

# Somatic Engineering of Oncogenic Chromosomal Rearrangements: A Perspective

Danilo Maddalo and Andrea Ventura

## Abstract

The ability to engineer specific mutations in mice has proven essential to advancing our understanding of the molecular basis of cancer. Chromosomal rearrangements, a common and clinically relevant class of cancer-causing mutations, have however remained difficult to faithfully recapitulate *in vivo*. The development of genetic tools for *in vivo* somatic genome editing has recently

overcome this limitation and led to the generation of more sophisticated and accurate preclinical models of human cancers. Here, we review the potential applications of these new technologies to the study of tumor biology and discuss their advantages over more conventional strategies, their limitations, and the remaining challenges. *Cancer Res*; 76(17); 4918–23. ©2016 AACR.

## Introduction

More than a century ago, Theodore Boveri suggested that chromosomal abnormalities were at the core of cancer development (see ref. 1 for an English translation of his original work). This idea, initially met with skepticism, was finally proven correct nearly 50 years later with the identification of the "Philadelphia Chromosome" (2), a reciprocal translocation involving chromosomes 9 and 22 (3) that leads to the formation of an oncogenic gene fusion between *BCR* and *ABL1* (4, 5). Initially considered a characteristic of hematologic malignancies, recurrent chromosomal rearrangements are now recognized as a common cause of many tumors. In fact, the number of newly identified gene fusions has rapidly increased due to widespread application of high-throughput sequencing methods (6, 7).

The event that initiates chromosomal rearrangements is the concomitant occurrence of double-strand DNA breaks in two distinct regions of the genome followed by canonical non-homologous end joining (NHEJ) of the two breakpoints (8, 9). The breaks can be caused by DNA-damaging agents, during cell division, or enzymatically, for example, by crossing over between repetitive sequences or during V(D)J recombination. On the basis of the relative position of the breaks, and on how they are repaired, rearrangements can be classified as interchromosomal (translocations) or intrachromosomal (large deletions, inversions, and duplications).

Oncogenic chromosomal rearrangements can promote tumorigenesis by leading to supraphysiologic expression levels of a cellular oncogene, for example, by bringing it under the control of a powerful promoter or enhancer, or by removing sequences

important for its transcriptional or posttranscriptional repression (10). A classic example of the first mechanism is the t(8;14) (q24;q32) rearrangements that is commonly observed in Burkitt lymphomas. As a consequence of this translocation, the *MYC* oncogene is brought under the control of the immunoglobulin-heavy chain locus (11, 12) and becomes expressed at supraphysiologic levels in the B-cell lineage. In other cases, best exemplified by the Philadelphia chromosome discussed earlier, the rearrangement leads to the in-frame fusion of two distinct genes, with the resulting fusion oncoprotein acquiring novel functions or signaling constitutively (6). The clinical relevance of the gene fusions is now widely recognized because they are often excellent therapeutic targets. Indeed, the first targeted anticancer drug to be approved for clinical use was the *BCR-ABL* inhibitor imatinib, and it has since radically changed the prognosis of CML patients (13–15).

## Available Strategies to Model Chromosomal Rearrangements

*In vivo* models of human cancers have proven indispensable to dissect the molecular mechanisms of cancer initiation and progression and to test and develop novel anticancer therapeutics. It is, therefore, not surprising that a variety of strategies to express oncogenic gene fusions in mice have been devised. These can be grouped in three broad categories: transgenic, knock-in, and Cre-lox-based methods. Each has specific advantages and limitations.

### Transgenic methods

This is by far the simplest and therefore the most widely used approach to rapidly characterize the oncogenic potential of a gene fusion. It consists of generating transgenic mice in which the fusion oncoprotein is expressed under the control of an exogenous promoter. The first example was a model of B-cell lymphoma induced by the translocation between the *MYC* gene and the IgH enhancer (16). A variation of this approach that can be easily applied to model hematologic malignancies involves using retroviral vectors encoding the gene fusion to transduce *ex vivo* hematopoietic stem cells that are then used to reconstitute sublethally irradiated mice (for example, ref. 17).

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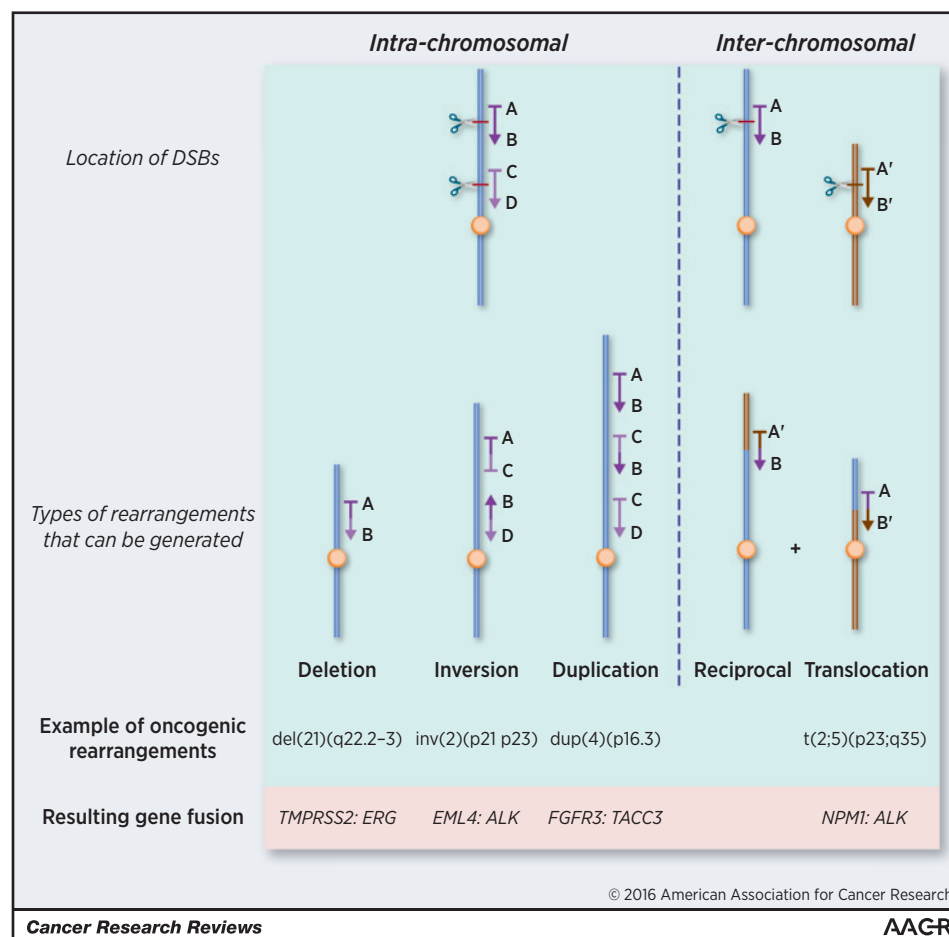
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**Figure 1.**

Modeling oncogenic chromosomal rearrangements by genome editing. Schematic representation of the possible chromosomal rearrangements generated upon introduction of two concomitant double-stranded breaks. Only stable rearrangements are shown for simplicity, but dicentric and acentric chromosomes will also be generated in the process. For each type of rearrangement, a representative example of a corresponding oncogenic gene fusion found in human cancers is also shown.



Although ectopic transgene expression is certainly useful to study oncogenic fusions, caution must be used in interpreting the results of these experiments. Although this approach leads to expression of the gene fusion, the endogenous genes involved in the rearrangements are not modified. Thus, this strategy does not model the reduced dosage of the wild-type genes that accompany expression of the gene fusion in human tumors. Such reduced dosage might contribute to the oncogenic potential of the rearrangements. Classic examples are the translocations resulting in the *NPM1-ALK* and the *PML-RAR $\alpha$*  gene fusions, because both *NPM1* and *PML* have been shown to be tumor suppressors (reviewed in refs. 18 and 19).

Many rearrangements, including inversions and translocations, often result in the generation and expression of reciprocal gene fusions (i.e., gene fusion A::B and its reciprocal, B::A). Although in many cases it is clear which gene fusion possesses oncogenic potential, there are examples in which both gene products contribute to tumor formation (20 and 21). Testing this possibility using conventional transgenic approaches is challenging and requires the generation and interbreeding of two separate transgenic strains (21).

The last major limitation of conventional transgenic approach is that the fusion oncoprotein is generally expressed ubiquitously and at nonphysiologic levels. This can alter its ability to induce cellular transformation (22, 23) and can be incompatible with

postnatal survival, thus preventing the generation of genetically engineered animals (24).

#### Knock-in strategies

Knock-in-based strategies in which the gene fusion is inserted via homologous recombination into the locus of its 5' component provide an effective and elegant alternative, as the fusion oncoprotein remains under the control of the endogenous promoter, and is therefore more likely to be expressed at physiologic levels (25). Initially applied to model the *MLL-AF9* translocation frequently observed in acute myelogenous leukemia (25), this strategy has been used over the past 20 years to successfully generate mouse models of hematologic and solid tumors (for example, refs. 26, 27). An important additional benefit of this approach is the potential to generate conditional, Cre-inducible, knock-in alleles, for example, by placing a transcriptional stop cassette flanked by loxP sites just downstream of the promoter. By restricting expression of the gene fusion to a specific tissue, or to a subset of cells using an appropriate Cre-expressing strain, this strategy can be used to overcome embryonic lethality and define the nature of the cell of origin for the particular tumor type under investigation (28).

Despite these advantages, however, knock-in-based strategies incompletely recapitulate chromosomal rearrangements because they do not model the reciprocal gene-fusion and leave the

endogenous locus of the gene at the 3' of the fusion intact. Furthermore, generating the knock-in strain is expensive, time-consuming, and technically complex, and therefore does not lend itself well to the rapid functional validation of newly discovered putative oncogenic rearrangements.

#### Cre-lox-based chromosomal engineering

The latter limitation is even more relevant with respect to Cre-lox-based methods to model chromosomal rearrangements. With these approaches, loxP sites are inserted at the desired chromosomal breakpoints via sequential gene targeting in mouse embryonic stem cells and the engineered cells are then used to generate mice. Upon expression of the Cre-recombinase (generally from a tissue-specific promoter), the loxP sites recombine, leading to the desired rearrangements (ref. 29; see ref. 30 for an excellent review). Depending on the experimental design, and on the relative orientation of the loxP sites, using this approach it is possible to model large deletions, duplications, inversions, and even reciprocal chromosomal translocations (31). Using this strategy, the *AML1-ETO*, *MLL-AF9*, and *MLL-ENL* translocations have been successfully modeled in mice (32–35), although only the latter has been shown to be sufficient to induce leukemia in the engineered animals.

### Use of Site-Specific Endonucleases and the CRISPR-Cas9 Revolution

Despite initial successes using Cre-lox-based approaches, the technical complexity of these methods has prevented their widespread use and recently alternative and simpler approaches based on the use of site-specific endonucleases have been proposed. Using initially rare-cutting endonucleases, and later Zinc-finger endonucleases, TALENs, and CRISPR/Cas, it was shown that introducing simultaneous cuts at the desired breakpoints it possible to induce specific intra- and interchromosomal rearrangements in mammalian cells (36, 37).

The CRISPR/Cas system, in particular, is ideally suited for *in vivo* somatic genome editing because of its simplicity and efficiency. Initially discovered as an adaptive immune defense system against invading nucleic acids in bacteria, the system has been engineered for use in mammalian cells (reviewed in refs. 38–40). With this technology, a single endonuclease (Cas9) and a short RNA molecule providing substrate specificity (the guide RNA or gRNA) are sufficient to induce site-specific double-strand DNA breaks. Because small indels are often introduced at the cut site by the endogenous DNA repair machinery, the CRISPR/Cas technology can be used to efficiently generate inactivating mutations (41). Of great interest for cancer research, several groups have shown that *in vivo* delivery of Cas9 and gRNAs to adult mice using naked DNA or viral vectors can be used to inactivate tumor-suppressor genes and induce tumor formation (42–46).

Building upon these initial results, our group has recently shown that simultaneous delivery of Cas9 and an appropriately designed pair of gRNAs can induce chromosomal rearrangements in adult mice (Fig. 1). As proof of concept, we chose a chromosomal inversion involving the short arm of human chromosome 2 and resulting in the generation of a gene fusion involving *EML4* and *ALK*. The *EML4-ALK* rearrangement is observed in a subset of human non-small cell lung cancers

and is clinically relevant because it confers sensitivity to ALK inhibitors (47, 48).

In a first series of experiments, we showed that intratracheal delivery of a recombinant adenovirus designed to express the Cas9 and two guide RNAs targeting the desired break points on *Eml4* and *Alk* (that in mouse are located on chromosome 2) is sufficient to induce the *Eml4-Alk* rearrangement in the lung of adult wild-type mice and, importantly, leads to the formation of lung adenocarcinomas with 4–8 weeks after infection. These tumors not only histologically resemble the human counterparts, but invariably harbor the *Eml4-Alk* rearrangement and respond dramatically to the ALK-inhibitor crizotinib (49). Similar results were reported shortly thereafter by another group who used recombinant lentiviruses to induce the same rearrangement (50). More recently, lentiviral delivery of the Cas9 and the pair of gRNAs in mouse myoblasts has been successfully used to generate a mouse model of rhabdomyosarcoma driven by the chromosomal translocation *Pax3-Fox1* (51).

#### Advantages and limitations of *in vivo* somatic genome editing

This CRISPR-based approach to somatically model chromosomal rearrangements offers obvious advantages over previously available methods. Perhaps the most important is that new mouse models of human cancer can be generated rapidly, within a couple of months, and at a fraction of the cost. An added benefit, of virus-mediated *in vivo* somatic genome editing to model chromosomal rearrangements is that by modulating the timing of infection and the viral titer used, the investigator can easily control tumor initiation and tumor load to more closely model the stochastic nature and the natural evolution of human cancers. In principle, this should streamline the process of determining the functional relevance of newly discovered rearrangements and provide an efficient way to generate preclinical models for drug testing and development. In addition, this strategy can in principle be adapted for use in organisms in which conventional gene-targeting methods are not feasible, resulting in even more accurate cancer models.

There are, however, significant limitations, some of them unique to CRISPR-Cas-based somatic genome editing, that need to be considered when planning these studies. Even though many rearrangements have been modeled in cells with CRISPR-Cas9 (52, 53), and a few of them have been reproduced *in vivo*, it is still unclear how easily *in vivo* somatic genome editing can be applied to model the hundreds of other recurrent chromosomal rearrangements. This is particularly true of chromosomal translocations, whose efficiency is likely to be dependent on the physical proximity of the two breakpoints. And even if somatic genome editing ends up meeting our most optimistic expectations, a significant fraction of chromosomal rearrangements found in human cancers will still be challenging to model in mice due to incompatible orientation of the murine orthologs of the human genes. For example, the t(15;17) translocation leading to the formation of the *PML-RARα* gene fusion that is characteristic of human promyelocytic leukemia cannot be modeled in mice because *Pml* and *Rar-α* have opposite orientations such that the fusion rearrangement would result in a dicentric chromosome that would eventually be lost. In principle, this limitation could be overcome by first inverting one of the two loci, but of course

such an approach, although feasible in principle, presents significant challenges (54).

Another consideration is that the feasibility of somatic genome editing strictly depends on the availability of effective means to deliver the guide RNAs and the Cas protein to the tissue and cell of interest. Hydrodynamic injection of naked DNA in the tail vein has been successfully used to edit the murine liver (43), but for other organs viral transduction methods are needed. Recombinant adenoviruses are ideal because they have a wide tropism, can carry a payload large enough to accommodate both Cas9 and the guide RNAs, and do not integrate into the host genome. Adenovirus-mediated somatic genome editing has already been successfully demonstrated in several tissues, including lung, liver, and brain (refs. 49, 55, 56; and our unpublished results).

Retroviruses and lentiviruses can also be used to deliver the CRISPR machinery directly to adult animals (42, 50, 57) or to model hematologic malignancy by transducing, *ex vivo*, hematopoietic stem cells that can then be transplanted into host mice. However, two limitations need to be carefully considered when using these vectors to induce chromosomal rearrangements. First, including two guide RNAs and Cas9 in a single retroviral vector is challenging due to the large size of the insert that results in reduced viral titer (Wu and Ventura; unpublished data). Another important consideration is that repetitive sequences in retroviral and lentiviral vectors have a propensity to recombine. It is, therefore, important that the expression of the two guide RNAs is driven by sufficiently distinct promoters (58).

Appealing alternatives are also being developed. For example, although recombinant adeno-associated viruses cannot accept inserts large enough to contain the entire Cas9 protein, they have been successfully engineered to express gRNAs. When used to infect transgenic animals expressing the Cas enzyme in the cell of interest, AAV can be highly effective at inducing somatic genome editing (45).

Novel nonviral delivery methods will likely be developed in the near future to allow for the efficient editing of organs that are not easily targeted by currently available tools. An interesting possibility is to deliver the gRNAs to Cas-expressing mice by co-opting methods used to deliver miRNA agonists and antagonists, by using *in vivo* electroporation (44), or by expressing the entire CRISPR-system (Cas and guide RNAs) from inducible transgenes, as recently demonstrated by the Lowe group (59).

#### Undesired effects of CRISPR-based somatic genome editing

In interpreting the results of genome-engineering studies using CRISPR/Cas9 it is also important to keep in mind that concomitant expression of two guides will induce not only the desired rearrangement, but also undesired and possibly confounding rearrangements. For example, guide RNAs designed to produce a chromosomal inversion will inevitably generate deletions and duplication of the region of interest in a subset of cells. In addition, off-target cuts can also lead to other

rearrangements or affect other genes involved in cancer formation, thus complicating the analysis of these studies.

Finally, the efficiency of editing, the ease in the design of highly specific gRNAs, and the possibility of coupling the CRISPR/Cas technology with viral delivery systems raise biosafety concerns that cannot and should not be ignored (60). Various strategies to minimize risks for the investigators should be employed. When working with model organisms, for example, care should be taken in designing guides that are not predicted to cut in the human genome. Although this is generally easy to do when the guides are designed to cleave poorly conserved regions such as introns, it can be much more difficult, if not impossible, when the target site is in a highly conserved coding region. Because Cas enzymes can only induce cleavage in the presence of a gRNA, a useful and safe alternative consists in delivering the two components separately or in taking advantage of genetically engineered organisms already expressing the Cas protein (45, 57, 59).

#### The Future

The availability of new and powerful tools for somatic genome editing is rapidly and irreversibly changing the way we study cancer in model organisms. The ease with which specific genetic changes can be induced directly in somatic cells not only drastically cut the cost and the time required to engineer new mouse models of human cancers, but also offers unprecedented opportunities to accurately recapitulate cancer's genetic complexity and multistep nature in a physiologic context. Combined with the large numbers of constitutive, inducible, and conditional knock-out and knock-in strains already available, somatic genome editing will allow the scientific community to model the role of the order of mutations (61), the evolution of metastatic clones, and the emergence of drug resistance with unprecedented accuracy. It is reasonable to predict that this will translate into a better understanding on the biology of human cancer and into more effective treatments.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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