AMD1 is essential for ESC self-renewal and is translationally down-regulated on differentiation to neural precursor cells

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The gene expression networks governing embryonic stem cell (ESC) pluripotency are complex and finely regulated during differentiation toward specific lineages. We describe a new role for Amd1 (adenosyl methionine decarboxylase), a key enzyme in the polyamine synthesis pathway, in regulating both ESC self-renewal and differentiation to the neural lineage. Amd1 is highly expressed in ESCs and is translationally down-regulated by the neural precursor cell (NPC)-enriched microRNA miR-762 during NPC differentiation. Overexpression of Amd1 or addition of the polyamine spermine blocks ESC-to-NPC conversion, suggesting Amd1 must be down-regulated to decrease the levels of inhibitory spermine during differentiation. In addition, we demonstrate that high levels of Amd1 are required for maintenance of the ESC state. We show that forced overexpression of Amd1 in ESCs results in maintenance of high Myc levels and a delay in differentiation on removal of LIF. We propose that Amd1 is a major regulator of ESC self-renewal and that its essential role lies in its regulation of Myc levels within the cell.

[Keywords: translational regulation; stem cells; neural precursor cells; miRNA; Amd1; polyamines]

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Mouse ESCs can be efficiently differentiated into neural precursor cells (NPCs), and this has been used in the identification of regulators of neural differentiation (Cai and Grabel 2007; Johnson et al. 2008; Tay et al. 2008). mRNAs play a role in many aspects of neuronal development and function (Saba and Schratt 2010). Only a small number of post-transcriptionally controlled mRNAs have been studied in differentiating ESCs, but it is clear that there are many more whose regulation and function are not yet known (Sampath et al. 2008; Tay et al. 2008). The same is true for the miRNA and RNA-binding protein regulators. Despite the many miRNAs and RNA-binding proteins known to be differentially expressed in ESCs, the targets of only a few have been described (Houbaviy et al. 2003; Suh et al. 2004).

By taking advantage of the sedimentation differences in sucrose gradients of mRNAs that are differentially loaded with ribosomes, we used microarray analysis to identify mRNAs that are translationally regulated on differentiation of ESCs to NPCs. Here, we describe the mechanism and functional significance of translational control of one such mRNA: Amd1 (adenosyl methionine decarboxylase). Amd1 is a critical enzyme required for the synthesis of the polyamines spermine and spermidine. Polyamines are required for a wide range of cellular processes, including differentiation and cell proliferation, and their levels are tightly regulated (Persson 2009; Igarashi and Kashiwagi 2010). We demonstrate that Amd1 is targeted for translational repression by miR-762, whose expression increases during NPC differentiation. We show that manipulation of Amd1 levels can influence both the ESC state and NPC differentiation and that this phenotype is mimicked by regulation of spermine levels. We suggest that precise regulation of polyamine levels by Amd1 is critical for both ESC self-renewal and directed differentiation. Finally, we demonstrate that in the absence of the self-renewal factor LIF, high levels of Amd1 promote elevated Myc levels, which allows ESC self-renewal independent of STAT3 activation.

Results

Identification of translationally regulated mRNAs on differentiation of ESCs to NPCs

We sought to determine the translational controls operating during the conversion of ESCs to NPCs. We used an established monolayer differentiation protocol with an ESC line carrying Sox1, an NPC marker, tagged with GFP (Aubert et al. 2003; Ying et al. 2003; Pollard et al. 2006). After 6 d of differentiation, we obtained >80% Sox1-positive NPCs (Supplemental Fig. S1A–E). To identify the translational changes occurring during ESC differentiation, we used microarrays to interrogate the ribosomal load of individual mRNAs isolated from sucrose gradients from ESCs and NPCs [Fig. 1A]. mRNAs were isolated into three groups—nontranslated (NT), low translation (LT), and high translation (HT)—dependent on their sedimentation through the sucrose gradient. These groups of mRNAs, along with the total cytoplasmic RNA, were analyzed on illumina microarrays. The microarray data were validated using quantitative RT–PCR (qRT–PCR) for a selection of 40 primers and showed a high rate of correlation, suggesting the microarrays give an accurate representation of mRNA distribution within the gradient (Supplemental Fig. S2).

Analysis of the data showed that the majority of gene expression changes occur at the total mRNA level [Fig. 1C]. We identified >182 mRNAs that are translationally controlled on NPC differentiation, with 134 mRNAs being up-regulated and 48 mRNAs down-regulated [Fig. 1B,C]. When overlaid with the transcriptome data set, we saw that many of the translationally controlled mRNAs are also under transcriptional regulation. Over 55% of mRNAs that are translationally up-regulated in NPCs are also up-regulated at the total mRNA level. Over 70% of mRNAs that are translationally down-regulated in NPCs also show a decrease in total mRNA [Fig. 1C]. This suggests there could be coordination between transcriptional and translational controls in these cells.

Validation of translationally regulated candidates

We selected a range of translationally regulated mRNAs for further validation. Polysome gradients were separated into 12 fractions, and the percentage of each mRNA within these fractions was determined by qRT–PCR. mRNA shifts to the heavier polysome fractions in NPCs were confirmed for Pdelb, Rcsd1, and Tchp as shown. Amd1 showed a shift to the lighter polysome fractions in NPCs, confirming the microarray results [Fig. 2A]. We also measured changes in total mRNA levels for each of the genes. Rcsd1 and Tchp both showed corresponding increases in total mRNA in NPCs, suggesting coordination between transcription and translation. Pdelb and Amd1 showed no significant change in mRNA levels [Fig. 2B], suggesting these mRNAs may be under exclusive translational control.

The regulatory sequences dictating translational activation or repression are generally located within the untranslated regions (UTRs) of an mRNA. To demonstrate the translation changes resulting from the ribosomal shifts, we used the luciferase reporter plasmid pGL3 carrying the luciferase mRNA under the control of the 3′ UTR from the translationally regulated mRNAs. These were transfected into ESCs and NPCs, and the levels of luciferase were measured as an indication of translation efficiency [Fig. 2C]. Pdelb, Rcsd1, and Tchp show decreased luciferase activity in ESCs compared with NPCs, while Amd1 shows a decrease in luciferase activity in NPCs compared with ESCs. Luciferase mRNA levels were measured in all cases and were found to be not significantly different between ESCs and NPCs, suggesting the decrease in luciferase activity is a result of translational repression (Supplemental Fig. S3). Taken together, these validations demonstrate that a significant number of mRNAs are translationally regulated on differentiation of ESCs to NPCs and that polysome profiling provides an effective means of identifying them.

Amd1 is translationally down-regulated during NPC differentiation

Amd1 is a highly conserved enzyme required for the production of the polyamines spermine and spermidine.
Polyamines are important for growth and proliferation, but their role in ESCs is currently unknown. To determine the kinetics of translational control of Amd1, we performed Western blots of ESCs and NPCs 2, 4, and 6 d after induction of differentiation. Amd1 protein levels are high in ESCs and are reduced by day 4 and further reduced by day 6 of NPC differentiation (Fig. 3A). Total Amd1 transcript levels did not change significantly during the 6 d of differentiation to NPCs (Fig. 3B), suggesting the change in protein level is independent of mRNA abundance. High-resolution sucrose density gradient fractionation showed that Amd1 mRNA is associated with the heavier polysome fractions (fractions 8–10) in ESCs. After 4 d of differentiation, Amd1 mRNA shifts partly to the lighter fractions and by day 6 is found almost exclusively in the lighter fractions (fractions 5–7) (Fig. 3C). These data strongly suggest that Amd1 starts to be translationally repressed from day 4 and is most dramatically repressed by day 6 of NPC differentiation.

To address the specificity of Amd1 down-regulation, ESCs were differentiated into embryoid bodies (EBs) or in the presence of N2B27 medium with BMP4. BMP4 functions to inhibit the differentiation of NPCs [Ying et al. 2003]. Western blot analysis showed that Amd1 protein levels were most dramatically reduced in NPCs compared with EBs or BMP4-treated cells (Fig. 3D). Amd1 mRNA levels remain the same (Fig. 3E). This suggests that the translational repression seen on NPC differentiation is specific for this lineage.

The Amd1 enzyme functions to decarboxylate S-adenosylmethionine (AdoMet) to produce AdoMet-DC. This provides the aminopropyl donor for the synthesis of spermidine from putrescine and of spermine from spermidine. We predicted that in NPCs with lower levels of Amd1 protein, the ratio of putrescine to spermidine and spermine would change. We measured the levels of all three polyamines in ESCs and NPCs. As expected, the ratio of the three polyamines changes dramatically in NPCs. The levels of putrescine are increased threefold. Interestingly, the levels of spermine are decreased in NPCs (Fig. 3F). These data suggest that the functional consequence of decreased Amd1 levels may be a change in the ratio of the three polyamines and a decrease in the levels of spermine.

Down-regulation of Amd1 protein is required for NPC differentiation

To establish the significance of Amd1 down-regulation for NPC differentiation, we overexpressed the Amd1 ORF using the EF-1a promoter in the pTracer vector. Amd1 protein levels start to decrease between 2 and 4 d of NPC differentiation [Fig. 3A], so we transfected NPCs that had been differentiating for 3 d with the pTracer-Amd1 or empty vector. Overexpression of Amd1 was demon-
Amd1 levels were similar to those found in ESCs, while cells transfected with the empty vector showed decreased protein levels, similar to the untransfected cells (Fig. 4A). Interestingly, continued expression of Amd1 resulted in a failure in the accumulation of the NPC marker Sox1. This was further demonstrated using flow cytometry. In the presence of the empty vector alone, >80% of Sox1-GFP-positive cells were recovered after 6 d of differentiation. In the presence of high Amd1 protein levels, however, <30% of cells were Sox1-positive (Fig. 4B,C). These data were further confirmed with immunofluorescence, showing that overexpression of Amd1 resulted in a failure of Nestin and Sox1 accumulation after 6 d of differentiation (Supplemental Fig. S4). qRT–PCR confirmed that on overexpression of Amd1 in differentiated NPCs, NPC markers Nestin and Sox1 failed to accumulate. Differentiation markers Hnf4, T, Foxa2, and Gata1 were elevated on Amd1 overexpression, suggesting the cells are differentiating into alternative lineages (Fig. 4D).

We showed that NPCs with low Amd1 protein levels have reduced levels of the polyamine spermine and an altered polyamine ratio (Fig. 3F). We show that overexpression of Amd1 in differentiating NPCs restored the polyamine ratio and that spermine levels are elevated to those seen in ESCs (Fig. 3F). To determine whether NPC differentiation requires lower levels of spermine, we added spermine to NPCs after 3 d of differentiation, the same time that Amd1 starts to be down-regulated, and assessed the effect on accumulation of Sox1-positive cells after 6 d. Strikingly, we saw a reduction in the number of Sox1-positive cells, suggesting that spermine is inhibitory to NPC differentiation (Fig. 4E,F). Taken together, these data suggest that down-regulation of Amd1 during NPC differentiation may be required to decrease the levels of inhibitory spermine in the cells.

Amd1 is translationally regulated by miR-762

To identify the cis-acting elements responsible for the translational repression of Amd1, we constructed a firefly luciferase construct carrying the Amd1 5’ UTR, 3’ UTR, or both the 5’ and 3’ UTRs and assessed their ability to regulate translation in a dual luciferase reporter assay. Luciferase constructs were transfected into ESCs and NPCs after 4 d of differentiation (Fig. 5A). The 5’ UTR had no effect on luciferase activity in either cell type, while the 3’ UTR promoted strong luciferase repression in NPCs. This suggests that the cis-acting elements responsible for the translational repression seen on NPC differentiation reside within the 3’ UTR. To map these cis-acting elements, we made a series of 3’ UTR deletions, Amd1-D1–5 (Fig. 5B), and tested them for their repressive effect on luciferase levels (Fig. 5C). Amd1-D1, Amd1-D2, and Amd1-D3 retained their ability to repress luciferase activity in NPCs, while this was lost in D4 and D5, suggesting the cis-element lies within the 528 nucleotides [nt] retained in D3 but lost on D4. There was no significant effect on the levels of luciferase mRNA between the two cell types, suggesting that these 528 nt are promoting translational repression and not transcript destabilization (Fig. 5D).

We performed miRNA microarrays on ESCs and NPCs to identify miRNAs that are differentially expressed between the two cell types. The microarray showed that a number of miRNAs were up-regulated in NPCs com-
pared with ESCs (Fig. 5E). One of these, miR-762, a little-studied but conserved miRNA, has a target site within the 528 nt responsible for \textit{Amd1} translational repression (Miranda et al. 2006). miR-762 was up-regulated threefold in NPCs compared with ESCs, making it a likely candidate for translational repression of \textit{Amd1} (Fig. 5F). miR-762 up-regulation was specific to NPCs, as no increase in levels was seen in EBs or BMP4-treated NPCs (Supplemental Fig. S5A). We coexpressed the \textit{Amd1} 3' UTR luciferase construct with the miR-Vec vector carrying the miR-762 miRNA or nontargeting miR-155 in HeLa cells and saw a decrease in luciferase activity of >40% in the presence of miR-762 (Fig. 5H). To confirm the 528-nt region was being targeted by miR-762, we tested the ability of miR-762 to repress luciferase activities of the five \textit{Amd1} 3' UTR deletion constructs (Fig. 5B). Constructs carrying the full-length \textit{Amd1} 3' UTR or D1, D2, or D3 showed strong repression of luciferase activity, while constructs D4 and D5, which do not contain the miR-762 target site, were not repressed in the presence of the miRNA (Supplemental Fig. S5B). There was no significant decrease in normalized luciferase mRNA levels between the constructs in the presence or absence of the miRNA (Supplemental Fig. S5C). To confirm that the miR-762 target site was solely responsible for the translational repression, we made mutants in the miRNA target site (Fig. 5G). Constructs carrying two different triplet target site mutations showed a complete restoration of luciferase activity when cotransfected with miR-762 into HeLa cells, confirming that the \textit{Amd1} 3' UTR is a direct target of miR-762 (Fig. 5H). To further confirm that miR-762 targets \textit{Amd1} in NPCs, we transfected the mutated \textit{Amd1} 3' UTR luciferase constructs into NPCs and again saw a complete restoration of luciferase activity (Fig. 5I). To confirm the specificity of \textit{Amd1} repression in NPCs, we transfected BMP4-treated NPCs with the \textit{Amd1} reporter vector. We saw no luciferase repression in these cells, confirming that the translational repression is specific for NPCs (Supplemental Fig. S5D). These data demonstrate that miR-762 targets the \textit{Amd1} 3' UTR for translational repression in NPCs and that miR-762 is sufficient for the translational repression of \textit{Amd1} on NPC differentiation.

\textit{Amd1} is required for ESC self-renewal

miR-762 is expressed at low levels in mouse ESCs compared with NPCs, so we determined whether its
overexpression could promote differentiation of ESCs. ESCs were transfected with control or miR-762 mimics and maintained in ESC culture conditions. Cells were stained with alkaline phosphatase (AP) 4 d after transfection, and cells transfected with the miR-762 mimic showed a clear decrease in AP-stained colonies, suggesting that the cells are differentiating and losing their self-renewal capacity (Fig. 6A). qRT–PCR showed a decrease in the pluripotency markers Oct4, Sox2, and Nanog and an increase in the differentiation markers Nestin, T, and FoxA2 (Fig. 6B). Decreased pluripotency markers were confirmed by Western blot (Fig. 6C). The miR-762 mimic also promoted the down-regulation of Amd1 protein. We postulated that miR-762 targeting of Amd1 could be responsible for the differentiation phenotype observed. To test this, we expressed the Amd1 ORF, which cannot be targeted by miR-762, in ESCs with the miR-762 mimic and assessed the effect on pluripotency. We saw an almost complete rescue of the differentiation phenotype in the presence of the Amd1 ORF. AP staining is retained in the presence of the Amd1 ORF (Fig. 6A), and qRT–PCR shows that pluripotency markers Oct4, Sox2, and Nanog are not affected and that differentiation markers Nestin, T, and FoxA2 remain low (Fig. 6B). These data suggest that the differentiation phenotype seen on expression of miR-762 in ESCs is due to the reduction of Amd1 protein.

To confirm a role for Amd1 in the maintenance of ESC pluripotency, we used a pool of siRNAs to transiently knock down Amd1 mRNA in ESCs (Fig. 6D). Following knockdown of Amd1, ESC colonies showed a reduction in AP staining, indicative of a differentiated state (Fig. 6E). To confirm the loss of pluripotency, we compared the levels of the pluripotency markers Oct4, Sox2, and Nanog after Amd1 knockdown (Fig. 6F). We saw significant reductions in mRNA levels for all three transcripts and a corresponding increase in the differentiation markers Nestin, T, and FoxA2 (Fig. 6F). This was further confirmed by Western blot for Oct4 and Sox2 (Fig. 6G). Immunofluorescence analysis shows that Oct4 and Sox2 are reduced in the majority of ESCs after Amd1 knockdown (Fig. 6H; Supplemental Fig. S6), suggesting that all ESCs are responsive to decreased Amd1 levels and not just a subset. To determine whether Amd1 is required in ESCs to maintain high levels of spermine, we added it to cells that had been treated with siRNAs to Amd1. Strikingly, we saw that the addition of spermine can rescue the differentiation phenotype seen on knockdown of Amd1, as shown by PCR of the pluripotency markers (Fig. 6F). We also demonstrated that addition of spermine can suppress the differentiation phenotype seen on addition of the miR-762 mimic to ESCs. The effect is specific to spermine, however, as spermidine had no effect (Supplemental Fig. S6A). These data clearly demonstrate that...
high Amd1 protein levels are required to maintain ESC self-renewal, implicating Amd1 as an essential self-renewal factor. Spermine can rescue the Amd1 knockdown phenotype, suggesting that Amd1 is required to promote the levels of spermine for ESC self-renewal.

Forced expression of Amd1 can delay differentiation of ESCs

Having established that Amd1 is required for maintenance of the ESC state, we wanted to determine whether its overexpression could delay differentiation. We overexpressed Amd1 in ESCs and differentiated the cells in the absence of LIF for 4 d. Control cells lose AP staining rapidly, but strikingly, AP staining remains high on Amd1 overexpression (Fig. 7A). This is further demonstrated by the maintenance of pluripotency markers Oct4 and Sox2 on removal of LIF when Amd1 is overexpressed (Fig. 7B). This demonstrates that increased Amd1 levels can delay ESC differentiation. Polyamines have been shown to regulate gene expression of a number of different proteins, one of which is Myc (Wang et al. 1993; Patel and Wang 1997; Liu et al. 2009), and maintenance of high levels of Myc promotes ESC self-renewal in the absence of LIF (Cartwright et al. 2005). We show that on removal of LIF, Myc protein levels are reduced dramatically by day 4 of differentiation, whereas when Amd1 is overexpressed, Myc levels are maintained at high levels (Fig. 7C). LIF signaling promotes the phosphorylation of STAT3, which in turn activates Myc transcription (Niwa et al. 1998; Matsuda et al. 1999; Cartwright et al. 2005). We demonstrate that Amd1 promotes elevated Myc levels in the absence of STAT3 activation, as shown by a decrease in pSTAT3 on removal of LIF in both control and Amd1-overexpressing cells (Fig. 7C).

We propose that regulation of Amd1 protein levels by miR-762 is essential to control polyamine ratios in ESCs and in differentiating NPCs (Fig. 7D).
ESCs, high levels of Amd1 are required to maintain the ESC state through promotion of Myc levels. During NPC differentiation, up-regulation of miR-762 results in targeting of Amd1 for translational repression. This leads to decreased levels of spermine, allowing for efficient NPC differentiation. Taken together, our data demonstrate that Amd1 is an essential enzyme for ESC self-renewal and that its translational regulation is critical for early neural specification.

**Discussion**

In this study, we undertook a genome-wide screen to identify new regulators of ESC differentiation to NPCs. Using polysome profiling coupled to microarray analysis, we identified a range of mRNAs that are under translational control. We focused our studies on one candidate, Amd1, that has been implicated in early mammalian development [Nishimura et al. 2002]. The role of Amd1 and polyamines in ESC differentiation had not been addressed, prompting us to investigate the significance of Amd1 regulation in ESC maintenance. We demonstrate that Amd1 is translationally repressed by miR-762 on NPC differentiation. Amd1 has been shown to be translationally controlled by a uORF [Law et al. 2001; Ivanov et al. 2010] in its 5′ UTR; however, our luciferase data suggest that the regulation seen on NPC differentiation is mediated entirely through the 3′ UTR. miR-762 is a recently identified, conserved miRNA, and Amd1 is the first validated target to be described. Mutation of the miRNA target site completely restores luciferase activity in NPCs, suggesting that targeting by miR-762 is sufficient for Amd1 down-regulation. Our data clearly show that miR-762 promotes the translational repression of Amd1. We demonstrate this by showing both a shift in ribosomal load for Amd1 in NPCs and a miR-762-induced reduction in luciferase activity that is independent of RNA levels. miR-762 is up-regulated in NPCs, but it is currently not clear what drives this. Further analysis is required to understand the transcriptional controls governing miR-762 expression in ESCs and NPCs.

The levels of polyamines in the cell are regulated on many levels, including synthesis, uptake, and export. The two rate-limiting enzymes in their synthesis, Odc1 and...
Amd1, are under tight control, and Amd1 has been shown to be regulated at the levels of transcription, RNA stability, translation, enzyme activity, and protein degradation (Pegg et al. 1998). Our demonstration that Amd1 is regulated by the 3' UTR targeting miRNA miR-762 is the first demonstration of miRNA-mediated regulation of the polyamine pathway. It is currently not clear how widespread this miRNA-mediated translational control is for polyamine homeostasis and whether it is specific to early differentiation of neural progenitors. The levels of polyamines are regulated at such a tight level in all cells that in order to override that control in a given cellular context, an additional dominant level of regulation may be required. This may be the reason that this mechanism of control exists during NPC differentiation in an already tightly regulated network. Increased polyamine levels have been shown to be coincident with cancer progression, and Amd1 has been targeted for the development of cancer treatments. A more detailed understanding of the role of miRNA-mediated regulation of polyamine levels in cancer cells could lead to novel therapeutics aimed at targeting polyamine levels through down-regulation of its enzymatic regulators.

Analysis of the levels of the three polyamines in ESCs and NPCs suggests that down-regulation of Amd1 results in a change in the ratio of polyamines; notably, spermine levels are decreased. The selective effect on spermine and not spermidine levels likely reflects additional regulation of the enzymes controlling spermine and spermidine synthesis, degradation, and export. Interestingly, Amd1 down-regulation only becomes apparent after 3 d of NPC differentiation, suggesting that its down-regulation is not required for the early stages of ES differentiation. We demonstrate that overexpression of Amd1 after 3 d of differentiation results in a dramatic decrease in NPCs and an increase in differentiation markers for other lineages. These data suggest that high levels of spermine are inhibitory to NPC differentiation and that Amd1 down-regulation may be required to promote a change in the polyamine ratio and a decrease in intracellular spermine levels.

Amd1 is expressed at high levels in ESCs, and we demonstrated that it is essential for maintenance of the ESC state. Knockdown of Amd1 by siRNA resulted in a loss of AP staining and a decrease in the mRNA and protein levels of the pluripotency markers (Fig. 6). Markers for all three lineages were increased on Amd1 knockdown, suggesting that the differentiation may not be lineage-specific. Addition of spermine can rescue the differentiation phenotype seen on Amd1 knockdown, suggesting the essential role of Amd1 lies in maintaining...
high levels of spermine. Previous reports have demonstrated that embryos devoid of Amd1 die during early gastrulation and that ESCs can only be recovered from Amd1−/− blastocysts in the presence of exogenous spermidine [Nishimura et al. 2002]. This is in agreement with our finding that Amd1 is essential for ESC self-renewal.

To further demonstrate the critical role of Amd1 in self-renewal, we show that forced overexpression of Amd1 can delay differentiation, as shown by the prolonged expression of pluripotency markers in conditions that promote differentiation [Fig. 7]. Notably, Myc levels are maintained in the absence of LIF when Amd1 is overexpressed. It has been demonstrated that nondegradable Myc can maintain ESCs in an ESC state with high Oct4 levels in the absence of LIF [Cartwright et al. 2005]. We propose that the mechanism by which Amd1 maintains ESC self-renewal in the absence of LIF is through promoting high Myc levels. LIF signaling is required in part to activate STAT3, which in turn promotes Myc transcription [Niwa et al. 1998; Matsuda et al. 1999; Cartwright et al. 2005]. Phosphorylated STAT3 is diminished equally in control and Amd1-overexpressing cells in the absence of LIF, showing that Amd1 promotes Myc levels independent of STAT3. Reports in the literature have demonstrated that polyamines can enhance Myc translation by promoting phosphorylation of the RNA-binding protein HuR [Liu et al. 2009]. It is possible that a similar level of activation is operating on overexpression of Amd1 in differentiating ESCs. On removal of LIF, Myc is also regulated at the protein level following phosphorylation on Thr58, which triggers protein degradation mediated by GSK3β [Sato et al. 2004]. While it is possible that Amd1 is functioning to promote Myc protein stabilization, it is less likely, as polyamines are believed to largely exist in RNA and DNA complexes regulating gene expression at the transcription or RNA level [Igarashi and Kashiwagi 2010].

Our data demonstrate that on removal of LIF, increased Amd1 can sustain the high levels of Myc needed for self-renewal. It is therefore likely that the differentiation phenotype we see on down-regulation of Amd1 is due to decreased Myc levels. We propose that in order for LIF to promote self-renewal, it requires appropriate levels of the polyamines. The levels of Amd1, and thus the polyamines, are likely tightly regulated during ESC self-renewal and differentiation. Small changes in the ratio of the polyamines could function to signal the cell to differentiate or self-renew. Our data suggest that maintaining an appropriate ratio of polyamines and ESC signaling networks maintains ESC self-renewal, but disruption of any of these will result in differentiation of the cells.

Polyamines function in a wide range of cellular processes, and each of the polyamines can have different functions in different cell types. Manipulation of polyamine levels have been linked to differentiation of F9 teratocarcinoma cells and the enhancement of cardiac differentiation [Frostesjo et al. 1997, Sasaki et al. 2008]. There have been reports of both neuroprotective and neurotoxic effects of all three polyamines on neurons [de Vera et al. 2008; Igarashi and Kashiwagi 2010], and disruption of polyamine homeostasis has been linked to a number of neurological disorders [Casero and Pegg 2009]. The role of polyamines in ESC self-renewal and differentiation is clearly complex, and it is likely that they function in different ways depending on the developmental window and the cellular context.

Our identification of Amd1 as a crucial regulator of ESC self-renewal highlights the importance of polyamine regulation in ESC biology. While Amd1 regulation and polyamine levels have been linked to cancer and proliferation, their significance had never been appreciated in ESCs. Here we demonstrate that Amd1 has an essential role in maintaining ESC self-renewal through promoting high Myc levels. Polyamines have the capacity to regulate a wide range of targets, and this work opens the door to exploring a new level of regulation in ESC self-renewal and differentiation. Understanding the role of polyamines in ESCs will greatly broaden our knowledge of the regulatory pathways operating to maintain or direct the differentiation of ESCs.

Materials and methods

Cell culture

ESCs were cultured on 0.1% gelatin in DMEM (GIBCO) supplemented with 15% fetal bovine serum (FBS) (GIBCO), 0.2 mM β-mercaptoethanol, 2 mM L-glutamine (GIBCO), 1× MEM non-essential amino acids (GIBCO), and LIF. EBs were formed by culturing ESCs in the absence of LIF on low-attachment plates as described [Sampath et al. 2008]. NPC differentiation was performed in N2B27 medium as described [Ying et al. 2003]. BMP4 (10 ng/mL; R&D, catalog no. 5020) was used to block NPC differentiation [Ying et al. 2003]. Spermine (Sigma, S3256) and spermidine (Sigma, S0266) were used at a concentration of 100 μM for all experiments. For neuronal differentiation, NPCs were replated onto laminin-coated plates for 3 d in N2B27 medium. For astrocyte differentiation, NPCs were replated on gelatin-coated plates for 8 d in N2B27 plus 1% FBS [Ying et al. 2003].

Polysome fractionation

For polysome fractionation, 20 million cells were incubated with 100 μg/mL cycloheximide [Sigma, catalog no. C4859] for 10 min. The cell pellets were resuspended in 2× RSB buffer (20 mM Tris-HCl at pH 7.4, 20 mM NaCl, 30 mM MgCl2, 200 μg/mL cycloheximide, 0.2 mg/mL heparin [Sigma, catalog no. H4787], 1000 U/mL RNasin), lysed with 2× lysis buffer (20 mM Tris-HCl at pH 7.4, 20 mM NaCl, 300 mM MgCl2, 1% Triton X-100, 2% Tween-20, 1% deoxycholate), and incubated on ice for 8 min, followed by centrifugation at 12,000g for 3 min to remove the nuclei. Supernatants were further centrifuged at 12,000g for 8 min at 4°C. Equal OD units were loaded onto 10%–30% linear sucrose gradients [prepared in 10 mM Tris-HCl at pH 7.4, 75 mM KCl, 1.5 mM MgCl2] and centrifuged at 36,000 rpm for 120 min at 8°C in a SW41 rotor (Beckman Coulter). Twelve fractions were collected from the top of the gradient using a piston gradient fractionator (BioComp Instruments). The absorbance at 254 nm was measured with a UV-M II monitor (Bio-Rad). Following fractionation, 110 μL of 10% SDS and 12 μL of protease K (10 mg/mL Invitrogen) were added to each fraction and in-
Translational control of AMD1 in ESCs

cubated for 30 min at 42°C. Fractions 1–5, 6–8, and 9–11 were combined as groups NT (nontranslated), LT (low translation), and HT (high translation), respectively.

Unfractionated cytoplasmic RNA and different groups or fractions of polysomal RNA were purified with phenol chloroform isoamyl extraction, followed by purification on an RNeasy column with on-column DNase digestion. Affymetrix GeneChip spike-in poly[A] RNAs (Ambion, catalog no. 900443) were added to each group of RNA. For the microarray, 300 ng of RNA from each group was amplified using the TargetAmp Nano-g Biotin-aRNA labeling kit (Epicentre Biotechnologies, catalog no. TAN07924), and the resultant cRNA was purified using the Picopure RNA isolation kit (Invitrogen, catalog no. 0202). Illumina Mouse Ref-8 v2 BeadChip arrays were loaded according to manufacturer’s instructions.

For the miRNA microarray, total RNA from ESCs and NPCs was purified using Trizol. Triplicate samples of RNA were profiled using the miRCURY LNA microarray (Exiqon).

Microarray analysis

For total cytoplasmic RNA, the background-subtracted intensities were first normalized using the cross-correlation method (Chua et al. 2006) and then log2-transformed. Identification of differentially expressed genes coding for cytoplasmic proteins was performed using the following criteria, with a P-value of <0.05 and a fold change of ≥1.5. For miRNAs, a cutoff mean of a twofold change and P-value of <0.001 were flagged as differentially expressed. The data for ribosomal profiling were first scaled using the external spike-in controls (thrB, pheA, and lysA) to account for the variations between different groups isolated based on sucrose gradients. As the volume of each isolation group varied, the scaled intensities were adjusted accordingly with their respective group volume. Genes coding for cytoplasmic proteins were assumed to be translationally differentially regulated when the translational rate between NPCs and ESCs was a ≥1.5-fold change and a P-value of <0.05. The translation rate is defined as the maximum of the ratios of scaled intensities between any two isolation groups.

qPCR analysis

cDNA was synthesized using a reverse transcription kit (SuperScript III, Invitrogen) according to the manufacturer’s instructions. For fractionated RNA, the same volume of RNA was used, and for total RNA analysis, the same quantity of RNA was used. SYBR Green was used with gene-specific primers, or TaqMan probes were used for qRT–PCR on an ABI PRISM 7900 sequence detection system. For polysome fractions, CT values were normalized to spike in control RNAs dap and thr. Quantification of miRNA levels was done using the TaqMan miRNA reverse transcription kit. The expression level of miRNAs was measured using gene-specific predesigned TaqMan primers. U6 was used as normalization control.

Western blot and polyamine quantification

Thirty micrograms of protein extract was separated on a NuPAGE 4%–12% Bis-Tris gel and transferred to a PVDF membrane. Amd1 (Santa Cruz Biotechnology, catalog no. sc-98569), Oct4 (Santa Cruz Biotechnology, catalog no. sc-2572), GAPDH (Abcam, catalog no. ab-9484), STAT3 (Santa Cruz Biotechnology, catalog no. sc-8019), pSTAT3 (Santa Cruz Biotechnology, catalog no. sc-8059), and Sox2 (R&D Systems, catalog no. MAb2018) antibodies were used at 1:1000 dilution for overnight primary antibody incubation, c-Myc (Santa Cruz Biotechnology, catalog no. sc-764) was used at 1:50. Polyamine levels were measured using high-performance liquid chromatography (HPLC) as previously described (Igarashi et al. 1986).

Immunofluorescence

Cells were fixed with 4% PFA for 20 min and then blocked (2% BSA, 0.5% Triton X-100 in PBS) for 15 min at room temperature. The primary antibody Sox2 (Mab2018) or Oct4 (sc-5279) was used at 1:200 dilution and incubated for 1 h at room temperature. Alexa488- or Alexa568-coupled secondary antibody (1 mg/ml Invitrogen) was added to the cells for 1 h. Cells were washed three times with 0.2% BSA and 0.05% Triton X-100 in PBS between each step. For AP staining, cells were stained using the Alkaline Phosphatase Detection kit (Millipore, SC004) according to the manufacturer’s instructions. For flow cytometry analysis, ESCs and NPCs were trypsinized and washed with PBS. The cells were analyzed for the intensity of GFP expression. FACS analysis was carried out using a BD FACS Calibur machine.

Luciferase assays

The 3’ UTR of candidate mRNAs were cloned at the 3’ end of the luciferase gene in pGLO3 vector. pTK was used as a cotransfection control. The 3’ UTR of Amd1 was cloned into PsfCHECK-2 vector (Promega) at the 3’ end of the Renilla luciferase coding sequence. Firefly luciferase was used for normalization. ESCs or NPCs after 4 d of differentiation were transfected using FuGENE HD (Roche) according to manufacturer’s instructions. Two days after transfection, cells were lysed, and Renilla and firefly luciferase activities were determined using the Dual-Luciferase Reporter Assay system (Promega). Amd1 3’ UTR deletion constructs were cloned into PsfCHECK-2. HeLa cells were transfected as for ESCs, and samples were collected 2 d later for luciferase assay. For miRNA analysis, mir-762 (genomic sequence NCBI M37:7:134851801:134852276) was cloned into the mir-Vect miRNA expression vector, mir-Vect carrying hsa-mir-155 was used as a control [kind gifts from Dr. Mathijs Voorhoeve (Voorhoeve et al. 2006)]. To generate the Amd1 3’ UTR mir-762 target site mutant, PCR-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions.

Overexpression and siRNA treatment of Amd1

The Amd1 ORF was cloned into pTracer-EF vector (Invitrogen) under the control of the EF1α promoter. Cells were transfected after 3 d of NPC differentiation with FuGENE HD according to the manufacturer’s instructions. Cells were analyzed at day 6 of NPC differentiation. For overexpression of Amd1 in differentiating ESCs, stable cell lines were made expressing Amd1 using the pCAG-GFP-IRESPuro vector [a gift from Dr. Stuart Avery (Liew et al. 2007)]. Cells were induced to differentiate through withdrawal of LIF. For the Amd1 knockdown experiments, ESCs were transfected with 100 nM siRNAs using DharmaFECT transfection reagent 1 d and 3 d after plating according to the manufacturer’s instructions. At day 6, cells were harvested. Amd1 siRNAs were purchased from Ambion (Amd1 siRNA AM 16708A, ID nos. 65502, 65596, and 160655). Scrambled siRNA was used as a control (Thermo Scientific, D-001810-10-05). miR762 mimics (Thermo Scientific, catalog no. 310780-01-0005) were transfected in a manner similar to the siRNAs.

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GENES & DEVELOPMENT 471
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References


AMD1 is essential for ESC self-renewal and is translationally down-regulated on differentiation to neural precursor cells

Dawei Zhang, Tianyun Zhao, Haw Siang Ang, et al.

*Genes Dev.* 2012, 26:
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