By Nick Lane PhD

Stem cells in ophthalmology – dawn of a new era?

While biologists in the 20th century focused on studying DNA and genes, culminating in the mapping of the human genome, the 21st century will be the century of cells" says Douglas Melton, PhD, Co-Director of the Harvard Stem Cell Institute.

If he's right, then ophthalmologists will be hogging the limelight. The eye is leading the way in stem cell research, because of its relative ease of access, neurological differentiation, and wide gamut of degenerative diseases. Limbal stem cell transplants have played an important role in corneal surgery, and recent research into retinal degeneration seems poised on the brink of a clinical breakthrough.

EuroTimes looks into just how close to the clinic retinal stem cell transplants really are, and whether embryonic stem cells will ultimately be needed.

From the front of the eye to the back

Limbal stem cell transplants were pioneered as long ago as the 1970s, but few were done until the last decade. There is no questioning their efficacy. In cases of severe limbal stem-cell deficiency (LSCD), such as Stevens-Johnson syndrome and thermal or chemical trauma, corneal transplants otherwise destined to fail can be saved by a preceding limbal stem-cell transplant.

There have been some notable clinical breakthroughs, such as the harvesting of limbal stem cells from cadaver eyes or from living relatives, along with stem-cell expansion in cell culture, but the basic transplant protocol has changed little in a decade. Many of the problems faced today are not related to the transplant itself, but to the environment that the limbal cells are transplanted into: transplants may fail as a result of lid deformities, underlying inflammation or dry eye. Even so, some recent surprises relating to the stem cells themselves are relevant to other types of procedure.

For example, Sheraz Daya MD, and his colleagues at the Queen Victoria Hospital and the Blond McIndoe Centre in East Grinstead, UK, reported a curious finding earlier this year (Ophthalmology March 2005): nine months after transplanting limbal cells in patients with profound LSCD, they could find no trace of the donor DNA by polymerase chain reaction (PCR) genotyping.

Despite the absence of donor cells, the ocular surface was generally stable at 1 year. Histology of the cornea showed a thickened multilayer epithelium, and 70% of patients were classified as a success in terms of various parameters of LSCD (conjunctivalisation, inflammation, vascularisation, etc), with some 40% experiencing improved VA. But what had happened to the stem cells?

"We don't know what happened to them. We found a mosaic of donor and host cells in the first few months after transplant, but by nine months only the host cells remained. We believe the donor cells recruited host stem cells, perhaps circulating bone-marrow stem cells, to the cornea. The transplant seems to have had a positive trophic effect on the cornea, altering the balance of cytokines, inflammatory mediators and growth factors from favouring degeneration to regeneration. One big practical implication is that we could in principle terminate immunosuppression by nine months post-op," Dr Daya told *EuroTimes*.

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> ***Sheraz Daya MD***

Another surprising finding from corneal transplantation (NEJM, Sept 2004) also highlights the importance of the trophic environment in determining the fate of stem cells and the ultimate clinical outcome.

This work, from the Japanese group headed by Yosuo Tano MD PhD, at the University of Osaka Medical School, shows that when fabricated in a suitable ex vivo environment, autologous oral mucosal epithelial cells can be developed into a transparent sheet capable of inducing complete re-epithelialisation of corneal surfaces within a week for up to 14 months, with remarkable increases in post-operative best-corrected VA (in one case from 20/2000 to 20/25).

This finding suggests a previously unanticipated degree of adult stem-cell plasticity. Though the Japanese team did not prove the involvement of oral stem cells, the duration of the clinical effect beyond 14 months implies continuous corneal regeneration (as the lifespan of transient amplifying cells committed to epithelial differentiation is less than a year).

Such regeneration is presumably attributable either to presence of donor stem cells or the recruitment of host stem cells, as suggested by Daya and colleagues. Clearly stem cell transplants have the power to shift the trophic environment in favour of regeneration; and adult stem cells have somewhat more plasticity and regenerative power than previously thought.

These findings are of immediate relevance to the more challenging arena of stem-cell transplants in the retina, which are all too easily confounded by their inhibitory trophic environment. By altering the retinal trophic environment, say researchers, stem cells might signal regeneration even in severe or chronic degenerative conditions such as retinitis pigmentosa and AMD.

The gentle art of stem-cell persuasion

In cold-blooded vertebrates such as teleost fish and frogs, the retina retains a population of stem cells that are active throughout adult life, enabling the retina to regenerate itself continuously. Such powers of regeneration are greatly restricted in mammals, and until a few years ago retinal regeneration was considered a pipe dream.

Then in 2000, in a seminal Science paper, Derek van der Kooy PhD, and colleagues at the University of Toronto, demonstrated the existence of a small population of retinal

The politics of embryonic stem cells

Stem cells have extraordinary power to polarise opinion. While the US was the driving force behind the genetic research in the 20th century, its position in the 21st century is less clear-cut. In 2001, George W Bush restricted federal NIH funding of embryonic stem cell research to the few stem cell lines created prior to August 2001. While not prohibiting privately funded commercial research, there is widespread consensus that US research is falling behind that in the Far East and parts of Europe.

On November 2, 2004, the day Bush was re-elected, the State of California passed Proposition 71 to establish and fund a stem cell agency, on the basis of a state referendum approved by 59% of voters. The measure sought to rectify the shortfall in federal funding by establishing the California Institute of Regenerative Medicine, funded by bonds to the tune of $3 billion over 10 years. The hope was to encourage a 'stem-cell gold rush', attracting top researchers and investment, and reinvigorating the moribund Californian economy. California's Republican governor, Arnold Schwarzenegger, supported the proposition.

Perhaps predictably, given the strength of the religious 'pro-life' lobby in the US, and the litigative environment, the project is currently mired in the courts, unable to raise funds until the lawsuits are settled. "We don't want our state tax dollars to be used to kill embryos," Susan Armacost, legislative director and chief lobbyist for one pro-life group said.

Other states, including Wisconsin and Massachusetts have tried to emulate California but have been similarly mired in lawsuits. A defiant vote in Congress in May, to loosen the laws governing stem cell research, is supported in the Senate but is almost certain to fall foul of presidential veto.

Stephen Minger, PhD, Director of the Stem Cell Biology Laboratory at Kings College London, is an American with a global take on stem cell research. Is he attracted by the California gold rush? "

"NO. I'm not convinced that the environment in California, even if the funds do materialise, is comparable to the scientific, regulatory and social environment that currently exist within the UK. There's been far more mature debate here in the UK about the science of stem cells; I think the public is much more informed about the merits of various stem cell populations and the public is much less polarised than in the US. There's a strong ethical regulatory framework governing the type of research that can be done, and a much broader consensus in society. The major problem here is that the level of funding is relatively low compared to the US, but I am not convinced that we need 3 billion dollars at the moment. The government has put new and considerable funding into stem cell research of all flavours in the UK and there is a strong commitment within the government to support stem cell research on a sustainable basis. Time will tell if they are serious about this, but my guess is this a major priority within the government."

So who will take advantage? "There is some very good research going on in the Far East, in Japan, Korea and China, and they do have good regulatory frameworks, equivalent to the UK. In Europe, Sweden and Denmark are doing good work, but other countries like Germany have taken a similar line to the US and frozen research to a limited number of cell lines" said Dr Minger.

stem cells in the pigmented ciliary margin of the mouse retina. Van der Kooy et al showed that these cells could differentiate into a range of retinal-specific cell types, including rod cells, bipolar neurones and Müller glia.

In 2004, the Toronto team went a stage further, showing that the human retina, too, contains viable retinal stem cells in the ciliary margin, perhaps 10,000 cells per eye, which, like the mouse cells, are capable of differentiating in culture into several retinal cell types. These cells seem to lose none of their vigour over seven decades of life: they can be cultured just as speedily when isolated from 70-year old cadavers as from early postnatal donors.

"We have transplanted both mouse and human retinal stem cells into neonatal immune-deficient mice, and their properties are remarkably similar, suggesting that their traits are highly conserved. In contrast, neural stem cells behave differently in mice and humans – the human cells grow much slower," Professor van der Kooy told *EuroTimes*.

"We transfected the cultured stem cells with green fluorescent protein (GFP), and injected the green cells into the vitreous cavity of 1-day postnatal mice. This is a permissive environment, when the host developmental factors induce neuronal differentiation, so we're giving the stem cells the best shot at survival. Then after 28 days we used the fluorescence microscope to visualise whether the GFP cells had integrated into the retina. In about half the cases, the human cells had indeed integrated well into different layers of the retina to form morphologically normal-looking photoreceptors and ganglion cells, expressing appropriate markers, such as the rod photoreceptor protein Rom-1."

But while promising much for the future, van der Kooy's work also highlights the serious difficulties involved. Their 2004 PNAS paper ended on a note of cautious optimism, heralding the next step as a genetic mouse model of retinal degenerative disease, but the reality has been less rosy. The trouble is exactly the trophic environment: in van der Kooy's experience, transplanting retinal stem cells into a degenerating environment leads to the degeneration of the stem cells.

"It may be that the retina was too aggressively degenerative. We were using the rd1 mouse model of retinitis pigmentosa, and it's really quite aggressive: the retina degenerates within a matter of weeks. Other models, such as transducer knockout mice, in which the photoreceptors don't work, but don't degenerate, may be easier to tackle; or maybe we can alter the trophic environment in some way to promote survival," said Dr van der Kooy."

One possibility, reported a couple of months back by Anne Calof PhD and her colleagues at UC Irvine (Science, June 2005), is to manipulate growth and differentiation factors such as the inhibitory protein GDF11 (part of the TGF-beta superfamily), which controls the 'window of opportunity', in which retinal precursor cells develop into retinal ganglion cells, amacrine cells and photoreceptors. Inhibiting GDF11 later in life has the potential to 'reopen' the window and allow stem cells to survive and differentiate, rather than degenerate.

Another possibility relates to the type of stem cell used. Martin Friedlander, MD, PhD, and colleagues at the Scripps Research Institute, La Jolla, last year reported (Nature Medicine, Sept 2004) success in two mouse models of retinitis pigmentosa – including the aggressive rd1 model that defeated van der Kooy's group – using lineage-negative hematopoietic stem cells (Lin HSC). These cells differentiate into at least six cell types including endothelial cells, which associate with astrocytes in the retina, and seem to participate in injury-induced angiogenesis.

Friedlander and colleagues found that injecting mouse or human Lin HSC into the vitreous cavity of immunodeficient mice not only halted degeneration of retinal vessels, but also produced a dramatic neurotrophic rescue of cones.

"The effect can't be direct, because we're not protecting the degenerating rods in either the rd1 or rd10 models – we're not correcting the genetic deficit, but we are modulating the consequences. The mouse retina consists mainly of rods, but the cells rescued after treatment with Lin HSC are nearly all cones. The rescue effect lasts for at least 6 months in mice, which if scaled up relative to lifespan might be the equivalent of a decade in humans," Dr Friedlander told *EuroTimes*.

Dr Friedlander doesn't know if this outcome is accomplished by preventing vascular degeneration, which is usually taken to be a consequence rather than a cause of retinal degeneration, or whether the Lin HSC are differentiating into some other cell type that is producing neurotrophic factors. But by using micro-array analysis the Scripps team did find a significant up-regulation of anti-apoptotic factors, including small heat shock proteins and transcription factors. "It does seem that we are altering the trophic balance of the retina."

Again, however, a relatively narrow window of opportunity limits Dr Friedlander's work. The bone marrow cells are injected into mice up to 2 weeks post-natally, but so far the stem cells have failed when injected into adult mice. They don't interact with the host astrocytes in the same way, and it may be that an inhibitory trophic environment is again to blame.

Furthermore, because the Lin HSC don't differentiate into neurones or photoreceptors, the technique can't protect against conditions that affect cones directly, or rescue photoreceptors that have already degenerated. So the approach offers hope for patients with early onset degenerative conditions, such as some forms of retinitis pigmentosa, but is unlikely to help people with age-related conditions like AMD. Are there other ways?

The power of young cells

Michael J Young PhD, at the Schepen's Eye Research Institute, Harvard Medical School, might have some answers. He and his colleagues are working with neural stem cells isolated from the neural retina of one-day postnatal mice, and more recently pigs. These are not embryonic stem cells, but they are ontogenetically immature – and that might make all the difference, for Young's group are the first to slow down degeneration of mature retinal dystrophic eyes.

"We really want to keep focussed on the main clinical challenge, and that is adult degenerative conditions like AMD. It's no use preventing retinal degeneration in neonatal mice if we can't transfer that technology to adult humans. And it's no use working only with acute, traumatic models of injury, like laser injury, when what we're dealing with clinically is chronic, atraumatic retinal degeneration," Dr Young told *EuroTimes*.

"In our hands – we're working with experienced retinal surgeons, not just squirting stem cells into the vitreous – the only cells that have both the migratory ability and developmental plasticity to integrate successfully into the mature dystrophic retina are ontogenetically immature neural stem cells."

Neural stem cells can differentiate into neurones if resident in the brain stem, while retinal stem cells can become a range of retinal cell types including rods and cones, if resident in the immature neural retina. When taken from the eye of GFP transgenic mice and transplanted into 4 to 8 week old rho/ or rd1 mice (the mouse retina matures fully within about 3-weeks of birth), Young and colleagues found that the green stem cells integrated into degenerating retinas (but not healthy mature retinas), and differentiated into morphologically normal-looking cells, expressing markers of rods (in the rd mice), cones and retinal interneurones.

Like Friedlander and colleagues, Young and colleagues also noted a positive neurotrophic effect of transplanting stem cells to the retina. In this case, stem cell transplants rescued host rods and cones that degenerated in control animals.

Crucially, Young and colleagues have demonstrated functional as well as morphological outcomes, using a simple behavioural assay. Mice are photophobic, and exposure to light inhibits their normal nocturnal patterns of behaviour. By dimming the intensity of lights, Young et al showed that transplanted mice responded to light even at the lowest levels, whereas control mice did not.

Since publishing their work last year (Invest Ophth Vis Sci, Nov 2004), Young et al have scaled up to pigs, which are closer to humans in that their eyes are a similar size, while pigs do not have a true macula, they do possess an "area centralis" that is rich in cones. Working with ophthalmologists at the Panum Institute in Copenhagen, their early results in injured pig retinas are encouraging and suggest that the mouse work does transfer. Progress in pigs with genetic retinal degeneration has been slow for various reasons, most particularly the slow speed of retinal degeneration and enormous size of pigs.

"By the time the rd pigs have developed serious retinal degeneration they weigh 1000 lbs (450 kilos) and need to be moved with a crane! We're now trying to work with mini-pigs, which plateau at about 100 lbs. The preliminary data looks encouraging, we're getting similar results to the mouse model."

This is an encouraging start, but there are practical drawbacks to using immature neural stem cells in the clinic, in particular sourcing them from humans. Culturing stem cells from the immature neural retina of neonates, even if considered ethically defensible, would not produce enough material for clinical use. Instead, retinal stem cells would need to be cultured up from early embryos, to a similar ontogenetic stage; but

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> ***Michael J. Young PhD***

that too poses problems that go beyond ethics.

"There have been reports of generating retinal stem cells by co-culturing on stromal cells like RPE, but you get an incredibly small number," says Stephen Minger PhD, Director of the Stem Cell Biology Laboratory at Kings College, London.

"The trouble is we haven't identified early markers for retinal stem cells, so it's very tricky to fish out the retinal progenitors from the myriad other cell types that are produced during the differentiation process. Remember these cells are pluripotent and therefore have the innate capability of forming every cell type in the human body. Until we have reliable early markers, the reproducibility and yield will be very low. As a means of producing a stable clonal population, we'd ideally engineer the cells to express retinal transcription factors early in the differentiation process and generate multipotent retinal stem cells. Ultimately we'd want to bank stable lines with differing HLA haplotypes, to lower the risk of rejection.

So when will this happen?

"I'm fed up of saying 10 years, it sounds too negative! But it's unlikely to happen any time soon. The truth is we know how to transplant organs, but we don't really know how to transplant cells yet. And although we know quite a lot about mouse embryonic stem cells, most stem cell research labs have only had access to human embryonic stem cells for less than four years. We've a lot to learn, and this is just the beginning," said Dr Minger.