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Induced pluripotency enables differentiation of human nullipotent embryonal carcinoma cells N2102Ep

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ABSTRACT

Embryonal carcinoma (EC) cells, which are considered to be malignant counterparts of embryonic stem cells, comprise the pluripotent stem cell component of teratocarcinomas, a form of testicular germ cell tumors (GCTs). Nevertheless, many established human EC cell lines are nullipotent with limited or no capacity to differentiate under normal circumstances. In this study, we tested whether an over-expression of Yamanaka's reprogramming factors OCT4, SOX2, c-MYC and KLF4 might enable differentiation of the human nullipotent EC cells N2102Ep. Using *OCT4* knockdown differentiated N2102Ep cells, we are able to derive reprogrammed N2102Ep cell lines. The induced pluripotency of N2102Ep allows the cells to differentiate toward neural lineage by retinoic acid; the expression of SSEA3 and SSEA4 is down-regulated, whereas that of neural surface markers is up-regulated. Consistent with the up-regulation of neural surface markers, the expression of the master neuroectodermal transcription factor *PAX6* is also induced in reprogrammed N2102Ep. We next investigated whether PAX6 might induce spontaneous differentiation of nullipotent stem cells N2102Ep. However, while an ectopic expression of PAX6 promotes differentiation of NTERA2, it induces cell death in N2102Ep. We nevertheless find that upon induction of retinoic acid, the reprogrammed N2102Ep cells form mature neuronal morphology similar to differentiated pluripotent stem cells NTERA2 as determined by TUJ1 expression, which is absent in N2102Ep parental cells. Altogether, we conclude that the nullipotent state of human EC cells can be reprogrammed to acquire a more relaxed state of differentiation potential by Yamanaka's factors.

1. Introduction

Testicular germ cell tumors (GCTs) are the most common malignant cancer in young men between 20 and 40 years old [1,2]. This pattern of the incidence occurring in young men is different from incidences of most cancers, which increase with age. One factor that might implicate the early onset of testicular GCTs is its embryonic cell origin from primordial germ cells (PGCs) which are recognized in human embryos at weeks 5–6. Seminoma and non-seminoma GCTs are the most predominant testicular GCTs. Seminoma is composed of cells that resemble PGCs, whereas non-seminoma testicular GCT may contain embryonal carcinoma (EC) cells together with elements of teratoma (together with EC cells, referred to as teratocarcinoma), yolk sac carcinoma and choriocarcinoma. The EC cells of teratocarcinoma are well recognized as pluripotent cancer stem cells capable of differentiating into somatic cells of all three embryonic germ layers [2]. Nevertheless, some GCTs are composed entirely of EC cells which have apparently lost their capacity for differentiation. Such apparently "nullipotent" EC cells may arise by mutation during cancer progression since a reduced capacity to differentiate would provide the cells with a selective growth advantage, leading to a more aggressive cancer phenotype [3].

The mechanism by which nullipotent EC cells arise is unknown. Some mouse EC cell lines established in vitro are nullipotent [4]. Several studies in which such nullipotent mouse EC cells were fused to somatic cells resulted in hybrid cells with pluripotent characteristics, suggesting that nullipotency results from a mutation leading to the loss of function of gene(s) required for differentiation [5–8]. A majority of human EC cell lines are nullipotent [9–12]. For example, most human EC cell lines do not differentiate in response to retinoic acid [13], which induces differentiation of mouse EC cells and the pluripotent human EC cell line NTERA2 [10]. Nevertheless, hybrids between a nullipotent human EC cell line N2102Ep and the pluripotent NTERA2 were capable of

Abbreviations: EC, embryonal carcinoma; ES, embryonic stem; iPS, induced pluripotent stem

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differentiation in response to retinoic acid, albeit without an ability to differentiate into mature neurons [14]. This finding suggests that the nullipotent state of human EC cells, like that of nullipotent mouse EC cells, results from the loss of some key differentiation inducers.

While the hybrid experiments point to mutations in the nullipotent EC cells limiting their capacity for differentiation, the nature of these mutations or the genes affected are unknown. Human embryonic stem (ES) cells in culture have been shown to acquire a variety of non-random genetic and epigenetic changes that also occur in human EC cells [15–17]. Hence, these genetic and/or epigenetic changes may arise because they confer similar selective advantages to such variant stem cells, whether in vitro or in vivo. In the case of N2102Ep human EC cells, which do not differentiate in response to retinoic acid, the cells nevertheless are capable of a response to retinoic acid since. For example, retinoic acid receptor beta (*RARB*) is induced in these cells by retinoic acid as it is in pluripotent NTERA2 EC cells [14,18]. Therefore, the block of differentiation must be downstream of RARB. One possibility might be a failure to up-regulate the expression of key master developmental regulators such as PAX6, which has been shown to directly suppress the expression of stem cell-associated genes in human ES cells [19], through genetic mutation of their regulatory or coding sequences. Another possibility is the genetic and/or epigenetic barrier within nullipotent EC cells, which does not allow the cells to differentiate. Such a barrier to differentiation has been suggested for mouse EC cells, which were unable to be reprogrammed by nuclear transfer [20].

Despite their nullipotency, human EC cell lines such as N2102Ep express the characteristic surface antigen patterns that characterize both human pluripotent EC and ES cells [21]. They also have gene expression profiles similar to human ES cells, including similar expression levels of pluripotency-associated genes such as those used for the generation of human induced pluripotent stem (iPS) cells (e.g. *OCT4*, *NANOG*, *SOX2*, *LIN28*, and *KLF4*) [22–25]. In the present study, we aimed to test whether the nullipotent stem cells N2102Ep could be induced to differentiate by reprogramming via Yamanaka's factors. We find that by reprogramming differentiated N2102Ep cells, which were generated by *OCT4* knockdown, we are able to derive reprogrammed N2102Ep cell lines which show an ability to differentiate into neural lineage by induction of retinoic acid. Our study therefore suggests that the nullipotent state might be maintained in part by epigenetic mechanism(s), which suppress differentiation of nullipotent stem cells.

2. Materials and methods

2.1. Cell culture

Human EC cell lines N2102Ep [26] and NTERA2 [9] were grown in DMEM with 10% FBS (Invitrogen), and placed at 37 °C under a humidified atmosphere of 10% CO_2 incubator. N2102Ep and NTERA2 were passaged every 3 days using 0.25% and 0.05% trypsin, respectively. Reprogrammed N2102Ep cell lines RepN5 and RepN13, which were generated by iPS reprogramming (see below), were cultured in human ES culture condition, i.e. Knockout-DMEM supplemented with 20% Knockout-Serum Replacement, 1 × non-essential amino acids, 1 mM glutamine, 0.1 mM beta-mercaptoethanol and 4 ng/ml bFGF (Invitrogen) seeded on 6×10^3 cells/cm^2 mitomycin C-treated MF-1 mouse embryonic fibroblasts (MEFs), and placed at 37 °C under a humidified atmosphere of 5% CO_2 incubator. Cells were passaged every 5–7 days using collagenase type IV (Invitrogen) and scraping with glass-beads (Sigma). NTERA2 and parental N2102Ep harboring *OCT4* inducible shRNAi system (see below) were cultured under this human ES condition for differentiation study. To induce differentiation, the culture medium was supplemented with 10 μM of all-trans retinoic acid (Sigma).

2.2. Generation of differentiated N2102Ep by OCT4 knockdown and induced pluripotency

N2102Ep cell line harboring tetracycline repressor (TetR) and short hairpin interfering RNA targeting *OCT4* was established by transfecting pCAG-TetRnls-IRES-puromycin and pSuperior-Neo (Oligoengine) containing *OCT4* siRNA [27]. Principally, inside the cells the plasmid pCAG-TetRnls-IRES-puromycin constitutively encodes the tetracycline repressor TetR, which will in turn bind to the shRNA promoter region of the plasmid pSuperior-Neo in the absence of doxycycline, thereby suppressing shRNA expression. In the presence of doxycycline, which is a ligand of TetR, the repressor is then evicted from the shRNA promoter leading to an expression of shRNA [27]. Stable transfected N2102Ep colonies were selected and expanded using 3 μg/ml puromycin and 750 μg/ml G418 (Invitrogen). Reverse transcription quantitative polymerase chain reaction and western blotting were employed to validate the expression of *OCT4* knockdown in cells grown with or without 1 μg/ml doxycycline.

To generate iPS cells, N2102Ep *OCT4* knockdown cells were treated with 1 μg/ml doxycycline for two weeks to derive differentiated cells. The medium was changed every 3 days. The expression of SSEA3 and SSEA1 and the cloning efficiency test were performed to assure a complete differentiation of N2102Ep. Differentiated *OCT4* knockdown cells were then transfected by *PmeI*-linearized four-in-one plasmid over-expressing OCT4, SOX2, c-MYC and KLF4 jointly expressed by 2A peptides together with puromycin resistant gene and a red fluorescence protein (RFP) (Supplementary Fig. 1). Transfected cells were immediately transferred to human ES culture condition. Colonies stably expressing the reprogramming factors, as indicated by RFP-positive expression, were manually selected [28]. Two reprogrammed N2102Ep (RepN) clones were subsequently tested for their differentiation potential, i.e. clones RepN5 and RepN13. The expression of SSEA3 and SSEA4 was confirmed in the reprogrammed N2102Ep cell lines by the flow cytometry analysis. The expression of stem cell-associated genes *OCT4* and *NANOG* was confirmed by qPCR.

2.3. Establishment of PAX6 over-expressing N2102Ep and NTERA2 cell lines

The human PAX6 coding sequence was amplified from a plasmid containing *PAX6* gene (kindly provided by Prof. Su-Chun Zhang, University of Wisconsin, Madison). A FLAG tag-containing primer was used to add the peptide at the 5′-position of the coding sequence to allow detection of the transgene by anti-FLAG antibody (Sigma). The FLAG-PAX6 DNA fragment was gel-purified using a DNA extraction kit from Qiagen. A red fluorescence protein (RFP) encoding gene of pCAG-RFP-IRES-puromycin was excised using *Xho*I and *Not*I, and was replaced by the FLAG-PAX6 DNA fragment. The plasmid pCAG-FLAG-PAX6-IRES-puromycin was then transfected into either N2102Ep or NTERA2 to establish the PAX6 over-expressing cell lines using electroporation. Briefly, human EC cells were dissociated using 0.25% trypsin. One million cells were then transfected with 3 μg of *Pvu*I-linearized plasmid DNA. pCAG-RFP-IRES-puromycin was also separately transfected in the parental N2102Ep and NTERA2 to establish control cell lines. The transfectants were immediately plated in DMEM-F12 plus 10% FBS (Invitrogen). Three days after the transfection, 3 μg/ml puromycin (Sigma) was added to the culture medium to select colonies which were resistant to the antibiotic. Western blotting was employed to test PAX6 over-expression in the human EC cell lines.

2.4. Western blotting

Cell pellets were resuspended in RIPA buffer (150 mM NaCl, 1% (w/v) sodium deoxycholate, 25 mM Tris–Cl pH 8.0, 1% (v/v) NP-40, and 0.1% (w/v) SDS, protease inhibitor cocktails), and were subjected to sonification. Protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific). Forty micrograms of