Researchers at the Paracelsus Medical University in Salzburg, Austria, prepare extracellular vesicles.

Inside the stem-cell pharmaceutical factory

Vesicles secreted by stem cells might give clinicians a safer and simpler alternative to cell therapy, but researchers are still grappling with how best to prepare and study these tiny particles. **By Michael Eisenstein**

It all seemed so straightforward at first. Stem cells are renowned for their capacity to develop into a wide range of other cell types, and researchers have spent decades exploring the notion that adult stem cells could be transplanted to form healthy new tissue in diseased or damaged organs.

But by the early 2000s, it had become apparent that stem-cell biology was more complicated than initially believed. Michael Chopp, a neuroscientist at the Henry Ford Health System in Detroit, Michigan, was among the first to explore the potential for adult stem cells — most notably a subtype known as either mesenchymal stem or mesenchymal stromal cells (MSCs) — to mitigate the effects of spinal-cord injury, stroke and other neurological trauma. "We looked at what's really going on, and we knew that the cells were not actually replacing the tissue," says Chopp. Rather, he and others hypothesized, these cells were repairing tissue by means of secreted factors.

Today, the evidence points strongly to exosomes — a class of tiny membrane bubbles known more generally as extracellular vesicles, which routinely bud off from cells and carry within them a cornucopia of biomolecules including RNA, proteins and lipids. "We found very quickly that we can recapitulate what the MSCs do, with the vesicles that are derived from MSCs," says Mario Gimona, head of good manufacturing practice at the Paracelsus Medical University in Salzburg, Austria.

Accordingly, many erstwhile cell-therapy researchers have shifted gear to explore whether exosomes might deliver the same clinical benefits without the potential risks associated with infusions of living cells, such as immune rejection or tumour formation. The early data hint at the potential to mitigate cardiovascular, neurological and immunological disorders. But exosome researchers are

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also coming to terms with the limits of their knowledge about how and why these little blobs work.

A medicinal mixture

Exosomes were first described in the late 1980s, and researchers subsequently teased out their role as a means of communication between cells. But it was only in 2010 that Sai-Kiang Lim, a cell biologist at the A*STAR Institute of Molecular and Cell Biology in Singapore, homed in on exosomes as the enigmatic secreted factor underlying MSC-mediated tissue repair[1].

Initially, Lim was surprised. She had expected the causative factor to be a protein or small molecule, so the identification of these strange vesicles sent her scrambling back to the literature. "The exosomes discovered us, rather than us discovering exosomes," she says. But the finding made sense: exosomes tend to be laden with non-protein-coding RNA molecules that can strongly modulate gene expression. "Any given type of extracellular vesicle might contain more than 30,000 different species of noncoding RNAs," says Eduardo Marbán, a cardiologist at Cedars-Sinai Medical Center in Los Angeles, California. This payload — alongside the diverse proteins and other biomolecules also found in exosomes — make these tiny droplets a potent engine for regulating cell biology.

Marbán's group demonstrated in 2014 that blocking the release of exosomes by heart-derived stem cells eliminated the cells' therapeutic effects in injured mouse hearts[2]. At around the same time, exosomes made their clinical debut[3]. Dietrich Beelen, a transplant doctor at the University of Duisburg-Essen in Germany, was interested in using MSCs to treat a patient with severe graft-versus-host disease (GVHD). This condition arises when transplanted bone marrow triggers a damaging immune response against the host tissue that can ultimately lead to organ failure and death. Some studies had indicated that MSCs might quell this immune backlash, but Beelen was concerned about the uneven track record of these cells in the clinic. So he teamed up with colleague Bernd Giebel, a stem-cell biologist, to dose a patient with MSC-derived exosomes instead. The results were remarkable: the patient's inflammation subsided dramatically, and she achieved a greatly improved quality of life that persisted until she ultimately died from possible steroid-related complications. "She was stable for more than four months," says Giebel.

The treatment was a one-off, permitted on compassionate grounds. In subsequent years, exosomes have seldom been tested in the clinic. But the preclinical data consistently indicate the feasibility of using exosome-based treatments to manage not just GVHD but a host of disorders.

Ashok Shetty, who studies regenerative medicine at Texas A&M University in College Station, has shown that MSC-derived exosomes could mitigate the damage from prolonged epileptic seizures in rodents[4]. According to Shetty, when exosomes are delivered nasally they permeate the animals' entire forebrain within six hours. "We found that we could rescue cognitive function and also prevent abnormal neurogenesis in the brain," he says. And, as in GVHD, the exosomes also seemed capable of modulating inflammation.

In their investigations of stroke and traumatic brain injury, Chopp and colleagues have found that exosome treatments in animals spur regeneration and remodelling of neural tissue[5,6]. "You'll find this whole restorative tapestry occurring throughout the central nervous system," he says. "We can actually restore neurologic, motor and cognitive function." Similarly, Marbán's group has tested cardiac stem cells and their exosomes as a treatment for people with Duchenne muscular dystrophy, to prevent the heart damage that is a major cause of death for those with the disease. Not only did both the stem cell and exosome treatments protect heart function[7], but they also promoted muscle repair throughout the bodies of treated mice. "The skeletal muscles from the leg were working more forcefully in animals that received the treatments in the heart," says Marbán. "It seems entirely consistent with the idea that systemic effects of exosomes are responsible for the benefits from the stem cells."

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Exosomes might be able to deliver the therapeutic benefits of stem cells without the baggage that has impeded the latter's translation into the clinic. Exosomes cannot self-replicate or form tumours. In addition, they are small enough for filtration to produce sterile material for use in patients, and stable enough for long-term freezer storage. Current data also suggest that the vesicles are remarkably safe. "We can use 10 or 20 times the therapeutic dose without seeing an adverse reaction," says Lim, referring to the number of exosomes required to deliver a clinical benefit.

Despite the many therapeutic benefits that have now been attributed to exosomes derived from MSCs, the mechanisms behind these effects remain frustratingly opaque. Exosomes seem to influence multiple components of the immune system, but to understand how they do this, researchers must carefully comb through their molecular cargo holds. Much of the focus so far has been on microRNA — short strands of RNA that encode no proteins themselves, but can modulate the amount of protein produced by other genes. Bioinformatic analysis of the material found within exosomes can help to identify strands of microRNA that act on disease-relevant cellular pathways. For example, Marbán found that one particular microRNA, miR-181b, accounts for many of the therapeutic effects of exosomes derived from cardiac stem cells[8].

Some researchers are seeking to manipulate MSC-derived exosomes to carry not just naturally occurring microRNAs, but also synthetic RNA drugs. Raghu Kalluri, a cancer biologist at the University of Texas MD Anderson Cancer Center in Houston, has extensively studied the natural role of exosomes in driving and impeding the progression and spread of tumours. Now, he is repurposing these vesicles to deliver engineered RNA molecules that selectively shut off genes that drive cancer growth. "We had a mouse which had pancreatic tumours, and those tumours were accumulating high numbers of exosomes, so we asked: what can we deliver there?" he says. They opted for a therapeutic RNA molecule that inactivates a gene called *KRAS*, a well-known driver of pancreatic cancer[9]. "We found dramatic responses," says Kalluri. "The tumours were smaller, and the mice lived significantly longer." His team is now looking to apply a similar strategy to glioma — another hard-to-treat tumour.

Unclassified information

Some controversy surrounds the therapeutic contributions of microRNA relative to other biomolecules carried in exosomes. Marbán has found that RNA-depleting chemical treatments eliminate many of the therapeutic effects of his exosomes, but he notes that microRNAs represent just a portion of the poorly understood RNA molecules in these particles, and that non-microRNA species could have a yet-underappreciated beneficial role. "There's a lot of things in there we don't even know how to classify," he says. Lim, in particular, has called the microRNA-centric model of exosome function into question[10], on the basis of initial evidence that proteins might exhibit more potent biological activity in therapeutic exosomes than doesRNA. She carefully profiled the molecular inventories of various

MSC exosome preparations, and concluded that "there was just not enough RNA to see an effect — most microRNAs are present at only about one copy per exosome". Because exosomes also contain many enzymes that can greatly accelerate biological processes with just a few molecules, she thinks these could exert a more rapid and direct therapeutic effect than microRNA.

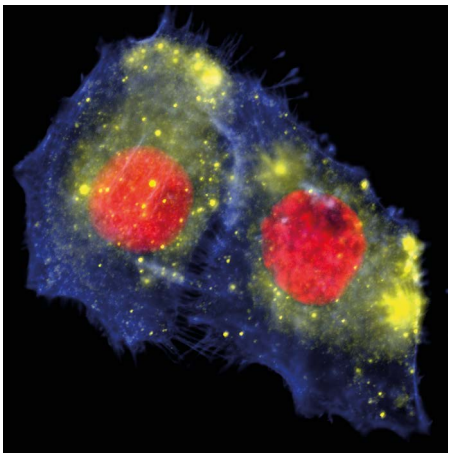
Giebel, too, is receptive to a more protein-oriented perspective. He points to several factors that could confound efforts to experimentally link an individual microRNA to an exosome's effects. For example, manipulations that knock out a microRNA gene in MSCs might also disrupt the function of those stem cells and perturb their exosome output to an extent that far surpasses just the loss of that single microRNA as a cargo molecule. But Giebel also hesitates to write off entirely the contributions of RNA relative to proteins and other molecules. "Very likely the truth is in the middle," he says.

Signal versus noise

Efforts to clarify these therapeutic mechanisms are further confounded by the considerable heterogeneity in exosome preparations. The term 'exosome' refers to a highly specific subset of extracellular vesicles, which are produced by a particular cellular pathway and exhibit diameters spanning roughly 30–150 nanometres. But this may be a misleading name for the preparations now being tested preclinically, which often contain a variety of non-exosomal vesicles. "Nobody should claim that they have achieved a 100% pure preparation," says Gimona.

Further variability between preparations can arise in a number of ways. Several studies have established that different types of stem cell — and mature cells, for that matter — produce cell-specific pools of vesicles with distinct contents. Some researchers are looking to exploit this therapeutically; for example, Shetty's lab has found evidence that vesicles from neural stem cells promote more-efficient neuronal repair than those from MSCs. But even different cultures of the same cell type may yield vesicles with different functional properties. "You can take the same MSC, raise it in different labs and it will behave differently," says Lim. These differences become yet more noticeable with MSCs from donors who differ in age, sex and other biological factors.

Organizations such as the International Society for Extracellular Vesicles are developing best practices for producing and characterizing exosome preparations for clinical research. The key objectives are ensuring that vesicle isolates are free from harmful contaminants and have a consistent set of functional properties. "If you want to treat a certain indication, you have to lay out how you think this would work," says Eva Rohde, a cell-therapy researcher at the Paracelsus Medical University. "We are looking for predictive assays." This can be complicated, given the myriad modes of action that vesicle preparations can exhibit; for example, Giebel notes that studies investigating exosomal treatments of GVHD would need to validate both their immunosuppressive activity and their capacity to promote repair in damaged tissues. But, by the same token, he thinks that clearing these hurdles should be sufficient to enable clinical testing even if the mechanism of action remains unclear. "If it has comparable activity to stem cells and is not harming the patient but reduces their symptoms, I'm good," says Giebel.



Exosomes (yellow) in cancer cells.

RAGHU KALLURI

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The processes required to produce uniform preparations of exosomes suitable for clinical testing are expensive. As a result, only a handful of academic centres are currently able to pursue human trials. Gimona and Rohde are working at their institution's clinical-grade manufacturing facility to optimize the medium- to large-scale production of trial-ready MSC exosomes. And Kalluri's team has garnered enough funding from MD Anderson and philanthropic groups to support the launch of a phase I clinical trial of exosome therapy for pancreatic cancer, which began accruing patients this March. But most clinical development is now occurring under the aegis of industry. For example, Capricor Therapeutics in Beverly Hills, California, is preparing to embark on a clinical trial based on Marbán's work with exosomes as a treatment for muscular dystrophy.

Unfortunately, disreputable commercial clinics are cashing in on the hype, peddling unproven "cures" for numerous conditions, based on exosome preparations of questionable provenance. "These exosome mills are sprouting up all over," says Chopp. Exosomes are much easier and cheaper to generate and handle than stem cells, and have fewer immediate safety concerns than cell therapies, he explains. And that makes them an appealing alternative for unscrupulous medical practitioners looking for an easy profit. And the steady trickle of exciting progress in preclinical research provides ready fodder for marketing. "I even get some of my papers cited in their brochures," says Lim. These unregulated clinics routinely make claims that go well beyond the available preclinical data, and sometimes border on the outlandish. Indeed, a New Jersey-based clinician and Kimera Labs, an exosome producer in Miramar, Florida, ran foul of the US Food and Drug Administration (FDA) in April 2020 for offering exosomes that they said could prevent or treat COVID-19 — claims that have since been struck from the company's website.

But, as with any therapy, a badly prepared batch of exosomes can pose an immediate threat. In December 2019, the FDA issued a public-safety warning about unregulated exosome clinics. The warning was based on reports of patients in Nebraska who developed sepsis after undergoing treatment. And although properly prepared exosomes have an excellent safety record, it will take a lot more testing, with careful clinical oversight, to identify any long-term risks. For example, Giebel notes that the same immune tolerance that keeps inflammation at bay and allows tissue to heal, could theoretically enable early-stage tumours to flourish and progress. "I think extracellular vesicles will be great therapeutic agents for many diseases," he says. "But if somebody is using them in a bad way, it could kill the field for many years."

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1. Chen, T. S. *Nucleic Acids Res.* **38**, 215–224 (2010).
2. Ibrahim, A. G.-E., Cheng, K. & Marbán, E. *Stem Cell Rep.* **2**, 606–619 (2014).
3. Kordelas, L. *et al. Leukemia* **28**, 970–973 (2014).
4. Kodali, M. *et al. Int. J. Mol. Sci.* **21**, 181 (2020).
5. Williams, A. M. *J. Neurotrauma* **36**, 54–60 (2019).
6. Xin, H. J. *Cerebr. Blood. F. Met.* **33**, 1711–1715 (2013).
7. Rogers, R. *et al. JCI Insight* **4**, e125754 (2019).
8. De Couto, G. *Circulation* **136**, 200–214 (2017).
9. Kamerkar, S. *et al. Nature* **546**, 498–503 (2017).
10. Toh, W. S., Lai, R. C., Zhang, B., & Lim, S. K. *Biochem. Soc. Trans.* **46**, 843–853 (2018).