ABSTRACT

Stem cells are being used for treating diseases such as leukaemia and Parkinson’s disease and can also be used to restore islet cell function in diabetes. Currently researchers are investigating the possibility of regenerating tissues using stem cells. Recent developments being successful bronchus transplants and the generation of skin for use in skin grafts for severe burns victims.

In theory stem cells could be used to grow more complex organs and eventually limbs. This paper discusses some of the evidence which points to the conclusion that although re-growing limbs using stem cells is a possibility in the future, this is not a practical proposition at the moment.
Introduction

Stem cells have the ability to undergo mitosis without a Hayflick limit which prevents all other cells (except cancerous cells) from dividing more than 52 times. Theoretically, they are able to self-replicate continuously throughout an organism's lifetime. They are unspecialised and so have the potential to differentiate into various new cells.

Stem cells come in different forms and can be found in a variety of places. The most widely recognised and perhaps most controversial source of stem cells is the embryo. First studied in 1998, embryonic stem cells are derived from the inner cell mass of the blastocyst (a hollow ball of about 70-100 cells) of a human embryo 4-5 days after fertilisation. Embryonic stem cells are pluripotent: they can differentiate into all three of the primary germ layers (ectoderm, endoderm and mesoderm). All of the 200+ different cells in the adult human body come from these three layers. This indicates that embryonic stem cells can potentially differentiate into any adult human cell if stimulated in the right way.

Adult stem cells are found in many places including blood, bone marrow and skin. Unlike embryonic stem cells, they are much scarcer and are harder to culture. Furthermore, whilst embryonic stem cells are pluripotent, adult stem cells are multipotent meaning that each can only produce limited cell types. For example, stem cells extracted from the bone marrow of an adult can differentiate into any type of blood cell but not a brain cell. This limits their utility when culturing new tissues as one would have to start with stem cells that were capable of differentiating into the type of cells needed for that particular tissue. There is some evidence to suggest that it is possible to alter the potency of an adult stem cell. This would enable us to change a multipotent bone marrow stem cell into a pluripotent cell. This would give a source of cells that could be an alternative to embryonic stem cells and would enable us to side step some of the ethical issues surrounding the use of the former.

There is a technique for inducing pluripotency in multipotent somatic cells. This process is called ectopic transcription and involves artificially expressing a gene in an abnormal place in an organism. A transcription factor is a protein which binds to a specific sequence of DNA and controls the transcription of genetic information. This means that genes can be manipulated so that somatic cells will display pluripotent characteristics like in an embryonic stem cell. These new cells are known as induced pluripotent stem cells (iPS) and have arguably the same function as embryonic stem cells.

New sources of adult stem cells are being discovered continually, for example the discovery of stem cells in the retinal pigment epithelium of eyes. These cells have been extracted and can be activated to produce growing cultures and have formed other cell types.

Cord blood and placental tissue are also sources of stem cells. These have recently been attracting a lot of attention as potentially large sources of the sought after cells. Like adult stem cells, cord blood cells are thought to be multipotent. However there is evidence to suggest that cord blood may in fact have pluripotent characteristics enabling them to differentiate into heart,
liver, brain and bone cells. Unlike adult cells however, they do not have to be extracted from a living person. Another advantage is that the embryo is not destroyed when they are harvested.

Stem cells can also be collected during pregnancy from amniotic fluid without the ethical issues related to embryonic stem cells. These stem cells have been applied to kidneys in mouse models where they have a beneficial effect on the area of damage\(^5\).

The ethical problems with research into the area of stem cells mainly involve embryonic stem cells. The principle of autonomy suggests that every embryo has the right to life. Extracting stem cells from an embryo results in its destruction and this is a cause for concern among many, especially in religious circles. However, those in favour of the use of embryonic stem cells use a utilitarian argument: the greatest good for the greatest number. All embryos used in stem cell research have been created in-vitro and the couple, whose egg and sperm were used to create it, have donated it to research. The alternative is for it to be destroyed which, to many, is a worse solution. Using unwanted embryos for research means that although they will still be destroyed, some good may come out of the process and eventually benefit many other people.

At the end of last year it was announced that 'Xeno-free' embryonic stem cells (uncontaminated by animal products) had been produced\(^6\). Two cell lines have been released into the Stem Cell Bank. A similar line of research in Manchester may enable embryonic stem cells to be used to restore cartilage damage in the 'next few years'. They have shown that the stem cells could be turned into cartilage cells in only 12 days. These cells could be used to treat injuries where the cartilage has been damaged.

Stem cells can also reduce scarring in wounds. Men with Peyronie’s disease whose treatment included extracted stem cells healed faster and with less scarring\(^7\). This is thought to be due to an increased presence of fibroblasts: cells which have a critical role in wound healing. It is likely that when presented with the new wound site some the stem cells differentiated into these fibroblasts and so the final result was one with less scarring. Another study\(^8\) using genetically engineered mice (called MRL) found that holes that had been punched into their ears for identification healed themselves rather than scarring. The mice formed blastemata, groups of cells which are also present in embryos. These cells have embryonic-like potential meaning that the wound heals rather than scars. Further investigation showed that these MRL mice could also heal damage to their hearts.

Stem cells have been used to create a transplanted trachea for a British boy\(^9\). A donor trachea was stripped down by enzymes to leave only the collagen scaffold. Stem cells taken from his own bone marrow, which had been programmed to turn into the appropriate tissue, were injected into the scaffold. These then coated the surfaces of the trachea. The modified trachea was then implanted into his body. Unlike an earlier trachea transplant, where cells were incubated outside the body before transplant, his stem cells will grow in the 'bio-reactor' of his body. This use of stem cells significantly reduces the chance of an immune response which means that the patients do not have to take immunosuppressant drugs.

Endometrial stem cells from menstrual blood have prevented the withering of limbs with restricted blood flow (in mice). They help to revitalise damaged limbs in the same way that stem cells derived from the bone marrow do\(^10\). Further trials on mice suggest that endometrial stem
cells can help to stimulate blood-vessel growth. These findings could be very important in the eventual re-growth of limbs as a means of creating new blood vessels to supply the tissue.

Some animals appear to have stem cells that are able to perform much more complicated functions. Salamanders have the unusual and very desirable characteristic of being able to replicate limbs. A salamander's limbs are very similar to ours: made up of a 'bony skeleton, muscles, ligaments, tendons, nerves and blood vessels' encased in skin. When a salamander loses a limb it does not form a scab. Instead a wound epidermis forms across the site which transforms into signalling cells which are known as apical epithelial cells. During this time, fibroblasts from the surrounding connective tissue travel across the surface to meet at the centre. Here they form a blastema. This knob of stem cells is then able to completely regenerate the limb. These blastemata like the MRL mice, act as embryonic stem cells.

A team of scientists have managed to make salamanders grow extra arms by making a cut on the salamander which then formed a blastema. In order to ensure the blastema would produce a new limb, a skin from the opposite side of the wound had to be grafted onto the wound site. This allows the fibroblasts from the other side of the wound to be involved in the healing.

Where humans form scar tissue, salamanders produce stem cells enabling them to re-grow limbs. Newts are also able to regenerate in this way and scientists have found that humans and newts share the genes for limb re-growth. However in humans these genes become switched off after birth whereas an adult salamander retains the embryonic potential to build an entire limb from scratch.

**DISCUSSION**

The current situation after limb loss is that most patients are given artificial limbs. It is possible to have a robotic limb however these are clumsy. According to neuroscience specialist Professor Wolpert of the University of Cambridge, stem cells are a "very promising area" and they "will be a far better solution than using robotics in the long run."

Future developments in stem cell research might make replacing or re-growing damaged limbs possible. There seem to be three potential pathways for development:

1. **Preventing the withering of limbs** after damage to the blood supply e.g. in a road traffic accident. This would prevent loss of the limb and so eliminate the need for regrowth in many cases.

2. **Repairing a damaged limb by replacing separate organs**, grown using stem cells. E.g. bone, muscle, skin, blood vessels, nerves.

3. **Re-growing the whole limb** in a human as in a salamander

Each of these three pathways will be considered in turn.

**1) Preventing the withering of limbs**

Current research has shown that stem cells from human menstrual blood can stimulate blood vessel growth in mice. Changing the source of stem cells from embryonic to this new source could be beneficial because there would be very few ethical issues with using menstrual blood
as a source of stem cells. It would benefit patients without compromising the life of any foetuses due to the fact that menstrual blood is a waste product. It would be an easy and continuous supply of stem cells and therefore a good replacement for embryonic stem cells. In this instance, cells do not even have to be pluripotent as they stimulate blood vessel growth as they are. This means it might be possible to avoid the need to convert them into iPS cells before use.

In order for this process to be realised, research would need to be carried out as to whether the same effect could be induced in humans as has been in mice. This has not always been easy e.g. the behaviour of neural stem cells in mice has yet to be replicated in humans\textsuperscript{13}, but with further research and understanding into how human stem cells interact in different situations, this is a possibility.

If the same effect could be generated in humans then the overcoming of disorders resulting in withered limbs such as arterial diseases becomes a possibility. This process, of repairing the blood vessels using stem cells, could also be implemented if a patient suffered severe arterial damage in an accident.

It would not be ethical to cut off the blood supply to a limb in a healthy human in order to test this. Human diseases can damage the blood supply such as atherosclerosis and diabetes so perhaps people who already have blood vessel damage would be willing to take part in trials. However, both atherosclerosis and diabetes are chronic diseases and so cause blood vessel damage slowly. It is therefore possible that such trials would need to be done on people from accidents where the damage is done quickly and the wounds are still fresh. Unfortunately, if the patient was unconscious, it would be difficult to gain consent to perform such a new procedure. This is an issue that would have to be overcome by creating some way for medics to identify someone who is happy to participate in a trial.

Theoretically these trials could be started right away as it is already possible to harvest adult endometrial stem cells from menstrual blood. A pure source of medical grade stem cells would be needed and there could be problems with matching if the stem cells were not the patient’s own. Obviously such stem cells could only be collected from menstruating females so it might be useful to have a bank of cells, similar to cord blood stem cell banks, from which cells could be taken when required. This would also be useful because even if the patient was female, it might not be possible to extract stem cells at the time of the accident.

2) Repairing a damaged limb

It is already possible to grow very simple organs using a template and stem cells. This has been demonstrated with the successful trachea transplants described in the introduction. However, re-growing a limb is much more complicated. The limb is made up of many different tissues encased in skin. This makes it a very complex entity especially when considering the possibility of separately re-growing each individual component.

Some of these components can already be made. For example, sheets of skin are already being made from stem cells. Umbilical cord stem cells are used to create sheets of skin that they use to treat serious burns\textsuperscript{14}. This method is less controversial than using embryonic stem cells. Furthermore, we know that stem cells from menstrual blood can stimulate blood vessel growth.
Further investigation into these methods may provide us with a quicker and more efficient process for mass-producing these components.

Scientists believe it will be possible to reconstruct facial bones and tissues from stem cells, so bypassing the need for immunosuppressant drugs. Although the face is, a ‘complex entity’, they believe it will be possible\(^{15}\). If this is true for the face, it may well be true for a limb as well.

However, even if all of these separate tissues could be created using stem cells, there is still the issue of how to make the tissues work together to become a functioning limb. The nervous system seems to be the answer to this problem. If it were possible to create new nerves from stem cells, then these would play an essential role in the communication between tissues.

It is known that adult canaries can re-grow brain cells and nerves. Mice seem to be able to do the same, using stem cell-like cells present in the brain. A similar process could happen in humans but it is not at all clear whether this is possible\(^{16}\). If scientists were able to induce adult nerve growth using stem cells, then these nerves could be part of the regeneration of the limb.

The obvious source of stem cells for all these tissues are pluripotent embryonic stem cells as they are able to differentiate into all three primary germ layers. However, the use of iPS cells helps avoid the ethical issues. Therefore, more research needs to be done in order to understand any differences in the behaviour of iPS cells compared to embryonic stem cells.

Instead of trying to create solely stem cell derived limbs, the first step for creating a limb in this tissue-by-tissue fashion might be to use artificial structures along with stem cell derived. Research into this area is already underway with artificial limbs already being covered with stem cell derived skin\(^{12}\). Muscles could be added to the combination next.

3) Re-growing the whole limb

Human cells don't retain the switched on genes for limb re-growth but turn these off after birth. If an embryo is damaged in utero then the baby is born without a scar. This is because the embryonic stem cells heal using a blastema. Amazingly, adult salamanders also heal in this way, somehow retaining the ability to produce a blastema even in adulthood.

In order for humans to be able to re-grow limbs they need to be able to keep the potential to form a blastema. Embryonic stem cells within the blastema may be responsible for its action in re-growing limbs. It may be possible to stimulate blastema formation from human embryonic stem cells. If we could insert embryonic stem cells into the site of damage in a limb and stimulate them to form a blastema, the entire limb could re-grow in a similar way to salamanders.

We need to find out which genes are involved in blastema formation and how to switch these on again once they have been turned off or even how to prevent them switching off in the first instance.

Human fibroblasts also would need to be studied to see how they differ from salamander fibroblasts and to work out if it is possible for humans to produce salamander like fibroblasts. Research is needed into whether it is possible to genetically alter human fibroblasts. Instead of scarring, human fibroblasts would need to behave like salamander fibroblasts.
In the study on salamanders it was concluded that all that is needed to re-grow a limb in this way in the presence of a blastema is ‘a wound epidermis, nerves and fibroblasts from opposite ends of the limb’. As the wound site would already have all of these features except the blastema, the major new area for research is into the production of a blastema.

There would also need to be studies undertaken in human embryos in order for us to understand human blastemas better but this comes with ethical issues as it involves the use and potential destruction of embryos.

CONCLUSION

As outlined above, the process of re-growing a limb is very complicated and there are many factors which, at the moment, are stopping us progressing further.

In thinking this idea through I have concluded that there are three different ways of approaching the problem: firstly, preventing the withering of injured limbs; secondly, building a limb following a tissue-by-tissue approach; lastly, re-growing the whole limb using a blastema.

There are problems with all three approaches.

In the first approach, even if it is possible to use human menstrual stem cells to stimulate blood vessel growth, we do not know whether these blood vessels would indeed save the limb as in mice. The issues surrounding real trials in humans are a challenge especially: harvesting, storing and matching the cells to patients who need them. In future, we need a way to mass harvest appropriate stem cells and store them in banks so that they can be accessed easily. We also need to be able to match cells to patients who need them or to find a way to prevent immune responses against non-self-cells. Trials could then be started on patients who have damaged blood supply.

In the second approach, although making individual tissues is becoming ever more possible, how to coordinate their action is a big problem. In the near future, we need to move away from simply coating artificial structures with new stem cell derived skin towards being able to add in other tissues such as muscles.

The third approach also presents problems, not least that we cannot yet make humans produce a blastema. If the embryonic blastemas were better understood and the genes for switching them on and off were located and studied, then future developments would be possible.

In summary the attempt to regrow human limbs using embryonic and other stem cells is an ambitious target. This does not seem to be an achievable target in the short term as there are too many difficulties. However, advances in medical procedures may well lead to a much better understanding of how this idea could be expanded upon in the longer term.
1 James Thompson

2 Pathology talk at Medlink 2011


5 Watts G. (2010) Leading stem cell scientists point to non-embryonic sources of cells. In BMJ;341;c3733

6 Hawkes N. (2011) Clinical grade stem cells are created by scientists in London. In BMJ;343;d8001


12 Personal communication


15 White C. (2008) Building people’s faces from stem cells may be possible in two decades. In BMJ;336;526.2

Other sources of information:


Ectopic Expression http://en.wikipedia.org/wiki/Ectopic_expression

Fibroblasts http://en.wikipedia.org/wiki/Fibroblast

Transcription Factor http://en.wikipedia.org/wiki/Transcription_factor


Types of Stem cells http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/S/Stem_Cells.html