bjh research paper

An activin receptor IIA ligand trap promotes erythropoiesis resulting in a rapid induction of red blood cells and haemoglobin

Soraya Carrancio,[1] Jennifer Markovics,[1] Piu Wong,[1] Jim Leisten,[2] Paola Castiglioni,[2] Matthew C. Groza,[2] Heather K. Raymon,[2] Carla Heise,[1] Tom Daniel,[2] Rajesh Chopra[3] and Victoria Sung[1]

[1]*Celgene Corporation, San Francisco,*
[2]*Celgene Corporation, San Diego, CA, and*
[3]*Celgene Corporation, Summit, NJ, USA*

Received 29 October 2013; accepted for publication 17 January 2014
Correspondence: Rajesh Chopra, MD, PhD, Celgene Corporation, 86 Morris Avenue, Summit, NJ 07901, USA.
E-mail: rachopra@celgene.com

Summary

Sotatercept (ACE-011), a recombinant human fusion protein containing the extracellular domain of the human Activin receptor IIA, binds to and inhibits activin and other members of the transforming growth factor -β (TGF-β) superfamily. Administration of sotatercept led to a rapid and sustained increase in red blood cell (RBC) count and haemoglobin (Hb) in healthy volunteers (phase I clinical trials), but the mechanism is not fully understood. Mice treated with RAP-011 (murine ortholog of ACE-011) respond with a rapid (within 24 h) increase in haematocrit, Hb, and RBC count. These effects are accompanied by an equally rapid stimulation of late-stage erythroid precursors in the bone marrow (BM). RAP-011 also induces a significant increase in erythroid burst-forming units and erythropoietin, which could contribute to additional, sustained effects on RBC production. Further *in vitro* co-culture studies demonstrate that BM accessory cells are required for RAP-011 effects. To better understand which TGF-β family ligand(s) mediate RAP-011 effects, we evaluated the impact of several of these ligands on erythroid differentiation. Our data suggest that RAP-011 may act to rescue growth differentiation factor 11/Activin A-induced inhibition of late-stage erythropoiesis. These data define the mechanism of action of a novel agent that regulates RBC differentiation and provide the rationale to develop sotatercept for the treatment of anaemia and ineffective erythropoiesis.

Keywords: haemoglobin, erythropoiesis, activin receptor IIA inhibition.

The regulation of erythropoiesis in the steady state is homeostatic, with the number of erythrocytes produced being equal to those lost through senescence; however, in the event of anaemia, the balance is offset, resulting in the activation of a stress response which acts to compensate for the drop in haematocrit (Hct) and haemoglobin (Hb) (Paulson *et al*, 2011). The regulation of early erythropoiesis requires stem cell factor (SCF, also termed KITLG) and erythropoietin (EPO) for the proliferation and survival of erythroid cells (von Lindern *et al*, 2004; Richmond *et al*, 2005). Anaemia, as defined by a decline in circulating red blood cells (RBCs), is one of the most common haematological pathologies in both adults and children. Treatment of anaemia ranges from dietary iron supplementation and other vitamins/minerals essential for erythropoiesis to blood transfusions and treatment with corticosteroids and/or EPO, the primary signal that drives stress erythropoiesis. However, current erythropoiesis-stimulating agents (ESAs) have limitations, including undesirable and serious side effects (cardiovascular and tumour-promoting), high cost of manufacture and non-convenient routes of administration (Lombardero *et al*, 2011). Most importantly, many patients with chronic kidney disease, myelodysplastic syndrome (MDS) and thalassaemia (characterized as 'ineffective erythropoiesis'), simply do not respond to EPO (Lindberg, 2005; Gardenghi *et al*, 2010; Ramanath *et al*, 2012). There is therefore a clinical need for novel agents that have a different mechanism of action from existing ESAs.

Members of the transforming growth factor beta (TGF-β) superfamily have been studied as potential regulators of

First published online 18 March 2014
doi: 10.1111/bjh.12838

erythropoiesis, iron regulation and globin expression, but data in the literature is mixed. Although Activin A was initially described as erythroid differentiation factor and shown to stimulate the maturation and differentiation of RBCs (Murata *et al*, 1988), the mechanism by which Activin A influences erythropoiesis remains unclear and, in fact, there are data from *in vitro* and *in vivo* studies that support both erythropoiesis-stimulatory (Shiozaki *et al*, 1989, 1992) and erythropoiesis-inhibitory effects (Nakao *et al*, 1991; Irion *et al*, 2010). Similar data have also been attributed to Activin B (Uchiyama & Asashima, 1992) and bone morphogenetic protein 4 (BMP4) has been described as a key regulator of response in stress anaemia (Lenox *et al*, 2009; Wu & Paulson, 2010).

Sotatercept (ACE-011), a recombinant fusion protein consisting of the extracellular domain of the human Activin receptor IIA (ActRIIA) linked to the human immunoglobulin G1 (IgG1) Fc domain, is a ligand trap which binds a number of TGF-β superfamily ligands including Activin A, Activin B, growth differentiation factor 11 (GDF11) and BMP10 (Table SI). The competitive binding of these ligands in blood and tissues by sotatercept results in inhibition of the ActRIIA receptor signalling pathway, impeding biological processes attributed to these pleiotropic proteins.

Administration of sotatercept induces an increase in RBC count and Hb in healthy human subjects, but the mechanism is not fully understood (Ruckle *et al*, 2009). The rapid onset of its erythrocytic effects suggests that sotatercept may target the later stages of erythropoiesis, differentiating it from EPO, which acts on earlier stages of erythroid development. A recent study has suggested that sotatercept may partially rescue inhibition of erythroid differentiation by bone marrow stromal factors (Iancu-Rubin *et al*, 2013). Here, we have explored the potential cellular and biochemical mechanisms by which sotatercept may regulate erythropoiesis. Defining specific erythroid populations that are targeted and stimulated by a murine ortholog of ACE-011 (RAP-011) will help to determine the role of TGF-β members in the regulation of erythropoiesis. The differential mechanism of action of sotatercept compared to ESAs provides an alternative for the treatment of anaemia associated with various pathologies.

Materials and methods

Compound

The extracellular domain (ECD) sequence of ActRIIA is completely conserved among numerous species including mouse, rat, cynomologus monkey and humans. However, in order to reduce the potential immunogenicity of the human molecule and to maximize the opportunity to maintain exposures in chronic models, a murine surrogate molecule was constructed by exchanging the human IgG1 Fc sequence portion of sotatercept with its murine IgG2a homolog. The resultant construct, referred to as RAP-011 (ActRIIA-mIgG2aFc) has similar *in vitro* binding characteristics as sotatercept and has been routinely employed in both cellular and pharmacology studies.

Mouse models

Six- to 8-week-old C57BL/6 female mice were purchased from Charles River (Morrisville, NC, USA). Animal handling and experimental procedures were conducted in accordance with national and institutional guidelines for animal care and use.

Phosphate-buffered saline (PBS) (vehicle control), RAP-011 (30 mg/kg) or recombinant human EPO (rhEPO) (600 iu/kg) were administered intraperitoneally on day 0. At 24, 48, 72, 96 h and 7 d post-treatment, animals were sacrificed and peripheral blood (PB), bone marrow (BM) and spleen were collected. PB was evaluated for complete blood counts (Quality Veterinary Laboratory, Davis, CA, USA) and plasma was saved to perform mouse EPO quantification by enzyme-linked immunosorbent assay (Quantikine; R&D Systems, Minneapolis, MN, USA; kit does not recognize rhEPO). BM cells were obtained by flushing both femurs and spleen-cell preparations were obtained by gently crushing the tissues to release the cells. Preparations were filtered to remove debris and washed twice in PBS and BM and spleen cells were assayed by flow cytometry for erythroid precursor (Figure S1) and reticulocyte counts as well as seeded in semi-solid media for erythroid burst-forming units/erythroid colony-forming units (BFU-E/CFU-E) quantification and RNA and proteins were collected to measure Hb content (Supporting information). The relative quantification of gene expression was performed using the cycle threshold increment method.

Direct culture of human cells

Human BM CD34+ cells from healthy donors were plated in Methocult media to assess BFU-E and CFU-E growth (Supporting information) (Lopez-Holgado *et al*, 2005). Human CD36+ cells were cultured in liquid media to evaluate RAP-011 effects on erythroid differentiation (Figure S2). In some experiments, Activin A, Activin B and GDF11 (R&D Systems) were added to the cultures with each fresh addition of media, at concentrations of 30 ng/ml for Activins or 50 ng/ml for GDF11.

Long-Term Bone Marrow Cultures

Long-Term Bone Marrow Cultures (LTBMC) were carried out according to the method of Gartner and Kaplan with slight modifications (Supporting information) (Gartner & Kaplan, 1981). After 3–4 weeks of culture, when all cellular components (confluent stromal layer, adipocytes and cobblestone areas) were present, RAP-011 (50 μg/ml) was added to cultures every 2 d for 5 d before co-culture. In some

experiments, Activin A (50 ng/ml), Activin B (50 ng/ml), BMP10 (50 ng/ml) or GDF11 (30 ng/ml) were added to the cultures for 20 min to evaluate Smad2/3 signalling by Western blot (Supporting information).

CD34+ or CD36+ cell co-culture with LTBMC

LTBMC media was replaced by erythroid differentiation media supplemented with rhEPO (2 u/ml). RAP-011 (50 μg/ml) was added to cultures every 2–3 d. For BFU-E/CFU-E studies, 10^4 CD34+ cells were seeded on the upper surface of the transwell plates for 3 d. Then, 5×10^3 cells were seeded in MethoCultTM H4435 to assess BFU-E and CFU-E as previously described (Lopez-Holgado *et al*, 2005). To evaluate late-stage precursors, 2×10^5 CD36+ cells were stained with carboxyfluorescein succinimidyl ester (CFSE; Life Technologies, Grand Island, NY, USA) according to the manufacturer's protocol and were seeded on the upper surface of the transwell plates. After 3 and 5 d, cells from the upper compartment were harvested for immunophenotypic analyses or staining with May-Grunwald-Giemsa (Merck KGaA, Darmstadt, Germany).

Statistical analysis

At least five independent samples were evaluated and data are expressed as a mean ± standard deviation (SD). A paired student *t*-test was used to evaluate the significance of the differences when only two groups were studied. Comparison between more than two groups was made by analysis of variance (ANOVA) and a *post-hoc* Bonferroni test was performed to confirm differences between groups. Statistical level was set at a level of $P < 0{\cdot}05$.

Results

ActRIIA pathway inhibition by RAP-011 rapidly increases RBC parameters in mice

RAP-011 was administered to C57BL/6 mice to evaluate the effect on the erythroid compartment over a 1-week time period and these effects were compared with those of rhEPO. After performing dose-response studies (data not shown), a dose of 30 mg/kg was selected for the current study; however it is not known whether the system is saturated following this single dose. A single dose of 30 mg/kg RAP-011 significantly increased Hct, Hb and RBCs and changes were first evident at 24 h post-treatment (Fig 1A–C). Small but significant changes in the mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) were observed at later time points despite an increase in cellular Hb content on day 3 and 4 post-RAP-011 treatment (Fig 1D–F). In contrast, the erythroid response after one dose of rhEPO (600 iu/kg) was slower, first appearing at 72–96 h post-treatment (Fig 1A–C). As previously described, rhEPO stimulated reticulocyte release into the circulation, starting approximately 24 h after treatment. In addition, rhEPO increased reticulocyte Hb content 48–72 h post-treatment; however, the effect of RAP-011 on the reticulocyte count was apparent only at the end of the study and there was no change in reticulocyte Hb content (Fig 1G–H). Overall, the kinetics, magnitude and RBC characteristics mediated by RAP-011 are distinct from rhEPO.

RAP-011 induces rapid RBC production by stimulating late-stage precursors in the bone marrow of mice

To elucidate the mechanism of action of RAP-011, the erythroid components from mouse BM and spleen were analysed. A single dose of RAP-011 increased the percentage of Ter119-positive erythroid precursors within 1–3 d post-treatment in the BM (Fig 2A) while, as expected, rhEPO effects were more evident in spleen cells (Figure S3). Neither rhEPO nor RAP-011 affected other haematopoietic populations (Figure S4). Further analysis of the BM Ter119-positive erythroid compartment after RAP-011 treatment indicated that while there was limited impact on early erythroid populations (ProE-Ery A), a higher percentage of the late precursors (Ery B/C), corresponding to polychromatic/orthochromatic erythroblasts (Fig 2B), were present as early as 24 h post-treatment. Flow cytometric analysis of mature BM RBCs revealed that there were $38{\cdot}5 \pm 1{\cdot}4\%$ enucleated cells within the Ter119+ cell compartment at 48 h in RAP-011-treated mice but only $30{\cdot}5 \pm 1{\cdot}6\%$ and $19{\cdot}1 \pm 2{\cdot}3\%$ enucleated cells in vehicle- or rhEPO-treated mice, respectively (Fig 2C), suggesting that RAP-011 promoted faster maturation of RBCs within the BM. Our observation was confirmed by visual examination of BM morphology (Figure S5). Hb concentration and induction of both alpha and beta chains in the BM cells of RAP-011-treated mice was also increased, consistent with the higher percentage of enucleation and late erythroid cells (Fig 2D and Figure S6). This is in contrast to the effects of rhEPO on earlier BM erythroid populations. The effects of RAP-011 on splenic erythropoiesis were less marked and not statistically significant (Figure S7). In contrast, rhEPO had greater effects on splenic compared to BM erythropoiesis, consistent with previous studies in murine models (Vogel & Gassmann, 2011; Elliott & Sinclair, 2012). EPO stimulated early erythroid precursors (ProE-EryA) in the spleen during the first days after treatment (24–72 h) (Figure S7).

RAP-011 promotes BFU-E expansion and EPO production in mice

To better understand the sustained effects of RAP-011 on RBC production that were observed in phase 1 clinical studies, bone marrow and splenic erythroid progenitors, BFU-E and CFU-E, were enumerated following drug treatment in mice. At 24–48 h after treatment, RAP-011 increased the percentage of bone marrow-derived erythroid

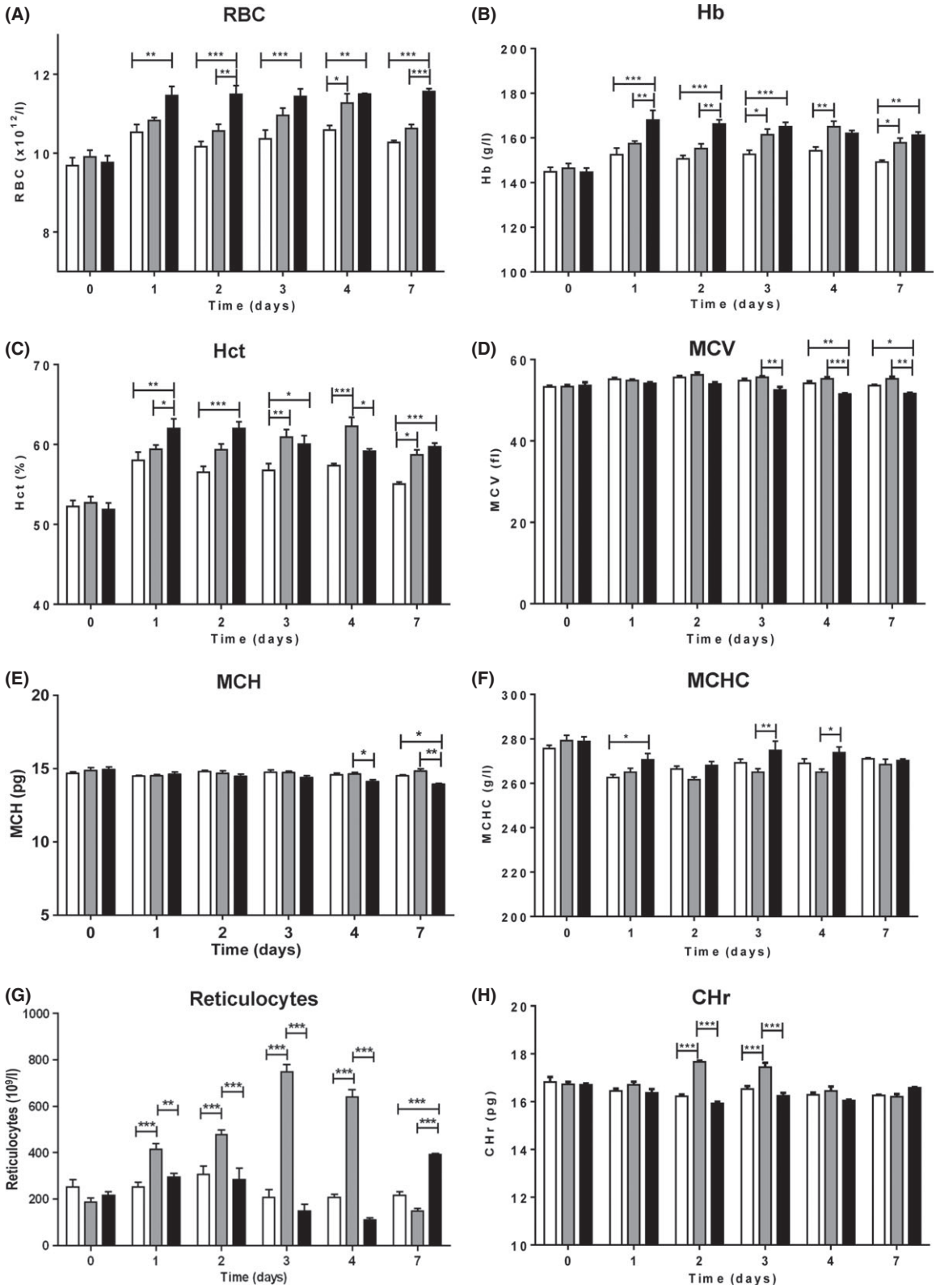


Fig 1. RAP-011 increases red blood cell parameters in wild type mice with faster kinetics than erythropoietin (EPO). (A) Peripheral blood red blood cell (RBC) count, (B) Haemoglobin (Hb), (C) Haematocrit (Hct), (D) Mean Corpuscular Volume (MCV), (E) Mean Corpuscular Haemoglobin (MCH), (F) Mean Corpuscular Haemoglobin Concentration (MCHC), (G) reticulocyte count and (H) Haemoglobin content in reticulocytes (CHr) during the first week after dosing revealed different kinetics in RBC parameters between Vehicle (white), EPO (grey) and RAP-011 (black) treatments. Data represent mean ± standard deviation. *, **, ***: $P < 0{\cdot}05$, $P < 0{\cdot}01$, $P < 0{\cdot}001$.

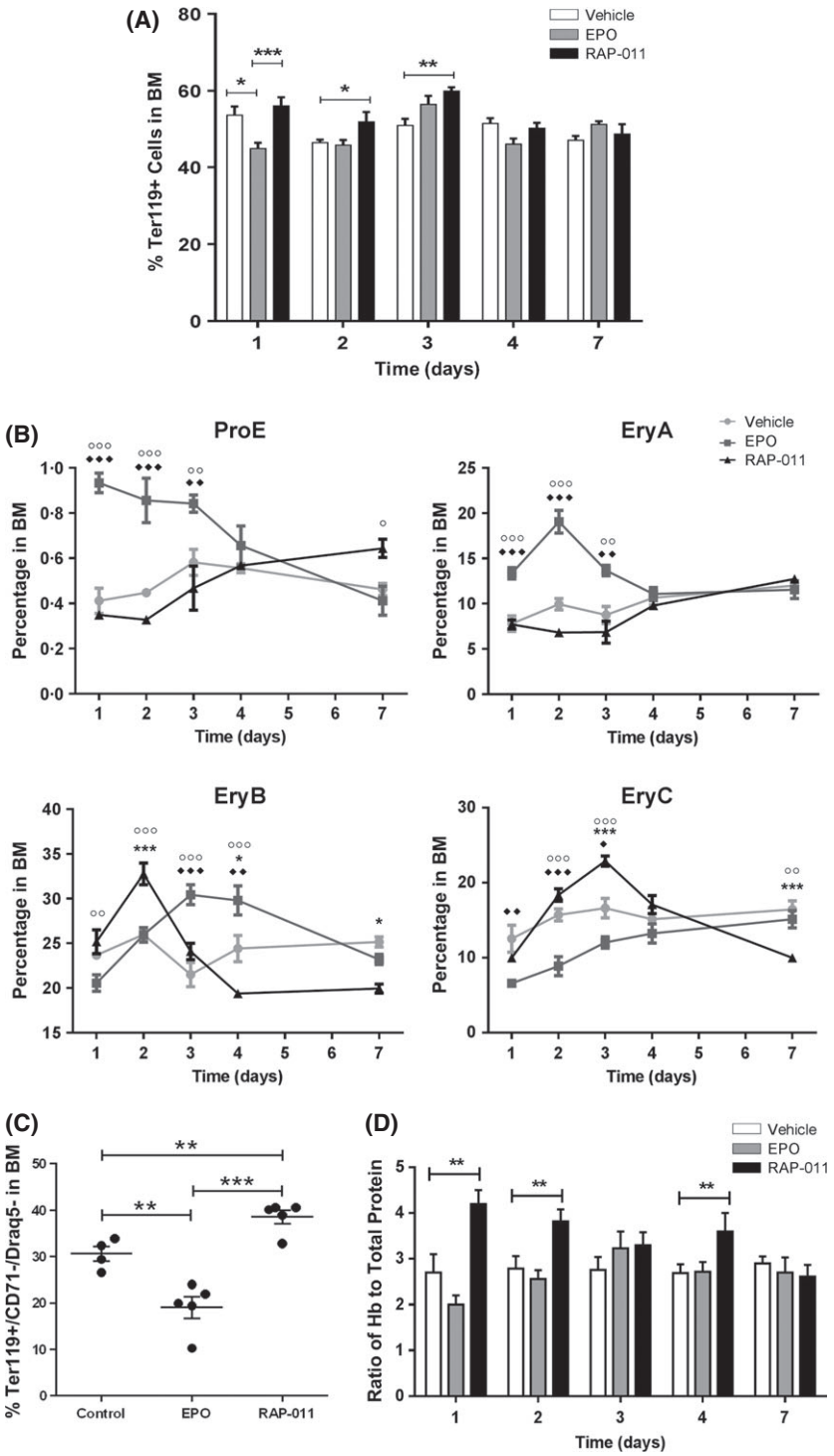


Fig 2. RAP-011 rapidly increases late-stage precursors and enucleated cells in bone marrow (BM). (A) Bone marrow cells from mice treated with vehicle (white), erythropoiein (EPO, grey) or RAP-011 (black) were harvested at different time-points and analysed by flow cytometry. RAP-011 treatment increased the percentage of erythroid committed Ter119+ cells in bone marrow at days 2 and 3. (B) Within the Ter119+ cells, erythroid precursors from proerythroblast to mature cells were characterized into four stages of differentiation, ProE, EryA, EryB and EryC, by flow cytometry using Infinicyt software. Quantification of these stages revealed a rapid increase in late-stage precursors from polychromatic erythroblast to mature red blood cells (EryB and Ery C) at 24–72 h after RAP-011 treatment. (C) The rapid erythroid differentiation induced by RAP-011 in late-stage bone marrow erythroid precursors was also reflected in a higher percentage of mature enucleated cells present at 48 h. (D) Consistent with this higher percentage of erythroid cells, the concentration of cellular haemoglobin was also increased during the first days after treatment in RAP-011-treated mice (black) when compared with Vehicle (white) or EPO (grey). *, **, ***: $P < 0{\cdot}05$, $P < 0{\cdot}01$, $P < 0{\cdot}001$ RAP-011 vs. Control; ♦, ♦♦, ♦♦♦: $P < 0{\cdot}05$, $P < 0{\cdot}01$, $P < 0{\cdot}001$ EPO vs. Control; ○, ○○, ○○○: $P < 0{\cdot}05$, $P < 0{\cdot}01$, $P < 0{\cdot}001$ RAP-011 vs. EPO.

progenitors (BFU-E) by 20–30% compared to vehicle control (Fig 3Ai) and induced the formation of larger colonies (Fig 3Aii and Figure S8) compared to vehicle- and rhEPO-treated mice. rhEPO increased CFU-E progenitors in BM (Fig 3Aii) and this effect was more evident in spleen-derived BFU-E and CFU-E cells (Fig 3B). CFU-E numbers after EPO treatment were increased greatly and then decreased progressively while they were differentiating to proerythroblasts (Figure S7).

Additionally, RAP-011 treatment increased murine plasma EPO levels starting at 4 d post-treatment and peaking with a threefold increase at 7 d. Likewise, we observed an increase in renal *EPO* mRNA expression approximately 4 d following treatment and persisting for 7 d (Fig 3C, D). This suggests

that the EPO stimulation is systemic and that it may be responsible for RAP-011-induced increase in splenic precursors as well as the increase in peripheral blood reticulocytes at 7 d post-treatment. Interestingly, while RAP-011-induced EPO did stimulate an increase in CFU-E colonies, it was not to the magnitude seen with rhEPO treatment (Fig 3B, Table SII). The effects of sotatercept, the human version of the drug, on EPO were also observed in healthy volunteers but it has not yet been confirmed whether the induction is physiologically relevant in humans (Ruckle *et al*, 2009). To summarize, RAP-011 may maintain sustained RBC effects following the initial, rapid increase by stimulating BFU-E formation and by inducing EPO.

Bone marrow microenvironment mediates RAP-011 effects on erythroid cells

In order to investigate the mechanism of action through which RAP-011 increases RBC parameters, we conducted a series of *in vitro* experiments to evaluate potential cellular effects of RAP-011 on human cells. We demonstrate that in liquid culture, RAP-011 does not act directly on erythroid progenitors or on precursors to stimulate growth/differentiation *in vitro* (Figure S9).

To evaluate if RAP-011 effects on erythropoiesis are mediated indirectly by cells in the BM microenvironment, human CD34+ or CD36+ cells (depending on whether the assay endpoint was enumeration of early progenitors or later-stages of differentiation) were co-cultured with BM long-term culture-initiating cell (LTC-IC) cultures (as described in the Materials and methods) which form an *in vitro* microenvironment containing adipocytes, stromal cells and macrophages that supports haematopoietic cell growth and differentiation (Figure S10). These LTC-IC BM cultures were treated with vehicle or RAP-011 and then co-cultured with either CD34+ or CD36+ cells to evaluate how RAP-011 impacts the BM cells which in turn, regulate RBC development.

In a first set of experiments, CD34+ cells were co-cultured with LTC-IC for 72 h with no impact on total cell number (Fig 4A). Then, cells were assayed for colony formation in semisolid medium. RAP-011 treatment for 72 h significantly increased the number of BFU-E (Fig 4B), consistent with our observation in the murine model at 24–72 h after dosing. There was no impact on CFU-E (data not shown).

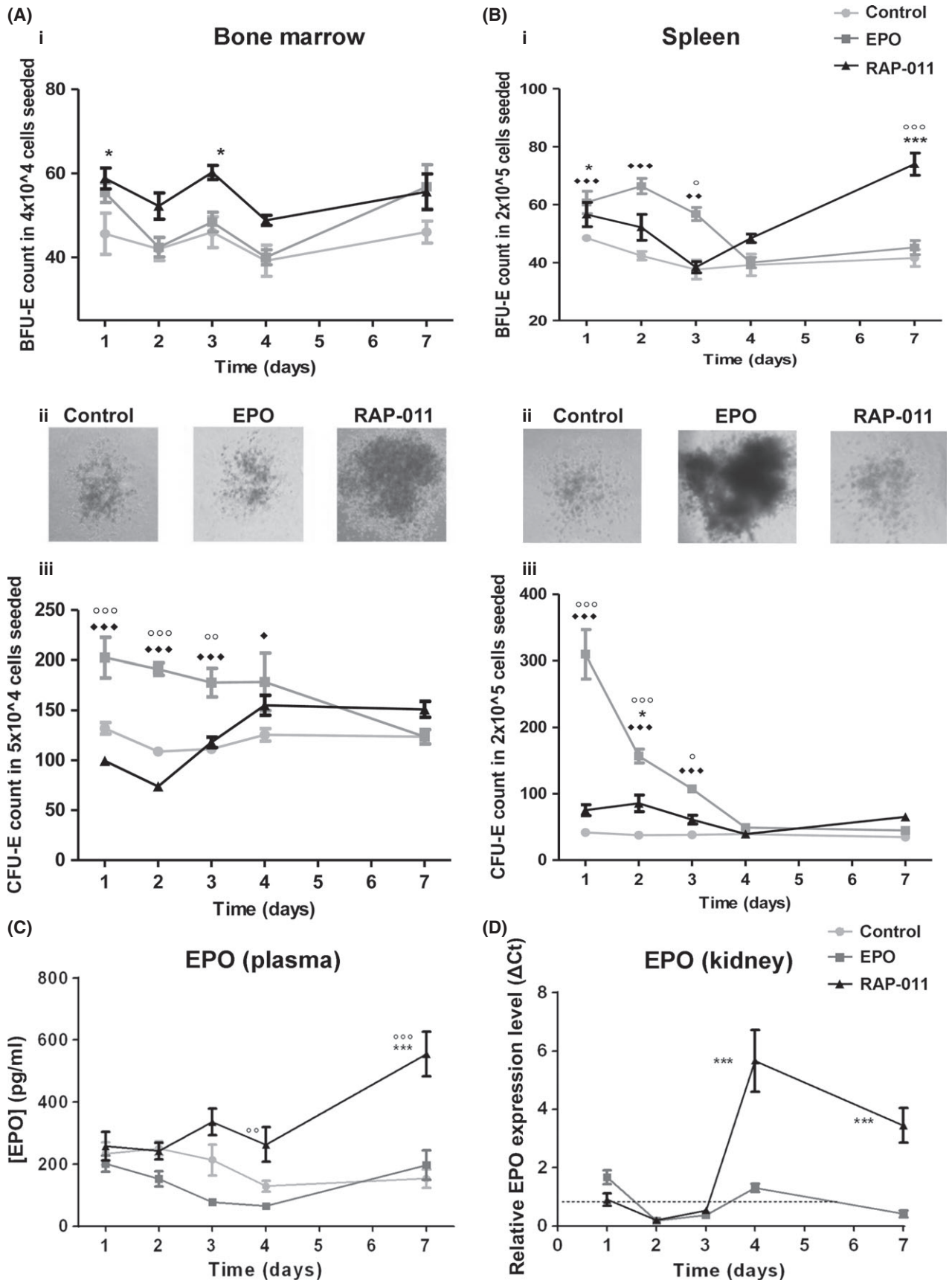
Next, to better evaluate RAP-011 effects on later stages of erythroid differentiation, CD36+ cells were used as a starting point and co-cultured with LTC-IC culture in liquid medium to support late stage erythroid precursor growth and differentiation. As seen in Fig 5A, no differences in cell number between treated and untreated cultures were observed. However, differentiation of glycophorin A positive (GPA+) cells was more pronounced following RAP-011 treatment (Fig 5B and Figure S11). Although untreated cells had the ability to fully differentiate, most cells were found at ProE/EryA. In RAP-011-treated cultures, similar erythroid kinetics were observed after 3 d in co-culture but by day 5, a statistically higher percentage of cells had shifted from Ery A to Ery C, pointing to the ability of RAP-011 to accelerate later stages of erythroid maturation. Finally, RAP-011 probably requires the presence of multiple BM cell types in the microenvironment to mediate its effect because co-culturing CD34+ cells with isolated cellular components (macrophages, stromal cells or haematopoietic cells) had little effect on stimulating proliferation or maturation of erythropoietic precursors (data not shown).

In order to determine if RAP-011 promoted proliferation or differentiation during erythropoiesis, the number of cell divisions was quantified by CFSE staining. During the 5 d in co-culture, both control and RAP-011-treated GPA+ cells underwent an average of three divisions (Fig 5C), indicating that RAP-011 did not preferentially increase the number of divisions; however, the percentage of cells that underwent these three divisions was higher in cultures treated with RAP-011, suggesting that RAP-011 induced faster cellular maturation/differentiation (Fig 5D). Moreover, we also analysed cell viability in these GPA+ cells. At the end of culture, when cell populations were most mature, the percentage of apoptotic death was higher in the control versus RAP-011-treated cells (Fig 5E). This suggests that RAP-011 may promote survival of late-stage precursors.

Collectively, these findings demonstrate that factors secreted by the BM microenvironment probably mediate RAP-011 effects on RBC differentiation. Furthermore, by evaluating erythroid maturation *in vitro*, we showed that RAP-011 exerts effects on both early erythroid progenitors (BFU-E) as well as on the latter stages of erythropoiesis and that this effect may be mediated by increased maturation rate and survival.

RAP-011 rescues TGF-β family ligand-induced inhibition of erythropoiesis in vitro

To assess potential candidates which may mediate the erythropoietic effects of RAP-011, we selected three high affinity RAP-011 ligands that were also highly expressed in mouse BM: Activin A, Activin B and GDF11 (Table SI) and evaluated their effects on Smad signalling in BM cells as well as on erythroid differentiation of CD36+ cells. First, we evaluated the ability of RAP-011 to block Smad2/3 phosphorylation induced by these ligands in LTC-IC cells. When Smad2 and Smad3 are phosphorylated they form a heteromeric complex with Smad4, allowing translocation of the complex to the nucleus. Once in the nucleus, Smads can target a variety of DNA binding proteins to regulate transcriptional responses. As shown in Fig 6, RAP-011 effectively blocked phosphorylation of Smad2/3 downstream of Activin A, Activin B and GDF11 but not BMP10 stimulation; inhibition was most evident following addition of Activin A or GDF11 to the cultures.



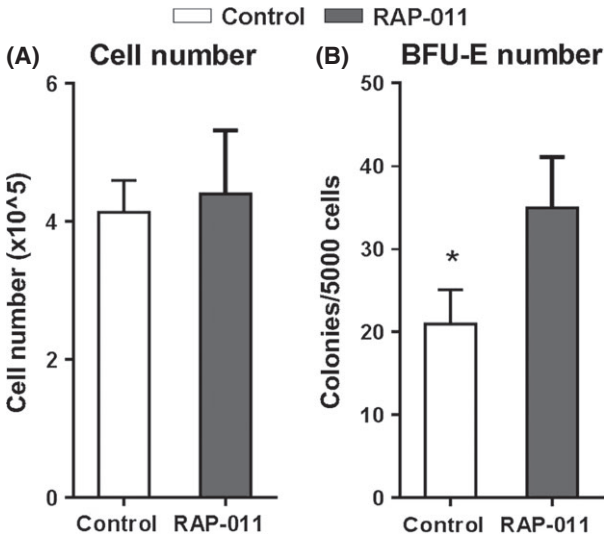


Fig 4. RAP-011 effects on erythroid burst-forming units (BFU-E) are mediated by bone marrow stroma. RAP-011 stimulation of *in vitro* erythroid differentiation requires co-culture with bone marrow cells. Although human CD34+ cells cultured for 72 h under these conditions showed no change in cell number (A), a significant increase in BFU-E number following RAP-011 treatment was observed (B). *: $P < 0{\cdot}05$.

In a different set of experiments, CD36+ cells were cultured in liquid media containing one of each of the three ligands. GDF11 and Activin A treatment significantly decreased the number of CD36-derived cells (Fig 7A). Concomitant treatment of these cells with RAP-011 effectively reversed the growth inhibition, but had no effect on untreated cells (Fig 7A). GDF11 and Activin A also significantly decreased the percentage of GPA-positive cells, while significantly increasing the percentage of CD45-positive cells. Again, RAP-011 blocked these effects (Fig 7B–C). Treatment of CD36+ cells with Activin B had no effects on growth or differentiation.

Finally, we also evaluated the effect of the TGF-β family ligands on early erythroid progenitors and demonstrated that activin A, but not GDF11 or activin B, inhibited BFU-E colony growth. Interestingly, RAP-011 treatment effectively reversed this inhibition (Fig 7D). BMP10 is a ligand to which RAP-011 binds but is not highly present in the BM environment (Table SI); BMP10 had no effect on BFU-E colony formation. These data suggest that inhibition of both GDF11 and Activin A may contribute to the erythropoietic stimulatory effects of RAP-011.

Discussion

We have demonstrated that inhibiting activin receptor IIA signalling can result in increased RBC, Hct and Hb within 24–48 h, a response more rapid than that following rhEPO treatment. Analysis of BM cells indicated that a faster rate of differentiation of late precursors contributes to the mechanism of action of RAP-011. RAP-011 treatment induced a significant increase in the pool of more mature erythroid precursors (poly/orthochromatic erythroblasts) in the BM while rhEPO targeted erythroid progenitors (CFU-E) and early precursors (ProE) from the ProE stages and exerted its effects primarily on the murine spleen. The lack of spleen response during early time-points (Figures S3 and S7) and the high increase of mature erythroid cells in BM after RAP-011 treatment clearly differentiate RAP-011 from EPO through their mechanisms of action and moreover, our results suggest that while EPO plays a more important role in the murine stress erythroid response [taking place in the spleen of the adult mouse (Socolovsky, 2007)], RAP-011 primarily modulates steady state erythropoiesis in the BM. Furthermore, RAP-011 induced a more rapid differentiation/enucleation of Ter119+ cells, giving rise to mature enucleated cells (almost 40% of erythroid cells) within 48 h with no detectable change in reticulocyte number in the peripheral blood. Because of technical limitations (differentiation of RBC precursors into a sufficient and measurable number of mature cells in culture is not possible) we could not study this finding *in vitro*; *in vivo* we did not detect increased numbers of reticulocytes even at earlier time points (2 and 10 h after dosing). This implies that treatment with RAP-011 induces reticulocytes to mature to RBCs in the BM. We believe that the faster maturation may occur in BM because we have consistently detected a higher percentage of mature erythrocytes in BM of RAP-011-treated mice than in controls (Fig 2C). It is possible that other mechanisms may mediate the rapid increase in Hb and Hct after RAP-011 treatment but additional experiments conducted to measure RBC half-life and plasma volume suggest that neither are probably responsible for the rapid, RAP-011-induced RBC effects (R. Kumar, R. Suragami, S. Pearsall, unpublished data).

In addition to stimulating late-stage erythropoiesis, we also observed that RAP-011 significantly increased BM BFU-E colony number and size. Interestingly, the larger-sized colonies resemble those found in the spleen of mice undergoing erythropoietic stress (Paulson *et al*, 2011) and suggest that RAP-011 may also induce an acute response in early BM

Fig 3. RAP-011 sustained effect may be mediated by BFU-E expansion and EPO production. BM (A) and spleen (B) cells from mice treated with vehicle (light grey), EPO (dark grey) or RAP-011 (black) were culture in semisolid media to evaluate the number (i) and morphology (ii) of BFU-E and CFU-E colonies (iii) to determine the effects of ActRIIA inhibition on erythroid progenitors. RAP-011 treatment increased BFU-E number and size in BM at early time-points and also impacted spleen progenitors by day 7. Late effects in spleen may be mediated by an increase in EPO production by RAP-011-induced plasma EPO (C) and increased EPO expression in kidney (D). Data represent mean ± SD. Images acquired with an Olympus microscope by phase contrast observation at 10× magnification. BFU-E, erythroid burst-forming units; CFU-E, erythroid colony-forming units; EPO, erythropoietin; BM, bone marrow. *, ***: $P < 0{\cdot}05$, $P < 0{\cdot}001$ RAP-011 vs. Control; ♦, ♦♦, ♦♦♦: $P < 0{\cdot}05$, $P < 0{\cdot}01$, $P < 0{\cdot}001$ EPO vs. Control; ○, ○○, ○○○: $P < 0{\cdot}05$, $P < 0{\cdot}01$, $P < 0{\cdot}001$ RAP-011 vs. EPO.

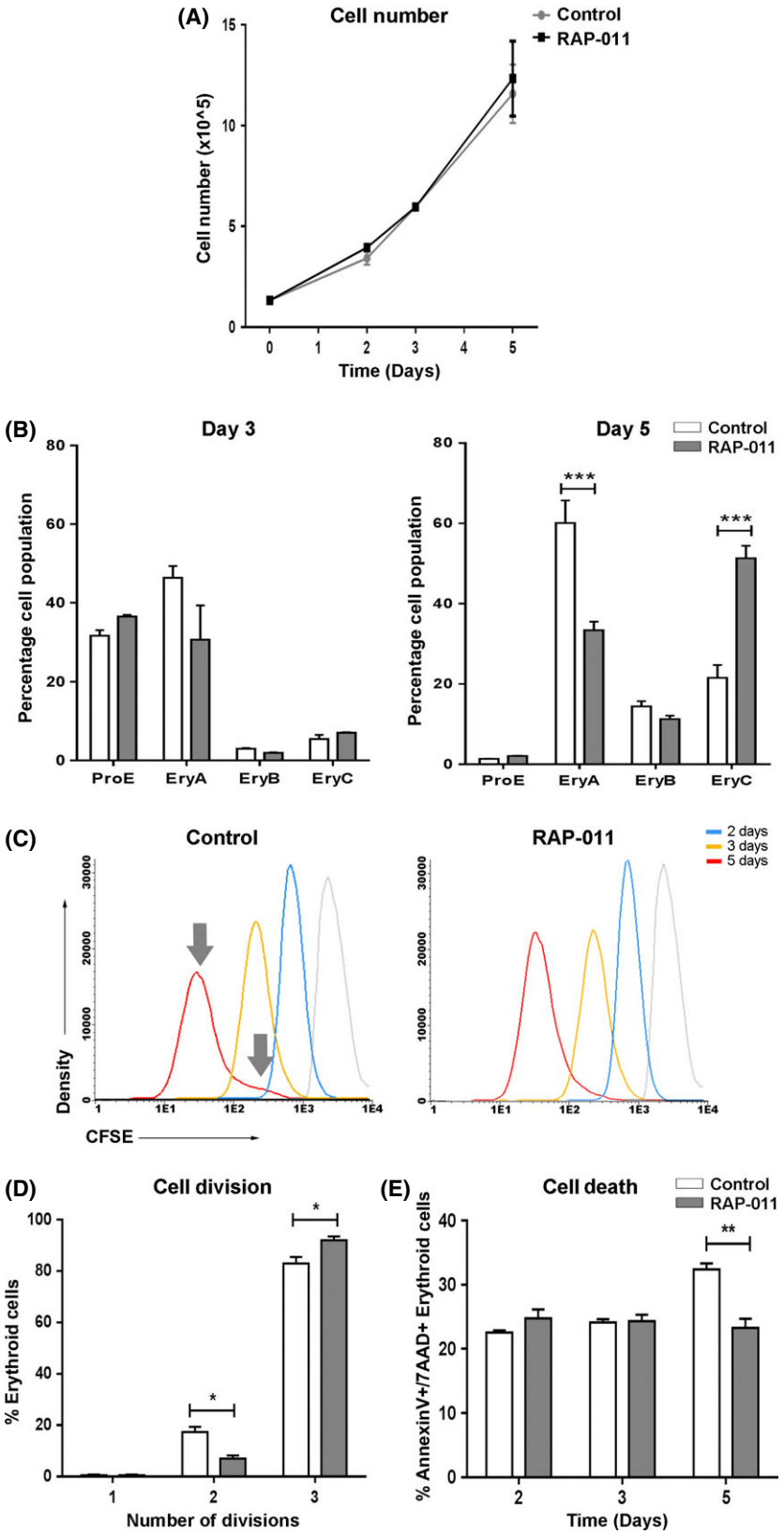


Fig 5. RAP-011 effects on late-stage erythroid precursors are mediated by differentiation and survival of late-stage erythroid precursors. CD36+ cells were used to assess RAP-011 effect on late-stage erythroid precursors in a co-culture system. Inhibition of ActRIIA ligands by RAP-011 did not affect cell number of CD36+ derived cells during co-culture time (A), but modified differentiation pattern by favouring late-stage differentiation after 5 d of culture when compared with vehicle-treated cells (B). At this time-point, CD36+ cells grown in the presence of RAP-011 contained a statistically higher percentage of EryC cells than the vehicle control group, where most cells were retained at an early stage of differentiation (B). Number of divisions of these CD36+ derived cells grown in co-culture conditions, was quantified by carboxyfluorescein succinimidyl ester (CFSE) staining. The *in vitro* expansion of late-stage erythroid cells mediated by RAP-011 is not due to an increase in the number of divisions but by a higher number of cells reaching the last division by day 5 (red line) (C). This may be due to faster maturation of cells as well as a decrease in apoptosis/death of most mature cells. After 5 d of culture, the percentage of cells undergoing the last division was higher when cells were treated with RAP-011 (D). At this time-point, cells grown in control conditions showed increased apoptosis/cell death, determined by Annexin V and 7AAD expression, but RAP-011 was able to rescue this effect and maintain viability of these mature cells (E). Data represent mean ± standard deviation. *, **, ***: $P < 0{\cdot}05$, $P < 0{\cdot}01$, $P < 0{\cdot}001$.

progenitors. The magnitude of increase was indeed similar to what was observed in spleen after rhEPO treatment. This finding may partly account for phase 1 clinical observations where, in addition to rapidly stimulating RBC parameters, sotatercept also sustained Hct and Hb increases for approximately 3 months after a single dose in healthy volunteers

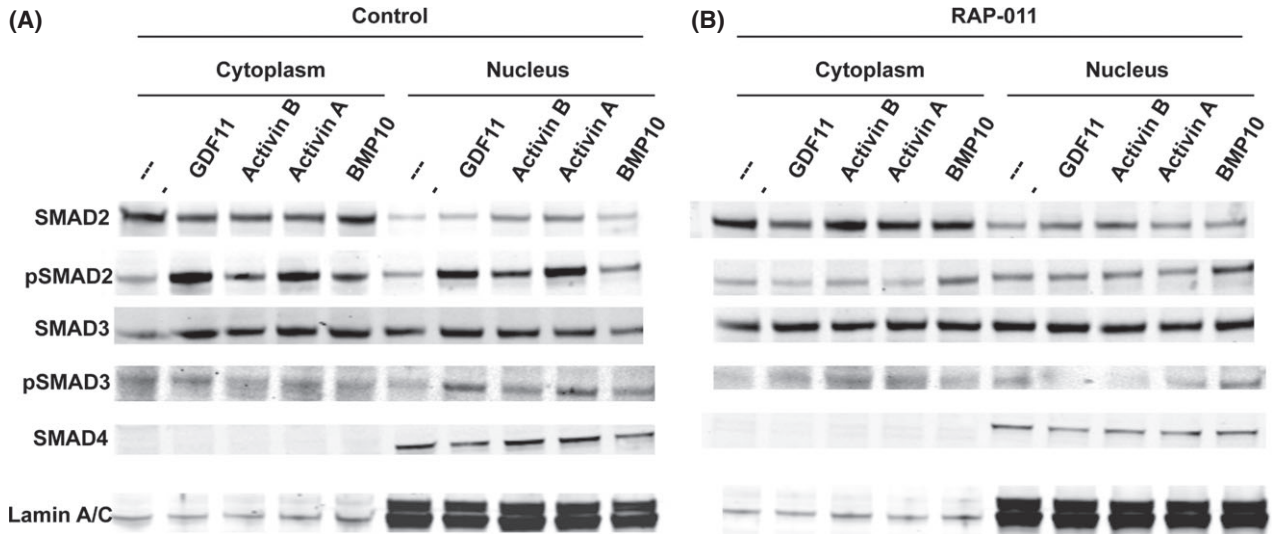


Fig 6. RAP-011 inhibits Smad2/3 phosphorylation in bone marrow cells. TGF-β ligands with high affinity for RAP-011 as well as high expression in mouse bone marrow (GDF11, Activin A and Activin B) were selected to evaluate their effects on Smad signalling in bone marrow cultures (A). RAP-011 blocked ligand-stimulated phosphorylation of Smad2 and Smad3 in bone marrow cells from LTC-IC cultures (B).

(Ruckle *et al*, 2009). Interestingly, we also observed an induction of plasma EPO levels following RAP-011 treatment. Although we have not yet verified the physiological relevance of this effect, it may also contribute to the drug's sustained effects. This EPO induction corresponded with increased peripheral blood reticulocytes and a modest increase in CFU-E, although the increase was not as high as observed after exogenous administration of human EPO. Also, a concordant increase of EPO message was observed in the kidney, implying that the regulation of erythropoiesis by RAP-011 may be systemic and not limited to activity within the haematopoietic environment. This sustained effect of RAP-011 as well as the mechanism responsible for EPO induction are very interesting but not fully understood. These are high priorities in ongoing experiments to better understand RAP-011 mechanism of action.

The production of mature RBCs is regulated by complex cell-cell interactions as well as extrinsic factors secreted by cells in the BM microenvironment (Molineux *et al*, 2009; Iancu-Rubin *et al*, 2013). Osteoblasts, vascular and most stromal cells are implicated in haematopoietic stem cell maintenance and controlling fate decisions to initiate cell proliferation and differentiation commitment (Walkley, 2011). Iancu-Rubin *et al* (2013) demonstrated that RAP-011 was able to reduce the inhibitory effect of conditioned media from stromal cells on human erythropoiesis. In our hands, neither stromal cells nor haematopoietic cells, alone, were sufficient to confer the effects of RAP-011 on erythropoiesis. For the full stimulatory effect, both cell types were required in co-culture with erythroid precursors. Using this human *in vitro* system for erythroid differentiation, we were not only able to reproduce the effects on erythroid progenitor and precursor cells observed in our murine studies but also could confirm a central role for the BM microenvironment in mediating effects of RAP-011. Furthermore, we have been able to better define the role of the TGF-β superfamily of ligands in the regulation of erythropoiesis. The precise role of these ligands is currently not well understood (Shiozaki *et al*, 1989, 1992; Nakao *et al*, 1991; Irion *et al*, 2010). RAP-011, a biological tool that can bind to and inhibit ActRcIIA ligands, is a reagent that enables a more careful study and understanding of the role of these ligands in erythropoiesis. Due to the role of Activin A on bone metabolism, the use of RIIA FC chimeras as well as ACE-011/RAP-011 in several models of skeletal diseases has been previously described (Pearsall *et al*, 2008). We attribute the novelty of our studies to the use of this drug, which inhibits Activin A, among other TGF-β family ligands, for the treatment of anaemia. We focused on the ligands with highest binding affinity and show that both Activin A and GDF11 but not Activin B or BMP10 play an important role in erythropoiesis. We demonstrated that GDF11 and Activin A exert an inhibitory effect on erythropoiesis and demonstrated that RAP-011 was able to abrogate the GDF11-mediated inhibitory effect. Our data suggest that RAP-011 activity may be mediated by, but probably not limited to, GDF11 and Activin A. Difficulties in detecting and measuring soluble ActRIIA/B affinity-purified native ligands have been previously described (Souza *et al*, 2008) and although we have not shown direct binding between RAP-011 and GDF11, Activin A or other ligands in these experiments due to technical limitations, these ligands are present (Table SI) and produced by BM microenvironment. Our results are in keeping with the fact that several members of the TGF-β superfamily of ligands have been implicated as negative growth regulators, or 'chalones'. They play an important homeostatic function to maintain the size

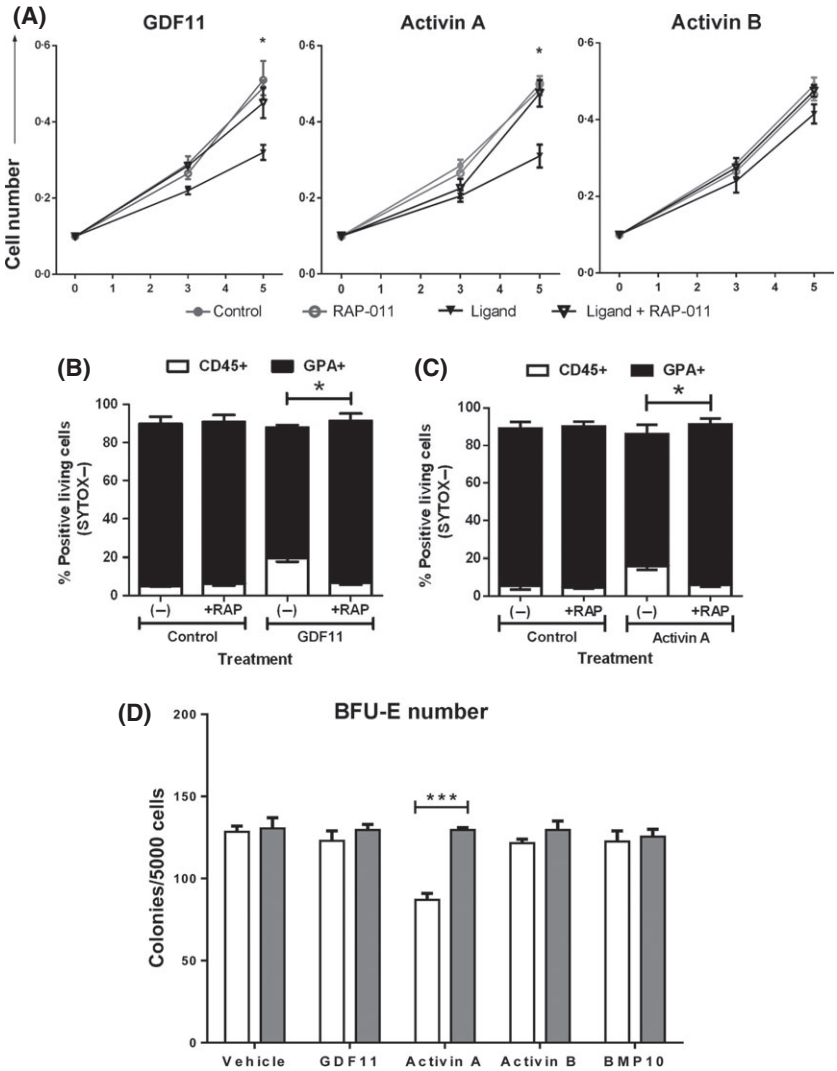


Fig 7. RAP-011 rescued TGF-β ligand-induced growth inhibition in erythroid cultures. High affinity RAP-011 ligands were selected to evaluate their effects on erythroid differentiation of CD36+ cells. Growth curves representing the total number of viable CD36+ derived cells at each time-point in culture revealed that both GDF11- and Activin A- inhibited growth and that RAP-011 reversed this effect. Activin B did not appear to impact cell growth (A). Flow cytometric characterization of erythroid and non-erythroid lineage cells elucidated by staining with antibodies against GPA or CD45, respectively, demonstrated that RAP-011 was able to rescue Activin A (B) and GDF11-induced (C) inhibition of erythroid commitment (GPA+ expression). Effect on erythroid progenitors was also evaluated by culturing CD34+ cells these ligands. The number of BFU-E progenitors was diminished following culture with Activin A. RAP-011 was able to rescue BFU-E numbers (D). BFU-E, erythroid burst-forming units; CFU-E, erythroid colony-forming units; GPA, glycophorin A. *, ***: $P < 0{\cdot}05$, $P < 0{\cdot}001$.

of mature tissue compartments (McKnight, 1997): such as muscle (McPherron *et al*, 1997), bone (Canalis, 2009), adipose tissue (Zamani & Brown, 2011), or olfactory epithelium (Lander *et al*, 2009). Regarding erythropoiesis, there exists controversial data related to Activin A functions. As occurs with many members of this pleiotropic family of ligands, slight differences in dosing scheme, duration of treatment, cell type and context can impact the results of the study. While initial publications described that Activin A promoted maturation of BFU-E (Nakao *et al*, 1991), CFU-E (Shiozaki *et al*, 1989) and more mature erythroid precursors (Okabe-Kado *et al*, 1991), more recent publications evaluating haematopoietic differentiation of embryonic stem cells or induced pluripotent stem cell point to a pivotal role for Activin A signalling in the maintenance of self-renewal and pluripotency (Vallier *et al*, 2005; Xiao *et al*, 2006) as well as in maintaining commitment to the mesodermal lineage (Nostro *et al*, 2008; Pearson *et al*, 2008). However, Activin A was shown not to be necessary, and was perhaps even inhibitory with regard to definitive erythroid differentiation (Nonaka *et al*, 2008; Kennedy *et al*, 2012). Moreover, in transgenic mice over-expressing a soluble form of its receptor an increase on RBC parameters was also described (Yamawaki *et al*, 2013). The results from our studies using the ActRIIA-Fc ligand trap, RAP-011, suggest that GDF11 and Activin A, as well as other ligands, may also be 'chalones' for the blood, specifically regulating the homeostasis of mature RBCs. We suggest that RAP-011 increases RBC parameters by blocking inhibitory signals induced by TGF-β superfamily members, and contributes to normal homeostatic mechanisms regulating erythropoiesis.

There are a number of therapeutic implications for our findings. Current dogma suggests that EPO is the major factor governing erythropoiesis. The first phase of CFU-E erythroid differentiation is highly EPO-dependent, whereas later stages are no longer dependent on EPO. RAP-011 increases erythropoiesis at the BFU-E progenitor level and also on later stage erythroid precursors after they lose responsiveness to

EPO. These effects are mediated by the BM microenvironment through targeting ActRIIA ligands, such as GDF11. This mechanism is completely different from how classical ESAs function, supporting a rationale for using sotatercept as a new therapeutic agent to manage anaemia in diseases of ineffective erythropoiesis, such as β-Thalassaemia, Diamond-Blackfan Anaemia (DBA), or MDS. In these conditions, the homeostatic mechanism is unbalanced, resulting in a hyperproliferative marrow with accumulation of mature and abnormal erythroid precursors and peripheral anaemia. Also, these patients have high EPO levels and as such, often respond poorly to EPO therapy. It is possible that in diseases where this homeostatic mechanism regulating RBC production is unbalanced, the effects of sotatercept may be even greater than in the normal, homeostatically-balanced environment. This hypothesis has been evaluated in preclinical disease models and is currently being investigated in β-Thalassaemia, MDS and DBA clinical trials.

In conclusion, we have explored the potential mechanisms by which RAP-011 may regulate erythropoiesis and have demonstrated that inhibition of ActRIIA signalling can rapidly stimulate RBC production, Hb and Hct in mice. Furthermore, the stimulatory effect of RAP-011 on BM erythropoiesis appears to be indirect and probably mediated by inhibition of bone marrow-derived factors, such as Activin A and GDF11. RAP-011 acts on both BFU-E progenitors and also on mid-late erythroid precursors, distinguishing it from EPO, which acts on CFU-E and early precursors in the erythropoiesis differentiation pathway. Finally, we propose that members of the TGF-β superfamily, such as GDF11 and Activin A, may be negative growth regulators acting on late stage erythropoiesis – 'erythropoietic chalones' – and that RAP-011 blocks these ligands to rapidly increase RBC parameters. These data have contributed to defining a regulatory checkpoint of erythropoiesis and support the rationale to develop sotatercept for the treatment of anaemia and ineffective erythropoiesis.

Acknowledgements

We would like to thank Ravi Kumar, Rajasekhar Suragani and Scott Pearsall for contributing unpublished data and much of the early work evaluating RAP-011 as well as for their helpful discussions. Thanks are also due to Professor Harvey Lodish for his excellent scientific guidance. All authors are full-time employees of Celgene Corporation and/or have ownership interest in the company.

Author contributions

S.C. and J.M. designed and performed experiments, analysed and interpreted data, and wrote the manuscript; P.W. performed experiments and revised the manuscript; J.L., P.C., M.C.G. and H.K.R. performed experiments; C.H. and T.D. interpreted data and revised the manuscript; R.C. conceived the study, designed research, interpreted data and revised the manuscript; V.S. conceived the study, designed research, interpreted data and wrote the manuscript.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table SI. Affinity of ACE-011 and RAP-011 for TGF-β ligands and ligand expression in mouse bone marrow.

Table SII. CFU-E number in mouse bone marrow and spleen.

Table SIII. Flow cytometry antibodies.

Fig S1. Analysis of erythroid precursors in mouse samples.

Fig S2. Gating strategy for *in vitro*-derived erythroid populations.

Fig S3. Erythroid precursors in spleen.

Fig S4. RAP-011 treatment does not affect other hematopoietic populations within the bone marrow.

Fig S5. RAP-011 has an early effect on bone marrow erythroid precursors.

Fig S6. RAP-011 increases haemoglobin expression in mouse bone marrow.

Fig S7. RAP-011 promotes spleen erythropoiesis after 1 week.

Fig S8. Larger bone-marrow-derived BFU-E colonies were observed in mice treated with RAP-011.

Fig S9. RAP-011 does not have direct effects on erythropoiesis *in vitro*.

Fig S10. Long term bone marrow cultures.

Fig S11. RAP-011 increases late-stage erythroid precursors *in vitro*.

References

Canalis, E. (2009) Growth factor control of bone mass. *Journal of cellular biochemistry*, **108**, 769–777.

Elliott, S. & Sinclair, A.M. (2012) The effect of erythropoietin on normal and neoplastic cells. *Biologics: Targets & Therapy*, **6**, 163–189.

Gardenghi, S., Grady, R.W. & Rivella, S. (2010) Anemia, ineffective erythropoiesis, and hepcidin: interacting factors in abnormal iron metabolism leading to iron overload in beta-thalassemia. *Hematology/Oncology Clinics of North America*, **24**, 1089–1107.

Gartner, S. & Kaplan, H.S. (1981) Long-term culture of normal and leukemic human bone marrow. *Haematology and Blood Transfusion*, **26**, 276–288.

Iancu-Rubin, C., Mosoyan, G., Wang, J., Kraus, T., Sung, V. & Hoffman, R. (2013) Stromal cell-mediated inhibition of erythropoiesis can be attenuated by Sotatercept (ACE-011), an activin receptor type II ligand trap. *Experimental Hematology*, **41**, 155–166.

Irion, S., Clarke, R.L., Luche, H., Kim, I., Morrison, S.J., Fehling, H.J. & Keller, G.M. (2010) Temporal specification of blood progenitors from mouse embryonic stem cells and induced pluripotent stem cells. *Development (Cambridge, England)*, **137**, 2829–2839.

Kennedy, M., Awong, G., Sturgen, C.M., Ditadi, A., LaMotte-Mohs, R., Zuñiga-Pflucker, J.C. & Keller, G. (2012) T lymphocyte potential marks

the emergence of definitive hematopoietic progenitors in human pluripotent stem cell differentiation cultures. *Cell Reports*, **2**, 1722–1735.

Lander, A.D., Gokoffski, K.K., Wan, F.Y., Nie, Q. & Calof, A.L. (2009) Cell lineages and the logic of proliferative control. *PLoS Biology*, **7**, e15.

Lenox, L.E., Shi, L., Hegde, S. & Paulson, R.F. (2009) Extramedullary erythropoiesis in the adult liver requires BMP-4/Smad5-dependent signaling. *Experimental Hematology*, **37**, 549–558.

Lindberg, E.H. (2005) Strategies for biology- and molecular-based treatment of myelodysplastic syndromes. *Current Drug Targets*, **6**, 713–725.

von Lindern, M., Schmidt, U. & Beug, H. (2004) Control of erythropoiesis by erythropoietin and stem cell factor: a novel role for Bruton's tyrosine kinase. *Cell Cycle (Georgetown, Tex.)*, **3**, 876–879.

Lombardero, M., Kovacs, K. & Scheithauer, B.W. (2011) Erythropoietin: a hormone with multiple functions. *Pathobiology*, **78**, 41–53.

Lopez-Holgado, N., Pata, C., Villaron, E., Sanchez-Guijo, F., Alberca, M., Martin, A., Corral, M., Sanchez-Abarca, I., Perez-Simon, J.A., San Miguel, J.F. & Del Canizo, M.C. (2005) Long-term bone marrow culture data are the most powerful predictor of peripheral blood progenitor cell mobilization in healthy donors. *Haematologica*, **90**, 353–359.

McKnight, S.L. (1997) Gatekeepers of organ growth. *Proceedings of the National Academy of Sciences of the United States of America*, **94**, 12249–12250.

McPherron, A.C., Lawler, A.M. & Lee, S.J. (1997) Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature*, **387**, 83–90.

Molineux, G., Foote, M.A. & Elliott, S. (2009) Erythropoietins and Erythropoiesis: Molecular, Cellular, Preclinical, and Clinical Biology, 2nd edn. Birkhauser Verlag, Basel, Switzerland.

Murata, M., Eto, Y., Shibai, H., Sakai, M. & Muramatsu, M. (1988) Erythroid differentiation factor is encoded by the same mRNA as that of the inhibin beta A chain. *Proceedings of the National Academy of Sciences of the United States of America*, **85**, 2434–2438.

Nakao, K., Kosaka, M. & Saito, S. (1991) Effects of erythroid differentiation factor (EDF) on proliferation and differentiation of human hematopoietic progenitors. *Experimental Hematology*, **19**, 1090–1095.

Nonaka, H., Watabe, T., Saito, S., Miyazono, K. & Miyajima, A. (2008) Development of stabilin2+ endothelial cells from mouse embryonic stem cells by inhibition of TGFbeta/activin signaling. *Biochemical and Biophysical Research Communications*, **37**, 256–260.

Nostro, M.C., Cheng, X., Keller, G.M. & Gadue, P. (2008) Wnt, activin, and BMP signaling regulate distinct stages in the developmental pathway from embryonic stem cells to blood. *Cell Stem Cell*, **2**, 60–71.

Okabe-Kado, J., Hayashi, M., Honma, Y., Hozumi, M. & Tsuruo, T. (1991) Inhibition by erythroid differentiation factor (activin A) of P-glycoprotein expression in multidruf-resistant human K562 erythroleukemia cells. *Cancer Research*, **51**, 2582–2586.

Paulson, R.F., Shi, L. & Wu, D.C. (2011) Stress erythropoiesis: new signals and new stress progenitor cells. *Current Opinion in Hematology*, **18**, 139–145.

Pearsall, R.S., Canalis, E., Cornwall-Brady, M., Underwood, K.W., Haigis, B., Ucran, J., Kumar, R., Pobre, E., Grinberg, A., Werne, E.D., Glatt, V., Stadmeyer, L., Smith, D., Seehra, J. & Bouxsein, M.L. (2008) A soluble activin type IIA receptor induces bone formation and improves skeletal integrity. *PNAS*, **105**, 7082–7087.

Pearson, S., Sroczynska, P., Lacaud, G. & Kouskoff, V. (2008) The stepwise specification of embryonic stem cells to hematopoietic fate is driven by sequential exposure to Bmp4, activin A, bFGF and VEGF. *Development*, **135**, 1525–1535.

Ramanath, V., Gupta, D., Jain, J., Chaudhary, K. & Nistala, R. (2012) Anemia and chronic kidney disease: making sense of the recent trials. *Reviews on Recent Clinical Trials*, **7**, 187–196.

Richmond, T.D., Chohan, M. & Barber, D.L. (2005) Turning cells red: signal transduction mediated by erythropoietin. *Trends in Cell Biology*, **15**, 146–155.

Ruckle, J., Jacobs, M., Kramer, W., Pearsall, A.E., Kumar, R., Underwood, K.W., Seehra, J., Yang, Y., Condon, C.H. & Sherman, M.L. (2009) Single-dose, randomized, double-blind, *placebo*-controlled study of ACE-011 (ActRIIA-IgG1) in postmenopausal women. *Journal of Bone and Mineral Research*, **24**, 744–752.

Shiozaki, M., Sakai, R., Tabuchi, M., Eto, Y., Kosaka, M. & Shibai, H. (1989) *In vivo* treatment with erythroid differentiation factor (EDF/activin A) increases erythroid precursors (CFU-E and BFU-E) in mice. *Biochemical and Biophysical Research Communications*, **165**, 1155–1161.

Shiozaki, M., Sakai, R., Tabuchi, M., Nakamura, T., Sugino, K., Sugino, H. & Eto, Y. (1992) Evidence for the participation of endogenous activin A/erythroid differentiation factor in the regulation of erythropoiesis. *Proceedings of the National Academy of Sciences of the United States of America*, **89**, 1553–1556.

Socolovsky, M. (2007) Molecular insights into stress erythropoiesis. *Current Opinion in Hematology*, **14**, 215–224.

Souza, T.A., Chen, X., Guo, Y., Sava, P., Zhang, J., Hill, J.J., Yaworsky, P.J. & Giu, Y. (2008) Proteomic identification and functional validation of activins and bone morphogenic protein 11 as candidate novel muscle mass regulators. *Molecular Endocrinology*, **22**, 2689–2702.

Uchiyama, H. & Asashima, M. (1992) Specific erythroid differentiation of mouse erythroleukemia cells by activins and its enhancement by retinoic acids. *Biochemical and Biophysical Research Communications*, **187**, 347–352.

Vallier, L., Alexander, M. & Pedersen, R.A. (2005) Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. *Journal of Cell Science*, **118**, 4495–4509.

Vogel, J. & Gassmann, M. (2011) Erythropoietic and non-erythropoietic functions of erythropoietin in mouse models. *Journal of Physiology*, **589**, 1259–1264.

Walkley, C.R. (2011) Erythropoiesis, anemia and the bone marrow microenvironment. *International Journal of Hematology*, **93**, 10–13.

Wu, D.C. & Paulson, R.F. (2010) Hypoxia regulates BMP4 expression in the murine spleen during the recovery from acute anemia. *PLoS One*, **5**, e11303.

Xiao, L., Yuan, X. & Sharkis, S.J. (2006) Activin A maintains self-renewal and regulates fibroblast growth factor, Wnt, and bone morphogenic protein pathways in human embryonic stem cells. *Stem Cells*, **24**, 1476–1486.

Yamawaki, K., Ueda, S., Okada, T., Oshima, T., Kakitani, M., Kato, T. & Tomizuka, K. (2013) Adult-specific systemic over-expression reveals novel *in vivo* effects of the soluble forms of ActRIIA. ActRIIB and BMPRII. *PLoS One*, **8**, e78076.

Zamani, N. & Brown, C.W. (2011) Emerging roles for the transforming growth factor-{beta} superfamily in regulating adiposity and energy expenditure. *Endocrine Reviews*, **32**, 387–403.