Using non-thermal irreversible electroporation to create an in vivo niche for exogenous cell engraftment

Tammy T. Chang¹, Vivian X. Zhou¹, and Boris Rubinsky²

¹Department of Surgery, University of California, San Francisco, CA and ²Department of Mechanical Engineering, University of California, Berkeley, CA

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The critical shortage of donor organs has spurred investigation of alternative approaches to either generate replacement organs or implant exogenous cells for treatment of end-stage organ failure. Non-thermal irreversible electroporation (NTIRE), which uses brief high electric field pulses to induce irreversible permeabilization of cell membranes, has emerged as a technique for tumor ablation. Here, we introduce a new application for NTIRE that employs in situ cell ablation to create a niche within a solid organ for engraftment of exogenous cells in vivo. We treated the livers of mice with NTIRE and subsequently implanted exogenous congenic hepatocytes within the zone of cell ablation. Donor hepatocytes engrafted and integrated with host liver parenchyma pre-treated with NTIRE. This new approach should have value for studying the effects of a native matrix scaffold on in vivo cell growth and may pioneer a new type of minimally-invasive regenerative surgery.

The availability of organ transplantation—the only treatment for end-stage organ failure—is severely limited by the shortage of donor organs. The critical, unmet need for alternative treatments has spurred development of tissue engineering and regenerative medicine approaches to generate replacement organs. Whole-organ decellularization has been developed to produce three-dimensional tissue scaffolds with native extracellular matrix (ECM) and preserved microvasculature (1). Here, we introduce a new

approach to generate a native decellularized ECM scaffold in situ, which serves to facilitate in vivo engraftment of exogenous cells into a solid organ. Our approach is based on the concept that an appropriate niche must first be created within a host organ in order for new cells to engraft efficiently.

Non-thermal irreversible electroporation (NTIRE) is a tissue ablation technique that employs high electric field pulses microseconds in length to irreversibly permeabilize the cell membrane, thereby causing cell death

(2). NTIRE treatment leaves behind an intact ECM in which new cells engraft, often without scar tissue formation (2-7). This unique feature of NTIRE suggests it could be used as a method for decellularizing organs (5,8,9).

Inspired by the earlier work, we hypothesized that NTIRE could be utilized to remove host cells from tissues while leaving behind an intact ECM, creating a supportive niche in situ for the in vivo engraftment of exogenous cells. To test this hypothesis, we treated the livers of mice with NTIRE and subsequently implanted exogenous congenic hepatocytes within the treated volume.

All animal procedures were performed in accordance to protocols approved by the Institutional Animal Care and Use Committee at UCSF. After induction of general anesthesia, the abdomen of a wild-type C57BL/6 mouse was aseptically prepared, and the liver was exposed by making a 1 cm midline incision below the xiphoid. Two needle electrodes, each 0.5 mm in diameter and spaced 4 mm apart, were connected to an ECM 830 Square Wave Electroporation System (BTX Harvard Apparatus, Holliston, MA) and inserted into the left lobe, perpendicular to the liver surface. Electrical pulses were applied using the following parameters: 500 V/cm (voltage over distance) and eight 100- μ s pulses with each pulse separated by 100 ms. These parameters were chosen because they represent the threshold for inducing irreversible electroporation (10). Other studies have employed different electrodes that applied higher electric fields and many more pulses (4,9). We used the lower threshold because recent studies showed that the larger numbers of pulses induced electrolysis and might not produce pure irreversible electroporation (11).

We found that the time course of tissue injury in response to NTIRE ablation in the mouse liver was comparable to that observed in pig and rat livers (3,4), blood vessels (5), and small intestines (12). At 3 hours

METHOD SUMMARY

Mouse liver was treated with non-thermal irreversible electroporation (NTIRE) electrical pulses in vivo. A few days later, the treated tissue was then implanted with exogenous hepatocytes, which successfully engrafted into the liver tissue.

Figure 1. Time course of non-thermal irreversible electroporation (NTIRE)-treated liver tissue shows hepatocyte injury, followed by recovery, without formation of scar tissue. There were no overt signs of cell injury at 3 h. By 24 h, there were injured hepatocytes with vacuolated, moth-eaten-appearing cytoplasm. By 3 d, hepatocytes showed ballooning degeneration. By 7 d, ballooned hepatocytes were no longer found. PT, portal tract; CV, central vein. Magnification: 20 $\times$. Scale bar = 100 μ m.

after NTIRE treatment, there were no overt signs of cell death or tissue injury (Figure 1). By 24 hours, the liver parenchyma showed injured hepatocytes with a vacuolated, moth-eaten-appearing cytoplasm. At 3 days, there was massive ballooning degeneration of hepatocytes throughout the NTIRE-treated tissue. The pathogenesis of ballooning degeneration is not completely known, but there is evidence that it represents terminally

Figure 2. Exogenous hepatocytes implanted into non-thermal irreversible electroporation (NTIRE)-treated liver in vivo show greater host parenchymal integration and larger clustering compared to controls without NTIRE pretreatment. (A) By Day 3 post-treatment, the livers of NTIRE-treated mice ($n = 3$) showed significantly greater numbers of X-gal $^{+}$ hepatocytes (blue) within and beyond the inflammatory zone compared to controls ($n = 3$); $P < 0.05$ by two-tailed Student's t -test. Error bars represent SEM. (B) By Day 14, individual X-gal $^{+}$ hepatocytes (arrowheads) or clusters (arrows) had integrated with the host parenchyma near or along the implantation scar (asterisks). Although total cell numbers were not significantly increased, there were larger clusters of X-gal $^{+}$ hepatocytes in the livers of NTIRE-treated mice ($n = 3$) compared to controls ($n = 3$). For all mice, serial cryosections were obtained through the entire implanted liver lobe from the anterior surface to the posterior surface, and X-gal $^{+}$ cells were manually counted in all sections. Scale bar = 400 μ m (5 $\times$) and 60 μ m (40 $\times$).

injured cells that cannot complete the apoptotic program (13). By 7 days, the NTIRE-injured tissue had repaired itself, and ballooned hepatocytes were no longer found. Importantly, there was no extensive infiltration of inflammatory cells, abnormal deposition of collagen, or scar formation after NTIRE treatment.

Time-course studies suggested that the optimal timing of in vivo exogenous hepatocyte implantation was 3 days after NTIRE treatment, a time point after host hepatocyte cell death had peaked. Therefore, we treated the livers of mice with NTIRE as described above and then introduced donor hepatocytes by direct parenchymal implantation 3 days later. Primary hepato-

cytes were isolated from B6.129S7-Gt(RSA)26Sor/J (ROSA26) mice, in which β -galactosidase is constitutively expressed in all cells so that implanted hepatocytes could be easily identified by X-gal staining in wild-type C57BL/6 recipients. ROSA26 mice were anesthetized, and primary hepatocyte isolation was performed by two-step perfusion (14). One million freshly isolated hepatocytes were loaded into a PE50 catheter (BD Biosciences, San Jose, CA) connected to a glass syringe with a screw-drive (Hamilton, Reno, NV). C57BL/6 mice pre-treated with NTIRE were anesthetized, and the previously made midline incision was re-opened. A hepatotomy was made using a 25-gauge needle in the left

liver lobe, in between the two needle electrode insertion sites. The catheter loaded with hepatocytes was inserted into the hepatotomy site, and donor hepatocytes were implanted by slowly turning the screw-drive of the syringe (15). Recipient wild-type mice that did not receive NTIRE pre-treatment were used as controls.

At 3 and 14 days post-implantation, serial cryosections were obtained through the entire recipient liver lobe from the anterior to the posterior surfaces. Hepatocytes that stained positive for X-gal (blue) were manually counted in each section to determine the total number of engrafted hepatocytes in each recipient mouse. The hepatotomy performed at the time of implantation created a space within the liver parenchyma (Figure 2A, 5× magnification). The majority of implanted cells within this space eventually die by necrosis (15). Exogenous cells that successfully engrafted were at the periphery of this space and had to migrate past the zone of inflammatory cells to incorporate within the host parenchyma (Figure 2A, 40× magnification). Livers from NTIRE-treated mice showed significantly greater numbers of X-gal⁺ hepatocytes within and beyond the inflammatory zone compared to livers from control mice that did not receive NTIRE pre-treatment (Figure 2A, bar graph). At 14 days after implantation, X-gal⁺ donor hepatocytes appeared integrated with the host parenchyma near or along the scar left behind by the implantation tract (Figure 2B, 5× magnification). Livers pretreated with NTIRE showed larger X-gal⁺ cell clusters compared to controls (Figure 2B, 40× magnification). These observations suggest that, with further optimization of electroporation protocols and surgical approach, improved donor cell engraftment may be achieved in NTIRE-treated liver tissue after direct exogenous cellular implantation in vivo.

We showed that exogenous hepatocytes engrafted into the niche prepared by NTIRE pre-treatment of the liver. Our study is the first to demonstrate the feasibility of this regenerative surgical approach that may be generalizable to other organs and cell types.

Author contributions

T.T.C. conceived the study, designed the experiments, performed the experiments, analyzed and interpreted the data, and wrote the manuscript. V.X.Z. performed the experiments and data analysis and prepared parts of the manuscript. B.R. designed the experiments and wrote the manuscript.

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Competing interests

B.R. is a co-inventor of the NTIRE technology. The intellectual property of NTIRE is assigned to the University of California, Berkeley. B.R. has no other financial interests except as a faculty member of the university.

References

1. Uygun, B.E., A. Soto-Gutierrez, H. Yagi, M.L. Izamis, M.A. Guzzardi, C. Shulman, J. Milwid, N. Kobayashi, et al. 2010. Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. *Nat. Med.* 16:814-820.
2. Davalos, R.V., I.L. Mir, and B. Rubinsky. 2005. Tissue ablation with irreversible electroporation. *Ann. Biomed. Eng.* 33:223-231.
3. Rubinsky, B., G. Onik, and P. Mikus. 2007. Irreversible electroporation: a new ablation modality--clinical implications. *Technol. Cancer Res. Treat.* 6:37-48.
4. Golberg, A., B.G. Bruinsma, M. Jaramillo, M.L. Yarmush, and B.E. Uygun. 2016. Rat liver regeneration following ablation with irreversible electroporation. *PeerJ* 4:e1571.
5. Phillips, M., E. Maor, and B. Rubinsky. 2010. Nonthermal irreversible electroporation for tissue decellularization. *J. Biomech. Eng.* 132:091003.
6. Narayanan, G., T. Froud, R. Suthar, and K. Barbary. 2013. Irreversible electroporation of hepatic malignancy. *Semin. Intervent. Radiol.* 30:67-73.
7. Maor, E., A. Ivorra, J. Leor, and B. Rubinsky. 2007. The effect of irreversible electroporation on blood vessels. *Technol. Cancer Res. Treat.* 6:307-312.
8. Phillips, M.A., E. Maor, B. Rubinsky, and J. Lavee. Extracellular matrix material created using non-thermal irreversible electroporation. US Patent 08835166, Sep 16 2014. The Regents of the University of California.
9. Sano, M.B., R.E. Neal 2nd, P.A. Garcia, D. Gerber, J. Robertson, and R.V. Davalos. 2010. Towards the creation of decellularized organ constructs using irreversible electroporation and active mechanical perfusion. *Biomed. Eng. Online* 9:83.
10. Miklavcic, D., D. Semrov, H. Mekid, and L.M. Mir. 2000. In vivo electroporation threshold determination. *Proc. 22nd Annual Internat. Conf. IEEE Engineering in Medicine and Biology Society.* 4:2815 - 2818.
11. Rubinsky, L., E. Guenther, P. Mikus, M. Stehling, and B. Rubinsky. 2016. Electrolytic Effects During Tissue Ablation by Electroporation. *Technol. Cancer Res. Treat.* 15:NP95-NP103.
12. Phillips, M.A., R. Narayan, T. Padath, and B. Rubinsky. 2012. Irreversible electroporation on the small intestine. *Br. J. Cancer* 106:490-495.
13. Kakisaka, K., S.C. Cazanave, N.W. Werneburg, N. Razumilava, J.C. Mertens, S.F. Bronk, and G.J. Gores. 2012. A hedgehog survival pathway in 'undead' lipotoxic hepatocytes. *J. Hepatol.* 57:844-851.
14. Desai, S.S., J.C. Tung, V.X. Zhou, J.P. Grenert, Y. Malato, M. Rezvani, R. Espanol-Suner, H. Willenbring, et al. 2016. Physiological ranges of matrix rigidity modulate primary mouse hepatocyte function in part through hepatocyte nuclear factor 4 alpha. *Hepatology* 64:261-275.
15. Zhou, V.X., M. Lolas, and T.T. Chang. 2017. Direct orthotopic implantation of hepatic organoids. *J. Surg. Res.* 211:251-260.

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Address correspondence to Tammy T. Chang, 513 Parnassus Ave., S549 San Francisco, CA 94143. E-mail: tammy.chang@ucsf.edu

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