Gene Therapy (2006) 13, 1617–1618

www.nature.com/gt

In vitro stem cell expansion

Stepping closer towards self-renewal

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Gene Therapy (2006) **13,** 1617–1618. doi:10.1038/sj.gt.3302804;
published online 1 June 2006

A recently published study by Zhang *et al*.[1] in *Nature Medicine* brings forward a new class of molecules serving as *ex vivo* expansion factors for hematopoietic stem cells (HSCs).

The continuous replenishment of mature blood cells relies on pluripotent HSCs, which can either self-renew and give rise to identical daughter stem cells or engage in differentiation and proliferation pathways involved in specific lineage formation. So far, mechanisms underlying cell fate decisions are not well characterized.

A symmetrical HSC self-renewal division generates two primitive stem cells, termed self-renewal of expansion (SR-E) (Figure 1a), whereas an asymmetrical type of division will generate an HSC and a differentiated progeny, preserving stem cell numbers, and named self-renewal of maintenance (SR-M) (Figure 1b).[2] Clearly, stem cells must undergo SR-E type of divisions to increase their numbers *in vivo*, as would be the case during ontogeny in the fetal liver and shortly after birth,[3] or following myeloablative bone marrow (BM) transplantation, where a critical interplay exists between intrinsic and extrinsic (e.g., micro-environmental) factors.

Attempts to achieve similar levels of HSC self-renewal *in vitro*, with optimization of the culture media with growth factors and cytokine or by retroviral gene transfer to BM cells,[4] have so far been modest, with up to a 40-fold net increase in HSC numbers in a 14-day culture period with BM cells engineered to overexpress HOXB4.[5] Indeed, most *ex vivo* culture conditions lead to symmetrical HSC divisions of differentiation, promptly leading to their depletion (Figure 1c). Maintenance and even modest *ex vivo* expansion of human HSCs would thus have significant impact for gene therapy protocols or for stem cell enrichment of grafts deemed unsuitable for transplantation purposes owing to scarce HSC content, as is often the case with cord blood samples. Avoiding the potential deleterious effects of insertional mutagenesis in doing so would have obvious clinical relevance.[6]

The work by Zhang *et al*.[1] provides further insight into conditions that favor survival and expansion of HSCs outside of their physiological niche. They have identified angiopoietin-like molecules, namely Angptl2 and Angptl3, through DNA microarray analysis of proteins differentially expressed in fetal liver CD3+Ter119 cells, which can support *ex vivo* expansion of HSCs,[7] as potential modulators of SR-M and SR-E divisions in culture. These proteins share similar characteristics with the angiopoietins, for instance, the coiled-coil and fibrinogen-like structural domains, but unlike them, they do not display binding affinity for the Tie tyrosine kinase receptor family, and their signal-transduction pathways are yet undefined.

Using competitive repopulation assays, 20 sorted side population (SP+) Sca+ (CD45.2+) BM cells, enriched in HSC content, were exposed for 5 days *in vitro* to either stem cell factor (SCF) alone or in combination with the conditioned medium of 293T human embryonic kidney cells transfected with Flag-tagged human Angptl2, and then co-transplanted in primary recipients (CD45.1+) together with 10 helper (CD45.1+) HSCs. Analyses of peripheral blood (PB) long-term reconstitution confirmed the *in vitro* depletion of HSCs in the presence of SCF alone, with no contribution from the donor (CD45.2+) cells to blood mature components. However, 10–20% reconstitution levels were observed when donor HSCs were concomitantly exposed to the conditioned supernatant of Flag-Angptl2-transfected cells. The experimental procedure was repeated using the serum-free STIF (*S*CF, *t*hrombopoietin, *i*nsulin-growth factor 2 and *f*ibroblast-growth factor 1) media and prolonging the culture period to 10 days, with or without Angptl2. Of note, the same group of investigators had previously reported eightfold *in vitro* increase in HSC numbers using this combination of growth factors alone.[8] Interestingly, cells exposed to both STIF media and Angptl2-containing supernatant for 10 days contributed more significantly to PB reconstitution (60–70%) at 9 months post-transplantation, than those exposed to STIF media alone (20–30%). These cultured cells retained terminal lymphomyeloid differentiation potential *in vivo* in primary and secondary recipients, with no evidence for malignant transformation.

To further strengthen the causal relationship between specific angiopoietin-like protein exposure and true stem cell expansion, the proteins were purified through affinity chromatography columns, and HSC frequency specifically calculated at

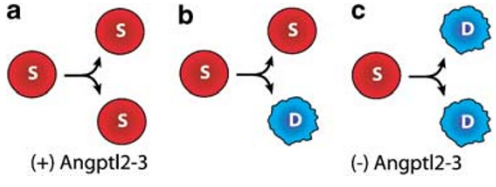

Figure 1 Symmetrical versus asymmetrical SR divisions. (**a**) HSCs can divide symmetrically with one parental cell giving rise to two identical daughter stem cells, hence increasing HSC numbers, a division termed SR-E, as occurs *in vivo* during ontogeny in the fetal liver, shortly following myeloablative BM transplantation, or *ex vivo* when HSCs are cultured in the presence of angiopoietin-like 2 or 3. (**b**) HSCs can also divide asymmetrically and generate both a primitive stem cell and a progeny committed to differentiation, thus preserving HSC numbers. This is termed SR of maintenance or SR-M, mainly the type of division believed to occur in the adult BM. (**c**) A stem cell can also divide symmetrically and give rise to two daughter cells committed to differentiation, a situation leading to HSC depletion, as is usually the case *in vitro* or *in vivo* in certain pathological conditions. Angptl2: angiopoietin-like 2; Angptl3: angiopoietin-like 3; D: differentiated cell; S: stem cell.

initiation and termination of culture, using transplantation at limiting dilution principles, thus enabling net HSC numbers increase to be calculated. At day 0 of culture, the HSC frequency, as evaluated by PB reconstitution at 6 months, was 1/39 for SP Sca+ freshly sorted cells. Upon exposure to Angptl2 and STIF for 10 days, this frequency rose to 1/1.6 cells, representing a net 24-fold *in vitro* expansion, or three times more than single use of STIF,[8] demonstrating the additive effect of this protein on HSC expansion. Considering the relatively modest and additive effect of Angpl2 ($\sim 3\times$) on HSC expansion compared to STIF media alone ($\sim 8\times$), the cellular mechanism (e.g., apoptosis, homing, cell division or self-renewal) affected by this orphan ligand cannot be ascertained until further experiments are performed.

Nonetheless, the authors provide compelling evidence regarding the pluripotency and the long-term repopulation potential of the expanded cells, confirming their stem cell nature. Comparable results, in terms of magnitude of HSC expansion, were obtained with purified Angptl3. The authors also underscore the importance of post-translational modifications present on human Angptl2, presumably glycosylation, and of the coiled-coil domain in carrying the reported effect on HSCs. Additional angiopoietin-like molecules, namely mouse Angptl3, human Angptl5, human Angptl7 and human microfibril-associated glycoprotein 4, also conferred an *in vivo* competitive repopulating advantage on *in vitro* exposed SP Sca1+ cells.

In conclusion, these studies provide an additional tool towards expanding HSCs, keeping in mind that the optimal setting or factor(s) that persistently trigger HSC self-renewal *in vitro* remain to be identified. ■

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Published online 1 June 2006

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