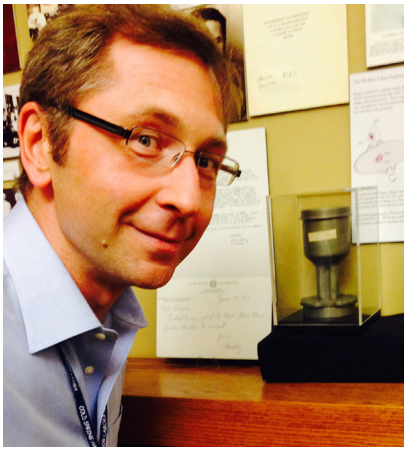
INTERVIEW

Dr Fyodor Urnov: The power and promise of zinc finger nuclease-mediated genome editing



Dr Urnov with the original Waring blender used in the Hershey-Chase experiment.

Fyodor Urnov, PhD, is Project Leader and Senior Scientist at Sangamo BioSciences, Inc. where he co-developed human genome editing with engineered zinc finger nucleases (ZFNs). Dr Urnov previously led the company's research and development efforts in deploying genome editing for crop trait engineering (in partnership with Dow Agrosciences) and in generation of engineered cell lines for manufacturing, improved generation of transgenic animals and as research reagents (in partnership with Sigma-Aldrich). In his current role as Project Leader for the Hemoglobinopathies, Dr Urnov heads Sangamo's collaboration with Biogen to develop genome editing as a one time, lasting treatment for beta-thalassemia and sickle cell disease. Dr Urnov is also an associate adjunct professor in the department of Molecular and Cell Biology at the University of California, Berkeley. Dr Urnov received his PhD from Brown University and holds a BSc in Biology from Moscow State University. He is an author on more than 60 scientific publications and an inventor on more than 90 issued and pending US patents related to ZFN technology.

Q You have been working in the field of gene editing for a number of years – can you briefly explain how you got into this area and your particular expertise?

I received my PhD from Brown University where I studied how proteins bind to DNA and turn genes on and off; following this I conducted my postdoctoral research at the NIH doing more of the same. Then 15 years ago I was brought into Sangamo with the goal of trying to build next-generation approaches to dealing with the challenge of genetic disease. As I'm a basic scientist and not a physician, at that time my background was not in any particular genetic disease; however, I found it thrilling that a general solution to the problem of how to change the sequence of DNA with high efficiency and precision inside the nucleus of living human cells in fact emerged out of the basic investigations of protein–DNA interactions, mechanisms of DNA repair, processes that most people think are rather fundamental and haven't an immediate applied relevance.

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This is probably the second time in the history of biomedicine where something this basic has had such a translational impact; the first instance was the discovery of recombinant DNA which emerged from studies of bacterial defence and resulted in the development of recombinant insulin and monoclonal antibodies. Genome editing arose from the studies of protein–DNA interactions, mechanisms of double strand break repair, understanding how to deliver nucleic acids to cells, and yet here we are with the conceptual equivalent of Microsoft Word for the human genome.

Q And why is it that we are now starting to see so much excitement and interest in gene editing?

In many ways we've always wanted to do this haven't we? The notion of improving the human predicament by changing DNA is an old one and probably on some level predates the discovery of DNA in the late nineteenth century. We first showed that human genes can be rewritten with high precision and efficiency nearly a decade ago and at the time it wasn't clear to us whether we would be able to make it sufficiently broad and useful, in other words could we make various types of edits, not just correct genes as we did initially for the genetic mutation that gives rise to bubble boy disease – or severe combined immune deficiency (SCID)? Would we be able to knock genes out? What about more than one gene at a time? Would we be able to integrate genes into specific locations to allow their sustained function? Or edit the genes of model organisms important in biomedicine such as the rat or pig? Over the 6 years that followed our initial discovery of human genome editing, we and our collaborators in academia as well as other academic groups who have been using zinc finger nucleases (ZFNs) demonstrated conclusively that the answer to those questions is an affirmative yes.

Yet whilst it was evident that genome editing will change the way people approach experimentation both in basic research and translational settings, the main question that remained was: how do you make that initial double strand break in the DNA?

Then two discoveries were made essentially back to back that expanded the access of the average researcher to the tools required to make that initiating break. The first was our discovery of a second nuclease class called TAL effector nucleases (TALENs) that are assembled using more Lego-like principles than ZFNs, which are more sophisticated set of molecular scissors. Then of course most recently Emmanuelle Charpentier and Jennifer Doudna made their landmark discovery that Cas9 is an RNA-guided nuclease. The field immediately realized that the previous 7 or 8 years

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of toolbox building that we'd generated with ZFNs and then with TALENS, could be taken and deployed wholesale to the cause of genome editing with CRISPR/Cas9. All the tools existed but the path to initiating the break was the question and that's what Cas9 has made so easy for everyone.

An additional factor that has also impacted the advancement of gene editing is that gene sequencing has become cheap and very efficient. We have the means of editing genes but of course we need to know what to edit and to what form. That part of the puzzle is what facile sequencing has really enabled.

You can sequence different organisms to understand the basis of trait differences or sequence the DNA of a patient who is presenting with a particular condition. Before the emergence of gene editing, you would just stare at that sequence and feel helpless; but now with not one but three different gene editing platforms available it is easy to understand why people empowered with the ability to not just read DNA but change it, are doing so.

Q At Sangamo you work with Zinc Fingers – what benefits are unique to using this specific type of nuclease?

As you can imagine this is a topic I could talk about at considerable length. The challenge in using a nuclease for the treatment or prevention of disease is twofold: potency and specificity. Zinc fingers are the best studied and most sophisticated nuclease platform for which both the potency and specificity metrics meet the demands of deployment at clinical scale. As an example, in collaboration with Biogen we are advancing genome editing of human hematopoietic stem and progenitor cells (HSPCs) as a potential treatment for the β-hemoglobinopathies – sickle cell disease and β-thalassemia. We discovered that the human genome contains a specific region which if disabled by genome editing creates a disease-protective phenotype in the erythrocyte progeny of the edited HSPCs. Remarkably, as we've shown in this work recently published in *Nature Methods*, it is a highly specific process – you have to cut to within one base pair of a specific position in the human genome to create that desired protective effect. Now this highlights a unique benefit of the ZFN platform in that it allows the placement of that double-strand break to that level of precision.

The other challenge is of molecular specificity and we've focused on ensuring that the nucleases we build attain clinical-grade specificity with respect to genome-wide action. ZFNs are of course Mother Nature's own invention for engaging specific loci in the human genome – they co-evolved with the human genome to allow the potent and specific regulation of specific gene loci. In many ways we are borrowing her invention and developing ways to engineer the zinc fingers to attain maximum potency and specificity of action within the nucleus of a human cell.

It is this ability to cut very precisely where we need to cut and to do so while maintaining clinical-grade specificity of action within the nucleus that drives our reliance specifically on the ZFNs rather than the other nuclease platforms.

Q Sangamo is one of the leaders in moving gene editing from the preclinical into the clinical setting – with a couple of INDs accepted by the FDA for HIV and β-hemoglobinopathies – can you tell us about some of the key challenges in making this translation step?

12 years ago, following the first demonstration that we could engineer ZFNs to create a double-strand break, we very quickly realized that there are numerous downstream considerations that you must address before this can become clinically actionable. The first consideration is that of deploying this approach in the cell type or setting that is clinically relevant. With specific focus on our programs in HIV and β-hemoglobinopathies, the challenge was how to genome edit at clinical-scale potency and specificity in a whole patient dose of cells – millions and potentially billions of human T cells or HSPCs. The challenge of cell husbandry and safe and effective delivery of the nuclease was a critical issue for us to resolve, but I'm delighted to report that after a great deal of work we have charted a path to *ex vivo* genome editing of T cells and human HSPCs.

The second consideration is building a panel of assays to evaluate the preclinical safety of genome editing prior to the cells being transplanted into patients in our trial. Here we have benefited greatly from an essentially collaborative effort with the regulatory authorities – FDA and NIH – in building a comprehensive panel of assays. These enable us to assess the safety of our genome editing approach in a way that is appropriately balanced relative to the risk–benefit profile in the context of a particular disease indication.

In summary, deploying your nuclease to make the DNA break in the right cell type and then assessing the specificity and safety of that editing in a manner that is commensurate with what you are trying to achieve clinically has been a formidable challenge that I'm delighted to report we have overcome. We are advancing to the clinic an approach for *in vivo* genome editing and have just received unanimous approval for our clinical study protocol for hemophilia B and MPS I (Hurler Syndrome) from the NIH Recombinant DNA Advisory Committee (RAC). Once reviewed by the FDA, this trial will be the first *in vivo* genome editing for any nuclease platform.

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Q As a trailblazer in clinical gene editing, Sangamo has paved an unchartered path through the regulatory landscape – what are the key learnings from this experience with regulatory bodies?

Dialogue is essential. The specific branch of the FDA that we work with is CBER – the Center for Biologics Evaluation and Research – they fully understand that cell and gene therapies are experimental, that we're not developing a small molecule for a particular disease indication with an existing history of preclinical and clinical development. The FDA has an established path for the progressive discussion of both assaying the safety as well as the clinical issues for proposed clinical deployment.

Over the past decade we have benefited tremendously from being able to engage the FDA in reviewing our proposals, receiving very constructive feedback and addressing that feedback. I'm very proud to report that not only are we in clinical trials with autologous edited T cells but that we have an open IND for the editing of HSPCs in HIV. We are also on track for filing an IND for *in vivo* genome editing before the end of 2015 as well as an IND for β-thalassemia in the first half of next year. The reason I mention these timelines is that when I talk about a dialogue with the agency this isn't hypothetical – we are in discussions and regulatory dialogue with them all the time and they have been a great partner to work with. And the other agency is of course the NIH RAC which is staffed by people who have been working in the field of cell and gene therapy their entire lives and so they have been really constructive with their feedback as well.

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Q When moving to the clinical setting, what are the potential safety risks and how do you mitigate these?

The first thing to understand is that this is very much disease indication specific and I can give you some insight into how we approach safety in our *ex vivo* therapy for beta-hemoglobinopathies. We have built a comprehensive panel of assays that assess the safety of genome editing to both the genome and the "stemness" phenotype of human HSPCs. What's interesting is that having watched this field over the last decade, the technology just does not stand still. The world around us continues to develop new approaches to evaluate biological systems that really didn't exist

even 10 years ago. When I was a graduate student in the early 1990s at Brown I used to perform a procedure called Sanger sequencing and it took me a month to determine the DNA sequence of 1000 base pairs; yet today we have next generation sequencing where for a fraction of the cost and within 48 hours you can determine the DNA sequence of thousands of loci or sequence the entire genome in a population of cells.

Assays evolve and our ways of looking at the safety of gene editing has also evolved as the technology becomes more sophisticated. An important thing to understand is that when we assess the safety of what we are doing, we don't really stand still with respect to what our clinical-grade reagents are. The research-stage reagents that we utilize to obtain initial read outs of efficacy with ZFNs both *in vitro* and potentially in animal models, we can assess their safety rather rapidly and then, if necessary, essentially optimize the reagents further to attain maximum on-target and genome-wide specificity read outs.

Q Sangamo's lead product for HIV targets the CCR5-encoding gene – can you briefly explain the scientific rationale behind the selection of this target and your approach to disrupt this gene?

The age of genomics has brought us this remarkable discovery that natural selection has non-uniformly distributed disease-relevant alleles in humans. We are all familiar with lactase-persistent alleles that are more prevalent in parts of the world where people drink milk. And in the mid-90s here in the San Francisco Bay area, the remarkable observation was made that some people who have been exposed to HIV appear to remain overt disease symptom free. It was very rapidly determined by DNA sequencing that these individuals are 'natural mutants' – namely they are homozygous for the loss-of-function allele of a gene called *CCR5* which encodes the co-receptor for HIV entry into the cell.

Timothy Ray Brown at this point is probably one of the best known names in biomedicine – but he's not a scientist, he is in fact the famous Berlin patient who has been effectively cured of his HIV infection by allogeneic bone marrow transplant of HSPCs that are homozygous for this disease-protective allele of *CCR5*. Whilst this is fantastic for Timothy, this approach is just not scalable worldwide to HIV patients. We reasoned therefore, that based on this very strong epidemiological and public health data indicating that people homozygous for the knock out allele of *CCR5* are protected from infection by R5 tropic HIV. Furthermore, looking at Timothy Ray Browns' experience we posited that you could attempt to recreate this HIV-protective genotype in the cells of HIV-positive individuals in the hopes of essentially creating a compartment of the immune system that is protected from HIV infection.

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Q In collaboration with the University of Pennsylvania, you have treated over 70 HIV patients with this ZFN-mediated gene editing approach – can you share with us some of your clinical experiences and outcomes to date?

We are excited to report that the treatment is well tolerated to date and that we have evidence of an antiviral effect including subjects that have demonstrated control of viral load for an extended period while remaining off anti-retroviral therapy (ART). For example in our most recent cohort, we have shown that two out of our three subjects have sustained functional control of viral load in the absence of ART. We also have a cohort of immunologic non-responders that we were happy to see have demonstrated a decrease in the size of their HIV reservoir at 36 months. We were greatly encouraged by how well this has gone and now have a Phase 1 study, for which we have an open IND, for the same approach but this time in HSPCs and this study is being conducted at the City of Hope in Southern California. And you may ask: "why go from T-cells to stem cells?" The logic here is that we are attempting to protect additional compartments of the hematopoietic tree from HIV infection, for example macrophages and dendritic cells.

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Q How durable does this response appear to be? Will there be a need or option to re-dose patients?

Re-dosing is an option and we are evaluating the delivery of ZFNs to the T cells in the form of *in vitro* transcribed messenger RNA (mRNA) which absolutely gives us the option to re-dose. With respect to the durability of response, we've seen modified cells persisting in our subjects out over 4 years post-transplant – the longest time point studied to date. Granted, 4 years is not a lifetime, but we are greatly encouraged by the durability we've observed so far.

Q HIV is renowned for its ability to evade immune detection through its presence in latent reservoirs in the body – can gene editing impact these reservoirs and cure patients vs functionally cure?

Looking at the cohorts 1 through 3 in one of our earlier studies, for whom we have up to 36 months of follow-up data, one could argue that the answer to this question is yes. We've observed a mean 0.9 log decrease in HIV reservoir in nine out of nine subjects and in some individuals this decrease is actually substantially greater. We are excited to observe an increase in genome editing in the $CD4^+$ central stem cell memory compartment and whilst I'm not an immunologist, my qualified immunologist colleagues assure me that this compartment of the immune system lasts the lifetime of a human. Therefore, being able to modify those cells and potentially protect them from HIV infection gives us great hope that we are in fact creating a lifetime effect.

Q Whilst HIV is a global healthcare issue, large patient populations are found within developing nations – do you think therefore that it's possible that gene editing technologies will one day replace existing ART which is currently a cheaper and comparatively easy to deliver to patients?

This as you can imagine is a topic that we think about a lot at Sangamo and the issue of cost is nuanced. ART really changed the prognosis of patients with HIV but it is a lifetime treatment and patients must take multiple pills daily. When I was in high school I was a huge fan of Queen and it was such a tragedy when Freddie Mercury succumbed to the disease; and it's fantastic – as a basketball fan – to see Magic Johnson is alive and well. But we must contend with the fact that the life-time cost of ART is not unsubstantial. The big hope of course with genome editing is that one creates a functional cure with a one-time treatment or a potentially short regimen of re-dosing.

The other issue to consider regarding ART is that it's evident that there are certain patients who are immunologic non-responders, whose immune system never completely recovered from the initial assault from the virus. Helping these people is a real challenge and that's why we enrolled them in some of our cohorts. Ultimately ours is an experimental therapy still in clinical development, but as I mentioned, to date the treatment has been well tolerated and we have some people with viral control in the absence of ART.

In addition to cost, a lifetime regimen of ART is clearly associated with side effects that in many patients, create serious non-compliance issues, namely they have to make a choice of whether to take the medication or not. Our hope is that this will be a non-issue with a genome editing approach because a human being once edited will not need to comply with a therapy anymore as the therapy will have been complete.

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Q Sangamo is also applying ZFN gene editing to the hemoglobinopathies – can you tell us about the selected target and how it was identified?

As I sometimes point out to my colleagues – I love being second. My colleague Michael Holmes, PhD, at Sangamo was the scientific leader responsible for taking our first genome editing approach to the clinic. As mentioned, the approach to editing *CCR5* in HIV patients was based upon epidemiologic and public health data that showed that there is a naturally occurring disease-protective genetic variation – namely the knockout mutation of *CCR5*.

In reference to my point of going second, I am the scientific leader in our collaboration with Biogen where we are looking to deploy the same fundamental principle – which is to rely on naturally occurring disease-protective genetic variation – to the hemoglobinopathies, the most prevalent genetic diseases globally. In Thailand alone there are 300,000 people with β-thalassemia; 100,000 people with sickle cell disease in the USA and that many neonates born annually with sickle cell disease in Nigeria alone. Therefore they truly represent a substantial public health burden.

One of the remarkable things about these diseases is the large variability in clinical presentation of the disease: some individuals are relatively disease free whilst others are severely ill despite having the same underlying genetic mutation. This led people to study this disparity and 4 years ago studies started to emerge that this is in fact due to the protective effects of mutations at other loci in the genome. These individuals are not single but double mutants; they have the disease-causing mutation and then they have a disease-protective mutation in addition. Researchers started to look at where that disease-protective variation lies and were greatly surprised to find it in the gene *bcl11a.* Now the name of this gene is actually a misnomer – it should really be called multi-functionally important human gene that happens to be the key regulator of human fetal globin! *In utero* or immediately post birth we produce a different β-hemoglobin to that which we make as adults. This hemoglobin is call fetal hemoglobin, which is quickly shut off and its synthesis in our erythrocytes is replaced by adult hemoglobin. In individuals with sickle cell disease or β-thalassemia, they are in this unfortunate position whereby Mother Nature doesn't realize that their adult hemoglobin is the mutant form and thus when they switch from a perfectly functioning fetal hemoglobin to mutant adult hemoglobin they develop the disease. That is unless they have this second mutation in *bcl11a* in which case the switch in hemoglobin production is incomplete and they synthesize sufficient levels of fetal hemoglobin throughout life which protects them from the fact that their adult hemoglobin is mutant.

Greatly encouraged by the fact that there are people with much milder or essentially no disease symptoms if they have this protective

mutation, our strategy is to recreate this disease-protective genetic variation in HSPCs of people with β-thalassemia and subsequently with sickle cell disease. I hope that that we will create a one-time approach that will be broadly applicable against both of these hemoglobinopathies where we take HSPCs from a person with either disease, genetically engineer them in a way that selectively eliminates expression of the bcl11a from the erythropoietic tree, transplant the cells back into the subject and cross all fingers other than zinc in the hope that we get a sustained elevation of fetal hemoglobin. If we achieve that elevation then we know that will confer protection against the disease.

Q You are also working on *in vivo* applications for diseases such as hemophilia and lysosomal storage disorders – what are the main differences and challenges in applying gene editing *in vivo* versus *ex vivo*?

Deployment *in vitro* is carried out in a more controlled environment: you harvest the T or stem cells from a subject and they are either in a bag or a cuvette in front of the operator at all times. Therefore you build non-clinical safety assays and efficacy assays that are focused on the fact that you are working with cells that are in front of you. With *in vivo* genome editing you develop your genome editing tool, deliver it to the subject but then Mother Nature takes her course so the challenge there, that we believe we have successfully met, is to build a comprehensive panel of preclinical safety and efficacy assays that adequately assess the potency and specificity of that approach.

One of the things that I find remarkable and most translationally exciting about the *in vivo* genome editing approach – and I must credit my colleagues Edward Rebar, PhD, and Michael Holmes, PhD, who are leading the development of our *in vivo* editing – is that we are potentially able to treat a range of monogenic disorders by the targeted editing of just one locus. In essence, we believe we are building an *in vivo* protein replacement platform and the strategy here was to identify a human gene that is expressed to a very high level in the human liver but is also non-essential so that its loss of expression causes no ill effects. And one such gene is albumin. The data we have generated thus far support the hypothesis that for monogenic diseases such as lysosomal storage disorders or hemophilia B, we will be able to replace the human albumin gene in the liver of an affected

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individual with the open reading frame of the gene which is disabled by mutation. Following genome editing with ZFNs, the hepatocyte, which is our natural 'engine' for the secretion of protein into the bloodstream, no longer secretes albumin but now faithfully secretes the protein we have just integrated into it. I find this truly thrilling – this notion that we have for lack of a more elegant term, a 'plug and play' locus on the human genome that we are hopeful we can develop as a broad approach for the treatment of monogenic diseases that are addressable in this way.

Q Sangamo is collaborating with Biogen and Shire on the development of couple of clinical products – what are the key considerations when looking to collaborate with industry partners?

In many ways the proverbial saying: "it takes a village to raise a child" is highly relevant here. We've discussed the tremendous efforts in academia upon which we have relied in building this gene editing platform. As we move toward the clinic, for the specific indications that we are targeting which affect a large number of people worldwide, being able to collaborate with a company like Biogen or Shire is wonderful because they bring the extraordinary might that big biotech and big pharma possess. Obviously it's good to be able to work with someone who is also focused on the same therapeutic areas that we are targeting, but more than that - they need to be excited about the fact that this is not a small molecule or a biologic. We are building genome editing as a therapeutic modality and we are hopeful to look for synergy – which is certainly the case with Biogen and Shire. We are the genome editing people, we live and breathe zinc finger nucleases, so it's incredibly important to be able to partner with organizations that have their own expertise that is relevant to the disease indication we are pursuing.

Q How do you envisage the field evolving over the next 5 years – what progress do you hope to see?

I am very much a glass half full kind of person – I am thrilled about the progress that has been made so far. Over the next 5 years we will have data, not only from the more advanced stages of clinical development of our genome editing in T cells in HIV but also of this approach in our trial of *CCR5* editing in HSPCs. Following on from this will be the application of genome editing of HSPCs for β-thalassemia or sickle cell diseases. Data from deploying editing in HSPCs for these three indications will really teach the field more broadly about what genome editing *ex vivo* can do to address the challenge of infectious and monogenic diseases of the blood. Should

the data look good, which I'm incredibly optimistic that they will, then this will fundamentally change the way we think about dealing with those diseases clinically.

Following closely on from this is our approach of genome editing *in vivo* and our initial approach to rewriting the gene expression programme of the hepatocyte to become an *in vivo* protein synthesis machine. Once again, if we are able to demonstrate that the liver genome was rewritable safely and effectively allows an improvement in the predicament of patients with hemophilia or lysosomal storage diseases, again I think the field as a whole will start to look very differently at how we approach the clinical management of these conditions.

Last but not least, if you had told me when I was a graduate student that 20 years from now you will be able to engineer molecular scissors that will be able to within base pair precision disable a locus in the human genome in a clinical-scale dose of HSPCs that would then retain every metric of viability and functionality to allow an autologous transplant for the treatment of HIV or beta thalassemia, I don't know how I would have reacted, but that would have sounded incredibly futuristic and yet here we are. The lesson from this and from other developments of technologies, such as deep sequencing, is that we should not underestimate the progress of technology. Engineering of nucleases, of cell husbandry, cell processing and of course delivery modalities is advancing at a pace that is just breath taking. Therefore good and accurate prognoses for the next 5 years are hard to provide because who knows what is currently being invented. My take on this is really formulated by Alan Kay who was one of the pioneers of computer science: "the best way to predict the future is to invent it". I am very much a believer in that paradigm – we are currently inventing what the next 5 years are going to look like. Exciting they will be, that's for sure.

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