The use of mixl1 in ex vivo and chimeric organ regeneration

- Anokhi Kashiparekh
- TA: Goheun Kim

Regenerative medicine is a field in biology that uses the underlying cell properties of differentiated growth to create entire tissues and organs from that cell. Regeneration in its true form, applies human pluripotent stem cell (hPSCs) differentiation, to make new organs like the natural regeneration of the human liver or that of the zebrafish heart (Mostoway *et al* , 2013). A very useful area for such a technique is the organ donation and replacement discipline. One of the greatest challenges for organ replacement is the shortage of organs donated for the cause. This is where the field of regenerative medicine can come in use. If the cells of the person in need of the organs can differentiate in a way that fills up the niche left by the diseased or missing organ, there could be a remarkable decrease in the need for organ transplantation and organ rejection. However, the development of the human organs is a gradual process and may take longer than the time the patient has to survive. A tactic to increase the speediness and the efficiency of organ regeneration is to manipulate certain genes to promote either ex vivo differentiation or differentiation in a chimeric host with a faster developmental time than humans. Specific genes can be engineered to perform specific functions, like prompting apoptosis using the *Bcl-2* gene or assessing mesodermal markers using *Wnt3* (Wu *et al* , 2016). Inducing *Mixl1* , the endoderm and mesoderm formation transcription factor, can play an important role in generating organs from induced pluripotent stem cells (iPSCs). *Mixl1* plays an important role in chimeric and *ex vivo* regeneration

models; although further research is required on the viability of these models.

The *Mixl1* transcription factor binds to the *Mix* gene and is a part of the hox gene family that codes for homeodomain proteins. The most important function of the *Mixl1* is the regulation of cell fate and differentiation during the developmental stages of an organism's life. It regulates the formation of the endodermal and mesodermal layers and consequently can be used to manipulate hPSCs towards a particular lineage of growth. This principal property of the *Mixl1* gene, as well its interactions with other genes, has been the focus of genetic regenerative medicine, in order to understand the role and consequent use of said genes.

Various experiments have tried to incorporate the *Mixl1* system in stem cell growth but two of the most widely known models are the *ex vivo* regeneration model and the chimeric model. Both of these models rely on the property of *Mixl1* to guide iPSCs towards either endodermal or mesodermal fate. The basic difference between these models is the environment in which these cells are allowed to differentiate.

The *ex vivo* model allows cell differentiation and growth outside an organism, generally in a laboratory. It is a widely preferred model due to the fact that cells from an organism can be extracted, cultured in a lab and placed back in the same organism. Each step in the experiment can be tracked and monitored and all the cells are cultured in a sterile environment. Thus, the cells placed back inside the animal are ' safe' from potential bacteria or viruses. However, this also means that the organs generated

from this may not be compatible with the surrounding tissue when introduced in an organism’s body, due to the lack of interaction with other cells.

Ex vivo culture of cells and ultimate organ regeneration is a step towards solving the problem of limited availability of desired cells. This *ex vivo* model of organ regeneration makes use of different substrates to recreate a ‘ natural’ differentiating environment for the cultured cells. However most times it is hard to push the hPSCs towards a particular lineage of growth, i. e. mesodermal or endodermal. The *Mixl1* gene with its property to establish cell fate, is useful in resolving this. The forced expression of *Mixl1* in hPSCs, in the right environment, with particular substrates and specific protein mediums, can promote *ex vivo* cell differentiation. *Ex vivo* culture, with connection to the *Mixl1* gene is efficient due to the control over time of forced expression of the *Mixl1* gene as well as external monitoring of the growth.

Liu et al (2011) [3] established this by using Lð°5Î²1 and Lð°6Î²1 protein ligands to promote *Mixl1* induced hPSCs, in a BMP4 medium. The procedure included purifying polypeptides, culturing the hPSCs and allowing them to differentiate. The results showed that the differentiation of cells peaked on the third (to) fourth day of culture when both the LÎ±5Î²1 and Lð°6Î²1 protein ligands were used. This gradual growth was tracked using immunofluorescence and analyzed by flow cytometry. The results of this experiment encompass both the usefulness as well as the disadvantages of an *ex vivo* regeneration of organs using *Mixl1* .

The biggest challenge encountered in an *ex vivo* organ regeneration model is the limited number of substrates that the hPSCs can use and differentiate into a mesodermal lineage. In various cases, *Mixl1* is induced unsuccessfully. This is due to the high substance substrate specificity of the iPSCs that do not survive long enough for *Mixl1* to express and differentiate. Another case seen in Liu et al was the very minute expression of *Mixl1* when cultured with various other individual ligands, showing that this procedure also requires the correct combination of substrates.

Various agencies have also raised ethical concerns over culturing animal cells in labs. The obtaining of cells, external media and substrates from animal bodies is cited as animal abuse (cruelty). To minimize the use of animal products, a more specific area of the *ex vivo* model has been developed, called the xeno-free culture. Typically, all components required for a xeno-free culture come from the same organism while taking care that it is completely free of animal or human elements, like bovine blood for culturing media, etc. As a replacement to these essential components, researchers are trying to synthesize new protein ligands that can function in a similar pattern [4] . To reiterate, genetic manipulation of the *ex vivo* model has the potential to save lives but requires a deeper study in the areas of limited substrate compatibility and availability.

In contrast to the *ex vivo* model, the chimeric model revolves around cell differentiation inside a living body. Chimeras are organisms made up of a combination of two or more zygotes and thus this model introduces extraction from and cell differentiation in two different individuals.

Generation of embryonic chimeras is of both practical and conceptual importance as it provides a method to assess the developmental competence of cells. The cells of the different individuals on the same embryo can be tracked and genes can be manipulated to create a chimeric organism that can act as a vessel for organ generation. Blastocyst complementation and target organ complementation are two important techniques in chimeric organ regeneration. While blastocyst complementation uses iPSCs transferred to an embryo of another species, generally a porcine embryo, target organ complementation is focused on the regeneration of specific organs of the body. Due to the unconventionality, the adherence to social and ethical limitations is of great importance and requires more research to be conducted.

Experiments combining this regenerative model and the forced expression of the *Mixl1* gene have been successful in producing organs in different hosts. By trying to reconcile the idea of targeted generation of organs derived by using the patient's own PSCs as seen in the case of the mice, Kobayashi et al (2016) makes use of blastocyst complementation to create pancreatogenesis–or nephrogenesis-disabled mice. Blastocyst complementation is a technique that makes use of induced human pluripotent stem cells (iPSCs) transferred to an embryo of another species; thus, following cell growth in another body.

This study used *Pdx1-LacZ* heterozygous mice as the chimeric hosts and injected humanized pancreatic cells in the blastocyst. This complementation was followed by the forced induction and expression of the *Mixl1* gene using the *pRosa26-tTA-Mixl1* vector. The data was analyzed by Western blotting

and ??? flow cytometry. The immediate result showed chimeric cells throughout the bodies of the mice. The mice were then introduced to a cell medium without Doxycycline (Dox). Immunofluorescence confirmed the distribution of cells being confined to just theguts, showing that specific target organ regeneration is possible with suitable environmental conditions and resources for the culturing cells. The forced expression of the *MIxl1* gene represses mesodermal fate determination and promotes endodermal fate determination, thus helping to induce the formation of target specific organs, including the pancreas or the liver, in the disabled mice.

In order to test for the amount of time *Mixl1* takes to express, these mice were injected with Dox at various time intervals and the results were examined using EpCAM, an endodermal genetic marker. The cell growth apex was noted on the 4???? $^{t\,h}$ day of Dox administration. This leads to the belief that time is an important factor in understanding cell growth in chimeric organisms. To understand the regulation of *Mixl1* based on biological functions, it was allowed to express under the influence of Oct3/4, a genetic marker seen to express in early development. The absence and presence of Dox in the host chimera was compared to establish that its absence would achieve specific target organ regeneration as compared to cell growth throughout the body. The data implied that *Mixl1* presence was necessary until the epiblast stage. This gives way to the inference that the time taken for *Mixl1* to express can be reduced, thus giving way to a quicker technique of organ regeneration.

This model, although promising, is questionable due to the ethical controversies like the formation of human neural cells or germ cells in the host animal. This is a cause for concern due to the fact that the idea of a human brain trapped inside a mute animal is disturbing. Proper manipulation of cell differentiating genes like *Mixl1* is essential to keep hPSCs from turning into cells that could ‘ humanize’ the host animal. While understanding and experimenting on techniques that help in human advancement, there has to be a larger focus on the social and ethical concerns of utilizing them. In conclusion, although it is limited by growing ethical concerns, genetic manipulation in chimera may help save lives with the advancement in understanding cell repair and regeneration. Thus, the idea of organ regeneration using chimerism should be looked into by science but in a way that can appeal to social principles.

Another problem associated with this model of regeneration is the low success rate of differentiation in non- rodent animals [2] . Majority chimera experiments include rodent species as the main focus, due to both the size and relatively easy manipulation of the rodents. Although recent experiments have shown (that) porcine hosts act as good carriers for human pancreatic growth [6] , there is a lot of research to establish pigs as conventional hPSC hosts in order to continue chimeric research to generate bigger organs like the human heart or the human lungs.

While comparing the advantages and the disadvantages of both these techniques of organ regeneration, the role and function of *Mixl1* itself cannot be overlooked. *Mixl1* has been shown to express within 4 days of being

induced. More research may lead to a quicker expression time. *Mixl1* has also helped to achieve a target specific organ regeneration by promoting mesodermal differentiation as required. The use of *Mixl1* is enormous in the field of regenerative biology and can be used in other projects as well as models of regeneration.

In conclusion, both *ex vivo* regeneration and chimeric regeneration have flaws but it is possible to refine them for better and more specific results. While the usefulness of *Mixl1* cannot be denied, better models of regeneration must be established to achieve maximum efficiency for its expression.

References:

1. Mostowy, S., Boucontet, L., Moya, M. J. M., Sirianni, A., Boudinot, P., Hollinshead, M., ...Colucci-Guyon, E. (2013). The zebrafish as a new model for the in vivo study of Shigella flexneri interaction with phagocytes and bacterial autophagy. *PLoS Pathogens* , *9* (9)
2. Wu, J., Greely, H. T., Jaenisch, R., Nakauchi, H., Rossant, J., & Juan Carlos Izpisua Belmonte. (2016). Stem cells and interspecies chimaeras. *Nature, 540* (7631), 51-59.
3. Yang, L., Wang, X., Kaufman, D., Shen W. (2011) A synthetic substrate to support early mesodermal differentiation of human embryonic stem cells. Biomaterials. 2011 Nov; 32(32): 8058-66.
4. Karnieli O., Friedner O., Allickson J., Zhang N., Jung S., Fiorentini D., Abraham E., Eaker S., Yong T., Chan A., Griffiths S., When A., Oh S. A consensus introduction to serum replacements and serum-free media for cellular therapies. Cytotherapy , Volume 19 , Issue 2 , 155 – 169.

5. Kobayashi, T., Kato-Itoh, M., & Nakauchi, H. (2015). Targeted organ generation using Mixl1-inducible mouse pluripotent stem cells in blastocyst complementation. *Stem Cells and Development, 24* (2), 182.
6. Matsunari, H., Nagashima, H., Watanabe, M., Umeyama, K., Nakano, K., Nagaya, M., . . . Nakauchi, H. (2013). Blastocyst complementation generates exogenic pancreas in vivo in apancreatic cloned pigs. *Proceedings of the National Academy of Sciences of the United States of America, 110* (12), 4557-4562.