Wnt and Nodal signaling simultaneously induces definitive endoderm differentiation of mouse embryonic stem cells

WA ZHONG, YU LAI, TAO YU, ZHONG-SHENG XIA, YU-HONG YUAN, HUI OUYANG, TI-DONG SHAN, QI-KUI CHEN

Department of Gastroenterology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, Guangdong, People’s Republic of China

Abstract

Induced differentiation of definitive endoderm (DE) from embryonic stem cells (ESCs) has been the recent focus of studies investigating regeneration and transplantation of organs of the digestive system. Poor cell survival is the most important challenge to DE differentiation from ESCs. This study aimed to optimize culture conditions to promote the differentiation of mouse ESCs into DE, and to investigate the roles of the Wnt and Nodal signaling pathways in the DE differentiation. The mouse ESCs were treated with or without leukemia inhibitory factor, Wnt3a and Activin A alone or together, and examined the DE differentiation by the DE marker CXCR4 and the ESC marker Oct4. The result showed the optimal induction of differentiation was achieved in cells simultaneously treated with Wnt3a and Activin A. Induction of CXCR4 was also earlier when there was simultaneous activation of Wnt and Nodal signaling compared to the groups treated with only Wnt3a or Activin A alone. These findings provide the basis for the induced differentiation of ESCs for the generation of functional, mature cells of gastrointestinal lineage, which can be potentially used for cell replacement therapy, disease modeling, as well as drug discovery studies.

Keywords: Activin A, definitive endoderm differentiation, mouse embryonic stem cell, Nodal, Wnt.

Introduction

Embryonic stem cells (ESCs) are characterized by their self-renewal properties, and their ability to differentiate into ectodermal, mesodermal and endodermal cell lineages [1, 2]. The endoderm has been shown to progressively differentiate to the definitive endoderm (DE) and the visceral endoderm (VE) [3, 4]. DE is thought to serve as the shared source of precursor cells of hepatocytes, pancreatic cells and intestinal epithelial cells, and is identified using markers such as CXCR4 (C-X-C chemokine receptor type 4), Sox17, E-cadherin (ECD), transmembrane 4 superfamily 2 (Tm4sf2), and goosecoid (Gsc) [5–9]. CXCR4 is a highly specific surface marker of DE expressed during early ESC differentiation [10], and CXCR4-positive cells have been shown to be capable of differentiating into endodermal cells [9, 11]. The induced differentiation of DE has been the focus of studies investigating regeneration and transplantation of organs of the digestive system [12].

Wnt3a belongs to the Wnt family of proto-oncogenes, which are homologues of Wingless. Wnt3a regulates a number of developmental pathways by binding to transmembrane receptor Frizzled (Fzd) and co-receptor low-density lipoprotein receptor-related protein (LRP5/6), and activating the Dishevelled (Dsh) protein to induce phosphorylation of glycogen synthase kinase 3 beta (GSK-3β) [13–15]. The subsequent dissociation of GSK-3β from Axin antagonizes the formation of β-catenin-Axin-APC-GSK3. Binding of free β-catenin to the Tcf/Lef family of transcription factors has been shown to regulate target genes such as c-myc and cyclin D1 and promote the proliferation or activation of cells [16]. It was recently shown that bone morphogenetic protein-4 (BMP-4) and activation of the Wnt signaling pathway induced differentiation of DE-derived precursor cells to hepatocyte-like cells [17]. Wnt3a, Wnt5a and Wnt8a have been shown to play an important role in early embryonic development [18, 19], and Wnt3a-deficient embryos, have been shown to exhibit defective development of the primitive streak, Hensen’s node and mesoderm, and early termination of epiblast differentiation [20]. Wnt signaling has been shown to play an important role in formation of the gut tube [E8.5], while Tcf4 and Tcf1 (downstream effectors of Wnt signaling pathway) have been suggested to promote an intestinal fate within the primitive gut. Indeed, suppression of Wnt signaling resulted in a reduction in goblet, entero-endocrine, and Paneth cells [21]. Additionally, mutation of Tcf1/Tcf4 during endodermal differentiation have been reported to cause abnormal differentiation of embryonic intestinal tissue [21]. These studies suggested that the Wnt signaling pathway plays an important role in early embryonic development, and organ development.

Early embryonic development is also regulated by the Nodal signaling pathway. Deficiency of Nodal [a member of transforming growth factor-beta (TGF-β) superfamily] in mouse embryos was associated with an inability to form the primitive streak, while low expression of Nodal in Nodal-hypomorph mice was associated with a deficiency in formation of the front ends of the primitive streak, mesoderm and endoderm [22]. Activation of the Nodal/TGF-β signaling pathway is triggered by Activin A, a member of TGF-β family, resulting in phosphorylation of SMAD2 and SMAD3, which subsequently bind to SMAD4, translocate to the nucleus, and regulate transcription of genes modulating cell proliferation or differentiation [23]. Activin A/Nodal signaling also plays a role in the formation of the primitive streak, and mesodermal
and endodermal differentiation [24]. Indeed, Activin A receptor inhibitor SB431542 along with Dkk1 (inhibitor of Wnt signaling pathway) completely blocked the serum-induced formation of primitive streak-like cells from human embryonic stem cells [25]. Additionally, differentiation of DE to embryoid bodies (EB) during early EB differentiation (within 24 hours after differentiation) was enhanced in the presence of Activin A [26]. Activin A and Wnt3a have been shown to act synergistically to promote differentiation of human ESCs to functional hepatic endoderm [27], while the Wnt and Notch signaling pathways were shown to play a role in patterning the anterior-posterior axis of the DE and control intestinal differentiation [28]. A recent study reported that a combination of TGF-β and Wnt activation along with BMP inhibition resulted in efficient DE induction in mouse ESCs [11].

The ability to use ESC-derived cells as a therapeutic tool in regenerative medicine has led to an intense focus on developing protocols for efficient differentiation of human and mouse ESCs. The most important challenge to DE differentiation from mouse ESCs has been poor cell survival, and this is a major limitation to implementing this technology in a clinical setting. Our study was designed to address an urgent need to optimize culture conditions in order to promote the differentiation of mouse ESCs into DE and maximize the production of seed cells for induced differentiation into gastrointestinal cells. We also investigated the roles of the Wnt and Nodal signaling pathways in the differentiation of mouse ESCs into DE.

Materials and Methods

Culture of mouse ESCs

ESCs (ES-E14TG2a) were purchased from the American Type Culture Collection (ATCC) and they were derived from the inner cell mass of 129/Ola Mus musculus blastocysts at three days post-fertilization. Mouse ESCs were maintained in high glucose Dulbecco’s Modified Eagle Medium (DMEM) (Gibco BRL, USA). Culture medium was supplemented with KnockOut™ Serum Replacement (KSR; Invitrogen, USA) rather than fetal bovine serum (FBS) in order to eliminate differences between different batches of serum, and because ES cells showed stronger proliferation and better maintenance of their undifferentiated state with KSR compared to FBS [29]. Medium was refreshed once daily, and cells were passaged once every 2–3 days. Cells were plated in 6-well plates, at a density of 1×10^5 cells/well and incubated at 37°C, in a humidified environment with 5% CO₂.

Induction of DE cells

Early preparations of ES-E14TG2a cells used mouse fibroblasts as trophoblast cells of ESCs, which was thought to inhibit the differentiation of mouse ESCs. Preparation of the feeder layer is very complex and the components of secretions are unclear. Later studies showed expression of leukemia inhibitory factor (LIF) receptor on the mouse ESCs, and fibroblasts were reported to inhibit the differentiation of mouse ESCs via LIF [30–33]. ESCs can therefore be cultured in the presence of LIF, which inhibits ESC differentiation in the absence of trophoblast cells, and preserves the multi-directional differentiation potential of ESCs.

Cells were cultured until they were adherent to the wall, after which they were divided into five groups: Group A: ESC culture medium [high glucose DMEM with 0.12% sodium bicarbonate (NaHCO₃), 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 0.1 mM non-essential amino acids, 10% KSR, 0.1 mM β-mercaptoethanol, 100 U/mL penicillin/100 μg/mL streptomycin, and 1000 U/mL LIF]; Group B: EB culture medium of high glucose DMEM with 0.12% NaHCO₃, 10 mM HEPES, 0.1 mM non-essential amino acids, 10% KSR, 0.1 mM β-mercaptoethanol, 100 U/mL penicillin/100 μg/mL streptomycin; Group C: EB culture medium + 25 ng/mL Wnt3a (R&D Systems, USA); Group D: EB culture medium + 50 ng/mL Activin A (R&D Systems, USA); Group E: EB culture medium + 25 ng/mL Wnt3a + 50 ng/mL Activin A.

When the cells were 70–80% confluent, the cells were trypsinized and pelleted at 1000 rpm, at room temperature, for 5 minutes. The cell pellet was resuspended in 6 mL of ESCs or EB culture medium, and the cell density was adjusted to 5×10⁴/mL. Cells were seeded into 6-well plates (2 mL per well for groups A–E) and cultured at 37°C, in the presence of 5% CO₂. The medium was refreshed once daily after seeding. The day of plating was designated as day 0 (d0). Cells were harvested on D1, D3, D5 and D7, and subjected to (1) Western blotting and immunohistochemistry to detect expression of CXCR4 and octamer-binding transcription factor 4 (Oct4) proteins, and (2) flow cytometry to quantify the percentage of CXCR4-positive cells.

Detection of CXCR4-positive cells by flow cytometry

After induction, cells were harvested at different time points, trypsinized and pelleted at 1000 rpm for 5 minutes. The cells were washed in phosphate-buffered saline (PBS) once, and re-suspended in 2 mL of PBS. Cell counts were determined in 20 μL of cell suspension. Then, 1×10⁶ cells were pelleted and re-suspended in 200 μL of PBS (control group), of which 30 μL of cell suspension was used as the experimental group. The experimental samples were incubated with phycoerythrin (PE)-conjugated anti-mouse CXCR4 monoclonal antibody (R&D Systems, USA; 10 μL/1×10⁶ cells) for 20 minutes, at room temperature. The cells were washed in PBS once, re-suspended in 200 μL of PBS, and the proportion of CXCR4-positive cells was determined at different time points (one, three, five and six days) by flow cytometry using FACSVerse (Becton Dickinson, USA). The data in each quadrant was recorded with EXPO32 MultiCOMP ver. 1.1C software, and analyzed with EXPO32 analysis ver. 1.2B software.

Immunocytochemistry

Cells were washed thrice with PBS and then fixed in 4% paraformaldehyde, at room temperature, for 30 minutes. Cells were washed again in PBS thrice (3 minutes each wash), and incubated at room temperature with normal rabbit serum for 20 minutes. The cells were then incubated with a 1:50 dilution of rat anti-mouse CXCR4 monoclonal antibody, at 4°C, overnight, washed in PBS thrice (3 minutes each wash), and then incubated...
in biotin-conjugated rabbit anti-rat immunoglobulin G (IgG) (Boster Co., China, 1:100 dilution), at 37°C, for 20 minutes. The cells were washed and incubated at 37°C, in streptavidin-biotin reagent, for 20 minutes. After four washes in PBS (5 minutes each wash), protein was visualized using the 3,3’-diaminobenzidine (DAB) ECL kit (Boster Co., China). Samples were washed in distilled water, dehydrated, transparentized and mounting, before observing under a light microscope.

Western blotting

Cell lysates were prepared by incubating cells on ice, for 30 minutes, in radioimmunoprecipitation assay (RIPA) buffer (50 μL/well; Thermo Scientific, USA) and a protease inhibitor cocktail (Roche, Switzerland), according to the manufacturer’s instructions. Samples were centrifuged at 4°C, for 20 minutes, at 14,000 rot/min. The supernatant was collected, and the protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit (CW Biotech, China). Protein samples (25 μg) were mixed with 25 μL of 5× loading buffer, denatured at 98°C, for 15 minutes, and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes, blocked in 5% non-fat milk, for one hour, and then incubated with a 1:1000 dilution of Oct4 or CXCR4 primary antibody at 4°C overnight. Membranes were washed in TBST (tris-buffered saline with Tween 20) (three washes of 5 minutes each) and incubated with a 1:1000 dilution of secondary antibody, at room temperature, for one hour. Membranes were washed thrice in TBST (10 minutes each wash), and proteins were visualized using an ECL kit. The protein bands were scanned and quantitated using Image J software (NIH, USA). β-actin served as an internal reference. All experiments were performed in triplicate.

Statistical analysis

Continuous variables were presented as mean ± standard deviation (SD). Differences between different culture groups at different culture times were compared using one-way analysis of variance (ANOVA). Additionally, differences in CXCR4 protein expression between the five groups at day 5 as determined by Western blotting were also compared. A post-hoc pair-wise comparison was performed using the Bonferroni’s test, with a significance level of 0.05. All statistical analyses were performed using the IBM SPSS statistical software ver. 22 for Windows (IBM Corp., Armonk, New York, USA). A two-tailed p<0.05 indicated statistical significance.

Results

Evaluation of CXCR4 expression in ES cells

Flow cytometry was used to determine CXCR4 expression in the five different experimental groups on days one, three, five and seven of culture (Figure 1A). CXCR4 protein expression was not detected in ES cells cultured in the presence of LIF (group A). However, CXCR4 expression was detected in ESCs cultured in basic medium, cells cultured in the presence of Wnt3a, cells cultured in the presence of Activin A, and cells cultured in the presence of both Wnt3a and Activin A (groups B–E).

![Figure 1](image-url)
CXCR4 expression was quantified in the five experimental groups on days 1, 3, 5 and 7 (Figure 1, B and C). Each experiment was repeated three times, and the mean ± standard deviation was calculated. Group A cells had baseline expression levels of CXCR4 (<1% CXCR4-positive cells) at each time point. However, there was a time-dependent increase in the number of CXCR4-positive cells in groups B–E from day 1 until day 5, and then a decrease on day 7. Group E had a significantly higher number of CXCR4-positive cells on day 1 compared to groups A–C. On day 3, the number of CXCR4-positive cells was significantly higher in group E compared to groups A and B. The number of CXCR4-positive cells was significantly higher in group E on days 5 and 7 compared to all the other groups.

![Graph showing comparison of CXCR4-positive cells in different groups over time](image)

**Figure 1 (continued) – (B) Comparison of the number of CXCR4-positive cells in the different groups (A–E) at different time points of culture. Error bar represents the standard deviation of the mean. Means with different small letters are significantly different by Bonferroni’s test (p<0.05). Letters represent a significant difference with group Aa, group Bb, group Cc, or group Dd. (C) Relative ratio compared to group B at the given time points.**

CXCR4 expression was evaluated using immunohistochemistry (Figure 2). CXCR4-positive cells exhibited brown or yellow-brown granules, and the staining pattern demonstrated that CXCR4 protein was mainly expressed on the cell membrane.

**Western blotting for Oct4 and CXCR4**

Protein expression levels of OCT4 and CXCR4 on days 1, 3, 5 and 7 were determined by Western blotting (Figure 3A). β-actin was used as a loading control. Western blotting data were quantified in triplicate after scanning and image analysis (Figure 3, F and G). Quantification of Oct4 expression showed that group A cells had represented decreased Oct4 protein expression from day 1 to day 7. However, it did not reach to the statistical significance. Cells from groups B and D had a significant decrease in Oct4 protein expression at day 7 comparing with day 1 and 3 (D7 < D5, D3 and D1). Group C cells had significantly lower expression of Oct4 protein on day 7 compared to days 1 and 3 (D7 < D1 and D3). Group E cells had significantly lower expression of Oct4 protein on days 5 and 7 compared to days 1 and 3 (D5 and D7 < D1 and D3) (all p<0.05; Figure 3F).

Although there was no significant difference in CXCR4 expression in group A cells over time, group B cells had significantly higher CXCR4 expression on day 5 compared...
to days 1 and 7 (D5 > D1 and D7). Cells from groups C–E had significantly higher levels of CXCR4 on day 5 compared to days 1, 3, and 7 (D5 > D1, D3, and D7). Comparison of CXCR4 expression between the five groups on day 5 showed that the dispersion of CXCR4 expression was group A < group B < groups C and D < group E. There was no significant difference in CXCR4 expression between groups C and D (Figure 3G).

Discussion

In this study, we optimized culture conditions to promote the differentiation of mouse ESCs into DE, and investigated the roles of the Wnt and Nodal signaling pathways during this differentiation process. There was a time-dependent increase in the number of CXCR4-positive cells, and a time-dependent decrease in OCT4 expression in cells cultured in basic medium, as well as cells cultured in the presence of Wnt3a alone, Activin A alone, or both Wnt3a and Activin A together. Comparison of CXCR4 expression between the different groups showed that cells treated simultaneously with Wnt3a and Activin A exhibited the highest levels of CXCR4. These data suggested that optimal differentiation of mouse ESCs to DE was achieved by the synergistic action of the Wnt3a and Activin A signaling pathways.

A number of reports showing that ESC-derived cells could be used for tissue repair and functional replacement therapy suggested that these cells could be a novel therapeutic tool for irreversible diseases such as cardiac insufficiency, genetic diseases, diabetes mellitus, neuronal degeneration and refractory intestinal epithelial injury [34–36]. A recent report described a three-step protocol for differentiation of human induced pluripotent stem (iPS) cells to generate hepatocyte-like cells [37]. IPS cells could also be differentiated into functional glucose-responsive insulin-producing cells [38]. However, the major challenge to using this technology in a clinical setting is the limitation in the number of differentiated ESCs obtained with the currently used protocols for induced differentiation.

![Figure 2 – CXCR4 expression at different time points (A–E). Group A: Serum free medium and LIF; Group B: Serum free medium; Group C: Serum free medium and 25 ng/mL Wnt3a; Group D: Serum free medium and 50 ng/mL Activin A; Group E: Serum free medium, 25 ng/mL Wnt3a and 50 ng/mL Activin A. CXCR4-positive cells had brown or yellow-brown granules (scale bar: 50 μm).](image-url)
Figure 3 – Representative Western blotting to evaluate expression of OCT4 and CXCR4 at different culture time points for each group: (A) Serum free medium and LIF; (B) Serum free medium; (C) Serum free medium and 25 ng/mL Wnt3a; (D) Serum free medium and 50 ng/mL Activin A; (E) Serum free medium, 25 ng/mL Wnt3a and 50 ng/mL Activin A; Quantitative analysis of Oct4 (F) and CXCR4 (G) at different culture time points for each group. The culture time points included Days 1 (D1), 3 (D3), 5 (D5), and 7 (D7). Experimental groups included groups A–E. Error bar represents the standard deviation of mean. Data were represented as mean with standard deviation (SD) as for the error bar of triplicate results. Means with the different small letters were significantly different by Bonferroni’s test (p<0.05). †‡§, represented a significant difference compared to D1 †, D3 ‡, and D5 § with group. abcd, represented a significant difference while comparing CXCR4 expression on D5 with group Aa, group Bb, group Cc or group Dd, respectively.
Studies investigating modulators of ESC differentiation have reported that the Wnt and Nodal/Activin A signaling pathways play key roles in DE differentiation, and in vitro differentiation of ESCs into primitive streak-like cells, and endodermal and mesodermal cells in different vertebrate models [39, 40]. Wnt signaling has been shown to be implicated in generation of the foregut [41], and this is mediated by a biphasic modulation of the Wnt signaling pathway [42]. Interestingly, recent data suggested that amplification and maintenance of high levels of Wnt signaling could be mediated by LGR4 and LGR5, which are important for stem cell maintenance [43]. Inhibition of Wnt signaling was shown to suppress the differentiation of ESCs into DE cells [44], and Wnt activation improved DE induction in suspension cultures of embryoid bodies [11]. Formation of DE during the natural differentiation of ESCs into EB was reported to be significantly enhanced in the presence of Activin A, especially in early EB differentiation (within 24 hours after differentiation) [26]. Mouse ESCs maintained in serum- and feeder-free adherent cultures were shown to respond in a dose-dependent manner to inducers of primitive streak formation by developing mesendoderm, which could further differentiate to cells resembling the foregut endoderm [45]. Interestingly, inhibition of Wnt-signaling suppressed Activin-induced development of endodermal cells at the late stages of differentiation, and Wnt3a and Activin acted additively on the expression of Mix1, an early mesodermal-specific marker [45]. The classical EB differentiation procedure does not involve the addition of Wnt3a and/or Activin A, and since this results in a limited amount of DE, it would not have generated enough cells for a transplantation procedure. Our final protocol was based on the fact that addition of Wnt3A and/or Activin A would result in a higher amount of DE, and a higher number of transplantable cells than a procedure without Wnt3A and/or Activin A. We previously reported successful induction of ESC differentiation into definitive endoderm, which was identified by detection of endoderm markers such as Gsc, Tm4sf2, Gpc1, Sdc4 and CXCR4 [33]. Our results showed that the proportion of endoderm peaked at five days after induction of differentiation. We also showed that endoderm cells could be induced to differentiate into small intestine stem cells, which could further differentiate into small intestine epithelium in non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice. Injection of small intestine stem cells via the tail vein into mice with radiation injury resulted in significant improvement of small intestine repair. Our present study was based on these findings, and aimed to investigate a way to increase the proportion of definitive endoderm differentiated from ESCs and explore potential mechanisms, which may provide experimental evidence on the collection of more seed cells.

We showed that endoderm cells accounted for about 10% of the cells on five days after natural differentiation of ESCs. Addition of Wnt3a and Activin A to the culture medium resulted in up-regulation of the Wnt and Nodal signaling pathways, which could promote the differentiation of ESCs into definitive endoderm, leading to the increased proportion of definitive endoderm. However, endoderm cells only accounted for 40% of the cells under these conditions, suggesting that it would be beneficial to sort cells after early differentiation. Using immunomagnetic bead sorting to separate the definitive endoderm cells positive for CXCR4, we obtained definitive endoderm cells with relatively high purity, which provided a basis for the following induction of differentiation into pancreas-like cells. CXCR4-positive cells were in the definitive endoderm stage during the differentiation of ESCs into organ cells, and the CXCR4-positive cells were further induced to differentiate after seeding.

In our present study, we showed that ESCs cultured in basic medium had a significantly higher number of CXCR4-positive DE cells at five days compared to ESCs cultured in the presence of LIF, which is an inhibitor of ESC differentiation. However, there was no significant difference in the expression of Oct4 between ESCs cultured in basic medium, and those cultured in the presence of LIF, suggesting that absence of LIF alone could only drive DE differentiation to a small extent. Decreased expression of Oct4, a marker of pluripotency, indicates a decrease in the pluripotency of the cells, and differentiation away from the embryonic cell properties.

We showed that addition of 25 ng/mL of Wnt3a resulted in a significant increase in the proportion of CXCR4-positive cells along with a significant decrease in the proportion of Oct4-positive cells at five days compared to cells cultured in basic medium, suggesting that this concentration of Wnt3a efficiently activated the Wnt signaling pathway to induce the differentiation of ESCs into DE. Similarly, addition of 50 ng/mL of Activin A resulted in a significant increase in the proportion of CXCR4-positive cells along with a significant decrease in the proportion of Oct4-positive cells at five days compared to cells cultured in basic medium, suggesting that this concentration of Activin A efficiently activated the Nodal signaling pathway to induce differentiation of ESCs into DE. Although Activin A has previously been used at a higher concentration (100 ng/mL) to activate the Nodal signaling pathway [10], our present data suggested that 50 ng/mL Activin A was sufficient to efficiently activate ESC differentiation. Our data also showed that there was no significant difference in the differentiation of ESCs in the presence of either Wnt3a or Activin A alone. However, simultaneous activation of the Wnt3a and Nodal/Activin A signaling pathways with 25 ng/mL Wnt3a and 50 ng/mL Activin A, resulted in a significantly higher proportion of CXCR4-positive cells and a lower proportion of Oct4-positive cells compared to cells treated with either Wnt3a or Activin A alone. Our data suggested that simultaneous activation of the Wnt and Nodal signaling pathways exerted a synergistic effect to induce the differentiation of mouse ESCs into DE. In the present study, the cells still survived at 21 days after seeded, and remained differentiated.

Differentiation of ESCs to DE was previously achieved with two-step protocols using (1) 200 nM of Stauprimide (Spd), a suppressor of pluripotency initially, followed by treatment with 50 ng/mL of Activin A for the next three days, or (2) 25 ng/mL of Wnt3a plus 100 ng/mL of Activin A for the first day followed by 100 ng/mL of Activin A for the next three days [9]. There was no significant difference in induction of DE
References


