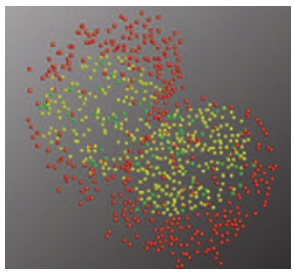
GENE EXPRESSION

Timing an intron's departure

An *in vitro* RNA-labeling technique with single-molecule resolution offers a look into the kinetics and the location of splicing.



Schematic of two cells expressing a splicing reporter. Pre-mRNA is shown in yellow, intron in green and spliced mRNA in red. Image courtesy of S. Tyagi.

Often a method and its application have a relationship similar to that of a chicken and an egg. It is not always clear which came first. Sanjay Tyagi and his team at the Public Health Research Institute can attest to this. For several years they studied the kinetics of splicing and refined a method to do so along the way.

"People believe, and it is generally true," explains Tyagi, "that most splicing takes place co-transcriptionally…, but much of splicing is alternative splicing, and this cannot happen if you remove the intron as soon as it is made."

A typical gene has long introns, several kilobases in length, and comparatively small exons of hundreds of base pairs. During standard processing, the introns are removed as soon as they are synthesized, but if the gene has several isoforms, in some of them certain exons will be skipped. For this process of alternative splicing the removal of introns must at least be delayed until all exons and intervening introns in a region are transcribed. The question Tyagi and his team asked was whether RNA polymerization just slows down, to provide sufficient time for the alternative splicing to finish or whether splicing happens at a location removed from the gene.

To answer this question, one needs to follow a single transcript as it is being synthesized. Initially the researchers used molecular beacons (probes that become fluorescent upon binding their target) to target two engineered introns, array 1 and 2, each with dozens of copies of

STEM CELLS

OPTIMAL STOICHIOMETRY FOR PLURIPOTENCY

Different ratios of reprogramming factors affect the epigenetic state of cells.

Ever since Shinya Yamanaka and Kazutoshi Takahashi showed that mature cells could be brought back to the pluripotent state of early embryos, researchers have been trying to perfect the recipe that launches reprogramming. Now Rudolf Jaenisch and collaborators at the Whitehead Institute show that supplying the key ingredients in the right ratio makes a big difference for making mouse induced pluripotent stem (iPS) cells that behave like embryonic stem (ES) cells.

Both Jaenisch's lab and the lab of his former graduate student Konrad Hochedlinger had created highly controlled systems to produce iPS cells from a variety of tissues. This involved making "reprogramming mice", transgenic mouse strains whose cells carry inducible versions of genes for all four of the standard reprogramming factors (Oct4, Sox2, Klf4 and c-Myc). Both labs made the two mouse strains by placing the same four factors together on a single genetic construct inserted into the same spot on the mouse genome. When cells from the mice were cultured, the reprogramming genes could be activated through the addition of the same small molecule.

Even though the systems were similar, the iPS cells produced from the two mouse strains were different. In the tetraploid complementation assay, pluripotent cells are injected into a special embryo that cannot develop normally and then implanted in a surrogate mother. Usually, the embryos die before birth. However, embryonic stem cells and very high-quality iPS cells occasionally give rise to live-born, breathing mouse pups. Although iPS cells containing the Hochedlinger construct passed many tests of pluripotency, they did not produce "all-iPS" mice in this assay. iPS cells containing the Jaenisch construct generated "all-iPS" mice at rates comparable to ES cells.

Given how alike the reprogramming systems were, the observed distinctions were surprising, says Jaenisch. They also offered a unique opportunity. "Because both systems were so well controlled, you could really compare apples with apples," he says.

50-nucleotide repeats, in the coding region of *GFP*. In parallel they also probed the 3′ untranslated region to distinguish spliced RNA from its unspliced counterpart. With array 1 they always saw the expected co-transcriptional splicing, but pre-mRNA from array 2 was dispersed in the nucleus, pointing to post-transcriptional processing.

Molecular beacons can be used in live cells, but their signal is not strong enough to detect single copies of endogenous transcripts. This roadblock triggered the development of a method that yielded enough signal to allow the imaging of a single copy of any mRNA *in vitro* (Raj *et al.*, 2008). The researchers then spent three years applying this method—in which at least 50 short, singly labeled oligonucleotides bind adjacently to a transcript—to follow the kinetics of splicing of endogenous genes (Vargas *et al.*, 2011).

They tested two endogenous genes, known to be alternatively spliced, by comparing the location of probes against constitutively spliced regions to that of probes against introns in an alternatively spliced cassette. Their results allowed the conclusion that during alternative splicing transcription and splicing are uncoupled, with the pre-mRNA still containing the cassette dispersed in the nucleoplasm.

Despite the fact that they saw hundreds of pre-mRNA molecules in the nucleus, not a single one escaped to the cytoplasm. "For post-transcriptionally spliced RNA there must be additional checkpoints," says Tyagi, "to ensure that the preRNAs are not exported." What these are remains to be discovered.

This approach may well provide answers to many questions about mRNA processing, cell-to-cell variation and the prevalence of post-transcriptional splicing. The next advance the field now needs is a technique to follow isoforms of a single transcript in real time *in vivo*.

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RESEARCH PAPERS

Raj, A. *et al.* Imaging individual mRNA molecules using multiple singly labeled probes. *Nat. Methods* **5**, 877–879 (2008).

Vargas, D.Y. *et al.* Single-molecule imaging of transcriptionally coupled ad uncoupled splicing. *Cell* **147**, 1054–1065 (2011).

Though both constructs encoded the same genes, the genes were placed in a different order and joined by different sequences. Both constructs produced similar amounts of messenger RNA, but protein expression was very different. Compared to the construct designed by Hochedlinger's lab, the construct from Jaenisch's lab produced 5 to 15 times more Oct4 and Klf4 and about half as much Sox2 and c-Myc. When Jaenisch's team added extra vectors producing Oct4 and Klf4 to cells expressing the Hochedlinger construct, the resulting iPS cells could produce "all-iPS" mice.

In addition, Hochedlinger's lab had previously noted a striking epigenetic signature distinguishing their iPS cells from the embryonic stem (ES) cells used to generate their reprogramming mice. The Dlk1-Dio3 gene cluster, which is usually 'imprinted', reflecting paternally inherited epigenetic patterns, was aberrantly silenced in the iPS cells. This was not consistently the case with cells from the Jaenisch reprogramming mice; however, follow-up experiments showed that high expression of Oct4 and Klf4 along with low expression of Sox2 and c-Myc were more likely to produce iPS cells that maintained normal imprinting in the Dlk1-Dio3 locus. (In contrast to work from other scientists, Jaenisch's experiments indicated that silencing at this locus is not absolutely associated with reduced pluripotency.)

What is clear, says Jaenisch, is that the relative ratios of reprogramming factors are important and that the standard methods for creating iPS cells leave these ratios to chance. "The stoichiometry at the very beginning of reprogramming really affects the quality of the iPS cells," says Jaenisch. "If you generate iPS cells by viral transduction, you can't control for that. We need to be aware of that." In fact, says Jaenisch, findings such as these may indicate that at least some differences observed between iPS cells and ES cells are not inherent to the reprogramming process but are instead due to technical aspects of current reprogramming protocols.

Monya Baker

Carey, B.W. *et al.* Reprogramming factor stoichiometry influences the epigenetic state and biological properties of induced pluripotent stem cells. *Cell Stem Cell* **9**, 588–598 (2011).