti-Nestin, anti-EphA2, anti-GFAP, anti-Nanog, anti-OLIG2 (Santa Cruz Biotechnologies, MA, USA), anti-β actin (Sigma Aldrich); anti-CD133 (Proteintech, Manchester, UK). Secondary antibodies were goat anti-rabbit and anti-mouse IgG (from Santa Cruz Biotechnology).

**RNA extraction and real-time PCR**- Total RNAs (miRNA and mRNA) were extracted using EuroGOLDTriFast (EuroClone, Milan, Italy) according to the manufacturer's protocol. All the RNAs were reverse transcribed as described previously\(^{39}\). To amplify genes of interest we used the following primers:

- β-ACTIN fw: 5′-TGCGTGACATTAAGGAGAAG-3′, β-ACTIN rv: 5′-GCTCGTAGCTCTTCTCCA-3′; NANOG fw: 5′-CAAAGGCAAAACAACCCACTT-3′, NANOG rv: 5′-TCTGGAACCAGGTCTTCACC-3′; GFAP fw: 5′-CTGCAGCTGATCTCCA-3′; OCT4 fw: 5′-ACCTGAGATGTCCCTCAGC-3′; NESTIN fw: 5′-GCCGGTTACAGAACCACACT-3′; NESTIN rv: 5′-ACAGGTGTCTCAAGGGTAGC-3′.

**Immunofluorescence analysis**- Mechanically disaggregated stem cells were forced to adhere on polylysine-coated glass coverslips for 15 minutes, or alternatively, differentiated cells were directly seeded on coverslips after treating them for 2 weeks with 200nM of A40s or Scrambled sequence. Fixed cells with 4% paraformaldehyde were permeabilized with PBS 0.5% Triton X-
100 for 15 minutes at room temperature. Thereafter, they were blocked in PBS 1% BSA for 30 minutes at room temperature, and, after two washes with PBS, incubated with PE mouse anti human anti-CD133 (BD Biosciences), mouse anti-Nestin and rabbit anti-EphA2 (Santa Cruz Biotechnologies) diluted in PBS 1% BSA min for 1 hour at 37 °C. Anti-Nestin and anti-EphA2 coverslips were treated with APC Goat Anti-Mouse Ig and FITC Goat Anti-Rabbit IgG (BD Biosciences) respectively for 30 minutes at 37 °C. Coverslips were washed three-times with PBS, mounted with Gold antifade reagent with DAPI (ThermoFisher Scientific) and the cells were visualized by confocal microscopy (LSM700, Zeiss, Milan, Italy). Images were captured at the same settings, enabling direct comparison of staining patterns.

**Proteome Profiler Array**- Human Phospho-RTK Array (R&D) was used according to the manufacturer's protocol. GSC#1 (45x10^4) cells were treated with 400nM of 40L or with the negative control G0 for 3 hrs at 37°C and then incubated with 20% FBS for 20 minutes at 37°C. Cells were harvested and lysed and 75 ug of proteins were incubated with the Human Phospho-RTK Array (R&D). Finally, EphA2 phosphorylation spots were evaluated by aligning them with the pairs of reference spots.

**In vitro limiting dilution assay**- The assay was performed as previously described by Adamo et al. A number of 1, 5, or 10 cells per well were seeded in stem cell medium into a 96-well plate. Two weeks after seeding, the number of wells containing spheroids for each cell plating density was counted, and extreme limiting dilution analysis was performed using software available at http://bioinf.wehi.edu.au/software/elda4. For clear and unambiguous understanding, the reciprocal of 95% confidence intervals for 1/(stem cell frequency) generated by ELDA software
was calculated and shown in the corresponding graph. Given the long period of treatment, aptamers were renewed in wells twice a week at a concentration of 100nM.

**Aptamer-based pull-down-** Protein identification was performed with an adapted protocol described previously by Berezovski *et al.* \(^{41}\). To isolate protein targets, 6 million GSCs (#1 and #83) were lysed with Sodium deoxycholate (Sigma Aldrich) 0.1% w/v in 10mM PBS with Ca2+, Mg2+ (Sigma Aldrich) and incubated with a biotinylated-scrambled oligonucleotide (TriLink Biotechnologies, San Diego, California, USA) at 200nM for 30 minutes at room temperature, as counterselection step. Then lysates were incubated with Streptavidine Magnesphere (Promega, Milan, Italy) for 30 minutes at 4°C. Afterwards, unbound proteins were incubated with biotinylated-A40s (TriLink Biotechnologies) at 200nM for 30 minutes at room temperature. A40s-protein complex were incubated with Streptavidine Magnesphere (Promega) for 30 minutes at 4°C. Collected beads were washed two times with cold 10mM PBS Ca2+, Mg2+ (Sigma Aldrich). Thus, incubation with 8M urea for 1h at 4°C led to proteins denaturation and release from the aptamer and magnetic beads. Pull-down proteins were separated by SDS-PAGE (10% polyacrylamide gel), transferred to nitrocellulose membranes (Millipore, Bedford, MA) and immunoblotted for EphA2 antibody (Santa Cruz Biotechnologies).

**Mass spectrometry identification of aptamer target-** Putative target of A40s in GSC#1 and GSC#83 were pulled-down by affinity chromatography as indicated in the “Aptamer-based pull-down” section. Samples were analysed by an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific) coupled to an Ultimate3000 nanoRLSC (Dionex, Thermo Fisher Scientific). Peptides were separated on an in-house column (Polymicro Technology), 15 cm x 70 μm ID, packed with Luna C18(2), 3 μm, 100 Å (Phenomenex) and employing a water/acetonitrile/0.1% formic acid gradient. Samples were loaded onto the column for 105 min at a flow rate of 0.30 μl/min.
Peptides were separated using 2% acetonitrile in the first 7 min and then using a linear gradient from 2 to 38% of acetonitrile for 70 min, followed by gradient from 38 to 98% of acetonitrile for 9 min, then at 98% of acetonitrile for 10 min, followed by gradient from 98 to 2% of acetonitrile for 3 min and wash 10 min at 2% of acetonitrile. Eluted peptides were directly sprayed into a mass spectrometer using positive electrospray ionization (ESI) at an ion source temperature of 250°C and an ionspray voltage of 2.1 kV. The Orbitrap Fusion Tribrid was run in top speed mode. Full-scan MS spectra (m/z 350–2000) were acquired at a resolution of 60,000. Precursor ions were filtered according to monoisotopic precursor selection, charge state (+2 to +7), and dynamic exclusion (30 s with a ± 10 ppm window). The automatic gain control settings were 4e5 for full FTMS scans and 1e4 for MS/MS scans. Fragmentation was performed with collision-induced dissociation (CID) in the linear ion trap. Precursors were isolated using a 2 m/z isolation window and fragmented with a normalized collision energy of 35%. Proteome discoverer 2.1 (Thermo Fisher Scientific) was used for protein identification. The precursor mass tolerance was set at 10 ppm and 0.6 Da mass tolerance for fragment ions. Search engine: SEQUEST-HT implemented in Proteome Discovery was applied for all MS raw files. Search parameters were set to allow for dynamic modification of methionine oxidation, acetyl on N-terminus and static modification of cysteine carbamidomethylation. The search database consisted of a nonredundant/reviewed human (20326 proteins) protein sequences in FASTA file format from the UniProt/SwissProt database. The false discovery rate (FDR) was set to 0.05 for both peptide and protein identifications.

**Aptamer-based ELONA-** Assays were performed as previously described. Nunc-immunoplate maxisorp 96 wells (Thermo Fisher Scientific) were left untreated or were coated
with 30nM of purified EphA2 extracellular domain (R&D Systems, Milan, Italy CF 3035-A2) over night at 4°C. Wells were blocked for 2 hours at room temperature with PBS containing 3% BSA, washed two times with PBS and, then, incubated for 2 hours at room temperature in PBS with 200 nM biotinylated-A40s aptamer (TriLinK Biotechnologies) or an unrelated aptamer (Scrambled) as a negative control. Following two washes with PBS, samples were incubated with horseradish peroxidase (HRP)-conjugated Streptavidin (Thermo Fisher Scientific) for 1 hour at room temperature and washed two times with PBS. Signals were revealed with TMB substrate solution (Thermo Fisher Scientific) and stopped with the stop solution for TMB substrate (Thermo Fisher Scientific). Absorbance at 450 nm was measured with a Multiskan FC Microplate Photometer (Thermo Fischer Scientific).

**Dermination of a dissociation constant –** Kd for A40s-GSC complex was determined by performing Custom TaqMan® Small RNA Assays (Thermo Fischer Scientific) or alternatively, A40s-Recombinant human EphA2 complex Kd was determined by performing Aptamer-based ELONA, as previously described. Fitting curves were designed by using GraphPad Prism 6 software.

**Stability in Human Serum–** 2’-F-RNA A40s was incubated until 7 days in 90% human serum (Type AB Human Serum, Euroclone) at starting concentration of 4 µM. At each time point, 16 pmoles of A40s were recovered and incubated for 1 hr at 37°C with 0.5 µL of proteinase K solution (600 mAU/mL) in order to remove serum proteins, which interfere with electrophoretic migration. Then, 9 µl of the denaturing gel loading buffer (1× TBE, 95% formamide, EDTA 10mM and bromophenol blue) were added to each sample before storing at −80°C. All time
point samples were loaded on 15% polyacrylamide/urea 7M denaturing gel. The gel was visualized by UV exposure after ethidium bromide staining.

**In vivo experiments**- BBB crossing was assessed with intracardiac injection of 1,600 pmol of biotinylated A40s or saline solution in healthy, housed athymic CD-1 nude mice (nu/nu). The aptamer amount was determined by performing RT-qPCR.

**Statistical analysis**- Continuous variables are given as mean ± 1 standard deviation (SD) or standard error of the mean (SEM). Statistical values were defined using GraphPad Prism 6 (San Diego, CA, USA) software, by student’s t-test (two variables), or one-way ANOVA (more than two variables). P value ≤ 0.05 was considered significant for all analyses.

**Plasmid transfections**- EphA2-YFP was a gift from Kalina Hristova (Addgene plasmid # 108852) \(^{42}\). EphA2-YFP or control vector were transfected by using Lipofectamine 3000 reagent (Thermo Fisher Scientific).

**Ethics Statement**- Animal studies were conformed to internationally accepted standards according to D.lgs. 26/2014 and approved by “Servizio Veterinario Area C”, ASL Roma E, with the authorization number n°259/2017-PR (prot. D9997.35 on 21/02/2017).
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Author Contributions

Experiments design and performance: C.Q. and AA (bindings and target discovery); technical support: C.G. and I.S.; data interpretations: S.N., A.A., C.Q., C.L.E and G.R.; in vivo assays: L.R.V. and R.P.; manuscript preparation: A.A., C.Q., G.C. and V.D.F.; figure assembly: A.A., S.N.; proteomic experiments: Z.M. and M.V.B. All the authors reviewed the manuscript.

Conflict of Interest

All authors have read and approved the manuscript, its contents and its submission to and disclose no potential conflict of interest.

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FIGURE LEGENDS

Figure 1. Functional activity of 40L. (A) 20 wells per doses of cells were treated with the aptamer and limiting dilution analysis (LDA) was performed. Confidence intervals for stem cell frequency is shown. Estimate stem cell frequency is reported in the graph; bars indicate lower and upper confidence intervals for stem cell frequency as calculated by ELDA software. (B) Stem cells were incubated with 400nM of 40L and cell viability evaluated by a MTT assay after 6 days of treatment. Results are presented as mean ± SD of three independent experiments. (C) Real-time PCR was performed to analyze GFAP and NANOG levels in GSCs#1 or GSCs#83 upon 2 weeks of treatment with 40L at 400nM. Representative experiments are shown and results are expressed relative to the background binding, detected with the starting pool of sequences used for selection. (D) Overnight cell migration was analyzed by a transwell migration assay upon 72h of treatment with 400nM of 40L aptamer. A representative experiment is shown. Vertical bars indicate standard deviation values. **, p ≤ 0.01; ****, p ≤ 0.0001.

Figure 2. A40s effects on GSCs stemness. (A-D) LDA was performed using ELDA. Confidence intervals for stem cell frequency is shown. Estimate stem cell frequency is reported in the graph; bars indicate lower and upper confidence intervals for stem cell frequency, as calculated by ELDA software. (E-H) Change in GSCs markers expression are shown after treating the cells for 2 weeks with 400nM of A40s sequences or negative control. (E-F) Real-time PCR was performed to analyze stemness markers levels in GSCs #1 and #83. Representative experiments are shown and results are expressed in relation to the background effect detected by using scrambled sequence, *, p ≤ 0.05; **, p ≤ 0.01. (G) Confocal
microscopic images display CD133 (red), Nestin (red) and EphA2 (green) staining in GSCs. Yellow arrows show CD133 negative cells and pink arrows indicate cell surface localization of EphA2. (H) Western blots show downregulation and upregulation of principal GBM stemness and differentiation markers respectively.

Figure 3. EphA2 is the target of 40L and A40s. (A) Reduction of EphA2 phosphorylation is shown through receptor tyrosine Kinase (RTK) proteome profile array after treating GSCs with 40L or G0. As control, reference spots of each array are shown. (B) A protein pull-down protocol is optimized for detection of A40s binding proteins in GSC#1 and GSC#83. Eluted proteins were analysed by mass spectrometry and the most significant common membrane proteins are shown in the table. (C) EphA2 levels in several stem cells are compared to a differentiated counterpart. (D) The EphA2 level is shown in U251 derived stem-like cells, U87MG derived stem-like and differentiated cells.

Figure 4. A40S binds EphA2 with high affinity. (A) Pull-down EphA2 expression is analysed by SDS-PAGE. (B) EphA2 overexpression in differentiated U87-MG results in increasing A40s binding ability. (C) ELONA is used to prove A40s binding to Recombinant Human EphA2 and (D) to calculate Kd of the A40s-EphA2 complex. Vertical bars indicate standard deviation values.

Figure 5. A40s in vivo distribution. (A) Time course analysed through denaturing polyacrylamide gel electrophoresis illustrates A40s stability in 90% human serum at 37 °C. (B)
Biotinylated A40s in mice brain were quantified 30 minutes and 1 hour after aptamer intracardiac injection in left (sx) and right (dx) brain hemisphere.
Figure 1
Figure 2
Figure 3
Figure 4
