A regulatory circuitry comprised of miR-302 and the transcription factors OCT4 and NR2F2 regulates human embryonic stem cell differentiation

Alessandro Rosa and Ali H Brivanlou*

Laboratory of Molecular Vertebrate Embryology, The Rockefeller University, New York, NY, USA

Multiple levels of control are in play to regulate pluripotency and differentiation in human embryonic stem cells (hESCs). At the transcriptional level, the core factors OCT4, NANOG and SOX2 form a positive autoregulatory loop that is pivotal for maintaining the undifferentiated state. At the post-transcriptional level, microRNAs (miRNAs) belonging to the miR-302 family are emerging as key players in the control of proliferation and cell fate determination during differentiation. Here, we show that the transcriptional factors OCT4 and NR2F2 (COUP-TFII) and the miRNA miR-302 are linked in a regulatory circuitry that critically regulate both pluripotency and differentiation in hESCs. In the undifferentiated state, both OCT4 and the OCT4-induced miR-302 directly repress NR2F2 at the transcriptional and post-transcriptional level, respectively. Conversely, NR2F2 directly inhibits OCT4 during differentiation, triggering a positive feedback loop for its own expression. In addition, we show that regulation of NR2F2 activity itself relies on alternative splicing and transcriptional start site choice to generate a full-length transcriptionally active isoform and shorter variants, which enhance the activity of the long isoform. During hESC differentiation, NR2F2 is first detected at the earliest steps of neural induction and thus is among the earliest human embryonic neural markers. Finally, our functional analysis points to a crucial role for NR2F2 in the activation of neural genes during early differentiation in humans. These findings introduce a new molecular player in the context of early embryonic stem cell state and cell fate determination in humans.

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Introduction

Holding the capacity of self-renewal and the potential to give rise to all cell types, human embryonic stem cells (hESCs) represent a powerful system for modelling early human

development and promising tools for regenerative medicine (Spagnoli and Hemmati-Brivanlou, 2006). Much effort has been spent in recent years to understand the molecular mechanisms underlying hESC pluripotency and differentiation, and it is now clear that both transcriptional and post-transcriptional levels of regulation have crucial roles.

At the transcriptional level, the pivotal players are the homeodomain transcription factors POU5F1/OCT4 and NANOG and the HMG-box transcription factor, SOX2 (Yuan *et al*, 1995; Nichols *et al*, 1998; Chambers *et al*, 2003). Interestingly OCT4, SOX2 and NANOG form a core regulatory circuitry (Boyer *et al*, 2005). The three factors co-occupy an extensive subset of their target loci, activating genes involved in the maintenance of the undifferentiated state. Moreover, in co-operation with Polycomb group proteins, the trio also repress the expression of development and differentiation genes (Bernstein *et al*, 2006; Lee *et al*, 2006). Finally, OCT4, SOX2 and NANOG also sustain each other's transcription in autoregulatory and feedforward loops (Boyer *et al*, 2005). The maintenance of such transcriptional regulatory circuitry is crucial to preserve the pluripotency of hESCs, as even slight variations in the levels of the core factors is sufficient to trigger differentiation (Hay *et al*, 2004; Zaehres *et al*, 2005).

At the post-transcriptional level, an important role has recently been shown for hESC-specific microRNAs (miRNAs; Rosa and Brivanlou, 2009). miRNAs are short non-coding RNAs able to repress translation and/or trigger degradation of target mRNAs. In animals, they are part of a silencing complex, which binds prevalently the 3' UTR region of the target for regulation (Bartel, 2009). Both human and mouse ESCs are characterized by the expression of a specific set of miRNAs, some of them being exclusively expressed in early embryos and ESCs (Suh *et al*, 2004). miRNAs are necessary for the formation of the embryonic stem cell pool, as shown by mutant mouse embryos lacking all miRNAs (*dicer*^{−/−} mice), which are devoid of OCT4⁺ cells and die before axis formation (Bernstein *et al*, 2003). Moreover, mutations in miRNA-processing factors impaired differentiation in mouse embryonic stem cells (Kanellopoulou *et al*, 2005; Wang *et al*, 2007). The hESC-specific miR-302/367 cluster is highly conserved in mammals and comprises four miR-302 variants (miR-302a–d, collectively referred to as miR-302) and miR-367. MiR-302, which is specifically and abundantly expressed in hESCs, has been shown to regulate hESC cell cycle and fate specification during differentiation (Suh *et al*, 2004; Landgraf *et al*, 2007; Bar *et al*, 2008; Morin *et al*, 2008; Barroso-del Jesus *et al*, 2009; Rosa *et al*, 2009).

It has also been shown that OCT4, NANOG and SOX2, together with Tcf3, bind miRNA promoters in hESCs (Marson *et al*, 2008). Specifically, they occupy the promoters of most

*Corresponding author. Laboratory of Molecular Vertebrate Embryology, The Rockefeller University, Box 32, 1230 York Avenue, New York, NY 10065, USA. Tel.: +1 212 327 8656; Fax: +1 212 327 8685; E-mail: brvnlou@rockefeller.edu

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miRNAs highly expressed in ESCs, including the miR-302/367 cluster and a subset of silent miRNA genes (Barroso-delJesus *et al*, 2008; Card *et al*, 2008; Marson *et al*, 2008). Thus, the core transcriptional regulatory factors are directly linked to the crucial miRNA cluster.

In this study, we show that OCT4 and miR-302 collaborate to regulate the activity of their target gene *NR2F2* (also known as COUP-TFII and ARP-1, a member of the NR2F, COUP-TF, nuclear orphan receptor family of transcriptional factors; Tsai and Tsai, 1997; Pereira *et al*, 1999, 2000; Chung and Cooney, 2003). In undifferentiated hESCs, *NR2F2* is silenced both transcriptionally by OCT4 and post-transcriptionally by miR-302. Conversely, *NR2F2* transcriptional activation occurs during differentiation when OCT4 and miR-302 levels decline. Alternative splicing and transcriptional start site choice allow the production of different *NR2F2* isoforms, which can increase *NR2F2* transcriptional activity. *NR2F2* in turn represses *OCT4* transcription, thus providing a positive feedback input to its own expression. Finally, we show that *NR2F2* has an important role in the specification of neural ectoderm during human development by sustaining the activation of *Pax6* and other crucial neural genes in differentiating hESCs.

Results

Reciprocal OCT4, miR-302 and NR2F2 expression during hESC differentiation

It was previously shown that *OCT4* and *miR-302* are pluripotency-associated genes highly expressed in undifferentiated hESCs (Suh *et al*, 2004; Boyer *et al*, 2005; Rosa *et al*, 2009). To compare the expression of *NR2F2* with pluripotency genes during hESC differentiation, we used two common *in vitro* differentiation assays, differentiation in adherent cultures as a two-dimensional monolayer or in three-dimensional embryoid bodies (EBs). In both situations, the levels of *OCT4* transcript (Figure 1A) and protein (Figure 1C) decreased on retraction of mouse embryonic fibroblasts-conditioned HUESM medium (MEF-CM), becoming almost undetectable after the first week of differentiation. In mammals, the miR-302 cluster, comprising of the four miR-302a-d and the unrelated miR-367, is hosted in the first intron of a non-coding gene (Barroso-del Jesus *et al*, 2009; Supplementary Figure 1), referred to as miR-302 HT (host transcript) in this paper. During hESC differentiation, levels of miR-302 HT faithfully mirrored the expression of *OCT4* (Figure 1A),

Figure 1 Temporal expression of *NR2F2*, *OCT4* and *miR-302* during hESC differentiation. RUES2 hESCs were differentiated in non-conditioned medium as a monolayer (nCM 2D; left panels) or embryoid body (EBs; right panels) for the indicated time points. CM: undifferentiated cells. (A) RT-PCR analysis of mRNA levels of the indicated genes. Lane -, negative control (no template). miR-302 HT indicates the host transcript of the miR-302/367 cluster. *ATP50* is a ubiquitous gene used for normalization. (B) Northern blot analysis of mature miR-302a expression. The ubiquitous U2 snRNA is used for normalization. (C) Western blot analysis of protein levels. The anti-*NR2F2* antibody recognizes an epitope in the N-terminal region of the full-length *NR2F2* isoform. α -Tubulin is used for normalization. (D) Relative quantification by real-time PCR of mRNA and miRNA levels in A, B and by densitometric scanning of western blots in C (arbitrary units).

supporting the notion that the *miR-302* gene is directly activated by OCT4 in ESCs (Marson *et al.*, 2008). However, northern blot analysis showed that mature miR-302 decline was slightly delayed (Figure 1B), probably because of an extended half-life of the mature miRNA form in differentiating cells. In the same conditions, *NR2F2* was expressed in an opposite way, being undetectable in undifferentiated cells and activated on differentiation (Figure 1A, C and D). Interestingly, NR2F2 protein accumulated around day 13, whereas high levels of its transcript were already detectable 3–5 days before (Figure 1D).

Taken together, these data indicate an inverse correlation between *NR2F2* transcription and the pluripotency factors OCT4 and miR-302, and possible post-transcriptional regulation of NR2F2 translation during hESCs differentiation.

NR2F2 is a direct target of miR-302

Given the reciprocal miR-302 and NR2F2 expression during hESC differentiation, and a possible post-transcriptional brake for NR2F2 accumulation, we asked whether NR2F2 could be a direct miR-302 target. To address this point, we first took advantage of HEK-293T cells, in which both miR-302 and NR2F2 are not expressed (Figure 2A; Rosa *et al.*, 2009). A full-length *NR2F2* transgene was transfected alone or in combination with either a synthetic miR-302a or a control miRNA (miR-Co). As shown in Figure 2A, we observed a strong decrease of NR2F2 protein in presence of miR-302, whereas the miR-Co had no effect. This suggests that miR-302a regulates NR2F2 expression. TargetScan software analysis of the *NR2F2* 3' UTR highlighted two predicted miR-302 target sites (Figure 2B and Supplementary Figure 2). To experimentally validate this bioinformatics prediction, we used a luciferase-based assay. Several forms of *NR2F2* 3' UTR were cloned into a luciferase reporter vector; wild type (WT) or variants in which the first (Mut1), the second (Mut2) or both (Mut1 + 2) predicted miR-302 target sites were mutated (Figure 2B). On co-transfection in HEK-293 cells, the WT 3' UTR was specifically repressed by miR-302, whereas mutations in either site relieve the inhibition, and the double mutation produced a nearly full rescue, indicating that both sites contributed to the negative regulation. Interestingly, both sites are extensively conserved in vertebrates (Supplementary Figure 2), suggesting a selective pressure to maintain the miR-302 regulation during vertebrate evolution, similar to the situation previously described for another miR-302 target, Lefty (Rosa *et al.*, 2009).

To address a specific role for miR-302 in the regulation of NR2F2 during hESC differentiation, we took advantage of a miR-302-overexpressing hES cell line (Rosa *et al.*, 2009). As shown in Figure 1A, NR2F2 levels increased during WT hESC differentiation. Conversely, in RUES2-302 cells, *NR2F2* activation was impaired (Figure 2D). Interestingly, in the same conditions, expression of OCT4 and other pluripotency markers was elevated (Rosa *et al.*, 2009; Figure 2D and data not shown). These results show a reciprocal effect of miR-302 expression on the levels of NR2F2 and OCT4.

Expression in mutually exclusive domains of miRNAs and their targets has been proposed to confer robustness to developmental genetic programmes by providing another level of inhibition to keep target expression quiet outside their functional domain (Stark *et al.*, 2005; Hornstein and Shomron, 2006).

NR2F2 expression is repressed by OCT4

As the *NR2F2* gene is silent during pluripotency and is turned on upon induction of differentiation, we asked whether the hESC core transcriptional factors might contribute to *NR2F2* regulation. The genomic regions bound by the core transcriptional factors in hESCs were recently mapped (Boyer *et al.*, 2005). Interestingly, a genomic region bound by OCT4, but not by SOX2 and NANOG, was mapped upstream of the *NR2F2* gene. However, the functional relevance of OCT4 binding was not analysed. Anti-OCT4 siRNAs, transfected in undifferentiated hESCs, efficiently reduced the levels of OCT4 mRNA and protein (Figure 3A and C). This led to significant inhibition of NANOG and SOX2 (Figure 3A), in agreement with previously described mutual activation of the three transcription factors (Boyer *et al.*, 2005). In the same samples, we observed a striking increase of NR2F2 mRNA, as shown in Figure 3B. As OCT4 knockdown promotes hESC differentiation (Hay *et al.*, 2004), the increase in NR2F2 mRNA may be due to either direct or indirect effect on the gene expression. However, in the same conditions, other early differentiation genes such as *Sox1* and *Brachyury* were not yet activated, suggesting a more direct inhibitory effect of OCT4 on *NR2F2* transcription. Interestingly, analysis of NR2F2 protein by western blot revealed only a modest increase compared with the transcript (compare panels B and C in Figure 3). As undifferentiated cells hold high levels of miR-302, such discrepancy might be due to the post-transcriptional inhibition of NR2F2 translation by miR-302.

We then analysed the binding of OCT4 *in vivo* in undifferentiated hESCs by chromatin immunoprecipitation (ChIP). The 1.6Kb region occupied by OCT4 (Boyer *et al.*, 2005) overlaps the first exon of the NR2F2-203 splicing isoform (Figure 3D). A significant enrichment was observed in the OCT4 (*POU5F1*) and miR-302 promoter regions, confirming previous observations (Chew *et al.*, 2005; Barroso-delJesus *et al.*, 2008; Card *et al.*, 2008). Moreover, OCT4 was also bound to the NR2F2 locus, downstream of Exon 1 of the NR2F2-203 isoform (Figure 3D and E). It was previously shown that many non-transcribed genes in ES cells carry chromatin marks that are otherwise associated with active transcription, such as high levels of acetylated H3 and H4 and di- and trimethylated H3K4. These epigenetic modifications allow the genes to be 'poised' for expression in response to appropriate developmental cues (Spivakov and Fisher, 2007). As shown in Figure 3E, we detected significant enrichment of acetylated histone H3, both on the active *OCT4* and *miR-302* promoters and on the silent *NR2F2* promoter.

Taken together, these results indicate that in pluripotent hESCs, the *NR2F2* gene is transcriptionally repressed by OCT4, but it can be promptly activated on release of OCT4 binding from the promoter. Moreover, NR2F2 is also repressed at the post-transcriptional level by the OCT4-activated miR-302.

Several NR2F2 isoforms are produced in differentiating hESC

A survey of the Ensemble Genome Browser (<http://www.ensembl.org/index.html>) revealed that alternative splicing gives rise to different transcripts from the *NR2F2* locus (Figure 4A). The transcriptional start site (TSS) and the coding sequence (CDS) are the same for NR2F2-001 and -201, the only difference being longer 5' and 3' UTRs in the

Figure 2 NR2F2 is a *bona fide* miR-302 target. (A) HEK-293T cells were transfected with a vector encoding for full-length NR2F2 (coding sequence and untranslated regions) alone or in combination with either a synthetic miR-302a or a control non-targeting synthetic miRNA (miR-Co). First lane: mock-transfected cells. The figure shows western blot analysis of the indicated proteins. α-Tubulin is used for normalization. (B) Schematic representation of the reporter construct containing the *Renilla* luciferase-coding sequence fused to the NR2F2 3' UTR. Numbers 1 and 2 refer to the predicted miR-302-binding sites. Sequences below illustrate the annealing of miR-302a to the wild-type NR2F2 (WT) and the point mutations (in red) introduced to generate the mutant NR2F2 3' UTR (Mut1 and Mut2). (C) Luciferase assay. HEK-293T cells were transfected with the WT, single mutants (Mut1 and Mut2) or double mutant (Mut1 + 2) NR2F2 reporter genes in combination with synthetic miR-302a or control (miR-Co). *Renilla* luciferase activity was normalized with a co-transfected Firefly luciferase construct. Numbers above histogram bars indicate the fold change in luciferase activity. (D) Real-time PCR analysis of endogenous NR2F2 (left) or OCT4 (right) in RUES2 cells (blue bars) or miR-302 overexpressing RUES2-302 cells (red bars), differentiated as embryoid bodies for the indicated days. Asterisks in C, D indicate significant difference compared with the respective controls (Student's *t*-test; *P* < 0.05).

NR2F2-001 mRNA. Conversely, other NR2F2 isoforms (NR2F2-202, -203 and -204) have different TSS and shorter CDS, resulting in transcripts lacking the first exon, which encodes for the DNA-binding domain (Qiu *et al*, 1995). The second and third exons, encoding the ligand-binding domain (LBD) and required for interaction with co-activators (Kruse *et al*, 2008), are shared among all isoforms. Taking advantage of isoform-specific primers, we aimed to validate and analysed the expression of the different NR2F2 variants during hESC differentiation and on OCT4 knockdown. As shown in Figure 4B, there was no expression of any isoform during pluripotency (lane 5), but we observed accumulation of the full-length as well as the shorter NR2F2-203 and -204 variants during hESC differentiation. Similarly, the NR2F2-001/201, -203 and -204 transcripts increased on OCT4 knockdown (Figure 4B), indicating that these isoforms share the same transcriptional regulation. Moreover, the sequences targeted by miR-302 in the 3' UTR are also shared by all the variants (data not shown), suggesting similar post-transcriptional regulation.

We then asked whether the short NR2F2 proteins might exert any activity. As expected, because they lack a DNA-binding domain, NR2F2-203 and -204 failed to activate the NGFI-A:Luc reporter containing the NR2F2-responsive

NGFI-A promoter (Pipaón *et al*, 1999; Kruse *et al*, 2008), whereas the full-length NR2F2-001 induced a five- to sixfold increase in the reporter activity (Figure 4C). However, when the short NR2F2 isoforms were transfected in combination with full-length NR2F2, they significantly increased its activity (Figure 4D), suggesting that they may enhance the effects of NR2F2 on downstream target genes during hESC differentiation.

Collectively, these results indicate that during hESC differentiation, the NR2F2 gene produces different protein isoforms, including shorter variants that can enhance the transcriptional activity of the full-length NR2F2.

NR2F2 is expressed in hESC-derived neuroectodermal tissues

To better define the pattern of NR2F2 expression, we analysed differentiating hESCs by immunostaining. As expected, undifferentiated hESCs colonies were highly positive for OCT4, whereas no signal was observed for NR2F2 (Figure 5A–D). Conversely, OCT4 strongly declined on differentiation, whereas groups of NR2F2-positive cells started to appear after 7 days (Figure 5E–H). Interestingly, as shown in Figure 5I–L, NR2F2-expressing cells are restricted in round 'neural rosette' structures, characterized by the expression of

Figure 3 OCT4 represses NR2F2 transcription in undifferentiated hESCs. (A, B) RT-PCR analysis of the indicated pluripotency (A) and differentiation (B) markers in untransfected (NT) RUES2 cells (green bars) or RUES2 cells transfected with non-targeting siRNAs (siCo, red bars) or anti-OCT4 siRNAs (siOCT4, blue bars). Note the log scale in B. Asterisks indicate significant difference compared with the respective NT controls (Student's *t*-test; $P < 0.05$). (C) Western blot analysis of OCT4 and NR2F2 protein levels in the same conditions as in A and B. The anti-NR2F2 antibody recognizes an epitope in the N-terminal region of the full-length NR2F2 isoform. α -Tubulin is used for normalization. (D) Schematic representation of the 1.6 Kb OCT4-binding region located upstream of the NR2F2 locus (see also Figure 4A). Arrows indicate the position of primer sets 1 and 2 used in ChIP analysis. (E) Chromatin immunoprecipitation (ChIP) analysis in undifferentiated RUES2 cells. Chromatin was immunoprecipitated with the indicated antibodies (AcH3 is the acetylated form of histone H3; Rb IgG is a control normal rabbit IgG). DNA was then analysed for the enrichment of specific sequences, indicated on the left of each panel. POU5F1 is the locus encoding for OCT4.

neural markers such as Pax6 (Pankratz *et al.*, 2007). NR2F2 expression in hESC-derived neural progenitors is reminiscent of NR2F2 expression in the mouse embryo, where it is first detected post-gastrulation at embryonic day 7.5 (E7.5) in the neural ectoderm (Qiu *et al.*, 1994; Tripodi *et al.*, 2004). Interestingly, we have previously shown that in mammals and frog embryos, members of the miR-302 family were highly abundant around gastrulation, in OCT4 expressing cells, and declined after gastrulation, at neurula stage, when the miRNA was specifically restricted to the neural territory, suggesting a role for this miRNA in neural specification (Rosa *et al.*, 2009).

To evaluate NR2F2 expression during neurogenesis, we used a previously established protocol to promote direct hESC neural differentiation (Pankratz *et al.*, 2007). Cells were first induced to form EBs for 4 days and then cultured in the presence of neural fate-promoting medium (N2M). Time-course analysis of gene expression indicated that NR2F2 is induced as early as cells exit from pluripotency, at the EB stage, and was strongly upregulated in subsequent time points (Figure 5M). Interestingly, NR2F2 expression faithfully mirrored the pattern of Pax6 temporal activation in the same conditions. Pax6 is among the earliest neural markers detectable during human neurogenesis *in vitro*, preceding the other early neuroectoderm marker Sox1 (Figure 5M; Pankratz *et al.*, 2007). Their similar temporal pattern of expression prompted us to address whether NR2F2 and Pax6 are also present at the same time in the same cells during neural differentiation. Immunostaining analysis

showed a consistent overlap in early differentiation (days 8–10), when most nuclei in the columnar cells of the neural rosettes are positively stained for both proteins (Figure 5N–Q and Supplementary Figure 3). Subsequently, whereas Pax6 remained restricted to neural rosettes, NR2F2 became more broadly expressed, detectable in both neural rosettes and in peripheral cells (days 12–14; Supplementary Figure 3). These analyses show that NR2F2 is activated at the beginning of differentiation in neural cells expressing Pax6. This specific pattern of expression is suggestive of a role for NR2F2 during early human neurogenesis.

NR2F2 is necessary for proper activation of early neural genes and repression of OCT4 during hESC differentiation

To discover the function of NR2F2 during differentiation, we performed loss of function analysis. Differentiating RUES2 cells were transfected with NR2F2-specific siRNAs and the expression of cell type-specific markers was analysed by real-time PCR (Figure 6A and Supplementary Figure 4). Genes affected by NR2F2 depletion included the earliest neural markers expressed during hESC neural differentiation, such as Pax6 and other forebrain-associated genes (Pankratz *et al.*, 2007). Six3 and Lhx2 are homeodomain transcription factors expressed early in the anterior portion of the CNS and known to bind Pax6 (Oliver *et al.*, 1995; Porter *et al.*, 1997; Mikkola *et al.*, 2001). Zic1 is a zinc finger transcription factor expressed initially throughout the neural plate and, at later stages, in the dorsal portion of the entire neural tube (Nagai *et al.*, 1997).

Figure 4 Alternative splicing generates several NR2F2 isoforms during hESC differentiation. **(A)** Schematic representation of the human NR2F2 locus (red box) on chromosome 15. Several isoforms generated by alternative splicing and alternative transcription start sites are depicted below. Black boxes indicate coding sequences (CDS), empty boxes indicate untranslated regions and lines represent introns. Arrows indicate transcriptional start sites. **(B)** RT-PCR analysis with isoform-specific primers in differentiated (nCM) and undifferentiated (CM) RUES2 cells. 2D, monolayer differentiation; EBs, embryoid bodies; NT, siCo and siOCT4 samples are the same as in Figure 3. ATP5O is used for normalization. **(C, D)** Luciferase reporter assay for transcriptional activation by NR2F2 isoforms in HeLa cells. In both panels, the Firefly luciferase activity is normalized with a co-transfected constitutive *Renilla* luciferase-encoding vector. **(C)** Cells were transfected with the NGFI-A:Luc reporter in combination with a control (GFP) or vectors encoding for the indicated NR2F2 isoforms. **(D)** Cells were co-transfected with the NGFI-A:Luc reporter and the full-length NR2F2-001 in combination with a control (GFP) or the NR2F2-203 or NR2F2-204-encoding vectors. In **C** and **D**, asterisks denote significant difference (Student's *t*-test; $P < 0.05$) and numbers indicate fold change compared with the respective controls.

N-cadherin is a crucial cell adhesion molecule involved in neural tube formation (Radice *et al.*, 1997). Interestingly, expression of the neural transcription factor Sox1, which is expressed at later stages during hESC neural differentiation (Figure 5M; Pankratz *et al.*, 2007), and the mesendoderm markers Brachyury and Mixl1 are not significantly affected by NR2F2 loss of function (Supplementary Figure 4), suggesting

a specific role for NR2F2 in the specification of early anterior neuroectoderm.

Our target analysis showed that miR-302 could inhibit NR2F2 expression (Figure 2). We therefore hypothesized that the decrease of miR-302 during hESC differentiation is crucial for NR2F2 activity and, as a consequence, for proper expression of early neural genes. To address this point, we took advantage of functional analysis by loss of miRNA function. As shown in Figure 6B, specific knockdown of miR-302 by antisense LNA molecules (Rosa *et al.*, 2009) resulted in increased expression of both NR2F2 and anterior neuroectodermal markers.

NR2F2 was previously shown to act as a negative regulator of OCT4 transcription in mouse embryonal carcinoma cells (Ben-Shushan *et al.*, 1995; Schoorlemmer *et al.*, 1995). However, the conservation of such inhibitory activity was never addressed in humans. We performed overexpression analysis using an inducible system derived from the ePiggyBac transposon (Lacoste *et al.*, 2009). RUES2 cells were stably transfected with a construct constitutively expressing the TET transactivator (TET-on), along with a doxycycline-inducible NR2F2 construct (Figure 6C), giving rise to the RUES2-TRE-NR2F2 cell line. The endogenous *NR2F2* gene is silent in undifferentiated hESCs, which normally express OCT4 (Figures 1 and 5). Untreated RUES2-TRE-NR2F2 cells showed uniform OCT4 expression, whereas NR2F2 was undetectable (Figure 6D–G). Conversely, in cells induced with doxycycline for 4 days, we observed large areas of OCT4-negative nuclei, which corresponded to groups of cells expressing high levels of NR2F2 (Figure 6H–K). These hESC colonies, despite being maintained in MEF-CM during the whole treatment, also presented signs of differentiation, such as flat cells with a low nuclear-cytoplasm ratio (data not shown). On the contrary, RUES2 cells transfected with a RFP-encoding construct (RUES2-TRE-tagRFP) did not show any difference in presence or absence of doxycycline (Supplementary Figure 5), providing a specificity control for our approach. These results indicated that overexpression of NR2F2 was sufficient to significantly reduce OCT4 levels in pluripotency conditions. Moreover, the reciprocal experiment showed that higher levels of OCT4 protein were detected in siNR2F2-transfected differentiating RUES2 cells (Supplementary Figure 4), suggesting a critical role for NR2F2 in the exit of hESCs from pluripotency.

Taken together, our functional analyses demonstrates that NR2F2 has a role in the proper induction of *Pax6* and other neural genes for initial specification of neural ectoderm and contributes to OCT4 decrease during differentiation, linking the exit from pluripotency with the acquisition of a specific cell fate.

Discussion

Both negative and positive regulation has fundamental roles during embryogenesis. Inhibitors of signalling pathways, transcriptional inhibitors and miRNAs are all examples of negative regulators. Moreover, multiple levels of inhibition regulate key events during cell fate determination. Here, we report that a negative feedback loop links OCT4 and NR2F2, which are able to reciprocally repress each other's transcription. The transcriptional repression is only the first level of

Figure 5 Spatial expression of NR2F2, OCT4 and Pax6 during hESC differentiation. (A–L') Undifferentiated and (A–D) differentiated (7 days in non-conditioned medium as a monolayer; E–L') RUES2 hESCs colonies were stained for the indicated proteins. (C, G, K, K') Nuclear counterstain with Sytox Orange. I'–L' are magnifications of white boxes in I–L, respectively. Scale bars represent 200 μ m in A–L and 50 μ m in I'–L'. (M) Real-time PCR analysis of the temporal expression of NR2F2 (blue line), Pax6 (red line) and Sox1 (green line) in RUES2 cells induced to differentiate into neural ectoderm. The protocol of differentiation is depicted below the histogram and described in detail in the Materials and methods section. Note the logarithmic scale. All measurements, except Sox1 D4, are statistically different compared with the undifferentiated (D0) sample (Student's *t*-test; $P < 0.01$). (N–Q) RUES2 cells differentiated in N2M for 10 days with the protocol showed in M were stained for NR2F2 (N) and Pax6 (O), and counterstained with Sytox Orange (P). Scale bars represent 50 μ m.

negative regulation for NR2F2, as inhibition of NR2F2 by OCT4 is reinforced by miR-302. Interestingly, NR2F2 is among those differentiation genes that are silent in undifferentiated cells but poised for activation. We show here that NR2F2 accumulation can be readily induced on release of OCT4,

even in pluripotency conditions. With this in mind, the second layer of regulation by a miRNA directly activated by OCT4 can be envisioned as a leakage-reducing mechanism. Indeed, uncontrolled accumulation of NR2F2 due to fluctuation in OCT4 levels could result in further reduction of OCT4,

Figure 6 Functional analysis of NR2F2 in hESCs. (A) Real-time PCR analysis of the indicated neural markers in differentiating RUES2 cells. Control mock-transfected cells (NT, blue bars) and cells transfected twice with non-targeting siRNAs (siCo, red bars) or anti-NR2F2 siRNAs (siNR2F2, green bars) were collected for analysis at day 7 of differentiation as a monolayer. (B) Differentiating RUES2 cells were transfected twice with control non-targeting antisense LNA oligonucleotides (LNA-C; blue bars) or with anti-miR-302 LNA (LNA-302; red bars). Cells were collected at day 8 and levels of indicated neural markers were assessed by real-time PCR analysis. Asterisks in A and B indicate significant difference compared with the respective controls (Student's *t*-test; $P < 0.05$). (C) Schematic representation of the ePiggyBac-based inducible system for NR2F2 ectopic expression. TRE, TET responsive promoter; pA, polyadenylation signal. Pubc, human Ubiquitin C promoter; BsdR, Blasticidin resistance gene. In the ePB-TET-on vector, the constitutive CAG promoter drives a fusion gene encoding for the doxycycline-activated TET protein fused to the hygromycin resistance protein through a self-cleavage T2A peptide. (D–K) Immunostaining analysis of RUES2 cells stably transfected with the NR2F2-inducible system and left untreated (D–G) or cultured in presence of doxycycline (H–K). Cells were stained for OCT4 (D, H), NR2F2 (E, I) and counterstained with Sytox Orange (F, J). The scale bar in D represents 100 μ m and can be applied to all panels.

triggering a loop potentially leading to differentiation. Thus, miR-302 may confer buffering against perturbation (robustness) in undifferentiated hESCs, as already shown for other miRNAs in different systems (Stark *et al*, 2005; Hornstein and Shomron, 2006; Li *et al*, 2009).

Our previous work showed that the Nodal inhibitor Lefty is a target of miR-302 in hESCs (Rosa *et al*, 2009). The Nodal pathway is pivotal for cell fate specification in vertebrate embryos and sustained Nodal activity inhibits neuroectodermal specification in hESCs, whereas Lefty overexpression has an opposite effect (Vallier *et al*, 2004; Smith *et al*, 2008). By targeting Lefty, miR-302 has an inhibitory effect on the specification of neuroectoderm (Rosa *et al*, 2009). Here, we show that miR-302 also targets NR2F2, another regulator of neural differentiation. The increase in neuroectoderm

derivative observed on loss of miR-302 function can be the result of the increase of both Lefty and NR2F2 activity.

While both inhibition of translation and destabilization of the mRNA are mechanisms used by miRNAs to block gene expression, in hESC differentiation, we observed an increase of NR2F2 mRNA levels, and protein levels remained low, suggesting that miR-302 primarily inhibits NR2F2 at the translational level. However, in miR-302 loss- and gain-of-function analyses, we observed changes in NR2F2 mRNA levels (Figures 2 and 7). As NR2F2 itself is a neural gene, the increase in NR2F2 mRNA levels observed in cells depleted for miR-302 and its decrease in miR-302-overexpressing cells, may be due to indirect effects on neuroectoderm specification. Moreover, we showed that miR-302 overexpression in differentiating hESCs led to an increase of OCT4 levels

Figure 7 Model. The model illustrates different levels of regulation of pluripotency and neural differentiation mediated by the OCT4/NR2F2/miR-302 molecular circuitry in human embryonic stem cells. NR2F2 is repressed transcriptionally by OCT4 and post-transcriptionally by miR-302 and, in turn, inhibits OCT4 transcription. OCT4 activates transcription from the miR-302 locus and other pluripotency genes. NR2F2 is necessary for proper induction of neural genes during differentiation.

(Figure 2) that, in turn, may inhibit NR2F2 expression at the transcriptional level. Thus, miR-302 may contribute to the observed lack of NR2F2 induction, directly or indirectly, at multiple levels of inhibition.

During hESC differentiation, alternative splicing also regulates NR2F2 expression. Among different NR2F2 splicing variants that are expressed in differentiating hESCs, three isoforms, NR2F2-202, -203 and -204 that lack the first exon and give rise to proteins missing the DNA-binding domain, unexpectedly enhance the activity of the full-length isoform. On the basis of this finding, two mechanisms can be envisioned to explain such positive effect. The shorter forms might directly enhance the full-length NR2F2 by formation of heterodimers, as they retain the protein–protein interaction domain (Pereira *et al*, 2000). Alternatively, the shorter forms might be in charge of eliminating inhibitors involved in intermolecular interactions. Regardless of the mechanism, this positive effect is in agreement with the overlapped expression of long and short isoforms during hESC differentiation. It will be interesting to investigate whether such a reinforcing system is peculiar to hESCs or is more broadly used by NR2F2 in other tissues. The activity of NR2F2 can also be enhanced by co-activators (Kruse *et al*, 2008). Interestingly, we noticed that miR-302 and miR-367, which are co-expressed as a single precursor molecule (Suh *et al*, 2004; Rosa and Brivanlou, 2009), target at least two of such co-activators, SRC-1 (NCOA1) and SRC-3 (NCOA3; data not shown). Therefore, the OCT4-activated miR-302/367 cluster might repress NR2F2 function not only directly by targeting NR2F2 mRNA but also indirectly by targeting NR2F2 partners.

Germ cell nuclear factor (GCNF, also known as RTR or NR6A1), an orphan nuclear receptor not related to NR2F2, also exhibits a positive effect on neural differentiation of mouse teratocarcinoma and neural stem cells (Zechel, 2005; Akamatsu *et al*, 2009). Both GCNF and NR2F2 have been previously shown to repress OCT4 transcription in mouse teratocarcinoma and ES cells (Ben-Shushan *et al*, 1995;

Schoorlemmer *et al*, 1995; Gu *et al*, 2005; Zechel, 2005); however, GCNF does not seem to be involved in the regulatory circuitry described here, as the GCNF 3' UTR is not responsive to miR-302 (Supplementary Figure 6).

While the biological function of NR2F2 during embryogenesis has been extensively studied in the mouse model, its role in human early development and cell fate determination remains poorly understood. In the mouse, both NR2F2 (COUP-TFII) and the related NR2F1 (COUP-TFI) are involved in late stages of neural development and organogenesis (Qiu *et al*, 1997; Zhou *et al*, 1999, 2001; Tripodi *et al*, 2004; Naka *et al*, 2008). Homozygous deletion of the NR2F2 gene leads to growth-retarded mouse embryos with defects in the head and heart, which eventually die around E10 (Pereira *et al*, 1999). In hESCs, in contrast, we find that NR2F2 mirrors the expression of the earliest neural markers and has a key role in regulating the very early neural specification genes before the emergence of individual neural structures. Such positive effect could be due to either direct activation of neural genes or indirect effects (activation of activators or repression of repressors) and it will be interesting in the future to identify which promoters are directly bound by NR2F2 during human neural development.

Direct binding by NR2F2 to the *Pax6* gene was recently shown in the context of mouse eye development (Tang *et al*, 2010). Interestingly, the binding of NR2F2 to the *Pax6* gene resulted in transcriptional repression in the mouse retina and in the human ARPE-19 cell line, which originated from retinal pigmented epithelium (Tang *et al*, 2010). While this may at first seem at odds with our analysis, two lines of evidence help explain this apparent discrepancy. First, a dual role for NR2F factors as both repressors and activators of transcription is well established (Pereira *et al*, 2000). Depending on the context, NR2F2 might act as a positive or a negative regulator of the *Pax6* locus. Second, the previous study (Tang *et al*, 2010) concerns later events taking place during eye development, while our functional analysis was performed at very early time points. Our analysis shows that NR2F2 and *Pax6* domains of expression are mostly overlapped at the beginning of differentiation, whereas many NR2F2-expressing cells are *Pax6*-negative at later time points. Thus, a distinctive regulation of *Pax6* by NR2F2, possibly involving other auxiliary factors, might exist in specific lineages and time points during human neural development.

In conclusion, we show how the interplay between a miRNA, miR-302 and two key transcriptional factors, OCT4 and NR2F2, is crucial for maintaining hESC pluripotency on one hand, while also ensuring the proper specification of neuroectoderm. In this view, pluripotency and neural differentiation circuits are closely connected, as depicted in the model of Figure 7. While OCT4 is at the top of the hierarchy for the maintenance of pluripotency, NR2F2 is necessary for the correct activation of crucial neural genes, whereas miR-302 may contribute to fine-tune the balance between the two.

With the recent discovery and characterization of hESC-specific miRNAs, such as miR-302, we are only starting to appreciate the importance of such regulatory hierarchies, involving regulation of gene expression at the transcriptional and post-transcriptional levels.

Materials and methods

hESC culture and transfection

hESC line RUES2 was cultured as previously described (James *et al.*, 2006) on plates coated with Matrigel (BD Biosciences) in MEF-CM supplemented with 20 ng/ml bFGF (Invitrogen). For differentiation in adherent cultures (2D differentiation), CM was replaced with non-conditioned HUESM without bFGF (nCM). EBs were generated as previously described (James *et al.*, 2006). The miR-302 over-expressing RUES2-302 line is described in Rosa *et al.* (2009). Directed neural differentiation was performed as described (Pankratz *et al.*, 2007). Briefly, RUES2 cells were cultured in suspension as EBs in nCM for 4 days and in N2M for 2 additional days. Aggregates were then allowed to attach in laminin-coated plates and maintained in N2M for 8 days, for a total differentiation time of 14 days. When indicated, cells were cultured in presence of 2 µg/ml doxycycline (Sigma).

MiR-302 knockdown experiments were performed as described in Rosa *et al.* (2009). Briefly, hESCs were transfected with lipofectamine 2000 (Invitrogen) using a mixture of antisense LNA oligonucleotides specific for the four human miR-302 miRNAs or with a standard control LNA (sense miR-159), at a final 75 nM concentration. All LNA oligonucleotides were purchased from Exiqon.

For OCT4 knockdown, undifferentiated RUES2 cells were transfected twice with lipofectamine RNAiMax (Invitrogen) with 75 nM anti-OCT4 siRNAs (siGENOME Duplex D-019591-05-0010) or control non-targeting siRNA no. 3 (both from Dharmacon) and harvested after 2 days for protein and RNA analysis. For NR2F2 knockdown, RUES2 cells differentiating in adherent cultures were transfected at days 3 and 6 of differentiation with 75 nM anti-NR2F2 siRNAs (siGENOME SMARTpool M-003422-00-0005; Dharmacon) or control non-targeting siRNA no. 3, and harvested at day 7 for protein and RNA analysis.

Plasmid construction

The ePB-BSD-TRE-NR2F2, ePB-TET-on, ePB-CAG-NR2F2-001, -203 and -204 plasmids are based on a modified version of the enhanced PiggyBac (ePiggyBac) vector described in Lacoste *et al.* (2009). The Blastidicin resistance cassette was derived from the pUB/Bsd vector (Invitrogen). The TET-on system is based on the pTRE-Tight Vector (Clontech). The full-length NR2F2-001 sequence was purchased from Open Biosystems (Clone Id 5177487) and subcloned in the ePB-BSD-TRE plasmid to generate ePB-BSD-TRE-NR2F2 or in the ePB-CAG plasmid to generate ePB-CAG-NR2F2-001. The cDNA of NR2F2-203 and NR2F2-204 was amplified by RT-PCR from RUES2 cells differentiated for 14 days and cloned into the ePB-CAG plasmid. The pXP2-NGFI-A:Luc plasmid was kindly provided by Dr Eric H Xu (Laboratory of Structural Sciences, Van Andel Research Institute, Grand Rapids, MI, USA).

For generation of the luciferase reporter construct, the NR2F2 3' UTR was PCR-amplified from human genomic DNA and cloned downstream of the *Renilla* luciferase CDS in the pRL-TK vector (Promega). To generate the mutant variants, point mutations in the two miR-302-binding sites were introduced by PCR.

RT-PCR and real-time qRT-PCR analysis

For RT-PCR analysis, total RNA was first treated with DNA-free kit (Ambion) and then retrotranscribed with the SuperScriptIII kit (Invitrogen). cDNA was then used as template for PCR with the GoTaq DNA polymerase (Promega). Real-time qRT-PCR analysis was performed with the LightCycler 480 SYBR Green I kit (Roche) and results were analysed with the REST-MCS2 software. A complete list of primers is provided in Supplementary Table 1.

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Northern blot

Of the total RNA, 1 µg was isolated with RNA Bee (Tel-TEST Inc.) was electrophoresed in a 10% polyacrylamide TBE-Urea Gel (Bio-Rad) and transferred by electroblotting onto Hybond-N+ membrane (Amersham Biosciences). Hybridizations and washes were performed as described (Fazi *et al.*, 2005). Signals were detected with a Typhoon 9400 Variable Imager (Amersham Biosciences). Sequences for the probes are provided in Supplementary Table 2.

Luciferase reporter assay

HEK-293T cells were purchased from ATCC and cultured in DMEM containing 10% FBS. Cells were transfected 24 h after seeding using the Lipofectamine 2000 reagent (Invitrogen). MiR-302a and standard negative control no. 1 synthetic miRNA (mimics) were purchased from Dharmacon and transfected at a 20 nM final concentration together with a combination of each pRL-TK reporter construct and pGL3-control in a ratio of 10:1. Luciferase activity was determined with the Dual Luciferase Assay System (Promega) and normalized by the co-expressed Firefly Luciferase. All luciferase assays were repeated at least three times and done in triplicate each time.

Immunostaining, western blot and ChIP assays

Undifferentiated and differentiated hESCs were cultured in Matrigel- or laminin-coated glass-bottomed culture dishes (MatTek Corporation) and immunostained with primary anti-Oct3/4 (BD611202; BD Transduction Lab), anti-NR2F2 (Ab64849, Abcam) and anti-Pax6 (DSHB) antibodies, and secondary anti-mouse Alexa 488 and anti-goat Alexa 647 antibodies (Molecular Probes). Sytox Orange (Molecular Probes) was used for nuclear counterstain. Cells were imaged using a Zeiss LSM 510 confocal microscope.

For western blot, primary mouse anti-NR2F2 (H7147; R&D Systems), anti-Oct3/4 (BD611202; BD transduction lab) and anti-alpha/beta Tubulin (Cell Signaling) and secondary HRP-anti-mouse IgG (GE Healthcare) antibodies were used. Densitometric quantification of western blot films in Figure 1 was carried out with the Quantity One software.

ChIP was performed with the EZ-Magna ChIP A kit (Millipore) according to the manufacturer's protocol, with rabbit α -Oct3/4 (H-134; Santa Cruz Biotechnology) and normal Rabbit IgG and anti-acetyl-Histone H3 antibodies (both from Millipore). Sequences of primers are provided in Supplementary Table 1.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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