nature chemical biology

ARTICLE

PUBLISHED ONLINE: 2 MAY 2010 | DOI: 10.1038/NCHEMBIO.364

Metabolic oxidation regulates embryonic stem cell differentiation

Oscar Yanes[1], Julie Clark[2], Diana M Wong[1], Gary J Patti[1], Antonio Sánchez-Ruiz[2], H Paul Benton[1], Sunia A Trauger[1], Caroline Desponts[2], Sheng Ding[2]* & Gary Siuzdak[1]*

Metabolites offer an important unexplored complementary approach to understanding the pluripotency of stem cells. Using MS-based metabolomics, we show that embryonic stem cells are characterized by abundant metabolites with highly unsaturated structures whose levels decrease upon differentiation. By monitoring the reduced and oxidized glutathione ratio as well as ascorbic acid levels, we demonstrate that the stem cell redox status is regulated during differentiation. On the basis of the oxidative biochemistry of the unsaturated metabolites, we experimentally manipulated specific pathways in embryonic stem cells while monitoring the effects on differentiation. Inhibition of the eicosanoid signaling pathway promoted pluripotency and maintained levels of unsaturated fatty acids. In contrast, downstream oxidized metabolites (for example, neuroprotectin D1) and substrates of pro-oxidative reactions (for example, acyl-carnitines), promoted neuronal and cardiac differentiation. We postulate that the highly unsaturated metabolome sustained by stem cells allows them to differentiate in response to *in vivo* oxidative processes such as inflammation.

Global gene-expression patterns of embryonic and adult stem cell populations have allowed the identification of important genes in stem cell biology. Parallel studies of epigenetic regulation have led to the discovery of a bivalent histone modification motif in embryonic stem cells (ESCs)[1,2]. At the proteomic level, studies have revealed protein expression patterns associated with stem cells[3]. Despite these important advances, the molecular framework that controls the balance between pluripotency and stem cell differentiation is still not fully understood. This may be partially due to the challenge of correlating gene and protein expression data with functional activity and cellular phenotype. Indeed, increases in mRNA levels do not always correlate with increases in protein levels[4,5]. Additionally, a variety of post-translational modifications can prevent a protein from being functionally active once it is translated.

A promising complementary approach to exploring the stem cell phenotype is metabolomics, defined as the metabolic complement of functional genomics. Metabolomics enables the characterization of endogenous small molecules that are the products of biochemical reactions, revealing connections between different pathways that operate within a living cell[6,7]. We quantitatively characterized pluripotent and differentiated cells from two germ layers (the ectoderm and mesoderm) using an untargeted metabolomics approach with liquid chromatography–electrospray ionization–time of flight MS (LC-ESI-TOF-MS). To our knowledge, this is the first study to characterize stem cells with an untargeted metabolomics approach. We compared two different lines of mouse embryonic stem cells (mESCs) that have greater than 99% homogeneity (46C mESC and R1 mESC). Their differentiated progenies were analyzed when more than 80% of the cells were neurons and when more than 70% were cardiomyocytes. On the basis of the mass-spectrometric metabolomics results, we selectively inhibited enzymes *in vitro* that we predicted would mediate differentiation. Similarly, we supplemented stem cell media with naturally occurring metabolites involved in and produced by oxidative pathways to determine their effects on pluripotency and differentiation.

Together, our results show that the phenotype of ESCs is characterized by the presence of structurally unsaturated metabolites whose levels decrease upon differentiation. We suggest that this highly unsaturated metabolome is susceptible to pro-oxidative events that ultimately influence cell fate.

RESULTS

Highly unsaturated metabolites in embryonic stem cells

Our MS-based platform (**Supplementary Fig. 1**) involves LC-ESI-TOF-MS profiling followed by data analysis with open-source software called XCMS (ref. 8). We quantified the relative abundance of metabolites in homogeneous populations of ESCs, neurons and cardiomyocytes (as determined by characteristic morphology and marker expression, **Supplementary Fig. 2**) by comparing the integrated area of each feature and assigned a fold value to indicate the level of differential regulation between populations (see full data in **Supplementary Table 1**).

To identify a 'metabolic signature' associated with the stem cell phenotype, we characterized metabolites that were differentially regulated (greater than twofold difference) in ESCs relative to neurons and cardiomyocytes, and for which the difference was statistically significant ($P < 0.01$). The high resolving power (~200,000) and high mass accuracy (<1 p.p.m. error) of Fourier transform ion cyclotron resonance MS allowed us to determine the molecular formulas of more than 150 metabolites. On the basis of the chemical formulas, we were able to calculate the index of hydrogen deficiency (IHD) for each metabolite, which we normalized by mass (**Supplementary Fig. 1b**). The IHD accounts for the degree of unsaturation of a specific molecule, determined by the number of ρ bonds and rings that the molecule contains. **Figure 1a** shows the distribution of IHDs for the endogenous metabolites for which we determined molecular formulas, indicating a substantially higher degree of unsaturation in the stem cell metabolome than in mature populations. More than 85% of the metabolite formulas in ESCs

[1]Department of Molecular Biology and Scripps Center for Mass Spectrometry, The Scripps Research Institute, La Jolla, California, USA. [2]Department of Chemistry and the Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, California, USA. *e-mail: siuzdak@scripps.edu or sding@scripps.edu

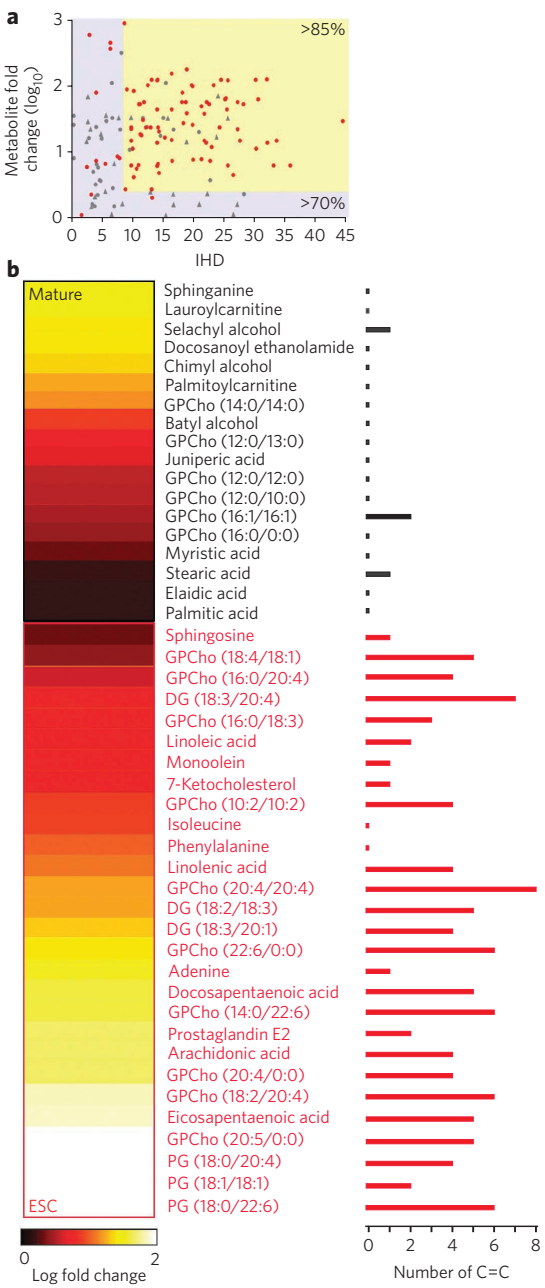

Figure 1 | Embryonic stem cells are characterized by abundant metabolites with highly unsaturated structures. (**a**) Degree of unsaturation in ESCs and mature populations. The plot shows the relative fold change of metabolites that are either upregulated in ESCs (red dots) or upregulated in mature populations (gray dots and gray triangles representing neurons and cardiomyocytes, respectively) versus the IHD of each metabolite. Of the molecular formulas we characterized in ESCs, 85% have IHD values ≥8 and fold increases ≥5 (yellow-shaded area). Of the molecular formulas we characterized in mature populations, 70% have IHD values ≤8 or fold increases ≤5, or both (blue-shaded area). (**b**) Heat map showing 46 metabolites whose structures were identified by tandem MS. Lighter colors (yellow and white) correspond to the largest fold differences between the integrated peak area of each feature in ESCs and the integrated peak area in mature populations. Metabolite names shown in black are upregulated in mature populations relative to ESCs ($P < 0.01$). Metabolite names shown in red are upregulated in ESCs relative to mature populations ($P < 0.01$). The number of carbon–carbon double bonds (C=C) for each metabolite is shown in the bar graph on the right.

show a high degree of unsaturation (IHD > 8) and a high relative abundance (over fivefold) in comparison to the mature populations. For metabolites that were over fivefold more abundant in mature populations than in ESCs, most of the calculated molecular formulas had IHDs <8.

Some of the most upregulated metabolites identified by tandem MS/MS (**Supplementary Fig. 1c**) in ESCs relative to mature populations are secondary lipid messengers and inflammatory mediators (**Fig. 1b**) such as arachidonic acid, docosapentaenoic acid, eicosapentaenoic acid, linolenic acid, diacylglycerols, glycerophosphocholines (GPCho) (**Supplementary Table 2**), glycerophosphoglycerols and eicosanoids (for example, prostaglandin E2). Sphingosine and 7-ketocholesterol—respectively, a sphingolipid and a cholesterol metabolite involved in signal transduction—were also identified. Mature populations showed increased levels of saturated free fatty acids together with their acyl-carnitines (primarily in cardiomyocytes), which consist of fatty acyl–CoA (coenzyme A) conjugated to carnitine, implicated in transport into the mitochondria for β-oxidation. Neurons were unique in that they contained saturated fatty alcohols (namely chimyl, batyl (**1**) and selachyl alcohol) not observed in ESCs. Little is known about these fatty alcohols, and to our knowledge they have not previously been described in mammalian neuronal cells. However, they have been associated with the inhibition of neurodegenerative diseases[9] and in bone marrow cell regeneration[10]. Docosanoyl ethanolamide, a saturated *N*-acylethanolamide (endocannabinoid) that modulates voltage-dependent Ca^{2+} channels[11], was found to be uniquely upregulated in differentiated cardiomyocytes.

Overall, the structures of the differentially regulated metabolites indicate that the high degree of unsaturation reflected by the IHD in stem cells is primarily a result of carbon–carbon double bonds (**Fig. 1b**). Consequently, as chemical unsaturations such as carbon–carbon double bonds are highly reactive under oxidative conditions (for example, arachidonic acid is a metabolic precursor for the biosynthesis of over 100 structurally diverse metabolites inside the cell), we hypothesized that the abundance of these species in ESCs is important in maintaining 'chemical plasticity' and ultimately in mediating differentiation through regulation of redox status and activation of oxidative pathways.

Redox status mediates embryonic stem cell differentiation

To confirm that the oxidation of the unsaturated metabolites we identified was associated with intracellular changes in redox status, we quantified the ratio of reduced and oxidized glutathione (GSH/GSSG) as a function of days of differentiation of ESCs using triple-quadrupole MS and multiple reaction monitoring (MRM). GSH is a reducing agent that protects the cell from oxidative damage, mainly through the catalyzed reduction of hydrogen peroxide to water by the enzyme glutathione peroxidase. When mammalian cells are exposed to increased oxidative stress, the GSH/GSSG ratio decreases as GSH is converted to GSSG. Our results show a substantial decrease in the GSH/GSSG ratio at day 4 as a consequence of GSSG accumulation (**Fig. 2**). At day 7, GSH/GSSG returned to the original ratio observed at day 0. Note that the GSH/GSSG ratio at day 0 (0.58), as determined from the integrated peak areas of product ions in MRM experiments, corresponds to a concentration of GSH 90- to 100-fold higher than that of GSSG. This was confirmed with GSH and GSSG standards. Ascorbic acid is another strong reducing agent that neutralizes reactive oxygen species (ROS) in cells. Notably, when we compared ascorbic acid with GSH and GSSG using MRM, we observed that the concentration of ascorbic acid increased at day 4, coinciding with the lowest GSH/GSSG ratio. This indicates that the responses of the GSH/GSSG ratio and of ascorbic acid levels to ESC differentiation are inversely related. Therefore, our findings suggest that redox regulation, probably as

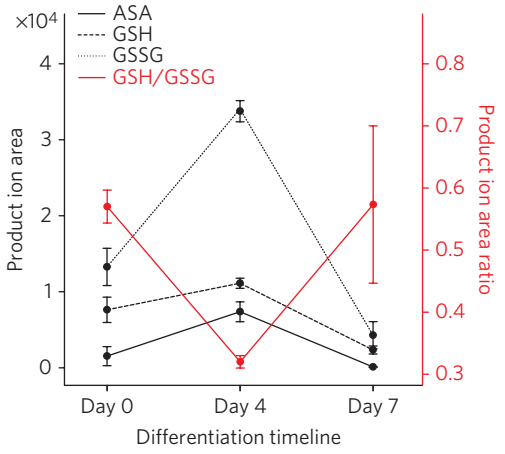

Figure 2 | GSH/GSSG ratio and ascorbic acid levels are inversely related in response to ESC differentiation. Abundance of ascorbic acid, GSH and GSSG, and GSH/GSSG ratio, as a function of days of differentiation of mESCs. Values shown are the integrated peak areas of product ions using triple quadrupole MS and MRM (see **Supplementary Methods** for details). Error bars represent mean values and s.d. for three independently prepared replicates (4 million cells) at each time point.

a result of events associated with oxidative stress occurring during differentiation, is important in mediating embryonic stem cell fate.

Eicosanoid pathway inhibition promotes pluripotency

To test our hypothesis that activation of oxidative pathways is important for differentiation, we inhibited the eicosanoid signaling pathway (**Fig. 3a**), a well-known pro-oxidative cascade in which the metabolic substrates (arachidonic acid (AA), docosahexaenoic acid (DHA), eicosa pentaenoic acid (EPA)) are characteristic of the pluripotent state, as revealed by our MS-based metabolomics results.

Desaturase inhibitors (sesamin (**2**), a selective inhibitor of 5Δ desaturase; and curcumin (**3**), an inhibitor of 5Δ and 6Δ desaturase), cyclooxygenase (COX) inhibitors (SC-236 (**4**), a selective COX-2 inhibitor; and SC-560 (**5**), a selective COX-1 inhibitor), lipooxygenase (LOX) inhibitors (AA-861 (**6**) and BW-A4C (**7**), selective 5-LOX inhibitors) and a phospholipase A2 (PLA_2) inhibitor (palmityl trifluoromethyl ketone, or PTK (**8**)) were tested, and each promoted the pluripotent state of mESCs when cultured in neuronal differentiation conditions. We measured pluripotency by monitoring the expression of the mESC markers *Nanog* and *Oct4* (**Fig. 3b**) and analyzing colony morphology (**Supplementary Fig. 3**) at days 2 and 4 of treatment with the inhibitors. Specifically, selective inhibition of these enzymes led to 15–60% increases in *Oct4* and *Nanog* expression levels relative to DMSO at day 4. Expression of *Oct4* and *Nanog* in cells grown in the absence of DMSO was not different from expression in those grown in DMSO control.

Inhibition of 5Δ and 6Δ desaturase with sesamin and curcumin resulted in a substantial reduction in neuronal differentiation with increasing dosages. Sesamin, curcumin or DMSO vehicle was added to mESCs cultured in N2B27 medium without leukemia inhibitory factor at day 1, and cells were analyzed for neuronal differentiation at day 10 (**Supplementary Fig. 4**). The number of βIII-tubulin–positive neurons, a widely used marker for early neuronal differentiation[12], decreased with increasing concentrations of sesamin and curcumin, with no detectable reduction in total cell number over the treatment range of 0–10 μM, as determined by DAPI staining (**Fig. 3c** and **Supplementary Fig. 5**). Compared to DMSO vehicle, 10 μM sesamin or 10 μM curcumin delayed neuronal differentiation, as evidenced by 78% and 83% reductions in the number of βIII-tubulin—positive neurons, respectively. The number of βIII-tubulin–positive neurons in the absence of DMSO was not different from the number in the DMSO control.

COX inhibitors were also administered to a separate culture at day 1, and cells were analyzed at day 10 of differentiation. Cultures treated with SC-236 (COX-2 half-maximal inhibitory concentration (IC_{50}), 5–10 nM; COX-1 IC_{50}, 17 μM) showed a reduction in both βIII-tubulin–positive neurons and microtubule-associated protein-2ab (Map2ab)–positive neurons with increasing inhibitor concentrations (**Fig. 3c** and **Supplementary Fig. 5**). Map2ab is a marker for mature neurons. Exposure to 10 μM of SC-236 reduced numbers of βIII-tubulin–positive and Map2ab-positive neurons more than 90% relative to DMSO-treated cultures. Treatment with SC-560 (COX-1 IC_{50}, 0.009 μM; COX-2 IC_{50}, 6.3 μM) also resulted in a dosage-dependent decrease in both βIII-tubulin–positive and Map2ab-positive neurons. Relative to the neuron counts of DMSO-treated cultures, the reduction of βIII-tubulin–positive neurons in cultures treated with 0.5 μM SC-560 was greater than 95%, and no Map2ab-positive neurons could be seen in cultures treated with more than 0.5 μM of SC-560 inhibitor. At the concentrations tested, both COX inhibitors reduced neuronal differentiation with no appreciable change in total cell number, as determined by DAPI staining (**Supplementary Fig. 6**). The levels of the ω-6 and ω-3 fatty acids (AA, EPA, DHA and linoleic acid), the metabolic substrates of the enzymes COX and LOX, were also analyzed at day 4 and day 7 of differentiation (**Supplementary Fig. 7**). Compared to cells treated with the DMSO vehicle, cells treated with AA-861 (5-LOX inhibitor) or SC-236 (COX-2 inhibitor) showed an accumulation of unsaturated fatty acids at day 7.

The results demonstrate that inhibition of the eicosanoid signaling pathway promotes the pluripotent state of ESCs, thereby delaying the loss of *Nanog* and *Oct4* expression and maintaining the high levels of ω-6 and ω-3 fatty acids that are characteristic of ESCs. Accordingly, we hypothesized that specific downstream endogenous metabolites produced by and involved in oxidative pathways (for example, hydrogenation and oxygenation) mediate stem cell differentiation.

Metabolites promote neurogenesis and cardiogenesis

To test the hypothesis that metabolites produced by and involved in oxidation metabolism mediate stem cell differentiation, we treated mESCs with eicosanoids (**Fig. 3a**) and metabolites that undergo mitochondrial β-oxidation (for example, fatty acids and acylcarnitines; **Fig. 4a**) and allowed them to differentiate in chemically defined neuronal- (ref. 13) or cardiac-permissive conditions.

For neuronal differentiation, the experiments were stopped at days 8 to 13 and differentiation was evaluated with βIII-tubulin and Map2ab staining at high magnification (×10 or ×20). Random-field counting of the number of neurons with βIII-tubulin–positive or Map2ab-positive staining, or both, was performed manually. The cells were determined to be neurons if they stained for βIII-tubulin and possessed correct neuron morphology (that is, a round cell body with processes two times longer than the diameter of the cell body). Differentiation medium was supplemented with 8 μM of one of the following: batyl alcohol, capric acid (**9**), lauric acid (**10**), myristic acid (**11**), caprylic acid (**12**), palmitic acid (**13**) or DMSO vehicle. The addition of each saturated metabolite increased the number of βIII-tubulin–positive neurons as compared to the DMSO-treated control. Palmitic acid resulted in the greatest increase in βIII-tubulin–positive neurons (400%), and capric acid caused the greatest increase in Map2ab-positive neurons (>300%) (**Fig. 4b** and **Supplementary Fig. 8**). It is also important to note that quantitative PCR revealed levels of *Oct4* and *Nanog* mRNA transcripts in palmitic acid– and capric acid–treated cells similar to those in DMSO-treated cells, indicating that palmitic and capric acid did not substantially promote ESC self-renewal (**Supplementary Fig. 9**).

On the basis of our mass spectrometric metabolomics results and the inhibition of COX and LOX enzymes, we suspected that

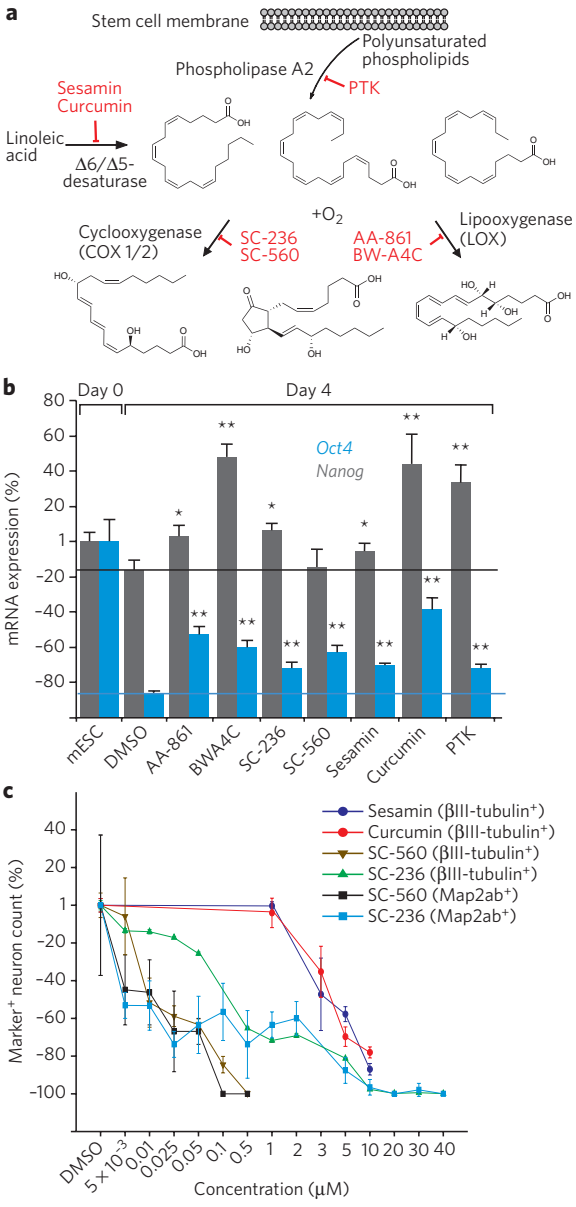

Figure 3 | Inhibition of the eicosanoid signaling pathway promotes the pluripotent state of embryonic stem cells. (**a**) The eicosanoid signaling pathway is mediated by the enzymes PLA_2, COX and LOX. PLA_2 hydrolyzes phospholipids from the cellular membrane, releasing AA, EPA or DHA, and lysophospholipids[38]. In the presence of O_2, COX and LOX oxidize carbon–carbon double bonds from AA, EPA and DHA, thereby producing important biological eicosanoid mediators that regulate the inflammatory response, such as thromboxanes, prostaglandins and prostacyclins, and yielding reactive ROS in the process[39]. (**b**) Inhibition of PLA_2, COX, LOX or desaturase delays the loss of pluripotency at day 4, as indicated by the higher expression of pluripotency markers *Oct4* (blue) and *Nanog* (black) relative to the DMSO control (horizontal blue and black lines). The sample labeled mESC corresponds to the levels of expression of *Oct4* and *Nanog* at day 0 (taken for normalization). $^*P < 0.05$. $^{**}P < 0.001$. (**c**) Fatty acid 5Δ and 6Δ desaturase inhibition by curcumin and sesamin, and COX inhibition by SC-236 and SC-560, substantially delay differentiation and neuron maturation. ESC cultures were treated with either curcumin, sesamin, COX inhibitors or a DMSO control at various concentrations from day 1 to day 10 of differentiation. Neuronal differentiation was evaluated with βIII-tubulin–positive neuron counts, or Map2ab and βIII-tubulin double-positive neuron counts, at day 10 of differentiation. Four cell culture replicates were analyzed for each inhibitor concentration. Data points and error bars represent mean values and s.d.

eicosanoids (produced by transformation of ω-6 and ω-3 fatty acids) were important to ESC differentiation. We supplemented neuronal differentiation medium with 50 nM of one of the following inflammatory-mediator eicosanoids: neuroprotectin D1 (NPD1) (**14**), leukotriene B4 (LTB4) (**15**) or leukotriene C4 (LTC4) (**16**). The addition of neuroprotectin D1 resulted in a >1500% increase in the number of βIII-tubulin–positive neurons (**Fig. 4c**) as compared to the ethanol-vehicle control. In contrast, leukotriene B4 and leukotriene C4 had no noticeable effect on neuronal differentiation.

To explore the generality of these findings, we conducted preliminary studies of human ESCs, which demonstrated that the metabolites we identified in mouse neurons similarly have neural-promoting activity in the human system. Specifically, we differentiated human ESCs via embryoid body differentiation in chemically defined media. Neural differentiation was determined with Pax6 expression as well as neural rosette structure and morphology[14] (**Supplementary Fig. 4**). Human ESCs underwent embryoid body differentiation, and 50 nM neuroprotectin D1, 8 μM capric acid, 8 μM palmitic acid or ethanol vehicle was added from day 4 through day 12 of differentiation. The addition of palmitic or capric acid increased neural rosette differentiation by 100% and 50%, respectively (**Supplementary Fig. 10**), suggesting that the role of these metabolites with respect to differentiation is conserved in mouse and human stem cells.

Mouse ESCs undergoing cardiac differentiation were also treated with different acyl-carnitines and allowed to differentiate in a chemically defined cardiomyocyte-inducing condition. The differentiation medium was supplemented with either 4, 8 or 16 μM of lauroyl- (**17**), caproyl- (**18**) or palmitoylcarnitine (**19**), or ethanol vehicle, for 9 d. On day 10, in the 24 h before analysis, no supplement was added. We then fixed cells and evaluated them for cardiac differentiation by staining with CT3, an antibody for the cardiac cell marker cardiotroponin, at day 10. The addition of each acyl-carnitine increased the number of CT3 clusters as compared to the ethanol-vehicle control. Palmitoylcarnitine resulted in the greatest increase of CT3 cardiac progenitors at 10 d (>40%) (**Fig. 4b** and **Supplementary Fig. 11**). Beating cell clusters (**Supplementary Video 1**) were visible in all treated wells at day 16 of differentiation. The addition of lauroyl-, caproyl- and palmitoylcarnitine in basal conditions alone, without growth factors, was not sufficient to increase CT3 expression at day 10 as compared to ethanol vehicle–treated cells.

These results suggest that certain endogenous metabolites are not merely final products of metabolic reactions but rather are involved in driving stem cell differentiation. Further studies will determine the roles of different eicosanoid signaling molecules and the detailed mechanism of this effect.

DISCUSSION

Significant effort has focused on using synthetic small molecules to control stem cell development[15], but endogenous (that is, naturally occurring) molecules have not been extensively examined. Our untargeted metabolomics analysis has revealed a unique metabolic signature in ESCs characterized by the presence of highly unsaturated endogenous molecules. The high degree of unsaturation makes these metabolites reactive and susceptible to oxygenation and hydrogenation reactions, conferring them with what may be interpreted as 'chemical plasticity'. Examples of such metabolites include polyunsaturated fatty acids such as AA, EPA and DHA, which are rapidly released by cell membranes in response to stress or altered homeostasis, making them available for oxidative metabolism by COX, LOX and P450 enzymes.

Our findings suggest that the redox status of ESCs is regulated during the process of differentiation, as revealed by measurements of GSH/GSSG ratios and ascorbic acid levels. The inverse relationship between the GSH/GSSG ratio and the ascorbic acid level may indicate that ascorbic acid compensates for the accumulation of GSSG to maintain homeostasis during ESC differentiation. This is

consistent with the closely linked antioxidant actions of glutathione and ascorbic acid previously observed in the liver of adult mice[16], where induction of glutathione deficiency is accompanied by a rapid increase in ascorbic acid (in contrast to other adult tissues or liver in newborn rats). Previous studies have revealed the importance of ascorbic acid in promoting differentiation when it is present during the early stages of stem cell differentiation[17,18].

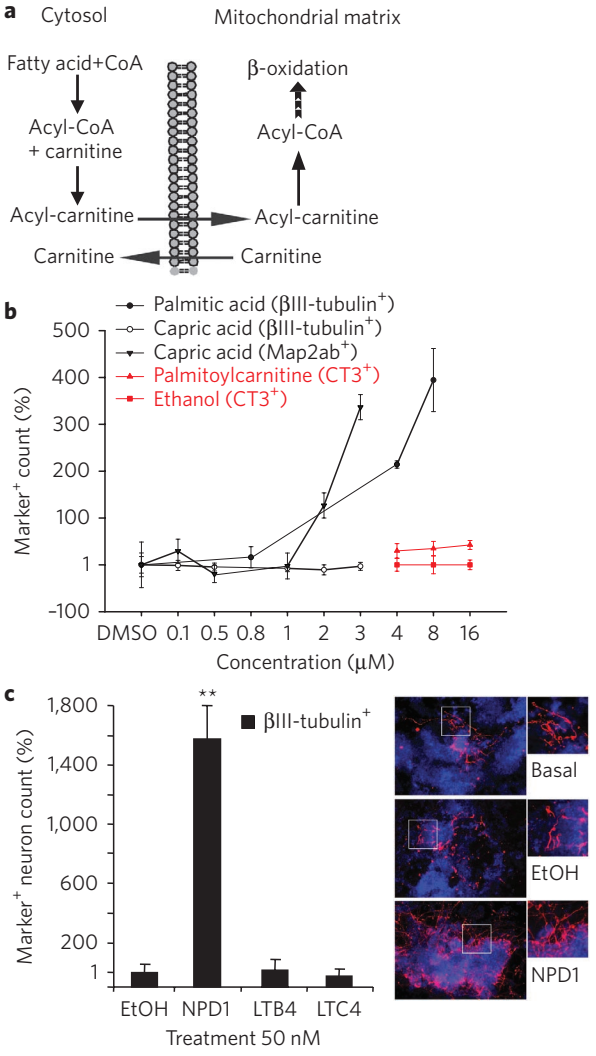

Figure 4 | Metabolites that undergo mitochondrial β-oxidation, and neuroprotectin D1, promote cardiac and neuronal differentiation. (**a**) Role of acyl-carnitines in fatty acid metabolism in the mitochondria. (**b**) Saturated fatty acids and acyl-carnitines enhance neuronal and cardiomyocyte differentiation, respectively. Map2ab and βIII-tubulin double-positive neuron count increases with higher concentrations of palmitic acid (0.8–8 μM) and capric acid (0.1–3 μM). An increase in CT3-positive clusters was observed with increasing concentrations of palmitoylcarnitine. Differentiation medium was supplemented with 4, 8 or 16 μM of palmitoylcarnitine for the first 9 d, and the metabolite was removed for the last day of differentiation. Cardiomyocyte differentiation was evaluated by staining with CT3, an antibody for the cardiac cell marker cardiotroponin. Marker-positive counts in ESCs differentiated in the absence of DMSO or ethanol were not different from those in ESCs differentiated with DMSO or ethanol controls. (**c**) Neuroprotectin D1 (NPD1) enhances neuronal differentiation. Differentiation medium was supplemented with 50 nM of NPD1, leukotriene B4 or leukotriene C4 throughout differentiation. $^{**}P < 0.001$ as compared to ethanol (EtOH)-treated cells. Photographs show neuronal differentiation evaluated with βIII-tubulin (red) staining. Nuclei were stained with DAPI (blue). Four independently prepared cell culture replicates were analyzed for each metabolite concentration. Data points and error bars represent mean values and s.d.

Our results also show that inhibition of 5Δ and 6Δ desaturases by curcumin and sesamin delays differentiation. These desaturases are key enzymes involved in the synthesis of 'plastic' ω-3 and ω-6 polyunsaturated fatty acids. Notably, curcumin is also known to act as an antioxidant[19]. Therefore, we cannot exclude the possibility that the increased levels of *Nanog* and *Oct4* caused by curcumin are partially due to its regulation of the redox status.

Our experimental results also show that supplementation of ESC media with essential, naturally occurring metabolites associated with oxidative metabolism in the mitochondria[20], such as saturated fatty acids and acyl-carnitines in β-oxidation, leads to a substantial increase in neuronal and cardiac differentiation. This is consistent with previous observations that increased production of mitochondrial proteins is associated with neurogenesis[21–23]. Activation of oxidative enzymes such as NADPH oxidase, and subsequent ROS generation, is also a prerequisite for cardiovascular differentiation of ESCs[24,25].

Overall, our results suggest that the activation of oxidation is a metabolic signature of stem-cell differentiation. Indeed, several independent lines of evidence have demonstrated that stem cells contain lower levels of ROS than their more mature progeny[23,26,27] and that ROS accumulation and signaling is required for differentiation[23]. This is consistent with previous observations that the intracellular oxidation state regulates the balance between self-renewal and differentiation[28]. Specifically, signaling molecules that promote pluripotency make stem cells more reduced, whereas those that promote differentiation make cells more oxidized. In addition, it is well known that hypoxia maintains the pluripotent and undifferentiated phenotype of stem or precursor cells both *in vitro* and *in vivo*[29,30]. We speculate that redox regulation, together with hypoxic conditions, allows stem cells to differentiate *in vivo* in response to oxidative processes such as inflammation.

Finally, our results raise the noteworthy possibility that specific endogenous inflammatory mediators might regulate the regenerative properties of stem cells. We found that inhibition of PLA_2, COX and LOX promotes the pluripotent, undifferentiated state of ESCs. In support of our results, a high-throughput screening assay has shown that anti-inflammatory drugs promote self-renewal in human ESCs[31]. For the first time, to our knowledge, we show that neuroprotectin D1 (ref. 32), a DHA-derived lipid mediator that activates inflammation resolution[33], accelerates neuronal differentiation. Pro-inflammatory lipid mediators such as leukotriene B4 and C4, in contrast, have no substantial effect on mESC differentiation. Previous studies have also revealed that stem cell fate is influenced by specific eicosanoids, such as prostaglandin E2 (ref. 34) and lipoxin A4 (ref. 35). These data suggest that specific molecular responses to injury and inflammation may regulate the proliferation and differentiation of stem cells; this may lead to the exploration of new avenues in understanding properties associated with regeneration. Further investigation will determine the mechanisms by which pro-inflammatory and pro-resolving endogenous metabolites activate proliferation and differentiation of quiescent stem or progenitor cells in response to tissue repair or wound healing.

METHODS

Metabolites and small molecules. Sphinganine, GPCho, sphingosine, diacylglycerols and glycerophosphoglycerols were from Avanti Polar Lipids. Fatty acids, acyl-carnitines, amino acids, sesamin, curcumin and BW-A4C were from Sigma-Aldrich. Docosanoyl ethanolamide, 7-ketocholesterol, prostaglandin E2, neuroprotectin D1, leukotrienes B4 and C4, palmityl trifluoromethyl ketone and SC-560 were from Cayman Chemical Company. SC-236 was from Calbiochem. AA-861 was from Biomol International. All chemicals were at a purity ≥94%.

Mouse embryonic stem cell culture. R1 and 46C mESCs were cultured according to routine protocols on 0.1% gelatin–coated tissue culture plates with irradiated CF-1 MEF feeder cells in growth medium (GM). Cells were passaged at the ratio of

1 to 10 every 3 d using 0.05% (v/v) trypsin EDTA (GIBCO). Before *in vitro* differentiation, cells were first cultured for one passage in either GM or chemically defined medium (CDM). See **Supplementary Methods** for GM and CDM composition.

Human embryonic stem cell culture. Hues9 human ESC lines were cultured according to routine protocols on irradiated CF-1 MEF feeder cells in ESC growth medium (DMEM/F-12 medium supplied with 20% (v/v) KnockOut Serum Replacement (GIBCO), 2 mM L-glutamine, 1.1 mM β-mercaptoethanol, 1 mM nonessential amino acids and 10 ng ml^{-1} basic fibroblast growth factor (bFGF) (Invitrogen)). Cells were passaged at the ratio of 1 to 6 every ~7 d using 1mg ml^{-1} dispase (GIBCO) solution.

Mouse neuronal differentiation. Neuronal differentiation is defined as the differentiation of embryonic stem cells into cells with (i) neuron morphology, namely a small round cell body consisting of multiple processes, and (ii) clear neuron-specific class III β-tubulin staining with Tuj1 antibody. Feeder-free mESC cultures were washed once with PBS and enzymatically dispersed with trypsin EDTA (GIBCO). Cells were replated on 0.1% gelatin–coated plates at 1.5×10^4 cm^{-2} and allowed to attach for 12–15 h in GM or CDM. Cells were washed once with PBS and the medium was replaced with chemically defined N2B27 medium[13]. The N2B27 medium was refreshed every day for 6–7 d. Cells were allowed to differentiate for an additional 5–8 d in N2B27 medium alone or with 20 ng ml^{-1} bFGF (Invitrogen), 100 ng ml^{-1} FGF8 (R&D Systems) and 400 ng ml^{-1} sonic hedgehog (R&D Systems), or with 1 μM of hedgehog agonist purmorphamine. Cells were collected for analysis once >80% of the culture showed βIII-tubulin expression with neuronal morphology, approximately at day 13. See **Supplementary Methods** for composition of neuronal-differentiation basal medium.

Human ESC differentiation. Human neural differentiation was accomplished via embryoid body formation. Hues9 human ESCs were lifted from feeder culture with 1 mg ml^{-1} dispase (GIBCO) solution and cultured overnight in low-attachment tissue culture plates. Human embryoid bodies were cultured in human growth medium for 4 d. On day 5, human growth medium was replaced with neuron induction medium[14,36] (100 ml F-12 medium, 300 ml DMEM medium, N2 supplement, 1 mM nonessential amino acids, 500 μl heparin solution (2 mg ml^{-1}); GIBCO) and 20 ng ml^{-1} bFGF (Invitrogen). At day 7, embryoid bodies were seeded on Geltrex (GIBCO)–coated plates and allowed to mature into neural progenitor structures termed rosettes.

Neuronal differentiation. Experiments were stopped at days 8 to 13 of differentiation, and neuronal differentiation was evaluated with βIII-tubulin and Map2ab staining at high magnification (×10 or ×20). We random field–counted neurons positive for βIII-tubulin, Map2ab or both, and statistical significance was evaluated with one-tailed Student's *t*-test using Excel software. Metabolites and inhibitors were not found to be autofluorescent.

Cardiac differentiation. Feeder-free mESC cultures were washed once with PBS and enzymatically dispersed with trypsin EDTA (GIBCO). Cells were plated on Matrigel (Invitrogen)–coated plates at 2×10^4 cm^{-2} in GM. After overnight attachment, cells were washed once with PBS, and GM was replaced with cardiac differentiation basal medium (BM) containing 20 ng ml^{-1} BMP4 (Stemgent) and 4 μM BIO (Stemgent). The BM with BMP4 and BIO was refreshed every other day for 6 d. Cells were then treated with BM supplemented with 100 ng ml^{-1} BIO DKK1 (R&D Systems). The BM with DKK1 was refreshed every other day for 6 d. DKK1 was then removed and cells were allowed to mature in BM alone until beating was observed.

Inhibition of PLA_2, COX, LOX, and 5Δ and 6Δ desaturase. COX was inhibited with SC-236 or SC-560. COX inhibitors were resuspended in DMSO solution and used at a range of 0.005 to 10 μM. Fatty acid desaturases were inhibited with sesamin or curcumin. Desaturase inhibitors were resuspended in DMSO solution and used at a range of 0.01 to 10 μM. LOX inhibitors, AA-861 and BW-A4C, were resuspended in DMSO solution and used at a range of 0.2 to 10 μM. PLA_2 inhibitor, palmityl trifluoromethyl ketone, was resuspended in DMSO solution and used at 3 μM. DMSO control (0.1% v/v) had no effect on stem cell fate.

GSH/GSSG ratio and ascorbic acid. Metabolite extraction was performed as described[37], with some modifications, as detailed here. Cells (4 million) at each time point ($n = 3$) were frozen in liquid N_2 and stored at −80 °C until analysis. The cell pellet was homogenized with 300 μl of cold (4 °C) extraction solution (5% (w/v) *meta*-phosphoric acid and 1 mM EDTA in 0.1% (v/v) formic acid). Samples were then mixed with a vortexer for 30 s and submerged for 1 min in liquid N_2, and then thawed in ice. This process was repeated three times, and samples were centrifuged at 13,000*g* for 15 min at 4 °C.

Received 6 July 2009; accepted 26 February 2010; published online 2 May 2010

References

1. Azuara, V. *et al.* Chromatin signatures of pluripotent cell lines. *Nat. Cell Biol.* **8**, 532–538 (2006).
2. Bernstein, B.E. *et al.* A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell* **125**, 315–326 (2006).
3. Graumann, J. *et al.* Stable isotope labeling by amino acids in cell culture (SILAC) and proteome quantitation of mouse embryonic stem cells to a depth of 5,111 proteins. *Mol. Cell. Proteomics* **7**, 672–683 (2008).
4. Foss, E.J. *et al.* Genetic basis of proteome variation in yeast. *Nat. Genet.* **39**, 1369–1375 (2007).
5. Gygi, S.P., Rochon, Y., Franza, B.R. & Aebersold, R. Correlation between protein and mRNA abundance in yeast. *Mol. Cell. Biol.* **19**, 1720–1730 (1999).
6. Kwon, Y.K. *et al.* A domino effect in antifolate drug action in *Escherichia coli*. *Nat. Chem. Biol.* **4**, 602–608 (2008).
7. Sreekumar, A. *et al.* Metabolomic profiles delineate potential role for sarcosine in prostate cancer progression. *Nature* **457**, 910–914 (2009).
8. Smith, C.A., Want, E.J., O'Maille, G., Abagyan, R. & Siuzdak, G. XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. *Anal. Chem.* **78**, 779–787 (2006).
9. Pruss, T.P. Treatment for the inhibition of neuro-degenerative disease states. US patent 5731354 (1998).
10. Brohult, A. Effects of alkoxyglycerols and especially selachyl alcohol on the bone marrow in connexion with irradiation treatment and in leukaemia therapy. *Nature* **181**, 1484–1485 (1958).
11. Oz, M., Alptekin, A., Tchugunova, Y. & Dinc, M. Effects of saturated long-chain N-acylethanolamines on voltage-dependent Ca^{2+} fluxes in rabbit T-tubule membranes. *Arch. Biochem. Biophys.* **434**, 344–351 (2005).
12. Song, H., Stevens, C.F. & Gage, F.H. Astroglia induce neurogenesis from adult neural stem cells. *Nature* **417**, 39–44 (2002).
13. Ying, Q.L., Stavridis, M., Griffiths, D., Li, M. & Smith, A. Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. *Nat. Biotechnol.* **21**, 183–186 (2003).
14. Yan, Y. *et al.* Directed differentiation of dopaminergic neuronal subtypes from human embryonic stem cells. *Stem Cells* **23**, 781–790 (2005).
15. Xu, Y., Shi, Y. & Ding, S. A chemical approach to stem-cell biology and regenerative medicine. *Nature* **453**, 338–344 (2008).
16. Martensson, J. & Meister, A. Glutathione deficiency increases hepatic ascorbic acid synthesis in adult mice. *Proc. Natl. Acad. Sci. USA* **89**, 11566–11568 (1992).
17. Tsuneto, M., Yamazaki, H., Yoshino, M., Yamada, T. & Hayashi, S. Ascorbic acid promotes osteoclastogenesis from embryonic stem cells. *Biochem. Biophys. Res. Commun.* **335**, 1239–1246 (2005).
18. Sato, H. *et al.* Collagen synthesis is required for ascorbic acid-enhanced differentiation of mouse embryonic stem cells into cardiomyocytes. *Biochem. Biophys. Res. Commun.* **342**, 107–112 (2006).
19. Cole, G.M., Teter, B. & Frautschy, S.A. Neuroprotective effects of curcumin. *Adv. Exp. Med. Biol.* **595**, 197–212 (2007).
20. Idell-Wenger, J.A., Grotyohann, L.W. & Neely, J.R. Regulation of fatty acid utilization in heart. Role of the carnitine-acetyl-CoA transferase and carnitine-acetyl carnitine translocase system. *J. Mol. Cell. Cardiol.* **14**, 413–417 (1982).
21. Cordeau-Lossouarn, L., Vayssiere, J.L., Larcher, J.C., Gros, F. & Croizat, B. Mitochondrial maturation during neuronal differentiation in vivo and in vitro. *Biol. Cell* **71**, 57–65 (1991).
22. Facucho-Oliveira, J.M., Alderson, J., Spikings, E.C., Egginton, S. & St. John, J.C. Mitochondrial DNA replication during differentiation of murine embryonic stem cells. *J. Cell Sci.* **120**, 4025–4034 (2007).
23. Tsatmali, M., Walcott, E.C. & Crossin, K.L. Newborn neurons acquire high levels of reactive oxygen species and increased mitochondrial proteins upon differentiation from progenitors. *Brain Res.* **1040**, 137–150 (2005).
24. Buggisch, M. *et al.* Stimulation of ES-cell-derived cardiomyogenesis and neonatal cardiac cell proliferation by reactive oxygen species and NADPH oxidase. *J. Cell Sci.* **120**, 885–894 (2007).
25. Li, J. *et al.* The NADPH oxidase NOX4 drives cardiac differentiation: Role in regulating cardiac transcription factors and MAP kinase activation. *Mol. Biol. Cell* **17**, 3978–3988 (2006).
26. Diehn, M. *et al.* Association of reactive oxygen species levels and radioresistance in cancer stem cells. *Nature* **458**, 780–783 (2009).
27. Ito, K. *et al.* Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells. *Nature* **431**, 997–1002 (2004).
28. Smith, J., Ladi, E., Mayer-Proschel, M. & Noble, M. Redox state is a central modulator of the balance between self-renewal and differentiation in a dividing glial precursor cell. *Proc. Natl. Acad. Sci. USA* **97**, 10032–10037 (2000).
29. Ezashi, T., Das, P. & Roberts, R.M. Low O_2 tensions and the prevention of differentiation of hES cells. *Proc. Natl. Acad. Sci. USA* **102**, 4783–4788 (2005).
30. Lin, Q., Lee, Y.J. & Yun, Z. Differentiation arrest by hypoxia. *J. Biol. Chem.* **281**, 30678–30683 (2006).
31. Desbordes, S.C. *et al.* High-throughput screening assay for the identification of compounds regulating self-renewal and differentiation in human embryonic stem cells. *Cell Stem Cell* **2**, 602–612 (2008).
32. Schwab, J.M., Chiang, N., Arita, M. & Serhan, C.N. Resolvin E1 and protectin D1 activate inflammation-resolution programmes. *Nature* **447**, 869–874 (2007).
33. Serhan, C.N. *et al.* Resolution of inflammation: state of the art, definitions and terms. *FASEB J.* **21**, 325–332 (2007).

34. Goessling, W. *et al.* Genetic interaction of PGE2 and Wnt signaling regulates developmental specification of stem cells and regeneration. *Cell* **136**, 1136–1147 (2009).
35. Wada, K. *et al.* Leukotriene B4 and lipoxin A4 are regulatory signals for neural stem cell proliferation and differentiation. *FASEB J.* **20**, 1785–1792 (2006).
36. Li, X.J. *et al.* Specification of motoneurons from human embryonic stem cells. *Nat. Biotechnol.* **23**, 215–221 (2005).
37. Rellan-Alvarez, R., Hernandez, L.E., Abadia, J. & Alvarez-Fernandez, A. Direct and simultaneous determination of reduced and oxidized glutathione and homoglutathione by liquid chromatography-electrospray/mass spectrometry in plant tissue extracts. *Anal. Biochem.* **356**, 254–264 (2006).
38. Farooqui, A.A., Horrocks, L.A. & Farooqui, T. Modulation of inflammation in brain: a matter of fat. *J. Neurochem.* **101**, 577–599 (2007).
39. Schneider, C., Pratt, D.A., Porter, N.A. & Brash, A.R. Control of oxygenation in lipoxygenase and cyclooxygenase catalysis. *Chem. Biol.* **14**, 473–488 (2007).

Acknowledgments

We thank G.G. Haraldsson and C.D. Magnusson (University of Iceland) for providing us with selachyl alcohol; C.T. McMurray and J.W. Trauger for helpful comments; and S. Hilcove, S. Ku and D. Watry for technical assistance. We gratefully acknowledge financial support from the California Institute for Regenerative Medicine, US Department of Energy, US National Science Foundation, US National Cancer Institute and US National Institutes of Health. A.S.-R. thanks Fundación Ramón Areces for his postdoctoral fellowship.

Author contributions

O.Y. and J.C. contributed equally to this work. O.Y. and J.C. designed and performed experiments, analyzed data and wrote the manuscript. G.J.P. analyzed data and wrote the manuscript. D.M.W. and H.P.B. analyzed data. A.S.-R. performed organic synthesis. S.A.T. and C.D. performed experiments. S.D. and G.S. oversaw the project, assisted in data analysis, wrote the manuscript and approved all intellectual content.

Competing financial interests

The authors declare no competing financial interests.

Additional information

Supplementary information and chemical compound information is available online at http://www.nature.com/naturechemicalbiology/. Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/. Correspondence and requests for materials should be addressed to S.D. or G.S.