STEM CELLS TRANSLATIONAL MEDICINE®

TISSUE ENGINEERING AND REGENERATIVE MEDICINE

Epidermal Growth Factor Tethered to β-Tricalcium Phosphate Bone Scaffolds via a High-Affinity Binding Peptide Enhances Survival of Human Mesenchymal Stem Cells/Multipotent Stromal Cells in an Immune-Competent Parafascial Implantation Assay in Mice

AUSTIN NUSCHKE,[a,b] MELANIE RODRIGUES,[a] JAIME RIVERA,[c] CECELIA YATES,[a,b,d,e] DIANA WHALEY,[a,f] DONNA STOLZ,[b,f] LINDA GRIFFITH,[c] ALAN WELLS[a,b,e]

Key Words. Multipotent stem cells • Mesenchymal stem cells • Surface-tethered epidermal growth factor • β-Tricalcium phosphate • Stem cell survival

Departments of [a]Pathology, [f]Cell Biology, and [d]Health Promotion and Development, and [b]McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, USA; [e]VA Pittsburgh Health System, Pittsburgh, Pennsylvania, USA; [c]Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA

Correspondence: Alan Wells, M.D., D.M.Sc., University of Pittsburgh, Department of Pathology, S713 Scaife Hall, 3550 Terrace Street, Pittsburgh, Pennsylvania 15261, USA. Telephone: 412-647-7813; E-Mail: wellsa@upmc.edu

Received November 2, 2015; accepted for publication April 8, 2016; published Online First on July 8, 2016.

1066-5099/2016/$20.00/0

http://dx.doi.org/10.5966/sctm.2015-0326

ABSTRACT

Mesenchymal stem cells/multipotent stromal cells (MSCs) are attractive candidates for cell therapies owing to their ability to differentiate into many lineages. However, these cells often fail to survive when implanted into a harsh wound environment, limiting efficacy in vivo. To improve MSC survival, we previously found that tethered epidermal growth factor (tEGF) molecules that restrict epidermal growth factor receptor (EGFR) signaling to the cell surface provide resistance to death signals. To adapt this system to wound healing, we tethered epidermal growth factor (EGF) to tricalcium phosphate (TCP) particle scaffolds, clinically used in bone healing. Human primary MSCs seeded on TCP and mixed into a collagen-based gel were injected in the perifascial space of immunocompetent mice with or without tEGF attached to the surface. We found that tethering EGF to the TCP scaffolds yielded approximately a fourfold increase in MSC survival compared with non-EGF scaffolds at 21 days, as well as significant improvements in survival in the short term at 2 and 7 days after implantation. Overall, our approach to sustaining EGFR signaling reduced MSC death in vivo and may be useful for future cell therapies where MSCs typically die on implantation. STEM CELLS TRANSLATIONAL MEDICINE *2016;5:1580–1586*

SIGNIFICANCE

Stem cells are limited as tissue replacements owing to rapid death induced in the hostile wound environment. It has been found that restricting epidermal growth factor (EGF) receptor signaling to the membrane provides a survival advantage. This report elucidates a method to tether EGF to bone induction material to improve the survival of mesenchymal stem cells/multipotent stromal cells in vivo.

INTRODUCTION

The use of mesenchymal stem cells/multipotent stromal cells (MSCs) has been studied in the clinical context of tissue regeneration and cell therapy for many years due to their ability to differentiate into multiple cell types [1–3]. In addition, MSCs secrete a variety of supportive cytokines and matrices that promote regenerative processes, particularly factors that induce neovascularization (vascular endothelial growth factor α [VEGF-α], insulin-like growth factor 1 [IGF-1], etc.) [4, 5]. These cells also have a documented immune privilege in an undifferentiated state, as well as the ability to modulate the local host inflammatory response itself, allowing them to temporarily quell host reactions before being rejected themselves [6–8].

Many therapeutically relevant contexts for MSCs involve implantation of MSCs into wound sites or tissues, in which the MSCs are immediately subjected to harsh conditions or degenerative tissue. Numerous studies have reported less than 50% survival of implanted cells after only the first 48 hours, with virtually no detectable MSCs remaining after 1–2 weeks [9–11], despite reports of greater resistance to death from high metabolic flexibility [12]. We have found that MSC survival can be enhanced by restricting signaling through the epidermal growth factor receptor (EGFR) to that subset of pathways activated from the cell membrane [13–15]. This approach of covalently tethering epidermal growth factor (EGF) has led to in vitro improvement of MSC survival in

the face of death stimuli such as Fas ligand [15, 16] while maintaining the functional capacity of the MSCs to differentiate [17]. For in vivo functionality, tethered epidermal growth factor (tEGF) needs to be functionalized to materials compatible with in vivo wound situations. Here, we applied this system of tethered EGF to a clinically relevant scaffold for bone healing, β-tricalcium phosphate (β-TCP), by using a high-affinity multimeric binding peptide to link soluble EGF to the scaffold. The effect on survival was then determined in a hypoxic, nonvascularized environment of a collagen plug in immunocompetent mice.

Materials and Methods

Scaffold With tEGF Binding Peptide

The high-affinity linker peptide used to bind soluble EGF to the tricalcium phosphate (TCP) surfaces was fabricated by Jaime Rivera in the Griffith Laboratory at the Massachusetts Institute of Technology (Cambridge, MA, http://web.mit.edu/) as described in detail in the online supplemental data [18]. β-Tricalcium phosphate powder consisting of 63–106 μM β-TCP particles were fabricated at Integra LifeSciences (Plainsboro, NJ, http://www.integralife.com) and were used for all studies as a scaffold for MSC seeding. Briefly, a human EGF sequence was inserted into a pMAL expression cassette (New England BioLabs, Ipswich, MA, https://www.neb.com) via polymerase chain reaction (PCR) mutagenesis to yield a library of multimer insertions fused to EGF. This protein was expressed in *Escherichia coli* and induced with isopropyl β-D-1-thiogalactopyranoside to yield protein for harvest via maltose binding protein affinity chromatography and purified using ultrafiltration and sterile syringe filtration. Reported purity of the full-length binding peptide ranged from 75%–90%. Protein concentration was measured via Nanodrop ND-2000 (Thermo Fisher Scientific, Minneapolis, MN, https://www.thermofisher.com) and provided to our group at the University of Pittsburgh for use in vivo.

β-Tricalcium phosphate powder consisting of 63–106 μM β-TCP particles (average 82 μM) was used for all studies as a scaffold for MSC seeding. For each individual plug, 10 mg of β-TCP powder was weighed and placed into separate tubes. For the experimental tEGF group, 10 mg β-TCP was incubated with 200 μl of 2 μM EGF/binding peptide solution for 24 hours at 4°C. Following incubation, the residual protein solution was aspirated and, for those scaffolds receiving MSCs, CM-DiI-tracked MSCs were trypsinized and added individually to both the tEGF and untreated β-TCP sample tubes at a concentration of 1.5×10^6 MSCs in 1 ml proliferation media per sample. Individual samples were rotated at 37°C for 90 minutes to allow attachment of the MSCs to the β-TCP scaffolds as previously described [19, 20]. Following cell attachment, samples were gently spun to collect all β-TCP particles, and proliferation media were aspirated. The samples were then gently mixed with a 1:1 volume mixture (0.5 ml each; 1 ml total plug volume) of growth factor-reduced Matrigel (BD Biosciences, San Jose, CA, http://www.bdbiosciences.com) and collagen I (3.84 mg/ml) to form a viscous injectable plug.

MSC Inoculations

Primary human bone marrow-derived MSCs, used before passage 7, were obtained from the NIH-funded core facility run by Dr. Darwin Prockop at Texas A&M University. Primary MSCs were tracked using a red CM-DiI cell tracker (Thermo Fisher Scientific, Minneapolis, MN, https://www.thermofisher.com); after 12 hours red fluorescence at the MSC cell membranes was confirmed in each dish before use in vivo. The CM-DiI-tracked MSCs were trypsinized and added individually to both the tEGF and untreated β-TCP sample tubes at a concentration of 1.5×10^6 MSCs as previously described [19, 20]. Following cell attachment, samples were gently spun to collect all β-TCP particles, and proliferation media were aspirated. Both MSC and non-MSC TCP and tEGF-TCP samples were then gently mixed with a 1:1 volume mixture (0.5 ml each; 1 ml total plug volume) of growth factor-reduced Matrigel (BD Biosciences) and collagen I (BD Biosciences) (3.84 mg/ml) on ice to form a viscous injectable plug.

Immunocompetent FVB mice that were 8–10 weeks old were obtained from Jackson Laboratories (Bar Harbor, ME, https://www.jax.org). For injection, the collagen I/Matrigel mixtures containing TCP (described in the previous section) were transferred into the perifascial space above the quadriceps on each leg, with 1 ml of gel injected transcutaneously on the anesthetized mice. Mice were treated at the time of inoculation with Buprenorphine SR (ZooPharm, Fort Collins, CO, http://wildpharm.com/zoopharm-home.html) to prevent pain; observational follow-up did not present signs of pain from the procedure. Plugs solidified immediately in vivo and were extracted at each time point following mouse euthanization via CO_2 asphyxiation. All animal work was reviewed and approved by the Pittsburgh VA Medical Center and the University of Pittsburgh Institutional Animal Care and Use Committees.

Results

To assess the ability of tEGF to improve immediate and long-term MSC survival, we applied tEGF to clinically relevant β-TCP particles and seeded MSCs on these scaffolds mixed into a gel plug for injection into the perifascial space of immunocompetent mice above the quadriceps muscle (Fig. 1A). MSC-TCP scaffolds seeded with CM-DiI-tracked MSCs were gently suspended in a 1:1 mixture of collagen I and Matrigel to provide a gel for scaffold injection into the perifascial site, with gel plugs removed after 1, 3, 7, and 21 days of incubation in vivo (Fig. 1B).

At each time point, half of each gel plug was fixed and cryosectioned to determine the distribution of the TCP particles and MSCs throughout each gel scaffold, as well as monitor the survival of the CM-DiI-tracked MSCs with time (Fig. 2). Sections showed a generally even distribution of MSCs throughout all implants, with tracked MSCs localizing in the vicinity of the β-TCP particle aggregates, as expected (Figs. 2A, 2C). Fluorescent signal from the tracked MSCs appeared to be more prevalent in the tEGF-modified TCP conditions compared with the standard TCP scaffold conditions. tEGF scaffolds also improved the apparent angiogenic character of the plugs, as can be seen in the extracted plugs in Figure 1B.

MSC survival was quantified via flow cytometry analysis of MSC presence using common human MSC markers CD73 and CD90 (Fig. 2A) following plug digestion. Percent positive MSC populations were derived from total cell harvests from all plugs at the aforementioned time points, and MSC percentages from flow cytometry were then applied to the total cell count from each plug to estimate the number of MSCs present in each half-plug; if the cells had fully differentiated, we would have not noted them, but their survival would not be expected due to the species difference in an immunocompetent animal. In the course of two experiments, results show that tEGF-TCP scaffolds provide a distinct survival advantages to implanted MSCs at all assayed time points. Days 3 and 7 showed an approximately

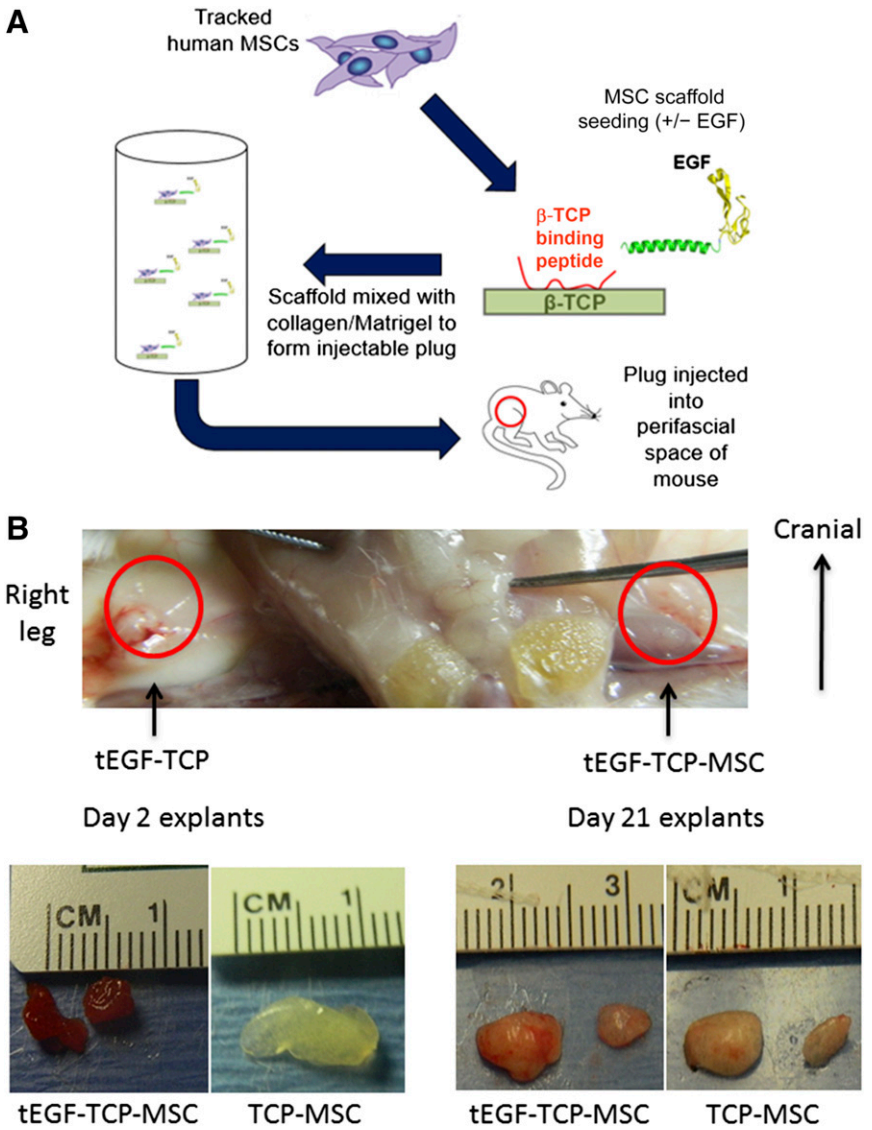

Figure 1. Experimental schematic for tEGF plug injections. **(A)**: Tracked (CM-DiI) primary bone marrow-derived human MSCs were seeded onto tricalcium phosphate particles with or without soluble EGF tethered to the scaffold surface via rotation in proliferation media (also see Methods). The seeded TCP scaffolds were mixed into a 1:1 collagen I/Matrigel gel mixture and injected into the perifacial space of FVB mice above the quadriceps. **(B)**: Plugs were extracted on days 2, 7, and 21 for cryosectioning and flow cytometry analyses. Abbreviations: β-TCP, β-tricalcium phosphate; EGF, epidermal growth factor; MSCs, mesenchymal stem cells/multipotent stromal cells; TCP, tricalcium phosphate; tEGF, tethered epidermal growth factor.

fourfold improvement in remaining MSCs over the TCP scaffold alone. This survival effect is perhaps most notable in the long term, where a significant portion of the MSCs persist out to 21 days.

Cellularity is often a point of interest in regenerative scaffolds, owing to the importance of invading $CD31^+$ cells and proliferation of any native cells that might be participating in the regenerative response. We assessed total cell counts from extracted plugs during the processing of each plug for flow analysis via automated cell counting. We found that the presence of tEGF on the TCP scaffolds significantly increased the total number of isolated cells within each plug, particularly at days 7 and 21; at these extended times the tEGF conditions showed a drastic increase in cellularity, well above the cellularity that would come from the implanted MSCs alone (Fig. 3A). This increased cellularity represented an almost twofold to threefold increase over the unmodified TCP scaffolds. At day 7, there were $3.89 \pm 0.13 \times 10^6$ cells on the tEGF particles at day 7 compared with $1.74 \pm 0.28 \times 10^6$ cells on TCP alone. Notably, the increased numbers of cells was noted with an apparent increase in the vascularity of each plug; unfortunately, the process of histochemistry for visualization of the TCP scaffolds prevented definitive immunohistochemical detection of the vessels. TCP scaffolds without tEGF showed a slight upward trend in cellularity within 21 days, roughly doubling the initial 1 million identified cells at the day 2 time point.

Differences in cellularity were potentially attributable to invading murine cells via innate immunity or paracrine functions of the MSCs themselves. We therefore analyzed whole cell extracts from the injected plugs for the presence of murine $CD11b^+$ (pan-leukocyte), $Nk1.1^+$ (natural killer T cells), and/or

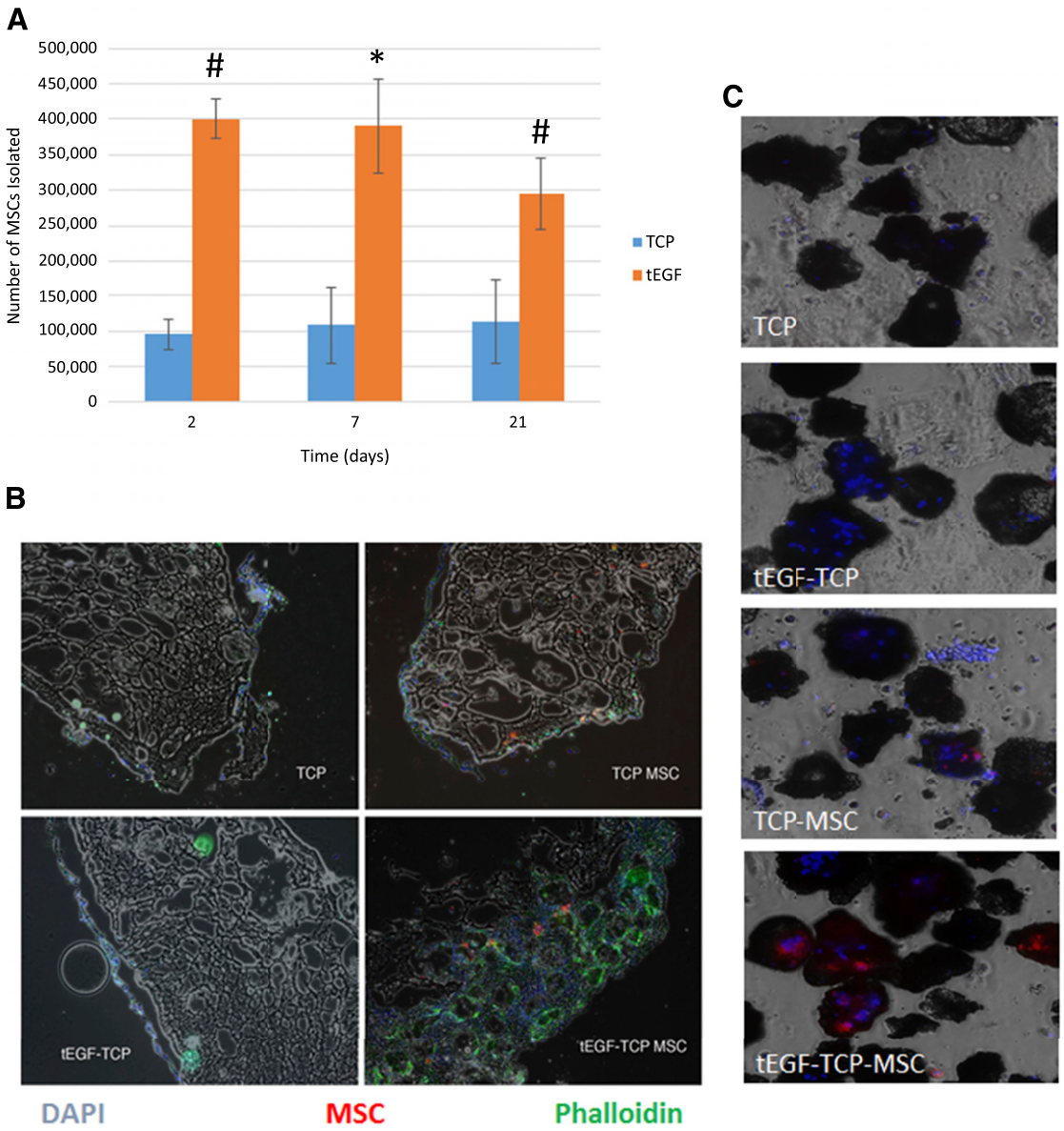

Figure 2. Surface tEGF improves MSC survival on β-tricalcium phosphate scaffolds. Extracted plugs were digested and cells were isolated for flow cytometry analysis, using MSC markers CD73 and CD90 to identify the human MSCs. **(A)**: Shown are mean $\pm$ standard deviation of five to six mice; $*$, $p < .01$, #, $p < .05$. Half of each plug was also cryosectioned and analyzed for presence of general cells (DAPI, actin stains) and visual presence of MSCs shown at 21 days at low magnification **(B)** and within the TCP particles themselves at higher magnification **(C)**. Shown are representative of plugs from five to dix mice, in two separate experiments. Magnification = $\times$20 objective for $\times$200-fold magnification. Abbreviations: MSC, mesenchymal stem cell/multipotent stromal cell; TCP, tricalcium phosphate; tEGF, tethered epidermal growth factor.

$CD3e^+$ (T-cell receptor) cells (Figs. 3B, 3C, 3D). Results showed that invading cells were primarily composed of murine cells positive for the pan-leukocyte marker CD11b (Fig. 3B), with a slight increase at early time points for $^+$tEGF injections. Nk1.1 and $Cd3e^+$ cells represented a minor portion of invading cells, with little difference demonstrated $^{+/-}$ tEGF in either of these subsets; this T-cell infiltration increase is as expected for acquired immunity rejection of the transplanted human cells.

Discussion

Our group has previously examined the benefit of EGF tethering largely in vitro, showing enhanced survival in the face of Fas ligand challenge [16, 17, 21]. Here, we have built on work by Alvarez and Rivera [18] in designing this peptide and examining effects in vivo, attempting to challenge MSCs in an implant site and hypothesizing that EGF tethering would provide a protective effect in this context. Results from this study demonstrate the utility of surface tethering soluble EGF to scaffolds for MSC implantation in such an environment. Our chosen implant site, the perifascial space above the quadriceps, was largely devoid of blood flow or a nutrient supply, highly hypoxic compared with the culture environment and thus similar to a wound bed. Thus, these cells were initially faced with a nutrient-poor and hypoxic environment within the plug itself, specifically chosen to mimic the harsh

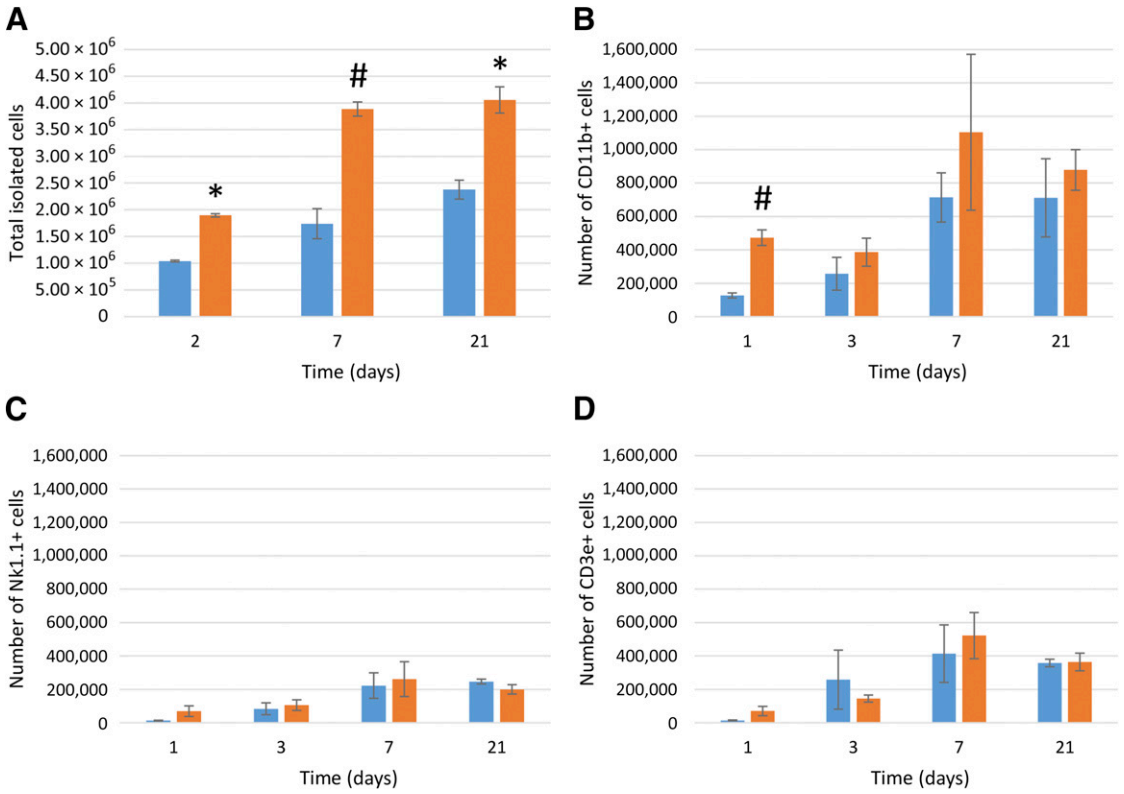

Figure 3. Improved MSC survival on tethered epidermal growth factor scaffolds increases overall cellularity with no overt effect on inflammatory infiltration. **(A)**: Digested plugs from each time point were counted using a Sceptre cell counter, and total cell counts were recorded and extrapolated to yield an estimated cell count for the entire extracted plug at each time point. Cell counts are reported here as those estimates, before MSC identification and thus including the whole cell extract from the mouse, as well as any MSCs left in the plug. In addition, percent positive for murine markers of inflammation including CD11b **(B)**, Nk1.1 **(C)**, and CD3e **(D)** were determined via flow cytometry and percentages were extrapolated back to cell counts to determine an estimated number of invading cells for each marker. A Student's *t* test was performed to determine significance, mean ± standard error, from five to six mice; ∗, $p < .01$, #, $p < .05$. Abbreviation: MSC, mesenchymal stem cells/multipotent stromal cell.

wound environment these cells would typically be exposed to upon injection. The immunocompetent nature of the animals was chosen for two reasons: first to demonstrate increased survival even in the face of immunological challenge, and second as a model for allogenic transplantation should the extended lifespan prove sufficient to "educate" a wound bed to improve healing from endogenous MSCs. We also subjected the cells to any relevant innate and acquired immune responses, as we used immunocompetent mice for our xenotransplant.

Our data show that a modest concentration of surface-tethered EGF is able to keep up to 30% of implanted MSCs alive even 21 days following injection into a region generally devoid of vessel growth and nutrients for the cells (Fig. 2), significantly more than clinical studies have shown for typical MSC survival in vivo [9–11]. Although a majority of the cells have still indeed died at the end of this study, a 3-week lifespan is sufficient for these cells to have started and proceeded well into the process of differentiation. Although we have shown that tEGF substrates have a positive effect on the osteogenic potential of MSCs in vitro [17], follow-up studies are necessary to look further at the efficiency of MSC osteodifferentiation in vivo and translate improved survival to a possible clinical benefit. The present study was not designed to determine whether the tEGF increased seeding, survival, and/or subsequent proliferation of the MSCs in vivo. Such information awaits further work, now that we found enhanced MSCs in the inoculated plugs. In addition, a significant limitation of this study is the lack of clarity on the fate of the surviving cells. Although we do show presence and persistence of the human MSCs long term, it is yet unclear if some of the inoculated cells migrated from the plug space or differentiated in situ. Future studies will require a more in-depth exploration of the fate of the surviving MSCs, as well as the more specific paracrine effects on invading host cells beyond those analyzed here to rule out any nontargeted EGFR activation.

Analyses of total cell counts from extracted plugs in this study showed increased cellularity in those scaffolds with the tEGF binding peptide, including all extracted murine and human cells. Although this is partially due to the improved survival of MSCs on these scaffolds as reported in Figure 2, these data also highlight the likelihood that MSCs maintain paracrine function with the constitutive EGFR activation induced on our tEGF scaffolds. For example, MSCs have long been known to be important in inducing angiogenesis locally via VEGF activity, improving vessel formation in tube formation assays [22] and also showing increased angiogenesis in implant sites in vivo in a wound healing context [23]. Flow cytometry analysis of several murine markers of inflammation demonstrated a clear cellular invasion after implant, largely composed of murine cells positive for pan-leukocyte marker CD11b. However, little difference in the number of invading cells

was seen between TCP controls and $^{+}$tEGF test groups, suggesting that another potential invading cell or another paracrine function of the implanted MSCs may be responsible for the trends in cellularity. Still, the numbers of MSCs and leukocytes combined account for much less than half of the total cell number in the presence of tEGF, although totaling up to approximately two thirds of the cells in the control condition; this suggests that other cells are involved. An untested cell type would be those of the vascular system, which would explain the seeming increased vascularity as noted by visual inspection of the implants that had tEGF. Our group has previously shown in vitro that sustained EGFR activation through tethering approaches may improve paracrine function of MSCs through upregulation of growth factor and/or cytokine production [24]. Future studies with EGF tethering and MSCs must discern the effects of EGFR tethering on the MSC secretome and associated outcomes in the implant site, both beneficial and otherwise.

Given its role in a wide array of cellular processes, it stands to reason that our persistent activation of EGFR may have effects beyond enhanced cell survival. Although survival signaling via EGFR functions primarily through the PI3K/Akt signaling arm [25, 26], there are other aspects to EGFR signaling that might benefit the MSCs in a clinical context. A variety of studies in EGFR tethering have shown, for example, enhanced MSC proliferation in concert with the improved survival we have seen in this study [18, 27]. Although enhanced proliferation may help to simply increase the number of available MSCs after implantation and improve overall survival outcomes, increased proliferation of MSCs long term may assist with MSC-derived osteoblast migration into a bone defect site or other comparable scenarios where proliferation is of importance. In addition, cell motility is intimately tied into the EGFR signaling cascade [28, 29], and a multitude of studies have shown the effects of EGFR activation on enhanced migration. Although surface-restricted EGFR signaling has shown to yield outcomes for improved survival preferentially over migration [27, 30], the benefits of any enhanced MSC motility from this construct may assist with cell engraftment and ultimately regenerative outcomes in the implant site.

Conclusion

Due to its relatively straightforward design and the ubiquitous expression of EGFR on many cell types, the EGF tethering system has a wide array of potential applications in regenerative medicine. Growth factor tethering (and specifically EGF tethering) has been used in a variety of contexts, including improving survival of other cell types such as vascular smooth muscle cells [31] or targeted therapeutic growth factor delivery [32, 33]. In the context of MSC clinical utility, although this study analyzed the effects of tEGF on a construct designed primarily for bone regeneration, the applications of EGF tethering may also extend outside of tricalcium phosphate scaffolds and bone repair in general.

Acknowledgments

We thank the members of the Wells' Laboratory at the University of Pittsburgh and Griffith Laboratory at the Massachusetts Institute of Technology for their helpful and insightful comments on this project. This project was supported by NIH Grants GM063569 and GM069668. A.N. was supported by the National Institute of Biomedical Imaging and Bioengineering-funded CATER T32 (EB001026) and the National Cancer Institute-funded Skin Biology and Cancer T32 (CA175294) training grants. Services were provided in kind by the VA Pittsburgh Health System research and development service line. M.R. is currently affiliated with the Department of Plastic and Reconstructive Surgery, Stanford University, Palo Alto, California, USA.

Author Contributions

A.N. and M.R.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; J.R.: conception and design, collection and assembly of data, data analysis and interpretation, final approval of manuscript; C.Y.: collection and assembly of data, data analysis and interpretation, final approval of manuscript; D.W.: collection and assembly of data, final approval of manuscript; D.S.: data analysis and interpretation, final approval of manuscript; L.G.: conception and design, data analysis and interpretation, financial support, final approval of manuscript; A.W.: conception and design, data analysis and interpretation, financial support, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

References

1 Pittenger MF, Mackay AM, Beck SC et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143–147.

2 Toma C, Pittenger MF, Cahill KS et al. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation 2002;105:93–98.

3 Mackay AM, Beck SC, Murphy JM et al. Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. Tissue Eng 1998;4:415–428.

4 Wang Y, Crisostomo PR, Wang M et al. TGF-alpha increases human mesenchymal stem cell-secreted VEGF by MEK- and PI3-K- but not JNK- or ERK-dependent mechanisms. Am J Physiol Regul Integr Comp Physiol 2008;295:R1115–R1123.

5 Chen L, Tredget EE, Wu PY et al. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. PLoS One 2008; 3:e1886.

6 Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 2005;105:1815–1822.

7 Ren G, Zhang L, Zhao X et al. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. Cell Stem Cell 2008;2:141–150.

8 Gebler A, Zabel O, Seliger B. The immunomodulatory capacity of mesenchymal stem cells. Trends Mol Med 2012;18:128–134.

9 Giannoni P, Scaglione S, Daga A et al. Short-time survival and engraftment of bone marrow stromal cells in an ectopic model of bone regeneration. Tissue Eng Part A 2010;16: 489–499.

10 Hu X, Yu SP, Fraser JL et al. Transplantation of hypoxia-preconditioned mesenchymal stem cells improves infarcted heart function via enhanced survival of implanted cells and angiogenesis. J Thorac Cardiovasc Surg 2008;135: 799–808.

11 Zimmermann CE, Gierloff M, Hedderich J et al. Survival of transplanted rat bone marrow-derived osteogenic stem cells in vivo. Tissue Eng Part A 2011;17:1147–1156.

12 Mylotte LA, Duffy AM, Murphy M et al. Metabolic flexibility permits mesenchymal stem cell survival in an ischemic environment. Stem Cells 2008;26:1325–1336.

13 Haugh JM, Schooler K, Wells A et al. Effect of epidermal growth factor receptor internalization

on regulation of the phospholipase C-gamma1 signaling pathway. J Biol Chem 1999;274: 8958–8965.

14 Iyer AKV, Tran KT, Griffith L et al. Cell surface restriction of EGFR by a tenascin cytotactin-encoded EGF-like repeat is preferential for motility-related signaling. J Cell Physiol 2008; 214:504–512.

15 Fan VH, Tamama K, Au A et al. Tethered epidermal growth factor provides a survival advantage to mesenchymal stem cells. STEM CELLS 2007;25:1241–1251.

16 Rodrigues M, Blair H, Stockdale L et al. Surface tethered epidermal growth factor protects proliferating and differentiating multipotential stromal cells from FasL-induced apoptosis. STEM CELLS 2013;31:104–116.

17 Platt MO, Roman AJ, Wells A et al. Sustained epidermal growth factor receptor levels and activation by tethered ligand binding enhances osteogenic differentiation of multipotent marrow stromal cells. J Cell Physiol 2009;221:306–317.

18 Alvarez LM, Rivera JJ, Stockdale L et al. Tethering of epidermal growth factor (EGF) to beta tricalcium phosphate (βTCP) via fusion to a high affinity, multimeric βTCP-binding peptide: Effects on human multipotent stromal cells/connective tissue Progenitors. PLoS One 2015;10:e0129600.

19 Kuznetsov SA, Cherman N, Robey PG. In vivo bone formation by progeny of human embryonic stem cells. Stem Cells Dev 2011;20: 269–287.

20 Kuznetsov SA, Mankani MH, Robey PG. In vivo formation of bone and haematopoietic territories by transplanted human bone marrow stromal cells generated in medium with and without osteogenic supplements. J Tissue Eng Regen Med 2013;7:226–235.

21 Rodrigues M, Yates CC, Nuschke A et al. The matrikine tenascin-C protects multipotential stromal cells/mesenchymal stem cells from death cytokines such as FasL. Tissue Eng Part A 2013;19:1972–1983.

22 Duffy GP, Ahsan T, O'Brien T et al. Bone marrow-derived mesenchymal stem cells promote angiogenic processes in a time- and dose-dependent manner in vitro. Tissue Eng Part A 2009;15:2459–2470.

23 Wu Y, Chen L, Scott PG et al. Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. STEM CELLS 2007;25:2648–2659.

24 Tamama K, Kawasaki H, Wells A. Epidermal growth factor (EGF) treatment on multipotential stromal cells (MSCs). Possible enhancement of therapeutic potential of MSC. J Biomed Biotechnol 2010;2010:795385.

25 Wang X, McCullough KD, Franke TF et al. Epidermal growth factor receptor-dependent Akt activation by oxidative stress enhances cell survival. J Biol Chem 2000;275: 14624–14631.

26 Downward J. PI 3-kinase, Akt and cell survival. Semin Cell Dev Biol 2004;15:177–182.

27 Platt MO, Wilder CL, Wells A et al. Multipathway kinase signatures of multipotent stromal cells are predictive for osteogenic differentiation: tissue-specific stem cells. STEM CELLS 2009;27:2804–2814.

28 Chen P, Xie H, Sekar MC et al. Epidermal growth factor receptor-mediated cell motility: Phospholipase C activity is required, but mitogen-activated protein kinase activity is not sufficient for induced cell movement. J Cell Biol 1994;127:847–857.

29 Xie H, Pallero MA, Gupta K et al. EGF receptor regulation of cell motility: EGF induces disassembly of focal adhesions independently of the motility-associated PLC gamma signaling pathway. J Cell Sci 1998;111:615–624.

30 Iyer AK, Tran KT, Borysenko CW et al. Tenascin cytotactin epidermal growth factor-like repeat binds epidermal growth factor receptor with low affinity. J Cell Physiol 2007; 211:748–758.

31 Lequoy P, Liberelle B, De Crescenzo G et al. Additive benefits of chondroitin sulfate and oriented tethered epidermal growth factor for vascular smooth muscle cell survival. Macromol Biosci 2014;14:720–730.

32 Wang TY, Bruggeman KA, Sheean RK et al. Characterization of the stability and bio-functionality of tethered proteins on bioengineered scaffolds: Implications for stem cell biology and tissue repair. J Biol Chem 2014;289:15044–15051.

33 Assal Y, Mizuguchi Y, Mie M et al. Growth factor tethering to protein nanoparticles via coiled-coil formation for targeted drug delivery. Bioconjug Chem 2015;26:1672–1677.

See www.StemCellsTM.com for supporting information available online.