Progress Report

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Title of Project: ***Novel agents for the targeting of abnormal blood vessels in the brain to prevent stroke.***
[Scientific title: DNA aptamers targeting endothelial RAS mutants for vascular drug delivery in brain arteriovenous malformations (AVMs).]

Summary:

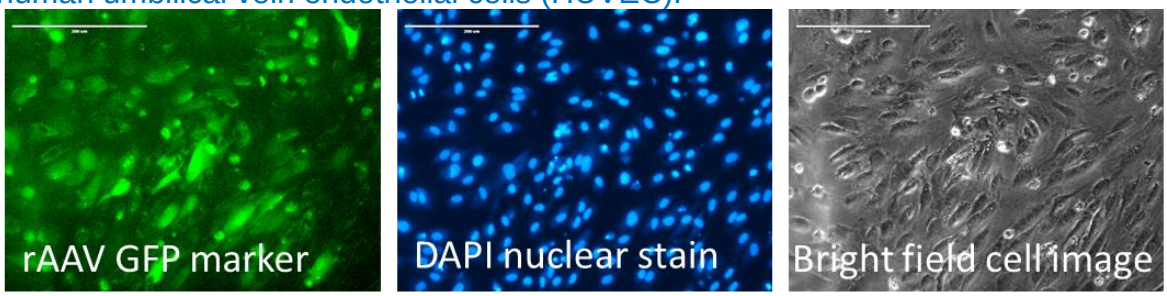
The aims of this study are:
1) to develop an AVM cell culture model in the laboratory that expresses a RAS-activating mutation characteristic of sporadic AVM endothelial cells; and
2) use it to generate novel targeting molecules (called DNA aptamers) through a process of cell-SELEX that can specifically recognise and bind molecular targets on the cell surface of these RAS mutant cells

Progress AIM 1 - Construct endothelial cell lines carrying RAS^{G12V}-activating mutations for use as an AVM cell model.
Our first aim was to construct an endothelial cell line that expresses RAS mutant protein. The original aim was to use lentiviral constructs however given the oncogenic nature of the RAS protein this was changed to adeno-associated viral constructs (AAV) as a safer means of protein expression. After acquiring approval for the use of this genetically-modified construct a $RAS^{(G12V)}$-expressing recombinant AAV construct was acquired from VectorBuilder USA in April. We designed this construct to specifically infect human endothelial cells and we chose to create constructs in primary human umbilical vein endothelial cells.
Different serotypes of AAV constructs require specific receptors on the cell surface for uptake so we tested various culture conditions and growth media for optimised uptake. Several matrix coatings were applied to tissue culture plates before seeding cells (such as collagen and gelatin) to ensure good cell adhesion as well as efficient virus uptake. Gelatin was found to be an optimal surface substrate that gave consistent high levels of infection. We determined that a relatively high MOI of 10,000 (multiplicity of infection = number of virus particles/number of cells) was required to obtain >80% infection rates suitable for subsequent experiments.
Figure 1 shows optimisation of infection uptake. The AAV construct expresses both the RAS protein and a fluorescent GFP protein (green) which is used to visually assess virus uptake using a microscope. Early experiments demonstrate that

infection with either a control or RAS-expressing virus does not cause significant induction of cell death.

Now we have established optimal conditions for infection we are further characterising and validating the cell model before aptamer selection begins. We will perform western blotting and immunofluorescent staining to assess protein expression of the RASG12V mutant specifically. We will also use these techniques to assess downstream activation of RAS-targeted pathways such as ERK and mTOR to validate that the model is acting appropriately. We will assess effects of RAS over-expression on cell proliferation, migration and apoptosis. Once this preliminary characterisation is done within the next month we will commence the cell-SELEX process for DNA aptamer selection on this cell model.

Figure 1: GFP imaging shows successful AAV infection and protein expression in human umbilical vein endothelial cells (HUVEC).



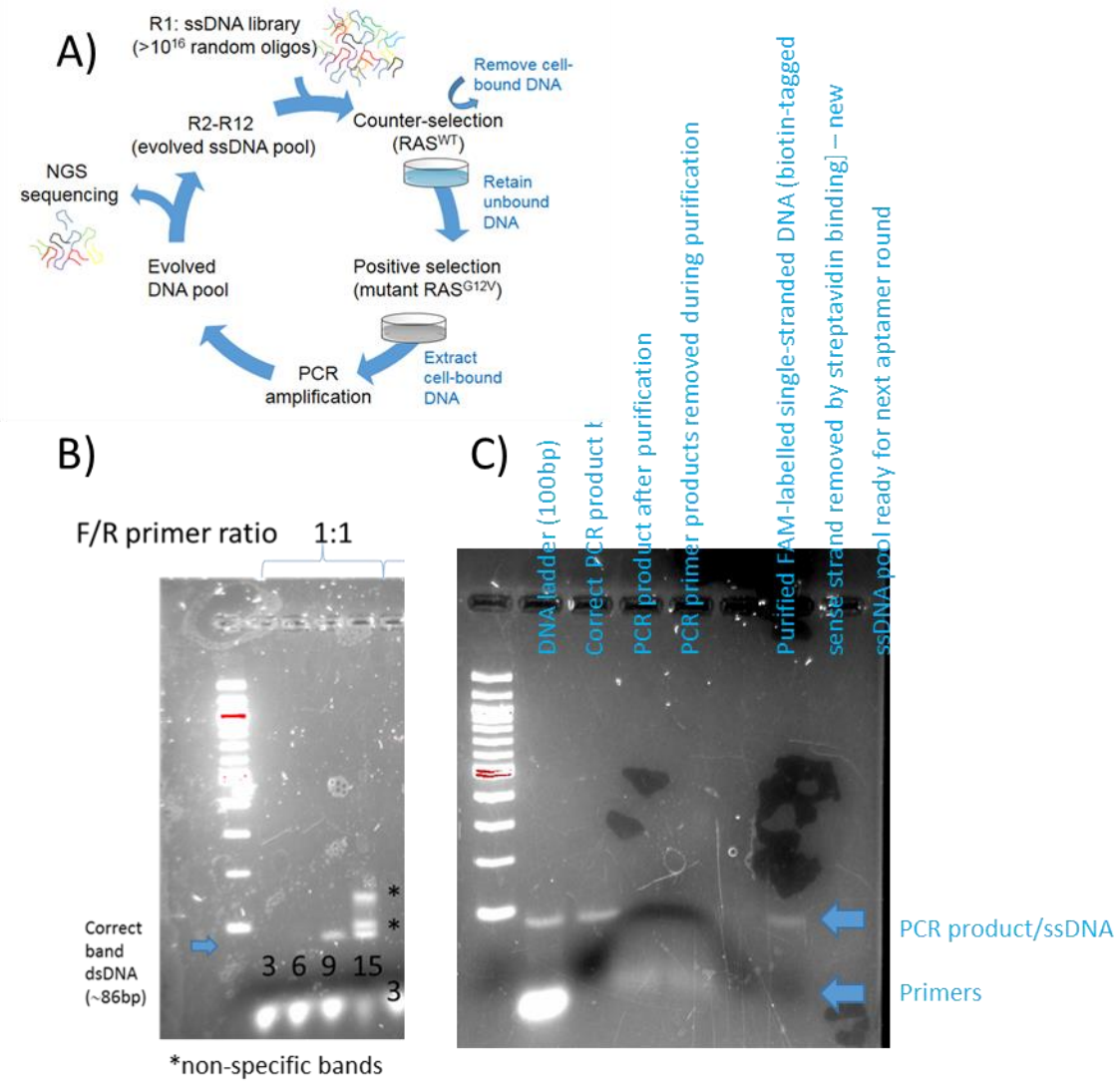
Progress AIM 2 - Generate novel targeting molecules (called DNA aptamers) that can specifically recognise and bind molecular targets on the cell surface of these RAS mutant cells.

While establishing the endothelial RAS cell model we have also been establishing the necessary protocols in the laboratory for DNA aptamer enrichment through cell-SELEX. The cell-SELEX process for high-affinity aptamer selection is shown in Fig. 2A. A pool of random DNA aptamers is enriched through repetitive rounds of selection on cells expressing the desired phenotype eg RAS stimulated proteins. As the RAS model has taken time to establish, we commenced work on optimising the PCR protocols for cell-SELEX selection using irradiated cells instead.

This process is more complex than standard PCR protocols as amplification of random pools tends to result in the accumulation of non-specific PCR products that interfere with selection. We have optimised the PCR amplification with two different DNA polymerases and by modifying parameters such as magnesium, annealing temperature, template concentration, extension time and cycle number, have been able to reproducibly amplify the heterogeneous pools between each cell selection round (Fig. B). We have also optimised protocols for the restoration of single-stranded DNA from the double-stranded PCR products through streptavidin-agarose bead enrichment to allow creation of next DNA aptamer pool for cell incubation and selection (Fig. 2C). To date we have performed 3 rounds of complete selection on the irradiated cells using non-irradiated cells as the negative selection step. This work to optimise protocols allows us to start directly on aptamer selection once the

RAS model is fully validated but will also be continued to search for potential aptamers suitable for binding radiation markers in brain AVMs.

Fig. 2 Aptamer enrichment using cell-SELEX



Hypothesis vs Findings

Hypothesis: We hypothesised that we could create endothelial cells expressing constitutive RAS as a cell model of brain AVMs and use them to enrich DNA aptamers as targeting agents.

Findings: So far we have been able to design adenoviral constructs and have demonstrated that they can efficiently infect primary human endothelial cells. We have optimised the conditions for PCR amplification of complex DNA aptamer pools in cell-SELEX. We have applied this to selection on irradiated cells while we await final validation of the RAS-expression cell model.

Unanswered Questions

Although we have achieved cell infection with the adenoviral constructs we must complete experiments to validate that the RAS overexpression is occurring as expected and that the viral infection itself has not significantly altered the phenotype of the cells. This will require a series of experiments (western blotting and immunostaining) to assess RAS (G12V) protein expression directly and to assess expression of intracellular downstream markers, we will also assess the effect of the expression on cellular activity such as cell growth and survival. Once the cell phenotype has been validated we can start the cell-SELEX process with the cell model and with the PCR protocols now optimised through pre-work performed using irradiated cells.

What these research outcomes mean

Although this work is ongoing in terms of results outcome, the work to date has allowed our group to establish several new techniques in the laboratory (adenoviral design and delivery; aptamer selection through PCR and cell-SELEX). These experiments are technically complex and require substantial optimisation. This funding allows us the opportunity to develop these skills within the group. With these techniques now established we can commence the process of aptamer selection through cell-SELEX to achieve our primary aim to enrich RAS-targeting ligands before the end of 2021.