Introduction

The first cell lineage specification in mammalian embryo development is the formation of trophectoderm (TE) and inner cell mass (ICM) of the blastocyst. TE cells will form a large part of the placenta, whereas ICM will give rise to the epiblast and primitive endoderm. After embryo implantation the epiblast generates the three germ layers, and hence all the tissues in the future body. ESC are derived from blastocyst-stage embryos and are thought to be functionally equivalent to the ICM.

Vitamin A (retinol) is obtained from the diet in the form of retinyl ester and oxidized in a two-step process, first to all-trans retinaldehyde and then, in irreversible fashion, to aTRA. The first step of aTRA synthesis is catalyzed by several members of two enzyme families: the alcohol dehydrogenases (ADHs) and retinol dehydrogenases (RDHs). Retinaldehyde is then further converted to retinoic acid by retinaldehyde dehydrogenases (RALDH). RA crosses the plasma membrane and is bound by cellular retinoic acid binding protein CRABP-I or CRABP-II. aTRA acts within the nucleus as a ligand to two nuclear receptor families (RAR and RXR), which regulate transcription of target genes by binding RA response elements (RARE) [1]. In placental embryos, autocrine retinoic acid synthesis begins at 7.5 d.p.c. and the major source of retinoids is maternal retinol [2]. aTRA plays important and pleiotropic roles in embryo development and cellular differentiation [3] and prolonged aTRA exposure promotes ESC differentiation into primitive endoderm or cells of the three primary germ layers, depending on culture conditions [4-6].

Recently, we identified Snai1 as direct target of retinoid receptors and found that Snai1 promotes ESCs differentiation into primitive endoderm and cells of the three primary germ layers through the direct binding and repression of pluripotency and self-renewal genes such as Nanog, Tcfcp2l1 and Nr5a2 [7]. Here we demonstrate that aTRA promotes early steps of ESC differentiation, and that ESC increase their capacity to synthesize atRA during spontaneous differentiation as EBs, up-regulating RDH1, RDH10, ADH3, RALDH2, and CRABP2. Among 35 transcription factors (TFs) regulated by aTRA in ESC, 3 TFs up-regulated (Snai1, Gala6, Cdx1) are known to be involved in ESC pluripotency exit and 3 TFs down-regulated (Otx2, Id2 and Arid1a) are involved in ESC pluripotency maintaining.

Results: Here, we demonstrate that aTRA promotes early steps of ESC differentiation, and that ESC increase their capacity to synthesize aTRA during spontaneous differentiation as EBs, up-regulating RDH1, RDH10, ADH3, RALDH2, and CRABP2. Among 35 transcription factors (TFs) regulated by aTRA in ESC, 3 TFs up-regulated (Snai1, Gala6, Cdx1) are known to be involved in ESC pluripotency exit and 3 TFs down-regulated (Otx2, Id2 and Arid1a) are involved in ESC pluripotency maintaining.

Conclusion: Cultivation and controlled differentiation of ESC has opened new frontiers both in regenerative medicine and biology of development. Here, we demonstrated that RA is synthesized by ESC during spontaneous differentiation as EBs and takes an active role to promote their own differentiation process.

Keywords: Retinoic acid; Retinol; Embryonic stem cells; Differentiation; Self-renewal; RDH1; RDH10; ADH3; RALDH2; CRABP2
Materials and Methods
ESC maintenance

Mouse E14 ESC were cultured as previously described [8] on gelatin-coated dishes in feeder-free culture system DMEM (4.5 g/l glucose) containing 15% Embryomax fetal bovine serum (FBS, Millipore), 1 mM sodium pyruvate (Invitrogen), 0.1 mM MEM non-essential amino acid (Invitrogen), 1000 U/ml LIF ESGRO (Millipore), and 50 µM 2-Mercaptoethanol. Mouse GFP-Bry ES cell line was kindly provided by Dr. Gordon Keller, and cultured in serum-free medium on gelatin-coated dishes in feeder-free culture system as previously described [9]. ESC-RARE-LUC cells were cultured and differentiated as wt E14 ESC.

ESC differentiation

For EBs formation, E14 ESC were plated at 2.5×10^5 cells/ml in ultra low attachment plates (Costar) in MEM ALPHA Medium (Invitrogen) supplemented with 10% FBS or Charcoal/Dextran Treated FBS (HyClone) and 50 µM 2-Mercaptoethanol. After 48 hours EBs were treated with 1 µM CD2665 (R&D Systems) or 0.1 µM Retinol (Sigma-Aldrich). GFP-Bry derived EBs were cultured in serum-free medium consisting of 75% IMDM (Invitrogen), 25% Ham’s F12 (Invitrogen) supplemented with 0.5x N2B27 (w/o retinoic acid) and 0.05% BSA, 2 mM glutamine (Invitrogen), 0.5 mM ascorbic acid (Sigma-Aldrich), 0.45 mM 1-thioglycerol (Sigma-Aldrich). After 24 hours GFP-Bry EBs were treated for additional 24 hours with 0.1 µM atRA (#R2625, Sigma-Aldrich). Subsequently, EBs were dissociated with trypsin and cells were reaggregated at 1.5x10^5 cells/ml in the same medium and new EBs were harvested 3 days later for flow cytometry analysis.

Luciferase assay

Lentiviral vectors pGREENFIRE1-mCMV-EF1-PURO (for background control) and pGREENFIRE1-RARE-mCMV-EF1-PURO (System Bioscience) were used to produce lentiviral particles as previously described [10]. E14-ESC were transduced with lentiviral particles and 24 hours after infection medium was changed and cells treated with Puromycin 6 µg/ml for 5 days. Selected cells were cultured for 8 hours in the indicated media, lysed and luciferase activity was measured by using the Dual-Luciferase® Reporter Assay (Promega) as described in [11].

RT-qPCR

Total RNA was extracted using TRReagent (Invitrogen) according to the manufacturer’s protocol. RT-qPCR was performed as previously described [12] using SuperScript III One-Step RT-PCR System and SYTO9 Green-Fluorescent Nucleic Acid Stain (Invitrogen) on Rotor Gene 6000 (Corbett Research). The primers used for RT-qPCR were designed using Primer3 software from a recent E14 genome assembly [13]. The oligonucleotides used are listed in Table 1.

Table 1: Primers sets used for RT-qPCR.

| GENES   | Forward sequence (5’→3’) | Reverse sequence (5’→3’) |
|---------|--------------------------|--------------------------|
| j-Actin | TCGTACTGCTGCTTCTTGGTTG | ACATGGAGGGAATACAGC       |
| Brachury| CTGTACGCTGCCTTCTGGAAG   | GGGTTTCTCTTCTTGGCATCAAG |
| Rdh1   | AAGCCCTTTCTCAGACAGAT    | GAGTGAAGGCAAAGACAGA      |
| Adh1   | TGTTACGCTTGTGGAGAAGG    | TTGCTGCCGTCCTAAAGTTT    |
| Adh3   | TGAGGTTGATGCTTCTGGAGG   | GTGCATGCTAGAAAGCTTGG    |
| Adh2   | AACAGTCCAGAAGTACCTTG    | AGGTTCTTCTTGCTGACTCA    |
| Raldh1 | CTCTGCGCTGTGGAAACATT    | CCATGGTGGTAACTCAAC      |
| Raldh2 | TTGGAGATGCTGCTTCTGGAGC  | TCTGAGGACCCCTCTGACCT    |
| Raldh3 | TGGATGTCGCTGCTTGAGAGG   | AGAAGCACTGGTTGATGAG     |
| Crbp1  | CTACTCGGACCGGAGACGTC    | GGAAATGGGTGGTCAAT       |
| Crbp2  | GGAAGTCGTCGCTCAGGTTGAA | GGAAGTCGTCGCTCAGGTTGAA |
| Crabp1 | CAGTGTGAGGAGGAGGAGG     | AGTGTACCTCCTGAGGCTT     |
| Crabp2 | TTGTCTTTCCTGAGGCTT     | TTGTCTTTCCTGAGGCTT     |
| Nanog  | CAGTTCGCTGACGGTGATCTC   | CGTTGTCATCGTACGACTC     |
| Oct4/4 | GGGTTTCTCTTGGAAAGGTT    | CTTGCGACACCTCTGAGGATT   |
| Snail1 | TGGGGCCCAATTCCCTTCAAG   | AGAAGAGGCTTCTCCCAAGGTT  |
| Aebp2  | ATGGGGATGATCAGGGAAGCA   | TGGTCCAGCAACAGTATTAGG   |
| Cdx1   | AAGACCTGGTCTGCTGAGTG    | TGGCTGAGGCTCCTGAGTCT    |
| Cyp26a1 | TCGACGCTCTGTAGGACTCG    | TCGACGCTCTGTAGGACTCG    |
| Foxa1   | AGACATTCAAGGCGACGACTA   | TCGTGTGCTCGCGTAATA      |
| Foxn4   | AACCTGCTGCTGCTGACAA     | TTTCCGGCGCGCTGAGTGA     |
| Gata6   | AAAAAAGCCACCCGCGAGAA    | AGGTGAGATGGCCTCGAGA     |
| On2    | TGGACACAGTGGCAGCTTCA    | AGAGTGAACAGGCGAGACAGA   |
| Arid1a  | TCAGCTGCTGTTGGAAGGAGA   | TCGGCTGCGTTGGAAGGAGA    |
| Neurog3 | CGGATGACGGCAACACTTCA    | TAGAAGCTGGTGTCGCGCTAT   |
| Meox1  | AACCCTGGTTGCTCCTCCA     | CTGTCGCTGGAAGTACCTCG    |
| Id2    | TCCAGCTGAAGGACTGTTG     | TGTAGACTGAACGAGGTG      |
| Mistl1  | TGCCGACGCTCTAAATATT    | TCCACAGGAGCACAAGACAT    |
| Hoxa1   | AGTGTGTGCTCAAGCTAGG    | ACCATGGAGGCTGAGAGGTT    |
| Hoxa5   | CGCCGCAACTACAGGACACA   | TGCCCTCAGGGAATCTGACCA   |
| Hoxb4   | AGAATCTCGAGAAGCAGGTTA  | GCGCTAGTGAGGCTGAGT      |
| Rarb   | AGACCGCCCATACGTCCTAA    | TTGTCCTGCGAAACAGGACA    |
Flow cytometry analysis

EBs generated from GFP-Bry cell differentiation were treated with Dissociation Buffer PBS-based (Invitrogen) for 1-2 min and arranged at 1x10^6 cells/ml in PBS. 1 µl of reconstituted fluorescence reactive dye LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen) was added and cells were incubated for 30 min protecting from light and analyzed by FACS CantoII (Becton Dickenson). Data analysis was performed by FlowJo software (Tree Star Inc.).

Microarray analysis

Microarray was performed on Illumina Platform and analyzed using BeadStudio Gene Expression Module (GX). Data were background adjusted and quantile normalized using default parameters in the BeadStudio Software. Probes with Log[FC]>1+ and p-value <0.05 were selected for downstream analysis. Heat-map plots were performed through the Bioconductor package in R. Differential expression analysis of the up- or down-regulated genes was performed by plotting genes on their Log2 expression value using Excel (Microsoft 19).

Results and Discussion

RA promotes ESC spontaneous differentiation

ESC have the ability to spontaneously differentiate and exogenous RA promotes their differentiation into primitive endoderm and a broad spectrum of derivatives of all three germ layers depending on culture conditions [4-6].

We used EBs as a model system recapitulating the embryogenesis to explore the early steps of RA signaling during embryonic development. For this set of experiments, we used the ES cell line with the green fluorescent protein (GFP) cDNA targeted to the primitive streak marker Brachyury locus (GFP-Bry), which does not differentiate in serum-free medium in absence of specific factors [9,14,15]. GFP-Bry ESC were differentiated as EBs in serum-free medium, and after 24 hours, when cells begin the transition from an ICM-like cells to an epiblast-like stem cell (EpiSC) [16], EBs were treated with a 24 hour pulse of atRA. At the end of day 2 EBs were dissociated to eliminate any trace of atRA and reaggregated in differentiation medium without atRA. Differentiation potential was evaluated by flow cytometry at day 5 (Figure 1A). In our experience, the EBs dissociation and reaggregation delayed differentiation timing and day 5 corresponded to day 3 of the original protocol without dissociation [14]. The pulse-type application of atRA induced a large GFP-Bry population compared to untreated cells (Figure 1B), demonstrating that atRA can act early in ESC differentiation, probably promoting loss of self-renewal, in agreement with the observations that atRA represses the expression of the pluripotency genes through direct activation of the transcriptional repressor Snail [7].

To evaluate the influence of RA pathway on spontaneous ESC differentiation in serum-containing medium, cells were allowed to differentiate as EBs, and treated from the end of day one with CD2665, a RAR antagonist [17], or vehicle alone (DMSO). mRNAs was collected at the end of day 3 and 4, and Brachyury (Bry) expression was analyzed as marker of early ESC differentiation state. In CD2665 treated cells Bry expression is down-modulated compared to control cells (Figure 1C), supporting the hypothesis that RA is involved in spontaneous ESC differentiation.

ESC increase their capacity to produce atRA during spontaneous differentiation as EBs

Because key components of the RA synthesis pathway are expressed at very low mRNA levels, Simandi et al. suggested that endogenous production of RA from serum-contained retinol is unlikely in undifferentiated ESC [18]. To verify this hypothesis and to assess the ESC ability to synthesize RA during spontaneous differentiation as EBs, we created a stable clone of ESC (ESC-RARE-Luc) carrying the Luciferase reporter gene under the control of RARE. ESC-RARE-Luc were cultured for 8 hours in growth medium conditioned by 24 hours culture of ESC or EBs at different times after LIF withdrawal. As shown in Figure 2A, ESC conditioned medium (ESC+LIF) does not induce significant increase of Luciferase signal with respect to unconditioned medium (p=0.157). To overcome the difficulty to estimate the retinol level in serum, we treated ESC-RARE-Luc with medium conditioned by DMSO-treated EBs at day 3 after formation. Data represent average ±SD of three independent experiments. *, P<0.05 (n = 3).

Figure 1: RA promotes ESC spontaneous differentiation. (A) Schematic representation of the experimental procedure used to test the ESC differentiation potential after a single pulse stimulation by RA. (B) FACS profiles of the atRA and DMSO treated cells analyzed for expression of GFP at day 5. Data are representative of three independent experiments. (C) Spontaneous differentiation of ESC as EBs is down-regulated by the RAR antagonist CD2665. ESC were let to differentiate spontaneously as embryoid bodies for 2, 3 or 4 days, in presence of 1 µM CD2665 or vehicle alone (DMSO). Differentiation was evaluated by quantitative reverse transcription-polymerase chain reaction (RT-qPCR) analysis of the early differentiation marker Brachyury. Values are normalized to β-Actin expression and represented as fold change compared with DMSO-treated EBs at day 3 after formation. Data represent average ±SD of three independent experiments. *, P<0.05 (n = 3).
levels, and the synthesis of RA increases during their spontaneous differentiation into EBs.

To further confirm that ESC can synthesize RA from retinol and use it to promote their own differentiation in serum-containing medium, we differentiated E14 ESC as EBs in medium containing Charcoal/Dextran absorbed serum to deplete the basal levels of retinoids. After 24 hours, 1 µM retinol was added, and RNA was extracted at 2 and 3 days later (corresponding to 72 and 96 hours of EBs differentiation). Retinol treatment enhances ESC differentiation evaluated by RT-qPCR for Brachyury expression (Figure 2B).

Expression of aTRA biosynthetic pathway components during spontaneous ESC differentiation

Since we observed an increase of RA synthesis in differentiating EBs, we wondered whether the expression of the RA biosynthetic pathway enzymes and retinoid transport proteins could be correlated with this finding. Thus, we quantified the mRNA levels of the enzymes that catalyze retinol oxidation to retinal (RDH1, RDH10 ADH1, ADH3 and ADH4, for the ADHs nomenclature used see [19]) and retinal oxidation to aTRA (RALDH1, 2 and 3), and for retinol (CRBP1 and 2) and RA (CRABP1 and 2) binding proteins (Figure 3A). As reference standards, we used RNA from primary mouse embryonic fibroblasts (MEF), mouse embryo fibroblast cell line NIH3T3 (3T3), and mouse liver. RDH1, ADH1 and ADH4 are undetectable in ESC and, among them only RDH1 significantly increases after 72 and 96 hours of EBs differentiation. RDH10 and AHD3 are the most expressed retinol dehydrogenases in undifferentiated ESC, and their expression increases during differentiation, especially for RDH1 and AHD3 (Figure 3B).

Our findings are consistent with the expression of these enzymes during embryonic development, where ADH3 is detected in mouse pre-implantation embryo [20], and RDH10 and RDH1 in early post-implantation embryo [21-23]. RDH10 mutant mice display embryonic lethality at 13.5 d.p.c., and this is the earliest phenotype observed for a single retinol-metabolizing enzyme-deficient mouse [24]. However, it was suggested that endogenous RA synthesis initiates in the mouse at 7.5 d.p.c. [2,25,26]. Only double-mutant mice will clarify if enzymes with redundant functions are presents in the early embryonic development.

Among retinal oxidizing enzymes, RALDH2 is the only one expressed in undifferentiated ESC and its expression increases during EBs maturation (Figure 3C), reflecting activation of the RARE-Luc reporter gene (Figure 2A) and in vivo data. Indeed, in the mouse embryo RALDH2 is the first enzyme to appear during gastrulation at 7-7.5 d.p.c. [27,28], and it is interesting to note that RALDH2 transcript was detected in all stages of bovine pre-attachment embryos, beginning from the oocyte through to the hatched blastocyst [29]. The two other RALDH enzymes (RALDH1 and RALDH3) appear later in development [27,30]. Thus, in EBs, resembling at 72 hours of differentiation the 7.5 d.p.c. embryo, the transcriptional activation of the genes involved in the retinoic acid synthesis pathway reflects the events observed in vivo.

Among the messenger RNAs for cytosolic retinoid binding proteins analyzed, only CRABP2 mRNA is up-regulated during EBs differentiation (Figure 3D). CRABP2 is a cytosol-to-nuclear shuttling protein, which facilitates RA transfer to the nucleus and binding to its receptor complex, suggesting that CRABP2 transcriptional increase could promote RA signaling. All the expression differences we observed between undifferentiated and differentiated ESC are due to their differentiation as EBs and not to the simple LIF withdrawal, as demonstrated by the values measured in ESC grown as monolayer without LIF for 4 days (ESC-LIF 4d). All together these data suggest...
Figure 3: Expression analysis of RA biosynthetic pathway during ESC spontaneous differentiation. (A) Schematic view of the major components of the RA biosynthetic pathway. Expression of the indicated retinol-oxidizing enzymes (B), retinal-oxidizing enzymes (C), and cellular retinoid binding proteins (D) was evaluated by RT-qPCR analysis. Values are normalized to β-Actin expression and represented as fold variation compared with primary mouse embryonic fibroblasts (MEF). Asterisk indicates undetectable levels. Data represent average ±SD of three independent experiments.
that RDH1, RDH10, ADH3, RALDH2, and CRABP2 are responsible of the increase in RA synthesis and signalling in ESC during spontaneous differentiation as EBs.

**Identification of early differentially expressed genes in atRA treated ESC**

To identify direct atRA target genes in ESC, we analyzed the gene expression profile of E14 ESC treated with 1 µM atRA for two hours. The analysis revealed that 176 genes were more than 2-fold up-regulated and 96 more than 2-fold down-regulated (Figure 4A, GEO accession No. GSE66043). Among these, we found 22 TFs up-regulated and 13 down-regulated (Figure 4B). Microarray data were independently validated by means of RT-qPCR analysis (Figure 4C). It is interesting to note that 3 TFs down-regulated (Otx2, Id2 and Arid1a) are involved in ESC pluripotency maintaining [16,31,32], and 3 TFs up-regulated (Snai1, Cdx1, Gata6) are known to be involved in pluripotency exit [7,33,34]. These data demonstrate the role of RA in promoting ESC differentiation acting directly on the regulation of RDH1, RDH10, ADH3, RALDH2, and CRABP2.

**Figure 4:** Identification of early differentially expressed genes in atRA treated ESC. (A) Heat map of microarray analysis showing the top up- and down-regulated genes following 2 hours atRA treatment (FC: Fold Change). (B) Heat map of microarray analysis showing the differentially expressed TFs. (C) The microarray data of 20 selected genes were validated by RT-qPCR analysis. Up-regulated genes are represented in red, down-regulated in green and negative controls (Nanog and Oct3/4) in black. Cyp26a1 was used as positive control. Gene expression was normalized to β-Actin expression and values obtained for DMSO or atRA treated cells are represented as fold change compared with corresponding values of untreated cells. Data represent average ±SD of three independent experiments *, P<0.001; §, P<0.005; #, P<0.01 (n=3).
of transcription factors involved in maintenance of, or exit from, pluripotency.

Conclusions

Cultivation and controlled differentiation of ESC has opened new frontiers both in regenerative medicine and biology of development. Many differentiation protocols use aTRA to promote formation of primitive endoderm or all three germ layers. Here, we demonstrated that RA is synthesized by ESC during spontaneous differentiation as EBs and takes an active role to promote their own differentiation process. In Luciferase reporter gene activation assay, we observed that also undifferentiated ESC produce RA from retinol, even if at low levels. We suggest that this low RA synthesis helps to maintain ESC self-renewal as demonstrated by Wang et al. [35], and only higher levels of RA synthesized by differentiating EBs trigger the differentiation process.

In EBs, the transcriptional activation of the genes involved in the RA synthesis pathway reflects the events observed in vivo, with RDH10, ADH3 and RAldh2 being the main enzymes providing RA for early organogenesis. Microarray gene expression profile of ESC treated with aTRA for two hours identified several TFs as direct target of RA receptors. Among them, Snai1 is one of the most up-regulated, confirming its role in organ and limb development. Our data suggest that the axis aTRA-Gdx1-Oct3/4 could be active also in murine or human ESC, but further experiments are necessary to confirm this hypothesis. This research is significant because it offers a comprehensive analysis of and demonstrates a role for endogenous RA synthesis during ESC cultivation and spontaneous differentiation. In the last years, a growing number of research groups turned to serum-free media. This research is significant because it offers a comprehensive analysis of and demonstrates a role for endogenous RA synthesis during ESC cultivation and spontaneous differentiation. In the last years, a growing number of research groups turned to serum-free media. In conclusion, our study points out the importance of RA synthesis in ESCs during differentiation, which is reinforced by the expression of RA receptors in murine and human ESCs. This study provides new insights into the role of RA in ESC differentiation and has implications for future research in the field.

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