Cholesterol synthesis-related enzyme oxidosqualene cyclase is required to maintain self-renewal in primary erythroid progenitors

C. Mejia-Pous, F. Damiola and O. Gandrillon

Bases Moléculaires de l'Autorenouvellement et de ses Altérations'' Group, Université de Lyon, Université Lyon 1, Villeurbanne, Centre de Génétique Moléculaire et Cellulaire, Lyon, France, Molecular Bases of Self-Renewal and Alterations, F-69622, University Lyon 1, Villeurbanne, CNRS, UMR5534, Center for molecular and cellular genetics and physiology

Received 29 January 2011; revision accepted 26 April 2011

Abstract

Objectives: Molecular mechanisms controlling cell fate decision making in self-renewing cells are poorly understood. A previous transcriptomic study, carried out in primary avian erythroid progenitor cells (T2ECs), revealed that the gene encoding oxidosqualene cyclase (OSC/LSS), an enzyme involved in cholesterol biosynthesis, is significantly up-regulated in self-renewing cells. The aim of the present work is to understand whether this up-regulation is required for self-renewal maintenance and what are the mechanisms involved.

Materials and methods: To investigate OSC function, we studied effects of its enzymatic activity inhibition using Ro48-8071, a specific OSC inhibitor. In addition, we completed this pharmacological approach by RNAi-mediated *OSC/LSS* knockdown. The study of OSC inhibition was carried out on both self-renewing and differentiating cells to observe any state-dependent effect.

Results: Our data show that OSC acts both by protecting self-renewing T2EC cells from apoptosis and by blocking their differentiation program, as OSC inhibition is sufficient to trigger spontaneous commitment of self-renewing cells towards an early differentiation state. This is self-renewal specific, as OSC inhibition has no effect on erythroid progenitors that have already differentiated.

Conclusions: Taken together, our results suggest that *OSC/LSS* expression and activity are required to maintain cell self-renewal and may be involved

in the self-renewal versus differentiation/apoptosis decision making, by keeping cells in a self-renewal state.

Introduction

The stem cell concept is sustained by the cells' self-renewal properties; this implies their ability to proliferate without losing developmental potential and without entering into differentiation processes (1). Despite the discovery of a range of molecular signatures of stem cells (2,3), molecular mechanisms responsible for choice between self-renewal and differentiation are still poorly understood.

In the haematopoietic system, self-renewal was initially thought to be restricted to the haematopoietic stem cells (HSCs), which generate all the blood lineages (1). However, the high number of erythrocytes produced, in either physiological (4) or pathological (5) conditions, cannot be sustained by HSCs self-renewal only. It has therefore been proposed that less immature progenitors can also self-renew (6,7), which led to use these progenitors in studying the molecular bases of self-renewal. As the balance between cell gain (self-renewal) and cell loss (apoptosis and differentiation) governs the size of the progenitors' compartment, this balance is critical to maintenance of haematopoietic system homeostasis and, consequently, it is tightly regulated. Thus, mechanisms regulating this decision making can be investigated in more detail by studying self-renewal in primary progenitors, which are less complex and more accessible than HSCs. In our study, we have used primary immature avian erythroid progenitor cells, T2ECs, obtained directly from chicken bone marrow. T2EC cells are normal, non-immortalized *ex vivo* cells that can be grown in an undifferentiated state – or in a self-renewal state – and can be induced to differentiate into mature erythrocytes, at any time, through a combination of external factors (6). In addition, their differentiation potential is restricted to the erythrocyte

Correspondence: O. Gandrillon, CGMC B.Mendel, 16 rue Dubois, 69622 Villeurbanne Cedex, France. Tel.: +33 (0)472448190; Fax: +33 (0)472432685; E-mail: olivier.gandrillon@univ-lyon1.fr

lineage, thus fate choice mechanisms (self-renewal vs. differentiation) are not blurred by mechanisms involved in lineage choices.

To characterize molecular mechanisms underlying the choice between self-renewal and differentiation, the transcriptome of T2EC cells in the self-renewal state and at the initial stage (commitment) of the differentiation process has been analysed (8). We identified nine candidate genes potentially involved in controlling T2ECs' cell fate decision making. Among them, the *OSC/LSS* gene, encoding 2,3-oxidosqualene:lanosterol cyclase, is the most significantly up-regulated gene in self-renewing cells, compared to differentiating cells. However, whether this up-regulation is required for maintenance of the self-renewal state and what mechanisms are involved, have remained to be elucidated.

OSC/LSS (EC: 5.4.99.7) catalyses cyclization of monooepoxysqualene to lanosterol, which is the first sterol in cholesterol biosynthesis (9). In addition, OSC also catalyses cyclization of diepoxysqualene to epoxylanosterol in the synthesis pathway of 24(S),25-epoxycholesterol, a natural ligand of nuclear liver X receptor (LXR) (10).

In this study, we demonstrate that OSC plays a key role in cell fate choice of immature erythroid progenitors. Our data show that OSC expression and activity in cholesterol biosynthesis are indeed necessary to maintain the self-renewal state.

Materials and methods

Primary cell culture and treatments

T2EC cells isolation. T2EC cells were generated from SPAFAS white leghorn chickens (PA12 line; INRA, Tours, France) and expanded as previously described (6).

Self-renewal and differentiation conditions. T2EC cells were maintained in the self-renewal state in LM1 medium [α -MEM medium, 10% foetal bovine serum (FBS), 1 mM Hepes, 100 nM β -mercaptoethanol, 100 U/ml penicillin, 100 μ g/ml streptomycin, 1 mM dexamethasone, 5 ng/ml TGF- α , 1 ng/ml TGF- β 1], as previously described (6). Differentiating cells were obtained by changing the T2EC cell culture medium from LM1 to DM17 medium [α -MEM medium, 10% foetal bovine serum (FBS)], 1 mM Hepes, 100 nM β -mercaptoethanol, 100 U/ml penicillin, 100 μ g/ml streptomycin, 5% anaemic chicken serum (ACS) (11), 10 ng/ml insulin.

Cell culture reagents and treatments. Ro48-8071 was a gift from Dr Johannes D. Aeby (Pharmaceuticals Division, Hoffmann-La Roche Ltd, Basel, Switzerland). Cholesterol was purchased from Sigma-Aldrich (Buchs, Switzerland)

as powder and was resuspended in ethanol according to the manufacturer's instructions. Drugs or cholesterol were added directly to cell culture media at concentrations and for time periods indicated. Untreated control populations were treated with an equal volume of molecule solvent or vehicle (DMSO for Ro48-8071 and ethanol for cholesterol).

Cell population size and growth measurements

Cell population size was determined either by counting living cells (negative after trypan blue staining) by the Mallassez cell method or by estimating numbers of cells by WST-1 assay (Cell Proliferation Reagent WST-1; Roche Applied Science, Mannheim, Germany), as previously described (12).

Cell cycle analysis

Cell division monitoring by CFSE staining. 5×10^6 cells were incubated for 15 min at 37 °C with CFSE (CellTrace CFSE Cell Proliferation Kit for flow cytometry; Molecular Probes, Eugene, OR, USA), according to the manufacturer's instructions, with the following modifications: cells were incubated with 5 μ M CFSE in LM1 culture medium instead of phosphate-buffered saline (PBS), 0.1% bovine serum albumin. Cells were then seeded at 5×10^5 cells/ml, as described above. Fluorescence was measured per culture aliquot every day by flow cytometry and cultures were diluted every 2–3 days according to cell density.

Cell cycle analysis by propidium iodide staining. For each state, 2×10^6 cells were washed in PBS and fixed in cold fixation solution (PBS; ethanol 2:3) with incubation period of 30 min on ice. Cells were then harvested, resuspended in PBS-RNase (1 mg/ml) and incubated for 30 min at 37 °C prior to being stained with propidium iodide (PI; Sigma-Aldrich) solution (15 min, 50 μ g/ml in PBS). Fluorescence was then measured using flow cytometry.

Cell surface antigen detection by immunofluorescence labelling

10^5 to 5×10^5 cells were labelled with the primary antibodies (MEP17 1:100; JS4 1:50), as previously described (12), and fluorescence was measured using flow cytometry.

Flow cytometric analysis

Fluorescence data acquisition was carried out using either FACScalibur or FACSCantoII flow cytometer. Data analysis was performed using CELLQuest software (data from

FACScalibur) or Diva software (data from FACSCantoII). Cytometers and software were all purchased from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). Overlay figures were constructed using FlowJo software v8.8.6 (<http://www.flowjo.com>). Positive fluorescence threshold was fixed to 99% negative control cells below this threshold.

Plasmid constructs

pSilencer-shRNA constructs. Sequences used to construct short hairpin RNA (shRNA) interference vectors, leading to pSilencer-shOSC.1, pSilencer-shOSC.5 and pSilencer-shLuc.2 vectors, were designed from chicken OSC (NM 001006514) and firefly luciferase (AY633557) cDNA sequences, using Whitehead software (<http://jura.wi.mit.edu/bioc/siRNAext/home.php>). Double strain fragments containing these targeting sequences were synthesized and are identified as follows (underlined: targeting sequence):

shOSC.1: 5'GCGGGCCCCGAGCCATCAGCGAGCTGTATTCAAGAGATACAGCTCCGTGATGGCTCTTTT-TTGAATTCG3'

shOSC.5: 5'GCGGGCCCCGTATCAGCATACGTCCGATTTCAAGAGAATCGGACCAATGCTGATACTTTT-TTGAATTCG3'

shLuc.2: 5'GCGGGCCCCGCGTTAATCTTAGAGGC-GATTCAAGAGATCGCCTCTTTGATTAAACGCTTTT-TGAATTCG3'

Fragments containing the shRNA coding sequences were cloned in *ApaI/EcoRI* into the pSilencer_1.0-U6 vector (Ambion, Austin, TX, USA).

pMACS.4.1. Vector purchased from Mitenyi Biotec (Bergisch Gladbach, Germany) and previously described (13).

Cell transfection and sorting

Transient transfections were performed using the nucleofection method (Amaza Technology, Lonza Cologne, Cologne, Germany), as previously described (14), with the following specification: cells were transfected with 10 µg DNA (7 µg pSilencer constructs + 3 µg pMACS.4.1). To increase magnetic cell sorting (MACS) efficiency, dead cells and fragments were removed before each MACS assay, as previously described (14). Transfected cells were then magnetically sorted using MACSelect-Transfected Cell Selection System (Mitenyi Biotec) as previously described (14).

Real-time qPCR

Total RNA extraction was performed using RNA Plus Mini Kit (Qiagen, Germantown, MD, USA), according to

manufacturer's instructions. Reverse transcription (RT) assays were performed as previously described (8). cDNAs were incubated overnight at -20 °C (70% ethanol, 500 mM NaCl), precipitated by centrifugation at 16 000 g at 4 °C, washed in 70% ethanol and resuspended in five times RT reaction volume. Real-time quantitative PCR (qPCR) was performed using LightCycler system, using LightCycler FastStart DNA Master SYBR Green I (both from Roche Applied Science), for *OSC/LSS* expression during differentiation and MX3000P system (Stratagene, Cedar Creek, TX, USA), using LightCycler 480 SYBR kit Green I Master (Roche Applied Science), for quantification of *OSC/LSS* and the *GB-β^A* expression in shRNA experiments. QPCR reactions were performed according to manufacturer's instructions, using 1/20th or 1/40th of RT reaction product as template. For information concerning specific PCR conditions, standard genes and primer sequences used, please contact the authors. Relative quantification for each gene, with respect to control conditions, was performed using a previously published mathematical method (15).

Statistical analysis

To determine whether performing a parametric test was possible or not, homoscedasticity of distribution (homogeneity of variance) was tested using a Fischer test. In our case, as values were homoscedastic for all comparisons, Student's test (*t*-test) was performed.

Results

OSC expression and activity are mandatory for proliferation of erythroid progenitors in the self-renewal state

To determine *OSC/LSS* function in self-renewal, we first studied effects of *OSC* enzymatic activity inhibition using the chemical inhibitor Ro48-8071. To determine the appropriate Ro48-8071 dose, initial dose-response testing was carried out (data not shown). Based on IC₅₀ (5.7 nM, information provided by Dr Johannes D. Aebi), three doses were tested: IC₅₀, 3xIC₅₀ and 10xIC₅₀. Although an effect was observed with IC₅₀ dose, intermediary 3xIC₅₀ (17.1 nM) was used for most experiments.

Addition of Ro48-8071 to self-renewing T2EC cells caused reduction in cell number, detectable at the beginning of treatment and becoming significant by fourth day after inhibitor addition, when treated populations showed reduction in cell number in the order of 40% compared to untreated control (Fig. 1: $1.0 \times 10^6 \pm 0.2$ and $1.7 \times 10^6 \pm 0.4$, respectively). This effect was amplified over time and after 7 days treatment, cell number of Ro48-8071-treated

Figure 1. OSC activity inhibition impairs self-renewal ability. T2EC cells were grown in self-renewing conditions in absence or presence (17.1 nM) of Ro48-8071, and cell number was determined by cell counting. Cell number evolution during treatment is represented using logarithmic scale, cumulative growth curve (Mean $\pm$ SD, $n = 3$), $P = 5.74 \times 10^{-2}$ (*) and $P = 1.80 \times 10^{-4}$ (**).

population was four times lower than that of the control group (Fig. 1: $8.6 \times 10^6 \pm 1.4$ and $3.2 \times 10^7 \pm 0.3$ respectively).

The Ro48-8071 inhibitor was initially designed with a therapeutic aim and its efficiency has been tested *in vitro* and *in vivo* (16,17). Co-crystallization of human OSC, in complex with Ro48-8071, clearly showed that this inhibitor binds at the active-site cavity and, thereby, acts

as a substrate structural analogue (18). As OSC, and in particular its active site, is extremely well conserved between species (avian and human OSC have 100% homology in active site amino acid sequence and position), we hypothesized that Ro48-8071 inhibits avian OSC as well as human or rodent OSC. However, to confirm that observed effects were strictly the consequence of OSC inhibition rather than result of non-specific inhibition of other protein functions, we used two different shRNAs, targeting two distinct regions of *OSC/LSS* mRNA (shOSC.1 and shOSC.5), which specifically down-regulate the gene at the mRNA level (Fig. 2a). Observed effects on cell population growth were even more striking than effects observed after OSC activity inhibition with Ro48-8071. Indeed, from the very first day after shRNA transfection, T2EC cells expressing either the shOSC.1 or the shOSC.5 already had growth impairment: population size, in both cases, represented less than 80% of the control population (Fig. 2b). Over the following days, differences between shOSC-expressing cells and the control population was accentuated and 3 days after transfection, there were only in the region of 15% cells left in the shRNA-expressing cell populations compared to the control group (Fig. 2b). That two different shRNAs, targeting two distinct regions of *OSC/LSS* mRNA, both induced the same dramatic effect on cell population growth strongly argues that this is specifically due to *OSC/LSS* mRNA knockdown. To further ascertain specificity of shRNAs inhibition, we designed a new shRNA that targets exogenous Luciferase mRNA (shLuc.2), and did not match any cell gene when querying its target sequence in the avian Ensemble database

Figure 2. OSC/LSS shRNA-mediated knock-down impairs self-renewal ability. T2EC cells expressing either shOSC.1 or shOSC.5 (each targeting a different region of *OSC/LSS* mRNA), or transfected with pSilencer empty vector (EV), were grown in self-renewing conditions. (a) *OSC/LSS* mRNA quantification in T2EC cells expressing either shOSC.1 or shOSC.5, compared to T2EC cells expressing shLuc.2 (shRNA targeting Luciferase mRNA). *OSC/LSS* mRNA was quantified by qPCR with respect to empty vector control. Data were normalized according to different standard genes ($n = 3$). (b) Number of shRNA-expressing living cells was assessed at each time point by WST-1 assay. Evolution of cell population growth with respect to transfection time is presented as percentage of empty vector control (Mean $\pm$ SD, $n = 2$).

Figure 3. Cholesterol reverses negative effect of OSC inhibition on self-renewal. T2EC cells were treated with Ro48-8071 (20 nM) either in absence or in presence (1 µg/ml) of cholesterol. Number of living cells was assessed at each time point by WST-1 assay. Evolution of cell population growth during treatment is presented as percentage of untreated control (Mean $\pm$ SD, $n = 2$).

(http://www.ensembl.org/Gallus_gallus/Info/Index). As expected, shLuc.2 affected neither *OSC/LSS* expression (Fig. 2a) nor cell population growth (data not shown).

Finally, as OSC is an enzyme of the cholesterol synthesis pathway, we examined compensatory effects of cholesterol addition, on Ro48-8071 treated cells. Our data clearly indicate that addition of exogenous cholesterol to culture media reversed the negative effect of OSC inhibition on cell population growth (Fig. 3). Data of this functional test therefore confirm specificity of Ro48-8071 effect on cholesterol synthesis-involved OSC activity. Moreover, this result suggests that disruption of cell self-renewal maintenance, observed with OSC inhibition, may be due to blockage of the cholesterol synthesis pathway.

Reliance on OSC activity is specific to the self-renewal state

Cell population growth impairment observed in self-renewing conditions could result from a global and non-specific negative effect on cell proliferation. For instance, cholesterol – whose synthesis pathway involves OSC activity – is an essential membrane component and membranes must be synthesized during cell division. To determine whether OSC inhibition would affect T2EC cells' proliferation independently of their state or not, we tested its effect before (self-renewal state) and after these progenitors had been induced to differentiate (Fig. 4). It is important to note that, during differentiation, T2EC cells still actively proliferated (Fig. 4a, Diff untreated control condition). Remarkably, in differentiating conditions, T2EC cells' proliferation no longer depended on OSC activity as treatment with Ro48-8071, even at a very high dose (100 nM is five times higher than the 17.1 to 20 nM dose used in the rest of experiments), had absolutely no effect on their proliferation (Fig. 4a, Diff Ro48 100 nM condition). In contrast, the same dose induced dramatic reduction in cell number of self-renewing populations compared to untreated controls (Fig. 4b).

In addition, we confirmed that during differentiation, the main parameters of erythroid differentiation were not affected by OSC inhibition (Fig. 5). First, we observed that haemoglobinization (haemoglobin can be considered as an intracellular maturity marker), evaluated by benzidine haemoglobin staining, was not affected by Ro48-8071 treatment (Fig. 5a). Also, percentage of T2EC cells expressing JS4 and MEP17 – respectively maturity (19) and immaturity (20,21) differentiation markers – at the cell surface, evolved according to normal differentiation kinetics (6). Indeed, as expected, we observed increase in number of cells positive for maturity marker JS4 and reduction of cells positive for immaturity marker MEP17, with no significant difference between untreated and treated populations (Fig. 5b,c). Finally, evolution of cell size

Figure 4. OSC inhibition has no effect on expansion in number of differentiating cells. T2EC cells were grown in absence or presence (100 nM) of Ro48-8071, either in differentiating (Diff, a) or self-renewing (SR, b) conditions. After 3 days treatment, number of cells was determined by cell counting (Mean $\pm$ SD, $n = 3$), $P = 2.7 \times 10^{-2}$ (**).

Figure 5. OSC inhibition does not affect the differentiation process. T2EC cells were grown in differentiating conditions in absence or presence (17.1 nM) of Ro48-8071, and phenotype parameters of erythroid differentiation were evaluated. (a) Haemoglobinization was quantified by acid benzidin staining at each time point during treatment, and percentage positively stained cells is shown (Mean $\pm$ SD, $n = 3$). (b, c) Differentiation markers [maturity: JS4 (b), and immaturity: MEP17 (c)] were quantified at each time point during treatment, by flow cytometry, and percentage of positive cells for either maturity or immaturity marker is shown (Mean $\pm$ SD, $n = 3$).

parameters, followed by flow cytometry, displayed the expected reduction in cell size of differentiating erythroid cells, similarly in both treated and untreated populations (data not shown). These observations confirm that OSC activity is crucial for self-renewal and suggest that this reliance is actually restricted to the self-renewal process alone and does not result from general impairment of cell population growth.

OSC activity protects cells from apoptosis

As OSC inhibition was seen to reduce cell population growth, we studied the effect of Ro48-8071 on cell cycle and apoptosis of our cells to determine the cause of these effects (Fig. 6). First, to monitor populations throughout cell divisions, T2EC cells were stained with CFSE, which integrates into cell membranes and is inherited by daughter cells after division, with decreased intensity. No differences were observed between Ro48-8071-treated

and untreated cells on transition time through the cell cycle phases, suggesting that OSC inhibition has no effect on transition time through the cell cycle. In particular, treatment did not result in emergence of a quiescent or slower-cycling population (Fig. 6a). This was confirmed by more detailed cell cycle analysis using PI-DNA staining, which showed no impact of Ro48-8071 treatment on S and G₂/M phases (Fig. 6b). In contrast, OSC inhibition led to significant reduction in percentage of cells in G₀/G₁ (Fig. 6b, untreated control $54.6 \pm 2.2\%$, treated populations $47.9 \pm 2.2\%$), due to inversely proportional increase in cell percentage in the SubG₁ fraction (Fig. 6b, untreated $22.2 \pm 1.6\%$, treated $29.9 \pm 3.1\%$). Cells in the SubG₁ fraction had lower DNA staining than G₀/G₁ cells and were often associated with apoptotic cells, as DNA compaction early steps of apoptosis hinders PI staining. Thus, augmentation of cell proportion in this fraction suggests increase in apoptotic cells. This hypothesis was confirmed by TUNEL assay, which showed increments of

Figure 6. OSC inhibition does not affect cell proliferation, but decreases survival. T2EC cells were grown in self-renewing conditions, in absence or presence (17.1 nM) of Ro48-8071, for 4 days. (a) T2EC cells, stained with CFSE, were monitored during the treatment period by measuring CFSE fluorescence by flow cytometry. Each peak represents cell count as a function of fluorescence intensity at a given time point. (b) Cell repartition (Mean $\pm$ SD, $n = 3$), determined by propidium iodide (PI) DNA staining, either in different cell cycle phases (G_0/G_1 , S and G_2/M) or in sub G_1 fractions (lower PI staining than G_0/G_1), $P = 2.12 \times 10^{-2}$ (**, sub G_1) and $P = 2.11 \times 10^{-2}$ (**, G_1). (c) Level of apoptotic cells determined by TUNEL assay (Mean $\pm$ SD, $n = 2$), $P = 7.51 \times 10^{-2}$ (*).

apoptotic cell levels as a result of OSC inhibition (Fig. 6c). Our data demonstrate that OSC inhibition had no effect on proliferation, but lead to increase in apoptosis in the self-renewal condition, suggesting that in physiological conditions, OSC activity protects self-renewing cells from apoptosis.

OSC controls balance between self-renewal and differentiation

Under normal differentiation conditions, *OSC/LSS* expression was down-regulated from the very first hours after differentiation induction, and was expressed four to five times less in differentiating cells than in self-renewing cells (Fig. 7). As this down-regulation took place particularly early, at a crucial moment of differentiation, we sought to determine whether it was upstream or downstream of the commitment to differentiate (that is, as a

necessary step or as a consequence of cells no longer needing OSC activity). Hence, we looked at the importance of OSC activity and expression in the commitment step and self-renewal/differentiation balance (Fig. 8). As expected, untreated cell populations growing in self-renewing conditions had a proportion of cells expressing the maturity marker JS4 lower than 15%, and a proportion of cells expressing the immaturity marker MEP17 in the order of 65%. In contrast, in cultures treated with Ro48-8071, proportion of cells expressing JS4 was more than three times higher (44%), and proportion of cells expressing MEP17 was halved (38%) (Fig. 8a). Interestingly, this observation was confirmed by *OSC/LSS* knockdown. Indeed, expression of shOSC.1, an *OSC* mRNA-targeting shRNA, resulted in up-regulation of *globin β^A* gene (Fig. 8b), suggesting increase in haemoglobinization of self-renewing cells when *OSC/LSS* expression was down-regulated. Preliminary results obtained by transient

Figure 7. *OSC/LSS* gene is down-regulated during normal erythroid differentiation. *OSC/LSS* mRNA expression in differentiating T2EC cells was quantified by qPCR, with respect to self-renewing T2EC cells (Diff/SR), at different time points. Evolution with respect to differentiation induction time is shown (Mean $\pm$ SD, $n = 3$).

over-expression of OSC protein tended to confirm this hypothesis (preliminary data not shown). These results indicate that inhibition of OSC activity in self-renewing

cells was a necessary step and not just a consequence of the differentiation process. Thus, reduced OSC activity lead to increase in spontaneous commitment to differentiation and affected both self-renewal maintenance and the self-renewal/differentiation balance.

Our data demonstrate that in self-renewing conditions, decrease in cell number induced by OSC inhibition is accompanied by increase in apoptotic rate and induction of spontaneous commitment to differentiation. Therefore, we propose that in physiological conditions, OSC activity is essential for self-renewal maintenance, and may be involved in the self-renewal vs. differentiation/apoptosis switch, by preventing both apoptosis and differentiation of self-renewing cells.

Discussion

T2EC cells are primary erythroid progenitors, capable of self-renewal without any obvious differentiation and without losing their differentiation potential (6). This cell model is therefore perfectly suited for studying molecular mechanisms controlling choice between self-renewal and differentiation *ex vivo* (6–8,12). Global transcription analysis, aimed at exploring the molecular mechanisms responsible for maintaining self-renewal ability, resulted

Figure 8. OSC inhibition and knockdown affect self-renewal/differentiation balance. (a) T2EC cells were grown in self-renewing conditions in absence or presence (100 nM) of Ro48-8071, and differentiation markers (maturity: JS4 and immaturity: MEP17) were quantified after 3 days treatment. Each figure represents cell count as function of intensity of marker labelling, and percentage of positive cells is presented either in bold (untreated control) or in italics (treated condition). Fold variation of Ro48-treated condition with respect to untreated control is presented as underlined. (b) T2EC cells either expressing shOSC.1 (targeting *OSC/LSS* mRNA) or transfected with pSilencer empty vector (EV) were grown in self-renewing conditions, and globine β^A (GB-BA) mRNA was quantified by qPCR with respect to empty vector control (EV), 48 h after transfection. Data were normalized according to standard genes ($n = 2$).

in a list of candidate genes potentially involved in this process (8). Among them, two have already been studied at the functional level:

- *Sca-2* gene, which we demonstrated to function in a dose-dependent manner to support the self-renewal state (22).
- *OSC/LSS* gene coding for 2,3-oxidosqualene cyclase that we report here to be essential for maintaining immature erythroid progenitors in a self-renewal state and is involved in the self-renewal/differentiation balance.

This leaves open the possibility that more of the differentially expressed genes that we isolated (8) might be functionally relevant for self-renewal regulation.

OSC/LSS expression and self-renewal maintenance

Our data demonstrate that inhibition of OSC enzymatic activity dramatically impaired self-renewing ability of primary erythroid progenitors, which resulted in a substantial reduction in cell number. Since both shRNA-mediated *OSC/LSS* knockdown and OSC enzymatic activity inhibition mediated by Ro48-8071 have similar effects on self-renewing progenitors, we can infer that the observed effect was specifically induced by OSC inhibition. Moreover, this impairment was firmly restricted to the self-renewal state, as once T2EC cells had undergone differentiation, OSC inhibition affected neither cell proliferation nor the differentiation process itself. We established that this effect was essentially due to increase in apoptosis. Therefore, we propose that in physiological conditions, OSC activity is necessary for survival of self-renewing cells, preventing them from dying by apoptosis. Finally, our data show that OSC activity inhibition, as well as its gene knockdown, induces T2EC cells' commitment to differentiation. Thus, besides protecting self-renewing progenitors from apoptosis, OSC also participates in preventing spontaneous differentiation and, hence, helps to maintain cells in the self-renewal state. We thus propose that in the self-renewal state, OSC is involved in a 'to exit or not to exit' decision, which leads cells either to stay in a self-renewal state or to exit and leave the self-renewal compartment and enter into a commitment phase (23). Once committed, these progenitors either terminally differentiate or die by apoptosis, and cell fate depends on presence or absence of external survival factors. Indeed, it has been reported that external factors, such as erythropoietin, are required for terminal erythroid differentiation by protecting differentiating cells from apoptosis through activation of survival pathways (24,25). Such survival signals, even if they are mandatory for late stages of erythropoiesis, are not required for commitment to the erythroid

lineage (26); in the absence of these survival factors, committed cells will undergo apoptosis. Thus, OSC activity, by preventing inappropriate commitment of self-renewing progenitors when conditions required for final differentiation (for example, presence of factors such as erythropoietin) are not present, blocks the resulting commitment-dependent apoptosis. The importance of OSC in this 'to exit or not to exit' model is highlighted by the observed effect of OSC enzymatic inhibition and shRNA-mediated knockdown on spontaneous differentiation.

OSC/LSS expression and cholesterol homeostasis

OSC catalyses cyclization of monoepoxysqualene to lanosterol, which is the first sterol in cholesterol biosynthesis (9). In addition, OSC catalyses cyclization of diepoxysqualene to epoxycholesterol, an intermediate of the synthesis pathway of 24(S),25-epoxycholesterol, which is a natural ligand of liver X nuclear receptor (LXR) (10). Once activated, LXR regulates expression of several genes involved in regulation of cellular lipid metabolism, including cellular efflux of cholesterol (10). Recent studies have reported a cause-and-effect relationship between *OSC/LSS* expression and cholesterol synthesis regulation, and it has been proposed that *OSC/LSS* down-regulation may constitute a novel mechanism involved in control of cholesterol metabolism (27,28). Because of the unique position occupied by OSC in both synthesis pathways, this mechanism probably involves a dual mechanism of action: reduced cholesterol synthesis and increased 24(S),25-epoxycholesterol synthesis (29).

Our results clearly show that OSC expression and activity are mandatory for the erythroid progenitors' population growth in self-renewal state. As addition of exogenous cholesterol reverses the negative effect of Ro48-8071, we suppose that impairment observed on self-renewing cells is due to a cholesterol synthesis-related effect, rather than non-cholesterol-related LXR activation. As cholesterol synthesis pathways can be modulated by *OSC/LSS* expression, we can speculate that cholesterol synthesis, controlled by *OSC/LSS* expression, is involved in maintenance of erythroid progenitors' self-renewal and in control of the self-renewal/differentiation balance. To qualitatively evaluate the effect of Ro48-8071 and cell cholesterol evolution, we decided to measure cholesterol levels. However, despite all our efforts (Amplex Red, Filipin staining), we were not able to quantify such cholesterol variations, perhaps because variations were below the sensitivity threshold of tests compatible with our cells. Moreover, consistent with the hypothesis of the importance of cholesterol rate regulation, our preliminary results on the study of cholesterol's role in self-renewal (work in progress) show that two

other inhibitors that block the cholesterol synthesis pathway upstream and downstream OSC, have similar effects to OSC inhibitor Ro48-8071 (Fig. S1), and suggest that OSC involvement in self-renewal maintenance relies on its cholesterol synthesis-related function. This is reinforced by addition of exogenous cholesterol experiments, which show that cholesterol has a positive effect on self-renewal (Fig. S2). An important cholesterol requirement by self-renewing cells could explain why cholesterol-rich lipids, added to StemSpan medium, improve human erythroblast growth (30).

The hypothesis of cholesterol synthesis involvement in the self-renewal/differentiation balance is in agreement with previous studies pointing out a link between cholesterol homeostasis and differentiation. First, it was shown that both HMG-CoA reductase inhibition (31) and, more specifically, cholesterol starvation (32) induce differentiation of human leukaemia HL-60 cells. Moreover, hypercholesterolaemia has been reported to result in blockage of erythroid differentiation (33). On the other hand, it has been reported that cholesterol redistribution is important during erythrocyte maturation (34). Besides, consistent with our own results, the authors also show that the cholesterol biosynthetic pathway is down-regulated during erythroid differentiation, and notably, that OSC is the first down-regulated enzyme of the pathway (34).

Cholesterol homeostasis and self-renewal

In the present work, both self-renewing and differentiation medium contain 10% FBS, which contains a fraction of lipoprotein-bound cholesterol (about 36 µg/ml, according to manufacturer's estimate). However, addition of free-cholesterol (1 µg/ml) to the culture medium reversed the effect of OSC inhibition. This free-cholesterol concentration is relatively low compared to lipoprotein-bound cholesterol already present in the medium; however, internalization of both sources is made through different molecular mechanisms. On the one hand, lipoprotein-bound cholesterol is transported inside a protein cage and its internalization requires expression of a cellular receptor. Cholesterol can be taken up from lipoproteins in the circulation by mechanisms involving receptor-mediated uptake. The best understood and quantitatively the most important process is the one involving serum low-density lipoprotein (LDL) and its LDL-receptor (35). Our transcriptome data show, however, no expression of avian LDLR, in either self-renewing or differentiating conditions [SQUAT database query (36)]. This absence of expression therefore suggests that even if this cholesterol source would be available, T2EC cells would not be able to use it as they cannot internalize it. On the other hand, free-cholesterol can passively diffuse through the phospholipid layers of

cell membranes, which could explain why a relatively low, but yet more accessible amount of free-cholesterol has such an effect and could explain why and how T2EC cells distinguish these two cholesterol sources.

Cholesterol is a key cellular element with both structural and functional roles. In particular, fluidity and mechanical stability of the cell membrane depends on its cholesterol content. Cell population growth impairment observed in self-renewing conditions could therefore result from global negative effect on cell proliferation. Along with the well-known requirement of cholesterol for mammalian cell proliferation (37,38), cholesterol has been shown to activate CDK1 (39,40), and to be essential for cytokinesis (41,42). In contrast with this, our data show that in differentiating conditions, in which T2EC cells continue to actively cycle, proliferation no longer depends on OSC activity. This observation thus suggests that the effect of OSC inhibition does not result from general impairment of cell proliferation. Yet, cholesterol is also involved in intracellular signalling, maintaining characteristic low fluidity in lipid rafts (43). It has been previously reported that Ras/Raf/ERK and TGFβ pathways, are both raft-dependent [respectively (44,45) and (46,47)], are essential for maintaining erythroid progenitor self-renewal (12,48,49).

In addition, cholesterol-mediated post-translational modification of Hedgehog proteins (50) is necessary for functional signal transduction. Indeed, reduction in cell cholesterol blocks signal transduction in the Hedgehog pathway (51,52). Interestingly, it has also been proposed that Hedgehog signalling is crucial to erythroid progenitor maintenance in stress-related and normal adult erythropoiesis (53,54). In agreement with this, our preliminary data suggest that Hedgehog signalling could be involved in T2EC cells' self-renewal. On the one hand, Sonic Hedgehog mRNA is present exclusively in self-renewing conditions, but absent in differentiating conditions [SQUAT database query (36)]; on the other hand, cyclopamine, a Hedgehog pathway inhibitor (55), has negative dose-dependent effects on T2EC cells' self-renewal, and the effect can be abolished by addition of cholesterol (our own unpublished data).

In conclusion, our data show that OSC activity in cholesterol synthesis is essential for survival of self-renewing erythroid progenitor cells, and we have demonstrated that this effect is essentially due to protection from apoptosis. Our results are consistent with the hypothesis that *OSC/LSS* expression, via OSC activity on *de novo* cholesterol synthesis, plays a physiological role in self-renewal vs. differentiation/apoptosis decision making, by maintaining cells in the self-renewal state. Further clarification of the mechanism underlying precise action of cholesterol, and its relationship with cell signalling, may provide

valuable information that would help in understanding cell self-renewal.

Author contributions

CMP: conception and design, collection and assembly of data, data analysis and interpretation, and manuscript writing; FD: Collection of preliminary data and final approval of manuscript; OG: financial support, administrative support and final approval of manuscript.

Acknowledgements

We are grateful to Dr Johannes D. Aebi for kindly providing the Ro48-8071 inhibitor, Corinne Bresson, Sandrine Gonin-Giraud, Claudine Faure and Gaël Leprun for their technical help, and to Valerie James (JW Business Services Ltd.) for language editing of the manuscript. This work was supported by grants from the LNCC, the CNRS, the UCBL, the Region Rhône Alpes and the ARC. CMP is a PhD fellow from the LNCC and UCBL.

Supporting Information

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

Fig. S1 Inhibition of cholesterol synthesis and self-renewal ability. T2EC cells were grown in absence or presence of increasing doses of either (A) Simvastatin or (B) AY-9944, in self-renewal conditions. Number of living cells was assessed at each time point through WST-1 and is presented as percentage of untreated control (Mean $\pm$ SD, $n = 2$). (C) T2EC cells were treated with (a) Simvastatin or (b) AY-9944, either in absence or presence (1 $\mu\text{g}/\text{ml}$) of cholesterol. Number of living cells was assessed at each time point through WST-1 assay and is provided as percentage of untreated control (Mean $\pm$ SD, $n = 2$).

Fig. S2 Cholesterol and self-renewal maintenance. (a) T2ECs were grown in absence or presence (10 $\mu\text{g}/\text{ml}$) of exogenous cholesterol for 3 days, in self-renewal conditions, and cell number was determined by cell counting (Mean $\pm$ SD, $n = 3$), $P = 1.4 \times 10^{-1}$. (b) T2EC cells were grown in absence or presence (100 $\mu\text{g}/\text{ml}$) of cholesterol for 3 days, in differentiating conditions, and maturity marker JS4 was quantified. This figure represents cell count as function of intensity of marker labelling, and percentage of positive cells is presented either in italics (untreated control) or bold (treated condition).

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

References

- Weissman IL (2000) Stem cells: units of development, units of regeneration, and units in evolution. *Cell* **100**, 157–168.
- Ivanova NB, Dimos JT, Schaniel C, Hackney JA, Moore KA, Lemischka IR (2002) A stem cell molecular signature. *Science* **298**, 601–604.
- Ramalho-Santos M, Yoon S, Matsuzaki Y, Mulligan RC, Melton DA (2002) “Stemness”: transcriptional profiling of embryonic and adult stem cells. *Science* **298**, 597–600.
- Hentze MW, Muckenthaler MU, Andrews NC (2004) Balancing acts: molecular control of mammalian iron metabolism. *Cell* **117**, 285–297.
- Bauer A, Tronche F, Wessely O, Kellendonk C, Reichardt HM, Steinlein P *et al.* (1999) The glucocorticoid receptor is required for stress erythropoiesis. *Genes Dev.* **13**, 2996–3002.
- Gandrillon O, Schmidt U, Beug H, Samarut J (1999) TGF-beta cooperates with TGF-alpha to induce the self-renewal of normal erythrocytic progenitors: evidence for an autocrine mechanism. *EMBO J.* **18**, 2764–2781.
- Dolznic H, Grebier F, Deiner EM, Stangl K, Kolbus A, Habermann B *et al.* (2006) Erythroid progenitor renewal versus differentiation: genetic evidence for cell autonomous, essential functions of EpoR, Stat5 and the GR. *Oncogene* **25**, 2890–2900.
- Damiola F, Keime C, Gonin-Giraud S, Dazy S, Gandrillon O (2004) Global transcription analysis of immature avian erythrocytic progenitors: from self-renewal to differentiation. *Oncogene* **23**, 7628–7643.
- Huff MW, Telford DE (2005) Lord of the rings—the mechanism for oxidosqualene:lanosterol cyclase becomes crystal clear. *Trends Pharmacol. Sci.* **26**, 335–340.
- Ory DS (2004) Nuclear receptor signaling in the control of cholesterol homeostasis: have the orphans found a home? *Circ. Res.* **95**, 660–670.
- Gandrillon O, Samarut J (1998) Role of the different RAR isoforms in controlling the erythrocytic differentiation sequence. Interference with the v-erbA and p135gag-myb-ets nuclear oncogenes. *Oncogene* **16**, 563–574.
- Dazy S, Damiola F, Parisey N, Beug H, Gandrillon O (2003) The MEK-1/ERKs signalling pathway is differentially involved in the self-renewal of early and late avian erythroid progenitor cells. *Oncogene* **22**, 9205–9216.
- Gaines P, Wojchowski DM (1999) pIRES-CD4t, a dicistronic expression vector for MACS- or FACS-based selection of transfected cells. *Biotechniques* **26**, 683–688.
- Mejia-Pous C, Vinuelas J, Faure C, Koszela J, Kawakami K, Takahashi Y *et al.* (2009) A combination of transposable elements and magnetic cell sorting provides a very efficient transgenesis system for chicken primary erythroid progenitors. *BMC Biotechnol.* **9**, 81.
- Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45.
- Dehmlow H, Aebi JD, Jolidon S, Ji YH, von der Mark EM, Hember J *et al.* (2003) Synthesis and structure-activity studies of novel orally active non-terpenoid 2,3-oxidosqualene cyclase inhibitors. *J. Med. Chem.* **46**, 3354–3370.
- Morand OH, Aebi JD, Dehmlow H, Ji YH, Gains N, Lengsfeld H *et al.* (1997) Ro 48-8071, a new 2,3-oxidosqualene:lanosterol cyclase inhibitor lowering plasma cholesterol in hamsters, squirrel monkeys, and minipigs: comparison to simvastatin. *J. Lipid Res.* **38**, 373–390.
- Thoma R, Schulz-Gasch T, D’Arcy B, Benz J, Aebi J, Dehmlow H *et al.* (2004) Insight into steroid scaffold formation from the structure of human oxidosqualene cyclase. *Nature* **432**, 118–122.

- 19 Schmidt JA, Marshall J, Hayman MJ, Doderlein G, Beug H (1986) Monoclonal antibodies to novel erythroid differentiation antigens reveal specific effects of oncogenes on the leukaemic cell phenotype. *Leuk. Res.* **10**, 257–272.
- 20 McNagny KM, Lim F, Grieser S, Graf T (1992) Cell surface proteins of chicken hematopoietic progenitors, thrombocytes and eosinophils detected by novel monoclonal antibodies. *Leukemia* **6**, 975–984.
- 21 Bradshaw AD, McNagny KM, Gervin DB, Cann GM, Graf T, Clegg DO (1995) Integrin alpha 2 beta 1 mediates interactions between developing embryonic retinal cells and collagen. *Development* **121**, 3593–3602.
- 22 Bresson-Mazet C, Gandrillon O, Gonin-Giraud S (2008) Stem cell antigen 2: a new gene involved in the self-renewal of erythroid progenitors. *Cell Prolif.* **41**, 726–738.
- 23 Gandrillon O, Ferrand N, Michaille JJ, Roze L, Zile MH, Samarut J (1994) c-erbA alpha/T3R and RARs control commitment of hematopoietic self-renewing progenitor cells to apoptosis or differentiation and are antagonized by the v-erbA oncogene. *Oncogene* **9**, 749–758.
- 24 Koury MJ, Bondurant MC (1990) Erythropoietin retards DNA breakdown and prevents programmed death in erythroid progenitor cells. *Science* **248**, 378–381.
- 25 Lesault I, Quang CT, Frampton J, Ghysdael J (2002) Direct regulation of BCL-2 by FLI-1 is involved in the survival of FLI-1-transformed erythroblasts. *EMBO J.* **21**, 694–703.
- 26 Dolznig H, Habermann B, Stangl K, Deiner EM, Moriggl R, Beug H *et al.* (2002) Apoptosis protection by the Epo target Bcl-X(L) allows factor-independent differentiation of primary erythroblasts. *Curr. Biol.* **12**, 1076–1085.
- 27 Dang H, Liu Y, Pang W, Li C, Wang N, Shyy JY *et al.* (2009) Suppression of 2,3-oxidosqualene cyclase by high fat diet contributes to liver X receptor-alpha-mediated improvement of hepatic lipid profile. *J. Biol. Chem.* **284**, 6218–6226.
- 28 Villagra A, Ulloa N, Zhang X, Yuan Z, Sotomayor E, Seto E (2007) Histone deacetylase 3 down-regulates cholesterol synthesis through repression of lanosterol synthase gene expression. *J. Biol. Chem.* **282**, 35457–35470.
- 29 Wong J, Quinn CM, Gelissen IC, Brown AJ (2008) Endogenous 24(S),25-epoxycholesterol fine-tunes acute control of cellular cholesterol homeostasis. *J. Biol. Chem.* **283**, 700–707.
- 30 Leberbauer C, Boulme F, Unfried G, Huber J, Beug H, Mullner EW (2005) Different steroids co-regulate long-term expansion versus terminal differentiation in primary human erythroid progenitors. *Blood* **105**, 85–94.
- 31 Kanno T, Kobuchi H, Kajitani N, Utsumi T, Yano H, Horton AA *et al.* (2002) Mevastatin, an inhibitor of HMG-CoA reductase, induces apoptosis, differentiation and Rap1 expression in HL-60 cells. *Physiol. Chem. Phys. Med. NMR* **34**, 1–15.
- 32 Sanchez-Martin CC, Davalos A, Martin-Sanchez C, de la Pena G, Fernandez-Hernando C, Lasuncion MA (2007) Cholesterol starvation induces differentiation of human leukemia HL-60 cells. *Cancer Res.* **67**, 3379–3386.
- 33 Holm TM, Braun A, Trigatti BL, Brugnara C, Sakamoto M, Krieger M *et al.* (2002) Failure of red blood cell maturation in mice with defects in the high-density lipoprotein receptor SR-BI. *Blood* **99**, 1817–1824.
- 34 Fan J, Rone MB, Papadopoulos V (2009) Translocator protein 2 is involved in cholesterol redistribution during erythropoiesis. *J. Biol. Chem.* **284**, 30484–30497.
- 35 Ikonen E (2008) Cellular cholesterol trafficking and compartmentalization. *Nat. Rev. Mol. Cell Biol.* **9**, 125–138.
- 36 Leyritz J, Schicklin S, Blachon S, Keime C, Robardet C, Boulicaut JF *et al.* (2008) SQUAT: a web tool to mine human, murine and avian SAGE data. *BMC Bioinformatics* **9**, 378.
- 37 Brown MS, Goldstein JL (1974) Suppression of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and inhibition of growth of human fibroblasts by 7-ketocholesterol. *J. Biol. Chem.* **249**, 7306–7314.
- 38 Chen HW, Heiniger HJ, Kandutsch AA (1975) Relationship between sterol synthesis and DNA synthesis in phytohemagglutinin-stimulated mouse lymphocytes. *Proc. Natl. Acad. Sci. USA* **72**, 1950–1954.
- 39 Martinez-Botas J, Suarez Y, Ferruelo AJ, Gomez-Coronado D, Lasuncion MA (1999) Cholesterol starvation decreases p34(cdc2) kinase activity and arrests the cell cycle at G2. *FASEB J.* **13**, 1359–1370.
- 40 Suarez Y, Fernandez C, Ledo B, Ferruelo AJ, Martin M, Vega MA *et al.* (2002) Differential effects of ergosterol and cholesterol on Cdk1 activation and SRE-driven transcription. *Eur. J. Biochem.* **269**, 1761–1771.
- 41 Fernandez C, Lobo Md Mdel V, Gomez-Coronado D, Lasuncion MA (2004) Cholesterol is essential for mitosis progression and its deficiency induces polyploid cell formation. *Exp. Cell Res.* **300**, 109–120.
- 42 Ng MM, Chang F, Burgess DR (2005) Movement of membrane domains and requirement of membrane signaling molecules for cytokinesis. *Dev. Cell* **9**, 781–790.
- 43 Lingwood D, Simons K (2010) Lipid rafts as a membrane-organizing principle. *Science* **327**, 46–50.
- 44 Rizzo MA, Kraft CA, Watkins SC, Levitan ES, Romero G (2001) Agonist-dependent traffic of raft-associated Ras and Raf-1 is required for activation of the mitogen-activated protein kinase cascade. *J. Biol. Chem.* **276**, 34928–34933.
- 45 Roy S, Luetterforst R, Harding A, Apolloni A, Etheridge M, Stang E *et al.* (1999) Dominant-negative caveolin inhibits H-Ras function by disrupting cholesterol-rich plasma membrane domains. *Nat. Cell Biol.* **1**, 98–105.
- 46 Ma X, Wang Q, Jiang Y, Xiao Z, Fang X, Chen YG (2007) Lateral diffusion of TGF-beta type I receptor studied by single-molecule imaging. *Biochem. Biophys. Res. Commun.* **356**, 67–71.
- 47 Zhou X, Johnston TP, Johansson D, Parini P, Funa K, Svensson J *et al.* (2009) Hypercholesterolemia leads to elevated TGF-beta1 activity and T helper 3-dependent autoimmune responses in atherosclerotic mice. *Atherosclerosis* **204**, 381–387.
- 48 Gonin-Giraud S, Bresson-Mazet C, Gandrillon O (2008) Involvement of the TGF-beta and mTOR/p70S6Kinase pathways in the transformation process induced by v-ErbA. *Leuk. Res.* **32**, 1878–1888.
- 49 Rubiolo C, Piazzolla D, Meissl K, Beug H, Huber JC, Kolbus A *et al.* (2006) A balance between Raf-1 and Fas expression sets the pace of erythroid differentiation. *Blood* **108**, 152–159.
- 50 Porter JA, Ekker SC, Park WJ, von Kessler DP, Young KE, Chen CH *et al.* (1996) Hedgehog patterning activity: role of a lipophilic modification mediated by the carboxy-terminal autoprocessing domain. *Cell* **86**, 21–34.
- 51 Guy RK (2000) Inhibition of sonic hedgehog autoprocessing in cultured mammalian cells by sterol deprivation. *Proc. Natl. Acad. Sci. USA* **97**, 7307–7312.
- 52 Incardona JP, Eaton S (2000) Cholesterol in signal transduction. *Curr. Opin. Cell Biol.* **12**, 193–203.
- 53 Cridland SO, Keys JR, Papathanasiou P, Perkins AC (2009) Indian hedgehog supports definitive erythropoiesis. *Blood Cells Mol. Dis.* **43**, 149–155.
- 54 Perry JM, Harandi OF, Porayette P, Hegde S, Kannan AK, Paulson RF (2009) Maintenance of the BMP4-dependent stress erythropoiesis pathway in the murine spleen requires hedgehog signaling. *Blood* **113**, 911–918.
- 55 Chen JK, Taipale J, Cooper MK, Beachy PA (2002) Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. *Genes Dev.* **16**, 2743–2748.