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SAFE AND EFFICIENT REPROGRAMMING OF SOMATIC CELLS INTO STEM CELLS IN LIVING TISSUE

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Stem cells, with their innate ability to differentiate into many types of specialized cells, hold tremendous potential for research and therapeutic applications. Stem cell research, however, has long been surrounded by controversy, primarily concerning the necessity of using human embryos as a source of pluripotent embryonic stem cells (hES cells). The discovery that fully differentiated somatic cells could be reprogrammed back into a pluripotent state by Yamanaka *et al.*[15] in 2006 thus looked to be paradigm-shifting, promising to resolve the ethical issues while presenting new research opportunities. Current methodologies in the generation of such induced pluripotent stem cells (iPS cells) are however not without limitations, especially with regards to the complexity of carrying out the reprogramming process *in vitro* and the subsequent risks concerning the genomic stability of such reprogrammed cells.[8,12] In their recent study, Yilmazer *et al.*[17] demonstrate that the reprogramming process can in fact be carried out *in vivo* following a nonviral gene delivery, with no lasting tissue damage. This could well represent a major leap forward in our understanding of stem cells, while opening up many exciting possibilities for the widespread application of iPS cells.

The basis of the original discovery by Yamanaka *et al.* revolves around the *in vitro* expression of four transcription factors collectively referred to as OKSM (Oct 3/4, Klf4, Sox2, and c-Myc), which was able to reprogram fully differentiated somatic cells into iPS cells. Such iPS cells express critical pluripotency markers such as Nanog and demonstrate many functional similarities to embryonic stem cells, including the capabilities of self-renewal and differentiation.[15] This transcription-mediated form of reprogramming necessitates some form of delivery system to transduce target cells with the specific transcription factors. This role was initially performed by viral vectors, a well-studied and established method of gene transfer that remains highly prevalent in reprogramming studies.[15,16,19] However, as the mechanism of viral delivery involves integration into the host cell genome, the risk of insertional mutagenesis and other complications such as the expression of oncogenes is significant and represents a serious obstacle in the therapeutic application of iPS cells generated in this manner.[4] On

the flip side, nonviral vectors such as plasmids have been shown to be generally safer, but suffer significantly in terms of reprogramming efficiency with reduced throughput compared to viral methods.[7] Importantly, conventional methods in iPS cell generation (whether through viral delivery or otherwise) required working on cell cultures under laboratory conditions.

In tackling this conundrum, Yilmazer *et al.* tested the hypothesis that a nonviral delivery of the OKSM factors directly into living tissue can also achieve the result of reprogramming towards pluripotency. Following a hydrodynamic tail vein injection of naked plasmid DNA into Balb/C mice (a method previously shown to effectively target liver cells),[2,14] Yilmazer *et al.* observed increased expression of select pluripotency markers as well as a decline in hepatocyte markers within two days of the treatment. These observations were separately verified visually via immunohistochemical staining of liver sections, leading to the conclusion that a reprogramming of target hepatocytes into iPS cells was achieved *in vivo* following a successful gene delivery into liver cells. To determine reprogramming efficiency, a transgenic TNG-A strain of mice with a fluorescent eGFP reporter gene inserted into the Nanog locus was treated with the same hydrodynamic injection procedure. Flow cytometry analysis of hepatocytes 4 days after the treatment indicated 6–15% eGFP-positive cells, translating to a similar rate of reprogramming efficiency. Taken together, these findings provide a proof of principle that reprogramming into pluripotency can take place rapidly and efficiently under *in vivo* conditions following a nonviral delivery, potentially circumventing many of the obstacles previously associated with the generation of iPS cells.

As the treatment is applied directly to living tissue within living organisms, the risk of tissue damage and other post-treatment abnormalities must be considered in order to ensure that such a procedure is safe and suitable for future study or clinical applications. In the same vein, iPS cells have also demonstrated increased propensity for teratoma formation following implantation compared to hES cells.[9] In their study, Yilmazer *et al.* analyzed liver histology and blood serum of treated mice up to 120 days post-treatment. Other than moderate liver damage immediately following treatment, no lasting tissue damage or loss in organ function was reported beyond day 2. Normal structure and function of treated livers was observed for the full 120 day duration of the study, and no teratoma formation was observed. This indicates that *in vivo* reprogramming of pluripotent cells is potentially stable and safe for the host organism. Thus we have an approach that appears to be safe, rapid and efficient in the generation of iPS cells, addressing previously identified challenges in the procedure.

In appreciating what may represent a significant breakthrough in the field of stem cell research, it is crucial to keep in mind that the paper is being presented as a proof of principle, with the findings also highlighting significant gaps in our current understanding of the intricacies of cell fate and plasticity. Notably, reprogramming into pluripotency was observed to be transient. Yilmazer *et al.* reported a peak of increased pluripotency marker expression and decreased hepatocyte marker expression at 4 days after the treatment, following which each indicator began to normalize. Hepatocyte markers reverted to control levels by day 8 and pluripotency markers displayed the same result by day 22. The authors postulate that the reprogrammed cells had re-differentiated into hepatocytes as a result of the tissue microenvironment (presence of growth factors and other signaling molecules) of the generated iPS cells, but the exact underlying mechanism remains unclear. Alongside existing evidence of *in situ* differentiation of implanted iPS-derived progenitors into living tissue,[5,13] the findings of Yilmazer *et al.* underscore the need for greater understanding of what drives and regulates cellular specialization before stem cells can be feasibly applied for modeling or therapeutic purposes.

Upon further scrutiny, we can see that the several other premises of the research in fact further demonstrate limitations related to the use of iPS cells on a more fundamental level. Firstly, it is important to recognize that the hydrodynamic injection method employed by Yilmazer *et al.* is only well-tested in rodents, and that current limitations associated with gene delivery in humans remain an obstacle for human studies of reprogramming.[14] Secondly, much more exhaustive work must be done to ensure the safety and genomic stability of cells reprogrammed in such a manner, beyond what is shown in the 120-day Yilmazer study. The biggest threat is the risk of cancer formation from the iPS cells.[4] Each transcription factor involved in the reprogramming process can be linked to cancer, with c-Myc in particular being a well-known oncogene.[6,10,18] Yet it is

c-Myc that was confirmed in the Yilmazer study to have a significant effect on increasing reprogramming efficiency, a reminder that the safety-versus-efficiency conundrum is not so easily solved. Finally, and perhaps most critically, Yilmazer *et al.* noted that the study does not characterize the reprogrammed iPS cells beyond the monitoring of specific markers. Knowledge of the exact extent of similarity between iPS and hES cells remains limited, but we do know at this point that iPS cells derived from different methodologies and even different source cells can exhibit fundamentally different characteristics.[8,11] This means that much more needs to be done to understand the exact properties, or "pluripotent-ness" of these *in vivo* iPS cells. Whether is it in gene delivery methods or the holistic understanding of stem cell properties and mechanisms, it is clear that our current knowledge remains lacking for human applications.

What we do know for sure, however, is that iPS cells have nearly limitless potential, and Yilmazer *et al.* in their study have provided a basis for which this potential can be unlocked. With evidence of the feasibility of *in vivo* reprogramming following a nonviral delivery of specific transcription factors, researchers now have access to a whole new platform for the study of cellular reprogramming and iPS cell applications. The ability to work with autologous pluripotent stem cells has already shown incredible potential in personalized disease modeling[3] and regenerative medicine,[5,13] and advantages of iPS cells generated *in vivo* rather than *in vitro* are already being discovered.[1] Where stem cells are concerned, the sky is truly the limit.

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