Angiopoietin-like 5 and IGFBP2 stimulate ex vivo expansion of human cord blood hematopoietic stem cells as assayed by NOD/SCID transplantation

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Hematopoietic stem cells (HSCs) are the basis of bone marrow transplantation and are attractive target cells for hematopoietic gene therapy, but these important clinical applications have been severely hampered by difficulties in ex vivo expansion of HSCs. In particular, the use of cord blood for adult transplantation is greatly limited by the number of HSCs. Previously we identified angiopoietin-like proteins and IGFBP2 as new hormones that, together with other factors, can expand mouse bone marrow HSCs in culture. Here, we measure the activity of multipotent human severe combined immunodeficient (SCID)–repopulating cells (SRCs) by transplantation into the nonobese diabetic SCID (NOD/SCID) mice; secondary transplantation was performed to evaluate the self-renewal potential of SRCs. A serum-free medium containing SCF, TPO, and FGF-1 or Flt3-L cannot significantly support expansion of the SRCs present in human cord blood CD133+ cells. Addition of either angiopoietin-like 5 or IGFBP2 to the cultures led to a sizable expansion of HSC numbers, as assayed by NOD/SCID transplantation. A serum-free culture containing SCF, TPO, FGF-1, angiopoietin-like 5, and IGFBP2 supports an approximately 20-fold net expansion of repopulating human cord blood HSCs, a number potentially applicable to several clinical processes including HSC transplantation.

Methods

Mice

Nonobese diabetic SCID (NOD/SCID; NOD.CB17-Prkdcscid/J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were

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maintained at the Whitehead Institute or University of Texas Southwestern Medical Center animal facilities. All animal experiments were performed with the approval of MIT or University of Texas Southwestern Committee on Animal Care.

Culture medium

STIF medium is StemSpan serum-free medium (StemCell Technologies, Vancouver, BC) supplemented with 10 μg/mL heparin (Sigma-Aldrich, St Louis, MO), 10 ng/mL mouse SCF, 20 ng/mL mouse TPO, 20 ng/mL mouse IGF-2 (all from R&D Systems, Minneapolis, MN), and 10 ng/mL human FGF-1 (Invitrogen, Frederick, MD). STIF medium is the same medium without IGF-2. When medium contained Flt3-L, we used 50 ng/mL human SCF, 10 ng/mL human TPO, and 50 ng/mL human Flt3-L. Indicated amounts of human Angptl5 (Abnova, Taipei City, Taiwan) or human IGFBP2 (R&D Systems) were added.

Human cell culture

Fresh and cryopreserved human cord blood cells were purchased from Cambrex (East Rutherford, NJ), StemCell Technologies, and AllCells (Berkeley, CA). All of cells were from pooled donors. CD34+ or CD133+ purity checked by flow cytometry was higher than 90%. After thawing, the cell viability tested by trypan blue exclusion was higher than 72%. The thawed cells were centrifuged and resuspended with StemSpan medium before being aliquoted for immediate transplantation or culture. In Figure 1, total human cord blood mononuclear cells were plated at 10⁶ cells/mL STIF medium, with 100 ng/mL Angptl3 or Angptl5. Medium volume was decreased to 105/mL (day 7).

Flow cytometry

For analyzing human hematopoietic engraftment in NOD/SCID mice, we followed a published protocol. Briefly, bone marrow cells from recipient NOD/SCID mice were stained with antihuman CD45-PE, CD71-PE, CD15-FITC, and CD66b-FITC to quantify the total human hematopoietic (CD45/71+CD15/66b+) population. Antihuman CD34-FITC was used to quantitate CD34+ cells in culture. All antihuman antibodies were purchased from Becton Dickinson (Mountain View, CA).

Results

Culture of total human cord blood cells in serum-free medium containing Angptl5 stimulates ex vivo expansion of SRCs

Recently we showed that culture of mouse bone marrow side population (SP) CD45 Sca-1+ cells in serum-free medium containing 10 ng/mL SCF, 20 ng/mL TPO, 20 ng/mL IGF-2, and 10 ng/mL FGF-1 (STIF medium) supplemented with one of several Angptls—Angptl2, Angptl3, Angptl5, Angptl7, and Mfap4—stimulated a dramatic expansion of LT-HSC numbers and activities. We sought to test whether this culture could also expand human cord blood HSCs. To this end, total human cord blood cells (Figure 1A) were
cultured in STIF medium containing Angptl5.\textsuperscript{18} We seeded $2.5 \times 10^7$ total cord blood cells at a density of $10^6$ cells/mL medium. After 23 days of culture, the number of total cells in the presence of Angptl5 increased to $2.2 \pm 0.3 \times 10^8$ cells (Figure 1A). The cultured cells were mostly in suspension with a minor adherent subpopulation.

We performed NOD/SCID repopulation assays\textsuperscript{19} to test whether ex vivo–expanded cells were capable of engraftment. Thus, $10^4$ uncultured cells or their cultured progenies were injected into sublethally irradiated NOD/SCID recipients (Figure 1B). When $9.2 \times 10^6$ cells cultured with Angptl5 for 19 days, that is, the progeny of $10^6$ initially plated total cord blood cells, were transplanted, an average human hematopoietic chimerism of 8.8\% was observed 2 months after transplantation (Figure 1B lane 2). This is much greater than the 0.6\% engraftment shown by the equivalent $10^6$ uncultured cells (Figure 1B lane 1; $P < .001$, Student $t$ test). Since the cells cultured in STIF medium with Angptl5 exhibited significantly higher engraftment than uncultured cells, we opted to use Angptl5 in our further experiments.

**Angptl5 and IGFBP2 individually stimulate ex vivo expansion of cultured human cord blood CD34\(^+\) and CD133\(^+\) cells**

We next sought to test whether we could expand freshly isolated human cord blood CD34\(^+\) cells, an enriched HSC population routinely used in clinical applications. We seeded $10^4$ CD34\(^+\) cells; as shown in Figure 2A, after 11 days of culture, the number of cells cultured in the presence of SCF + TPO + FGF-1 (STF medium) increased more than 250-fold. Addition of Angptl5 did not significantly change the overall extent of cell proliferation. We noticed that, at the end of the culture, most cells were in suspension but there was a minor adherent subpopulation.

Next, fresh CD34\(^+\) cells and their cultured progenies were transplanted into sublethally irradiated NOD/SCID mice. Ten thousand fresh CD34\(^+\) cells generated an average of chimerism of 0.4\% at 2 months after transplantation (Figure 2B lane 1). The progenies of 5000 initially plated cells that were cultured in STF medium had a modest engraftment of 0.4\% ($\pm$ 0.1\%; Figure 2B lane 2). Importantly, addition of Angptl5 to the culture medium significantly increased the engraftment to 6.2\% ($\pm$ 0.5\%; Figure 2B compare lane 3 vs lanes 1 and 2, $P < .05$). This demonstrates that Angptl5 supports ex vivo expansion of human SRCs.

In a recent study, we showed that the secreted protein IGF-binding protein 2 (IGFBP2) is able to support ex vivo expansion of mouse HSCs. IGFBP2 can replace IGF-2 in HSC cultures; a serum-free medium containing SCF, TPO, FGF-1, Angptl3, and IGFBP2 supports a dramatic increase in numbers of long-term repopulating mouse HSCs, as measured by competitive repopulation analyses (H.H., S.I., M.K., O. Kirak, H.F.L., and C.C.Z., unpublished data, September 2007).

To test the role of IGFBP2 on human HSCs, and to confirm the effect of Angptl5, we used a different serum-free medium containing human SCF, TPO, and Flt3-L, a commonly used cocktail for human cell culture, as the basal medium to culture cryopreserved CD133\(^+\) cord blood cells. The total number of cells increased more than 210–, 162–, and 220-fold in the basal medium, basal medium supplemented with IGFBP2, and basal medium supplemented with Angptl5, respectively, over the 10-day culture (Figure 3A). Next, $8000$ uncultured CD133\(^+\) cells, or their cultured progenies, were transplanted into NOD/SCID mice. Both IGFBP2 and Angptl5 significantly enhanced ex vivo expansion of SRCs (Figure 3B). Transplantation of cells cultured in the presence of SCF, TPO, and Flt3-L generated mice that had an average of 0.8\% human cells in the bone marrow. Addition of IGFBP2 or Angptl5 to the culture increased the chimerism to 11.3\% and 17.3\%, respectively (Figure 3B compare lanes 1, 5, and 9). Both IGFBP2 (Figure 3B lanes 6–8) and Angptl5 (Figure 3B lanes 10–12) led to increases in the formation of human myeloid cells (CD15/66b\(^+\)), B-lymphoid cells (CD34\(^–\)CD19/20\(^+\)), and primitive (CD34\(^+\)CD19/20\(^–\)) human cells in the bone marrow (Figure 3B). Thus either IGFBP2 or Angptl5, when added together with SCF, TPO, and Flt3-L, stimulated expansion of human cord blood SRCs.

**IGFBP2 and Angptl5 together stimulate extensive ex vivo expansion of SRCs of cultured human cord blood CD133\(^+\) cells**

In the experiment described in Figure 4, a representative of 3 independent experiments, we added IGFBP2 and Angptl5, together with SCF, TPO, and FGF-1 to our serum-free culture. A recent report suggested that hypoxia improves expansion of human SRCs\textsuperscript{20} and thus in parallel we sought to use a low O\(_2\) pressure (5\% O\(_2\)) to expand human HSCs. As shown in Figure 4A, we plated $10^4$ cryopreserved human cord blood CD133\(^+\) cells; after 11 days of culture in 5\% O\(_2\), the total numbers of cells increased greater
than 200-fold either in STF medium or STF medium containing Angptl5 and IGFBP2.

We transplanted cells before and after culture into sublethally irradiated NOD/SCID mice. Eight thousand uncultured CD133+ cells had an average chimerism of 0.2% at 2 months after transplantation (Figure 4B lane 1) and 15,000 uncultured CD133+ cells showed an increased but still modest engraftment—an average chimerism of 2.0% (Figure 4B lane 2). In striking contrast, 2.1 × 10^6 cells after 11 days of culture with SCF, TPO, FGF-1, Angptl5, and IGFBP2—that is, the progenies of 8000 initial cells—engrafted all recipient mice, and showed significantly increased chimerism relative to that from 8000 or 15,000 uncultured cells (average 39.5%; Figure 4B lane 4; P < .05, Student t test). In contrast, the cultured progenies of the same 8000 initial cells cultured in STF medium without Angptl5 and IGFBP2 (now 1.6 × 10^6 cells) exhibited poor engraftment, similar to that of their uncultured counterparts (Figure 4B lane 3).

Figure 4C shows human hematopoietic engraftment at 2 months in representative mice that received a transplant of uncultured or cultured human cord blood CD133+ cells. A mouse that received a transplant of cells cultured in STF medium containing both Angptl5 and IGFBP2 (Figure 4B lane 4) displayed a much higher engraftment of total hematopoietic (CD45.1+ 58%), myeloid (CD11b+ 8.1%), B-lymphoid (CD19/CD20+ 37%), and primitive (CD34+ 4%) human cells than the mouse that received the transplant of uncultured cells (Figure 3B lane 1: 0.19%, 0.03%, 0.1%, and 0.02%, respectively). A summary of multilineage engraftment of mice that received a transplant of uncultured cells (Figure 4B lane 2) and cells cultured in STF medium containing Angptl5 and IGFBP2 (Figure 4B lane 4) is shown in Figure 4D. The progenies of 8000 cells, after culture, repopulated myeloid and lymphoid lineages 2 months after transplantation, attesting to the expansion of human stem cell activity.

To measure the self-renewal potential of SRCs after culture in medium containing IGFBP2 and Angptl5, we collected bone marrow from the primary mice that received a transplant of uncultured cells (Figure 4B lane 2) or cells cultured in STF medium containing Angptl5 and IGFBP2 (Figure 4B lane 4) and transplanted the marrow into sublethally irradiated secondary recipients.15,21 While uncultured cells could not engraft secondary recipients (not shown), the cultured cells showed positive engraftment of myeloid, B-lymphoid, and primitive cells after the secondary transplantation (Figure 4E). These data indicate a net expansion of HSCs during the initial culture period, and we thus conclude that Angptl5 and IGFBP2 together support extensive ex vivo expansion of human SRCs.

We confirmed that we were able to dramatically expand human SRCs in our culture system in 2 additional independent experiments that also directly tested the response of HSCs to culture in low and ambient oxygen. In a representative study, we cultured 2 × 10^5 cryopreserved human cord blood CD133+ cells in STF medium containing Angptl5 and IGFBP2 under normal or low O₂
Figure 4. Culture of human cord blood CD133+ cells in the presence of Angptl5 and IGFBP2 stimulates ex vivo expansion of SRCs. (A) Culture of 10⁴ cryopreserved human cord blood CD133+ cells was initiated in serum-free STF medium (containing SCF, TPO, and FGF-1), or in STF medium supplemented with 500 ng/mL human Angptl5 and 500 ng/mL human IGFBP2, at 5% O₂. Total cell numbers were counted during the 11 days of culture period. (B) Extent of human chimerism in the bone marrow of NOD/SCID mice that received a transplant of 8000 or 15 000 uncultured human cord blood CD133+ cells, or the progenies of 8000 initial CD133+ cells cultured in STF medium with or without Angptl5 and IGFBP2 for 11 days. Each symbol represents the engraftment of a single mouse that underwent transplantation assayed at 2 months after transplantation (n=7-8). *Significantly different from lanes 1-3. Student t test, P<.05. (C) Representative fluorescence-activated cell sorting (FACS) plots of bone marrow cells from one mouse at the condition represented by lane 1 of panel B (after thaw), or at the condition represented by lane 4 of panel B (cultured in STF medium containing IGFBP2 and Angptl5) at 2 months after transplantation. Percentages of cells in each quadrant are listed. (D) Summary of multilineage reconstitution from mice in lanes 2 and 4 of panel B (n=8, respectively). Some mice that received a transplant of uncultured cells had 0% donor repopulation and these data points are not plotted. *Values are significantly different from values of the uncultured cells. Student t test, P<.05. (E) Bone marrow cells collected from mice represented by lane 4 of panel B were transplanted into secondary recipients; bone marrow aspirate from one hind leg from a primary recipient was transplanted into 2 secondary recipients. Multilineage engraftment in secondary NOD/SCID recipients was assayed at 5 to 8 weeks after transplantation (n=12 mice underwent transplantation).
conditions. After 10 days of culture, the number of total cells in both cultures had increased more than 200-fold (Figure 5A). We did observe a slightly higher number of CD34+/H11001 primitive cells after 5 days of culture at normal versus low O2 (Figure 5B). As part of the limiting-dilution assay to quantitate the SRC frequencies before and after culture, we measured the engraftment by 5000, 10000, and 20000 uncultured CD133+/H11001 cells, and the progenies of 1667, 5000, or 10000 CD133+/H11001 cells cultured in STF medium with Angptl5 and IGFBP2, or low or normal O2 levels (E). Plotted is the percentage of recipient mice containing less than 1% human hematopoietic populations in recipient mouse bone marrow 6 to 8 weeks after transplantation versus the number of input or input-equivalent cells injected. The progenies of 10000 input cells cultured at normal or low O2 repopulated all recipients and these data points (0% negative mice) are not plotted.

To make a comparison possible between cultured and uncultured cells, we arbitrarily chose to use 1% chimerism as the cutoff for positive engraftment. Based on this standard, Figure 5D shows that the frequency of repopulating cells (CRUs) for this particular sample of uncultured CD133+/H11001 cells is 1 per 64075 cells (95% confidence interval for mean: 1/23919 to 1/171643, n = 25). That is, as calculated from Poisson statistics, injection of an average of 64075 cells from this lot of uncultured human CD133+/H11001 cells is sufficient to repopulate 63% (1 - e^-1) of mice that underwent transplantation. Figure 5E shows there was an 8- or 20-fold increase in the number of SRCs after cultured in STF medium containing Angptl5 and IGFBP2 at normal or low O2 levels, respectively. Specifically, when cells were cultured in STF medium containing Angptl5 and IGFBP2 at low O2, the CRU frequency was 1/7871 normalized to the number of input cells (95% confidence interval

![Figure 5. Limiting-dilution analysis of human cord blood CD133+ cells transplanted into NOD/SCID mice after culture at normal or low oxygen levels.](image-url)

(A,B) Culture of 2 x 10^5 cryopreserved human cord blood CD133+ cells was initiated in serum-free STF medium supplemented with 500 ng/mL human Angptl5 and human 100 ng/mL IGFBP2. The numbers of total cells (A) and CD34+ cells (B) were counted. (C) Amount of human chimerism in the bone marrow of NOD/SCID mice that received a transplant of the indicated numbers (5000, 10000, 20000) of postthaw uncultured human cord blood CD133+ cells, or the progenies of 1667, 5000, or 10000 CD133+ cells cultured in STF medium with Angptl5 and IGFBP2 in ambient oxygen (lanes 4-6) or 5% oxygen (lanes 7-9) for 10 days. Each symbol represents the engraftment of a single mouse that underwent transplantation assayed at 2 months after transplantation. Horizontal dotted lines represent arbitrary cutoffs of 0.1% and 1% reconstitution. (D,E) Limiting-dilution analysis of the repopulating ability of cells before culture (D) and after culture for 10 days in serum-free STF medium containing 500 ng/mL Angptl5 and 100 ng/mL IGFBP2 at normal or low O2 levels (E). Plotted is the percentage of recipient mice containing less than 1% human hematopoietic populations in recipient mouse bone marrow 6 to 8 weeks after transplantation versus the number of input or input-equivalent cells injected. The progenies of 10000 input cells cultured at normal or low O2 repopulated all recipients and these data points (0% negative mice) are not plotted. (F) Multilineage engraftment in NOD/SCID recipients that received a transplant of 20000 postthaw uncultured CD133+ cells (n = 8) or cultured progenies of 5000 initial CD133+ cells at normal O2 (n = 10). Some mice that received a transplant of uncultured cells had 0% donor repopulation and these data points are not plotted.
for mean: 1/3460 to 1/17 903, n = 16), approximately 8-fold greater than that of the uncultured cells. Strikingly, when we cultured the cells at normal O2, the CRU frequency increased to 1/3235 input equivalent cells (95% confidence interval for mean: 1/1908 to 1/5486, n = 27). These cultured cells had much greater levels of multilineage engraftment than uncultured cells (Figure 5F).

To derive another estimation of the extent of ex vivo expansion of human HSCs, we combined data from Figures 4 and 5, and calculated CRUs using 0.1% and 1% engraftment as the cutoff, respectively (Table 1). As expected, the CRU values calculated based on the different cutoffs for positive engraftment are different. Nevertheless, and importantly, the increase in the CRU value of cells cultured at 5% O2 was again approximately 8-fold (= 43 992/3235), and at normal O2 was approximately 14-fold (= 43 992/3235). All these calculations indicate that the total number of functional human SRCs increased approximately 14- to 20-fold following culture in medium containing IGFBP2 and Angptl5 at normal O2. Table 2 summarizes the culture conditions and some of the key data presented in Figures 1 through 5.

### Discussion

Here, we describe a simple and robust culture system for ex vivo expansion of human cord blood HSCs. Our defined serum-free medium contains 3 well-studied hematopoietic growth factors, SCF, TPO, and FGF-1, together with 2 novel hematopoietic growth factors that we recently identified: Angptl5 and IGFBP2. As measured by transplantation in NOD/SCID mice, this culture supported a 20-fold increase of human SRCs.

Angptls are a family of secreted glycoproteins that share limited sequence homology with angiopoietins.22 However, given the paucity of publications on these Angptls, we presume that most of their physiological activities remain to be discovered. Recently, we showed that mouse fetal liver CD34+ cells, which support the ex vivo expansion of mouse HSCs, specifically expressed Angptl2 and Angptl3. A 24- or 30-fold net expansion of long-term mouse bone marrow HSCs was observed by reconstitution analysis when highly enriched HSCs were cultured for 10 days in serum-free medium in the presence of Angptl2 or Angptl3 together with saturating levels of SCF, TPO, IGF-2, and FGF-1. Among other Angptls tested, Angptl5 and Angptl7 also stimulated expansion of mouse HSCs.14 Interestingly, we showed here that Angptl5 stimulated ex vivo expansion of human HSCs. It is likely that other Angptls will also increase expansion of human SRCs and we are currently testing several.

IGF-binding proteins (IGFBPs) are a family of circulating proteins that bind IGF-1 and IGF-2 with an affinity equal to or greater than that of the IGF receptors. IGFBPs modulate the biologic effects of IGFs by controlling the distribution, function, and activity of IGFs.23 Human IGFBP2 is a secreted protein that

### Table 1. Combined data from Figures 4 and 5 for limiting-dilution analysis of human cord blood CD133+ cells transplanted into NOD/SCID mice before and after culture at normal or low oxygen levels

<table>
<thead>
<tr>
<th>Input equivalent cell no.</th>
<th>0.1% engraftment as cutoff</th>
<th>1% engraftment as cutoff</th>
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</thead>
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<tr>
<td>Uncultured CD133+ cells</td>
<td>Positive mice vs total mice</td>
<td>1/CRU</td>
</tr>
<tr>
<td>5000</td>
<td>6/9</td>
<td>10256</td>
</tr>
<tr>
<td>8000</td>
<td>2/7</td>
<td>—</td>
</tr>
<tr>
<td>10000</td>
<td>7/8</td>
<td>—</td>
</tr>
<tr>
<td>15000</td>
<td>4/8</td>
<td>—</td>
</tr>
<tr>
<td>20000</td>
<td>7/8</td>
<td>—</td>
</tr>
<tr>
<td>Cultured CD133+ cells at normal O2</td>
<td>&lt;1667</td>
<td>3235</td>
</tr>
<tr>
<td>1667</td>
<td>7/7</td>
<td>—</td>
</tr>
<tr>
<td>5000</td>
<td>10/10</td>
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<tr>
<td>10000</td>
<td>10/10</td>
<td>—</td>
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<tr>
<td>Cultured CD133+ cells at 5% O2</td>
<td>&lt;1667</td>
<td>5034</td>
</tr>
<tr>
<td>1667</td>
<td>6/6</td>
<td>—</td>
</tr>
<tr>
<td>5000</td>
<td>10/10</td>
<td>—</td>
</tr>
<tr>
<td>8000</td>
<td>8/8</td>
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</table>

— indicates not applicable.

### Table 2. Summary of culture conditions and SRC expansion based on data presented in Figures 1 through 5

<table>
<thead>
<tr>
<th>Figure no.</th>
<th>CB cells</th>
<th>Cytokines added and culture duration</th>
<th>O2</th>
<th>Average fold increase in nucleated cells</th>
<th>SRC expansion</th>
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<tbody>
<tr>
<td>1</td>
<td>Total nucleated cells</td>
<td>SCF + TPO + FGF-1 + IGF-2 + Angptl5 19d</td>
<td>Normal</td>
<td>19</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>CD34+ (fresh)</td>
<td>SCF + TPO + Flt3L 11d</td>
<td>Normal</td>
<td>257</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SCF + TPO + Flt3L + Angptl5 11d</td>
<td>Normal</td>
<td>283</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>CD133+</td>
<td>SCF + TPO + Flt3L 10d</td>
<td>Normal</td>
<td>210</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SCF + TPO + Flt3L + Angptl5 10d</td>
<td>Normal</td>
<td>220</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SCF + TPO + Flt3L + IGFBP2 10d</td>
<td>Normal</td>
<td>162</td>
<td>++</td>
</tr>
<tr>
<td>4</td>
<td>CD133+</td>
<td>SCF + TPO + FGF-1 11d</td>
<td>Low</td>
<td>203</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SCF + TPO + FGF-1 + Angptl5 + IGFBP2 11d</td>
<td>Low</td>
<td>266</td>
<td>++</td>
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<tr>
<td>5</td>
<td>CD133+</td>
<td>SCF + TPO + FGF-1 + Angptl5 + IGFBP2 10d</td>
<td>Normal</td>
<td>231</td>
<td>++</td>
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<td>SCF + TPO + FGF-1 + Angptl5 + IGFBP2 10d</td>
<td>Low</td>
<td>258</td>
<td>++</td>
</tr>
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</table>

The symbols denote relative, not absolute, values. ++ represents a greater expansion than +. We used freshly isolated CD34+ cells in the experiment depicted in Figure 2, and used cryopreserved cells in other experiments.
preferentially binds IGF-2 relative to IGF-1.\textsuperscript{24,25} In addition to modulating the biologic activities of IGF-1 and IGF-2, IGFBP2 may also have intrinsic bioactivities that are independent of IGFs.\textsuperscript{26} To our knowledge, this is the first report that demonstrates an effect of IGFBP2 on human HSCs.

It is intriguing that 5 factors are needed for maximal expansion of both mouse and human HSCs, though the specific factors may be different in the 2 species. As is well known, HSCs have several cell fates including self-renewal, differentiation, apoptosis, and quiescence. SCF and FGF-1 activate different receptor-protein-tyrosine kinases,\textsuperscript{12,27,28} while TPO signals through a member of the cytokine receptor superfamily that requires a Janus kinase to activate several intracellular signal transduction pathways.\textsuperscript{29} We previously suggested that the Angptls activate signal transduction pathways that cannot be activated by SCF, TPO, or FGF-1; however, until we clone and characterize the receptors for the Angptl proteins as well as the presumed IGFBP2 receptor(s) we will be unable to understand how these proteins synergize with the other growth factors to stimulate HSC expansion.\textsuperscript{30} It is possible that these individual protein factors affect different fates of HSCs, and it will be important to study how these factors synergize to stimulate a net expansion of HSC numbers. Some, for instance, could stimulate cell division, while others might prevent differentiation of the divided cells, and still others might prevent apoptosis.

Our ex vivo expansion system works for both freshly isolated and cryopreserved cord blood cells; the cryopreserved samples we used in our studies were purchased from commercial vendors. It is likely that multiple factors, including the cell-cycle status of the cells, storage medium, the procedures for centrifuging the cells, and the components of the resuspension medium, affect the ability of the stem cells to engraft a recipient mouse. Our limiting-dilution experiments strongly suggest that, under our culture conditions where there is a considerable enhancement of the engraftment of cultured hematopoietic precursor cells, there is a time- and cell division–dependent increase in the actual numbers of stem/precursor cells. Alternatively, we cannot exclude the possibility that one or more of our factors enhanced (eg, through induction of a specific set of HSC genes) the homing, survival, efficiency of engraftment, or some other properties of HSCs.

One report suggests that hypoxia improves expansion of human SRCs.\textsuperscript{20} We did observe a significant expansion of cord blood HSCs in a hypoxic culture, but unexpectedly SRC expansion was even greater under normoxic pressure. Whether our observation is related to the particular culture conditions we used needs to be clarified in future studies.

Numerous attempts have been made to improve the efficiency of ex vivo expansion of cord blood HSCs. These include the development of liquid culture systems, HSC–mesenchymal stromal cell cocultures, and continuous perfusion systems, and the use of transcription inhibitors, copper chelators, and glycogen synthase kinase-3 inhibitors (see review in Brunstein and Wagner;\textsuperscript{2} Robinson et al;\textsuperscript{3} and Hofmeister et al\textsuperscript{31}). However, all previous clinical trials using ex vivo–expanded cells in human bone marrow transplantation failed to show significantly improved hematopoietic recovery.\textsuperscript{5,32} Our use of Angptls and IGFBP2 for the ex vivo expansion of human SRCs may translate directly into increased clinical applications of cord blood for bone marrow transplantation. Our technology also likely will be useful for the development of novel strategies of cell and gene therapies that use HSCs.

Acknowledgments

We thank Dr Catherine J. Wu of the Dana-Farber Cancer Institute for advice in experiments, and Drs Oktay Kirak, Rudolf Jaenisch, and Robert Weinberg of the Whitehead Institute for providing the low O\textsubscript{2} incubator. We are grateful to Dr Ai Kotani Yoshida for critically reading the paper.

This work was supported by National Institutes of Health (NIH) grant K01 CA 120099-01 and the Michael L. Rosenberg Endowed Scholar Fund from University of Texas Southwestern (C.C.Z.). Support to H.F.L. is from NIH grant R01 DK 067356.

Authorship

Contribution: C.C.Z. contributed to design, experimental performance, interpretation, and writing; M.K., S.I., and H.H. contributed to experimental performance; and H.F.L. contributed to design, interpretation, and writing.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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