Molecular Human Reproduction Vol.7, No.2 pp. 155–161, 2001

Analysis of *Oct-4* expression and ploidy in individual human blastomeres

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Oct-4, a decisive factor that maintains totipotency in murine embryonic and germ cells, is exclusively expressed in such cells. In mice, different levels of *oct-4* expression in blastomeres predict development towards inner cell mass (ICM) (high oct-4) or trophectoderm (TE) (low oct-4). To address whether the mouse model also applies to human embryos, the cytoplasm of individual human blastomeres from normally and abnormally fertilized embryos was tested for *Oct-4* expression by reverse transcription–polymerase chain reaction (RT–PCR). The nuclei of the same blastomeres were subjected to fluorescence in-situ hybridization (FISH) to determine ploidy. A significant difference in *Oct-4* mRNA levels was revealed between blastomeres. The distribution of blastomeres with high *Oct-4* levels varied according to the cleavage stage of the embryo: the more blastomeres, the lower the percentage with high *Oct-4* levels. Aneuploid blastomeres did not exhibit lower *Oct-4* mRNA levels than diploid ones. Thus, differential *Oct-4* expression in individual human blastomeres appears to direct cells towards the ICM or TE lineages without regard to chromosomal status. *Oct-4* might be used as a marker in preimplantation genetic diagnosis to identify embryogenic blastomeres.

Key words: human blastomeres/Oct-4/Oct-3/preimplantation genetic diagnosis/totipotency

Introduction

In mammalian embryogenesis the first morphological indication of differentiation is the formation of the trophectoderm (TE) at the early blastocyst stage. While inner cell mass (ICM) cells remain totipotent, TE cells are restricted to extra-embryonic cell lineages. Data from various animal models suggests that, on a molecular level, this differentiation step could start in humans perhaps as early as the 2–4-cell stage and might involve the activation of maternally inherited determinants and/or embryo-specific genes, as well as environmental factors like cell allocation (Edwards and Beard, 1997). In humans, an understanding of the molecular mechanisms underlying this differentiation step may provide diagnostic benefits. Currently, blastomere biopsies of human embryos are performed for preimplantation genetic diagnosis (PGD) around the 8-cell stage and no prediction can be made regarding the future fate of these cells. However, in some instances, combined knowledge about ploidy and destination towards ICM or TE may be crucial in selecting the best embryos for transfer.

In mice, a key factor for the first differentiation step in embryogenesis is the POU domain transcription factor oct-4, also named oct-3 (Okamoto *et al*., 1990; Rosner *et al*., 1990; Scholer *et al*., 1990); oct-4 belongs to the sub-group of octamer-binding proteins that bind by the POU domain to promoter and enhancer regions of various genes with octamer sites. As with almost all POU domain transcription factors, oct-4 is developmentally regulated (for review, see Ovitt and Scholer, 1998).

In mice, oct-4 is exclusively found in totipotent embryonic cells and germ cells (Palmieri *et al*., 1994). A high level of oct-4 expression is thought to keep cells in a totipotent stage, whereas down-regulation is associated with differentiation (Palmieri *et al*., 1994); *oct-4* transcription occurs prior to any changes in known transcription factor levels (Brehm *et al*., 1997). In 8-cell stage murine embryos, five cells stain immunohistochemically positive for oct-4 protein and three negative (Palmieri *et al*., 1994). After day 8 of murine embryonic development, oct-4 is restricted to primordial germ cells. It is also expressed in murine embryonic stem (ES) cells (Rosner *et al*., 1990) and embryonal carcinoma (EC) cells (Okamoto *et al*., 1990).

In murine embryogenesis, *oct-4* has been shown to be essential for the development of totipotent ICM cells (Nichols *et al*., 1998). So far, approximately nine known and several unknown genes have been found to contain oct-4 binding sites; with some being positively, and others negatively, regulated

by oct-4 (Ovitt and Scholer, 1998). This includes repression of α and β subunits of human chorionic gonadotrophin (HCG) and activation of platelet-derived growth factor (PDGF) α receptor by oct-4.

In humans, Oct-4 is the product of the *OTF3* gene, which encodes two splicing variants designated *oct3A* and *oct3B* (Takeda *et al*., 1992). Human *Oct-4* (*Oct3a*) shares 87% sequence identity with mouse *oct-4* (Takeda *et al*., 1992). In human embryos, *Oct-4* is expressed throughout all stages from the unfertilized oocyte to the blastocyst stages, as detected by reverse transcription–polymerase chain reaction (RT–PCR) (Abdel-Rahman *et al*., 1995) and by PCR of cDNA libraries (Verlinsky *et al*., 1998). *Oct-4* is also expressed in human EC (Pera and Herszfeld, 1998) and ES cells (Reubinoff *et al*., 2000). We recently reported that *Oct-4* is up-regulated by ~31-fold in the ICM of human blastocysts compared with TE cells, making it a potential tool to produce and maintain human ES cells (Hansis *et al*., 2000).

In this study, we describe for the first time the distribution pattern of *Oct-4* mRNA in individual blastomeres of cleavage stage human embryos as well as its relationship to ploidy. To achieve this latter goal, we separated the cytoplasm and the nuclei of individual blastomeres of normally and abnormally fertilized human cleavage stage embryos. The cytoplasm was tested for *Oct-4* expression by RT–PCR and the nucleus was subjected to fluorescence in-situ hybridization (FISH).

Materials and methods

Embryo selection, grading and culture

Immature oocytes and embryos were donated to research with patient consent. A total of six immature (germinal vesicle stage) oocytes and 19 embryos at the 2–10-cell stage were discarded by nine patients. A total of 16 embryos developed from a two pronuclear (2PN) zygote, one from a 3PN zygote, one from a 1PN zygote and one displayed multinucleated blastomeres. Embryos were cultured in human tubal fluid (HTF) media (IVF Science Scandinavia, Santa Ana, CA, USA) from retrieval to fertilization check at 16–24 h (day 1), and then transferred to G1.2 (IVF Science Scandinavia) or HTF from days 1 to 3. Embryos were rinsed four times between each media change. Selected 2PN embryos displayed an anomalous morphology indicating a poor prognosis for pregnancy outcome. As such they were discarded from IVF cycles and were, therefore, appropriate for use in an Institutional Review Board (IRB) approved study (H-6902) to develop genetic diagnostic procedures.

In our IVF programme, embryos are routinely graded on a scale of 1–4 (1 = best grade) on the basis of fragmentation, cell size/shape and morphology. The 2–4-cell stage embryos had a mean grade of 3.3, the 5–7-cell stage embryos 2.7 and the 8–10-cell stage embryos 2.1. The 1PN embryo and the embryo with multinucleated blastomeres graded at 2.0 and 2.5 respectively; the 3PN embryo was not graded since these embryos are routinely discarded at fertilization check.

Separation of nucleus and cytoplasm

Embryos were placed in acid Tyrode's solution for 30–45 s until the zona pellucida appeared to dissolve. Embryos were then washed four times in G2.2 media and the blastomeres were separated by repeatedly pipetting. The blastomeres were then transferred to 5 µl 1× PCR Reaction Buffer (containing 0.1% Triton® X-100, no $MgCl_2$) as a cellular membrane lysis buffer (Promega, Madison, WI, USA). The nuclei of individual blastomeres were identified under phase contrast microscopy (Nikon Narishige, Tokyo, Japan) and transferred to a slide by micropipette. The cytosol was collected by a separate micropipette, transferred to a tube and directly submitted to a reverse transcription (RT) reaction. Some media samples were saved as negative controls for the PCR.

cDNA synthesis

Random oligo primer (0.2 µg) and reaction buffer (1 µl of 10×, SuperScript Preamplification System; Life Technologies, Rockville, MD, USA) were added to the cytosolic fraction of the blastomeres and heated to 70°C for 5 min. Samples were then placed on ice, centrifuged and 2.6 µl of a mix of 2.5 mmol/l $MgCl_2$, 10 mmol/l dithiothreitol, 0.5 mmol/l NTP and 5 IU RNAse inhibitor (Promega) was added. After incubation for 10 min at room temperature, Superscript II Reverse Transcriptase (100 IU) was pipetted into the sample; cDNA synthesis was carried out at room temperature for 5 min and 42°C for 50 min. The reaction was stopped at 95°C for 5 min.

PCR for Oct-4

The first PCR was carried out with 1 µl cDNA template, 500 nmol/l modified outer primer (Abdel-Rahman *et al*., 1995), 3′ primer GGAAAGGCTTCCCCCTCAGGGAAAGG, 5′ primer AAGAAC ATGTGTAAGCTGCGGCCC), 20 µmol/l NTP (Life Technologies) and 2 mmol/l $MgCl_2$. The second nested PCR used 2 µl of the first PCR, 50 nmol/l modified inner Primer (3′ primer TTCTGGCGCCGGTTA-CAGAAC CA, 5′primer GACAACAATGAGAACCTTCAGGAGA), 10 µmol/l NTP and 2 mmol/l $MgCl_2$. Both PCRs used a 'hot start' with 1 IU *Taq* DNA polymerase (Roche Diagnostics) at 94°C for 4 min followed by 40 cycles (first PCR) or 30 cycles (second PCR) of 94°C for 15 s, 62°C for 30 s, and 72°C for 30 s in a GeneAmp 9600 (Perkin Elmer, Norwalk, CT, USA). Final extension was 72°C for 10 min. Positive control reactions for β-actin were routinely carried out as described for the first PCR for *Oct-4*, except with 0.2 µl template and 50 cycles instead of 40 (3′ primer CGTGGGGCGCCCCAGGCACCA, 5′ primer TTGGCCTTGGGGTTCAGGGGGG). Water and media samples served as negative controls. PCR products were analysed on a 2% agarose gel with 0.5× Tris/borate/EDTA buffer (TBE). The usual precautions to avoid contamination were followed. A 50 bp ladder marker (100 ng, marker XIII, Roche Diagnostics) was routinely used as reference to estimate PCR product size.

A titration study was conducted with dilutions (1:2.5 to 1:25) of cDNA samples subjected to the above-described PCR conditions to examine the sensitivity of the PCR and the relative expression levels of *Oct-4* in blastomeres and oocytes. The sensitivity limit (up to a 1:10 dilution) for each blastomere was determined as the approximate mean between the last positive and the first negative value. For example, if a 1:2.5 dilution is positive for *Oct-4* expression and a 1:5 dilution is negative, the mean would be a 1:3.3 dilution indicating an expression of 3.3-fold higher than the sensitivity limit. The factor by which *Oct-4* is higher from the sensitivity limit of the applied PCR was calculated for each blastomere (range: 1.4–12.0-fold).

To determine whether *Oct-4* cDNA levels in *Oct-4*-negative blastomeres were above or below the detection limit, a second, highly sensitive PCR was also performed using the same PCR conditions as in the titration PCR except that 50 cycles instead of 30 were applied for the second round of PCR and 20 µmol/l NTP were used instead of 10 µmol/l.

In the titration experiments for the oocytes, the same PCR conditions were applied as in the blastomere titration studies. Since the nucleus was not mechanically removed from these oocytes, the RNA was prepared using caesium chloride centrifugation (Rappolee *et al*., 1989). Using this method, more RNA might be lost than with the

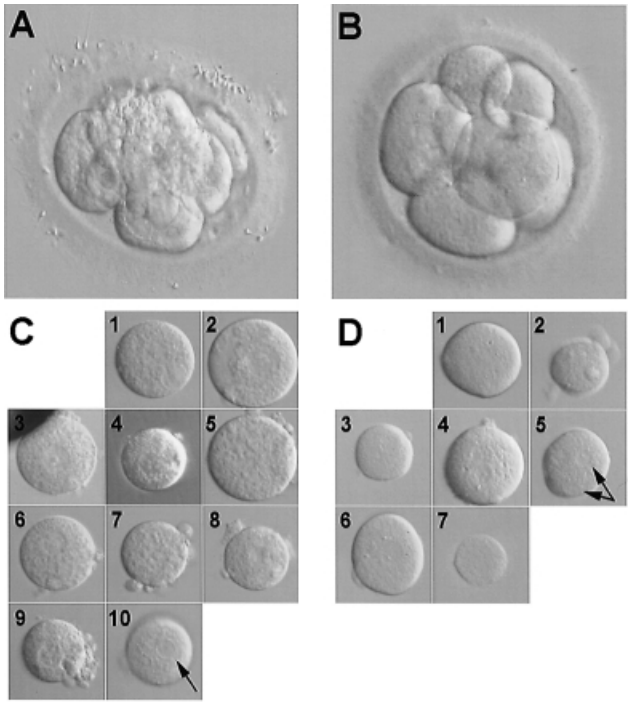

Figure 1. Separation of individual blastomeres from human cleavage stage embryos. (**A**) Two pronuclear (2PN) zygote from a 40 year old woman developed into this 10-cell embryo (embryo I) at day 4. Sperm cells are still visible bound to the zona pellucida. (**B**) Embryo II at day 3 which had developed from a 32 year old woman. (**C**) Individual blastomeres (nos. 1–10) from embryo I. (**D**) Individual blastomeres (nos. 1–7) from embryo II. Arrows point to nuclei.

blastomeres, so the results of the titration in oocytes should be considered as minimum values.

FISH

Blastomere nuclei were fixed on slides with methanol:acetic acid (3:1) and subjected to FISH using X,Y and 18 CEP direct-labelled probes (Vysis, Downers Grove, IL, USA) for 30 min in HYBrite (Vysis) according to the manufacturer's instructions. The nucleus was counter-stained with 4′6-diamidino-2-phenylindole (DAPI; Vysis). FISH signals were visualized using a fluorescent microscope (Olympus AX 70, Tokyo, Japan). Digital images were saved for analysis.

Results

A total of 19 embryos were examined for *Oct-4* expression and three were abnormal: one developed from a 3PN zygote, one from a 1PN zygote and a final one from a 2PN zygote, but with multiple multinucleated blastomeres beginning at the 2-cell stage. The embryo that developed from a 3PN zygote (Figure 1B) had one blastomere positive for *Oct-4* mRNA. In contrast, in the embryo that developed from a 1PN zygote, all blastomeres were negative for *Oct-4* and none had visible nuclei. Finally, the embryo with multinucleated blastomeres had no blastomeres with a detectable level of *Oct-4* mRNA. Of the 16 embryos that developed from 2PN zygotes (Figure 1A), one had no visible nuclei in all three blastomeres and another was negative for the expression of the control-gene β-actin in all four blastomeres. These two embryos, as well as the abnormal embryos described above, were excluded from further calculations.

Of the 14 remaining embryos, 12 displayed blastomeres with detectable *Oct-4* expression. The mean grade for *Oct-4*-expressing embryos was 2.6 (range 1–3.5), the mean grade for *Oct-4*-negative 2PN embryos was 2.3 (range 2–3). *Oct-4*-positive and -negative 2PN embryos were obtained from oocytes of women with a mean age of 36.3 years (range 30–43) and 38.0 years (range 31–41) respectively.

A total of 95 blastomeres were harvested from the 14 embryos. Five blastomeres lysed during separation and nine were considered to be fragments on the basis of size and the absence of a visible nucleus. Two blastomeres had neither β-*actin* mRNA expression nor a visible nucleus, whereas β-*actin* mRNA expression was not detected in three blastomeres and no nucleus was seen in 11 regular sized blastomeres. A final blastomere had a visible nucleus, but FISH did not reveal any signals. Each of these 31 blastomeres was excluded from further consideration.

Each of the remaining 64 blastomeres chosen to calculate the frequency of *Oct-4*-positive blastomeres were positive for β-*actin* mRNA (Figure 2B), had a clearly visible nucleus and showed a FISH signal. Of these blastomeres, 27 (42%) were positive for *Oct-4* mRNA expression (Figure 2A). Experiments for *Oct-4* expression were repeated for the blastomeres of five embryos and results were 100% in confirmation.

The blastomeres were obtained from 2PN embryos at different cleavage stages (2-cell, $n = 1$; 4-cell, $n = 1$; 5-cell, $n = 3$; 7-cell, $n = 2$; 8-cell, $n = 4$; 9-cell, $n = 2$; 10-cell, $n = 1$). The percentage of *Oct-4* mRNA positive cells varied according to cleavage stage: the higher the cell number, the lower the percentage of *Oct-4* expressing blastomeres. When organized in three main groups (Figure 3), the percentage dropped from 100% in 2–4-cell embryos ($n = 5$ analysed blastomeres from two embryos) to 44% in 5–7-cell embryos ($n = 16$ analysed blastomeres from five embryos) and to 35% in 8–10-cell embryos ($n = 43$ analysed blastomeres from seven embryos). Even in the advanced cleavage stages no more than four blastomeres per embryo were positive for *Oct-4*.

Taking all *Oct-4*-expressing blastomeres derived from 2PN zygotes together, *Oct-4* expression was, on average, 3.8-fold higher than the sensitivity limit of the PCR (Figure 2C and D). Relative expression levels in *Oct-4*-positive blastomeres dropped from 4.7-fold in 2–4-cell embryos to 3.0-fold in 5–7-cell embryos, but increased again to 3.7-fold in 8–10-cell embryos. The *Oct-4*-positive blastomere of the 3PN embryo only showed a relative expression of 1.4-fold.

When the highly sensitive second PCR was applied, five out of six *Oct-4*-negative blastomeres of Figure 2A with a visible nucleus remained negative (data not shown). Thus, although *Oct-4*-negative blastomeres might harbour some *Oct-4*-mRNA, the levels are, nonetheless, frequently below the detection limit of a PCR. Moreover, the calculated relative expression level of *Oct-4* in positive blastomeres is reasonably adequate and the difference between *Oct-4*-positive and negative blastomeres is more or less the difference between the *Oct-4* relative expression level and zero.

We also measured *Oct-4* expression in six discarded imma-

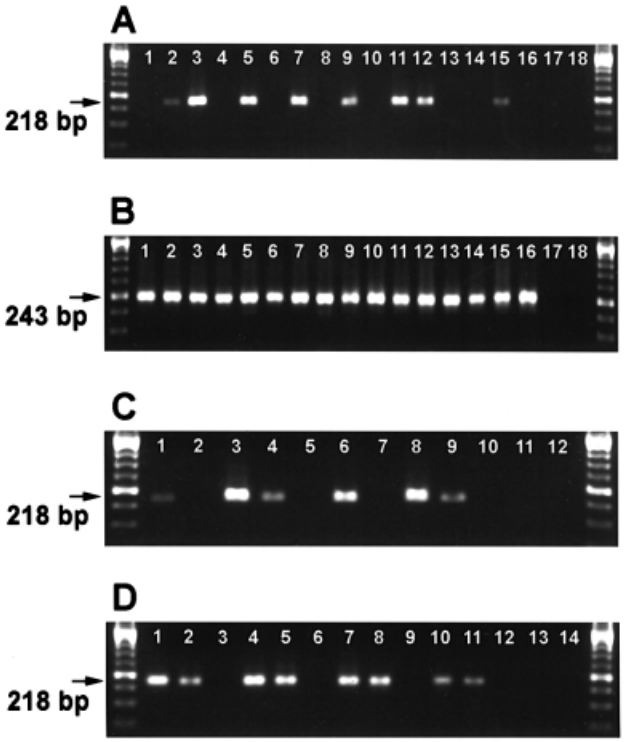

Figure 2. Analysis of *Oct-4* expression in two embryos that developed from two pronuclear (2PN) zygotes. (**A**) *Oct-4* expression in individual blastomeres. Lane 1–8 = blastomeres nos. 1–8 from embryo III (see Figure 4A); lane 9–16 = blastomeres nos. 1–8 from a day 3 embryo (embryo IV) from a 31 year old woman; lane 17 = water control, lane 18 = media control. All reactions with 1 µl template each were subjected to the same polymerase chain reaction (PCR) conditions in the same experiment. *Oct-4* nested PCR product = 218 bp. (**B**) β-actin control reactions for the blastomeres 1–8 of embryos III and IV; lanes correspond to those depicted in (A); 0.2 µl template for each reaction. β-actin product = 243 bp. (**C**) Determination of PCR sensitivity and relative levels of *Oct-4* expression in blastomeres nos. 2, 3, 5 and 7 of embryo III which correspond to lanes 2, 3, 5 and 7 of (A). Lanes 1–2 = blastomere no. 2 with 1:1 and 1:2.5 dilutions; lanes 3–5 = blastomere no. 3 with 1:1, 1:2.5 and 1:5 dilutions; lanes 6–7 = blastomere no. 5 with 1:1 and 1:2.5 dilutions and lanes 8–10 = blastomere no. 7 with 1:1, 1:5 and 1:7.5 dilutions; water control and media controls are in lanes 11 and 12 respectively. PCR conditions were identical to those applied in (A). Sensitivity limits translate into an ~3.0-fold induction for all four blastomeres. (**D**) Determination of PCR sensitivity and relative *Oct-4* expression levels of blastomeres nos. 1, 3, 4 and 7 of embryo IV which correspond to lanes 9, 11, 12 and 15 of (A). Lanes 1–3 = blastomere no. 1 with 1:1, 1:2.5 and 1:5 dilutions; lanes 4–6 = blastomere no. 3 with 1:1, 1:5 and 1:7.5 dilutions; lanes 7–9 = blastomere no. 4 with 1:1, 1:5 and 1:7.5 dilutions and lanes 10–12 = blastomere no. 7 with 1:1, 1:2.5 and 1:5 dilutions; water and media controls are in lane 14 and 15 respectively. PCR conditions were identical to those applied in (A). Expression levels were ~4.7 higher than the sensitivity limit. All gels: 2% agarose gel, 0.5× Tris/borate/EDTA buffer (TBE), 10 µl PCR product loaded, 50 bp ladder marker (100 ng, marker XIII, Roche Diagnostics).

ture (germinal vesicle stage) oocytes. Each displayed a high level of *Oct-4* expression, averaging 11.5-fold higher than *Oct-4* negative blastomeres (data not shown).

The results for the chromosomal analysis of all 14 embryos derived from 2PN zygotes and the relationship between ploidy

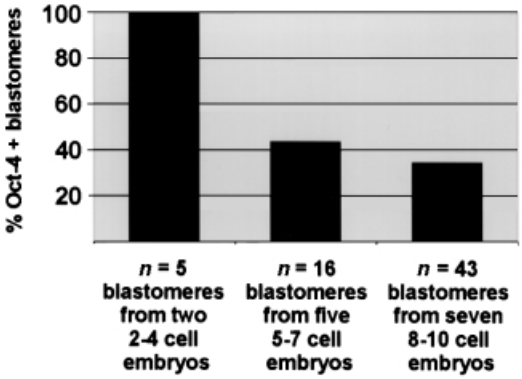

Figure 3. Percentage of blastomeres with a high level of *Oct-4* expression in embryos at three different cleavage stages. Mean value for all blastomeres at each stage: 100, 43.8 and 34.8%. Only blastomeres with a visible nucleus and a positive signal for β-actin were counted.

and *Oct-4* expression are shown in Tables I and II. Using FISH, three out of 14 embryos included in the study were euploid, while mosaic aneuploidies were found in eight, and three were totally aneuploid (Figure 4). Normal numbers of sex chromosomes and chromosome 18 were seen in the nuclei from 49 of the 64 blastomeres (77%). Five nuclei (8%) showed a loss of one or more chromosome(s), and three (5%) had a gain of one or more chromosome(s). Four blastomeres (6%) showed two nuclei with a normal set of chromosomes, whereas three blastomeres (5%) had two nuclei with an abnormal chromosomal set. Aneuploidy was observed in 23% of all blastomeres and in 22% of all low *Oct-4* mRNA level blastomeres. The only *Oct-4*-positive cell in the embryo that developed from a 3PN zygote was also the only cell of that embryo with normal numbers of X, Y and 18.

The mean grade of the chromosomally normal 2PN embryos (no abnormal cells detected, single nuclei) was 2.5 (range 2–3), the grade for the 2PN embryos with at least one abnormal cell, including binucleated blastomeres, was 2.5 (range 1–3.5). The mean age of the mothers of the chromosomally normal 2PN embryos was 34.5 years (range 31–38 years) and for the abnormal 2PN embryos, it was 36.5 years (range 30–43).

Discussion

In previous experiments we described procedures to monitor *Oct-4* expression in human blastocysts. Verifying *Oct-4* amplification and titration by PCR and comparing ICM and TE fractions, we found that *Oct-4* is expressed a mean of 31-fold higher in ICM than in TE (Hansis *et al.*, 2000). Using similar protocols for *Oct-4* amplification and titration, we describe here the temporal and spatial variations of *Oct-4* mRNA levels in human oocytes and between individual blastomeres of cleavage stage embryos.

The amount of *Oct-4* RNA was 3.8-fold higher in *Oct-4*-positive blastomeres than in *Oct-4*-negative blastomeres. This seems to be a rather high value for a transcription factor that initiates a signal cascade. In fact, recent results describe a much closer regulation of *Oct-4* expression in murine embryonic stem cells: relative expression levels of 0.5, 1.0 and 1.5 direct the

Table I. Chromosomal analysis for embryos derived from two pronuclear (2PN) zygotes ($n = 14$)

	All cells euploid, all nuclei visible	All cells euploid, some nuclei not visible	One cell aneuploid, some nuclei not visible	Two+ cells aneuploid, some nuclei not visible	All cells aneuploid, some nuclei not visible
No. of 2PN embryos	2	1	6	2	3

Both the embryo that developed from a 3PN zygote and the embryo with multinucleated blastomeres consisted of blastomeres with a chaotic chromosomal set, while the embryo derived from a 1PN zygote did not reveal any nuclei

Table II. Relationship between ploidy of chromosomes X, Y, 18 and *Oct-4* expression for all 64 blastomeres

	Euploid blastomeres	Blastomeres with loss of chromosomes	Blastomeres with gain of chromosomes	Binucleate blastomeres, each nucleus with normal set of chromosomes	Binucleate blastomeres, each nucleus with gain or loss of chromosomes
No. of *Oct-4*-positive blastomeres	20	0	2[a]	3	2[b]
No. of *Oct-4*-negative blastomeres	29	5[c]	1[d]	1	1[e]

[a]X, Y, 18, 18, 18 and X, X, Y, 18, 18.
[b]First blastomere: X, X, X, X, 18, 18, 18, 18 and X, X, X, X, 18, 18, 18, 18; second blastomere: X, Y, Y, 18, 18 and X, X, X, 18.
[c]X, Y, 18.
Y.
X, 18, 18.
X, Y.
X, Y, 18.
[d]X, X, Y, Y, 18, 18, 18, 18.
[e]X, 18 and X, Y, 18.

cells to trophectoderm, inner cell mass and primitive endoderm and mesoderm, respectively (Niwa *et al*., 2000). It appears that, in humans, variations of *Oct-4* induction may take place at relatively higher levels than in mice, because physiological *Oct-4* activity requires a higher level of expression. Thus, the relationship between *Oct-4* activity and the three cell destinations in mice could still take place in humans, but at higher levels of expression. Future quantification of *Oct-4* levels in individual cells of cleavage stage embryos, morulae and blastocysts are necessary to understand these processes. On the other hand, blastomeres with very high levels of *Oct-4* expression may be aberrant and destined to undergo apoptosis.

Taken together with our previous results, the present observations describe the temporal pattern of *Oct-4* expression during early embryonic life, from oocyte to blastocyst. The high levels of *Oct-4* mRNA in a mammalian oocyte suggests that this factor contributes to the restitution and maintenance of totipotency necessary for successful animal cloning experiments. *Oct-4* mRNA levels were detectable in every blastomere tested from 2–4-cell embryos. Moreover, the drop from an 11.5-fold relative expression level in oocytes to a 4.7-fold expression level per blastomere in 2–4-cell embryos could signify that maternal mRNA transcripts of *Oct-4* are equally distributed during the first two cell divisions. Clearly, such a pattern is at odds with a concept of a polarized distribution of *Oct-4* mRNA in human oocytes and early embryos (Edwards and Beard, 1997). The present findings of a mostly equal distribution of *Oct-4* mRNA from oocyte to 2–4-cell embryo also suggests that degradation of maternal transcripts of *Oct-4* does not occur prior to the 4–8-cell stage; in later stages this is difficult to assess since both de-novo transcription and degradation would probably occur simultaneously.

General genomic activation occurs during the 4–8-cell stage in human embryos (Tesarik *et al*., 1986; Braude *et al*., 1988). Thus, after a further drop to a 3.0-fold relative expression level in 5–7-cell embryos, genomic activation provides new *Oct-4* mRNA copies and the expression level rises to 3.7-fold in the 8–10-cell embryos. By the time the embryo reaches the blastocyst stage, *Oct-4* is expressed ~31 times higher in the ICM than in TE (Hansis *et al*., 2000). The calculated relative expression level for an individual ICM cell is ~2.4 (31/13) and is based on a report that there are ~13 cells in the ICM of a day 6 human blastocyst with poor morphology derived from a 2PN zygote (Evsikov and Verlinsky, 1998). The decline from a 3.7-fold *Oct-4* expression level in the 8–10-cell embryo to a 2.4- expression level in the blastocyst ICM is possibly due to the appearance of apoptotic cells in the ICM which lowers the calculated level of expression per cell (Hardy, 1999).

The physiological processes that trigger *Oct-4* down-regulation in some blastomeres are still unknown; location of cells in the embryo and formation of specific cell–cell contacts, e.g. a signal cascade from E-cadherin to β-catenin to lymphoid enhancer factor (LEF-1) (Pesce *et al*., 1998), might be one mechanism. Other possible mechanisms of repression, eventually connected with the E-cadherin signal cascade, involve retinoic acid responsive elements (Pikarsky *et al*., 1994), changes in chromatin structure (Minucci *et al*., 1996) and de-novo methylation (Ben-Shushan *et al*., 1993).

The temporal expression of *Oct-4* follows spatial expression; the higher the number of cells in an embryo, the lower the percentage of *Oct-4*-positive blastomeres. The presence of blastomeres with high *Oct-4* levels depends neither on the age of the mother nor the morphology or ploidy of the embryo. For each of the three groups of different cleavage stage embryos derived from 2PN zygotes, there was no apparent pattern for the age or chromosomal status of *Oct-4*-positive

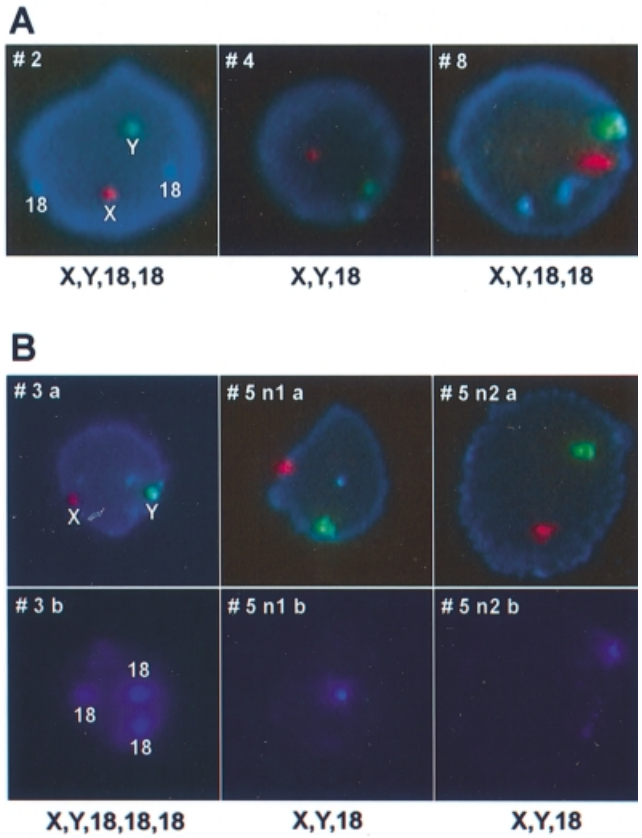

Figure 4. Fluorescence in-situ hybridization (FISH) for chromosomes X (red), Y (green) and 18 (blue); nuclei are counterstained with 4′6-diamidino-2-phenylindole (DAPI). (**A**) Blastomeres nos. 2, 4 and 8 from a 2PN zygote of a 40 year old woman that developed into an 8-cell stage embryo (III); blastomeres nos. 1, 3, 5, 6, and 7 had a normal karyotype (data not shown). (**B**) Blastomeres nos. 3 and 5 of 7-cell embryo II (see Figure 1B,D). Blastomere no. 5 displayed two nuclei. Blastomere no. 3 is shown in picture 3 of Figure 1D, blastomere no. 5 in picture 5. Chromosome 18 is also shown with the aqua band filter (no. 3 b, no. 5 n1 b, no. 5 n2 b). Blastomeres nos. 1 and 2 did not show a nucleus; blastomere no. 4: two nuclei, X X 18 18 and X Y 18 18; blastomere no. 6: X 18 18 18; blastomere no. 7: X Y 18 18 18 18 (data not shown).

blastomeres. However, grading comparison revealed that the higher the cell number, the better the mean grade of the embryo. This probably reflects the prolonged survival of higher-grade embryos, but could also mean that poor grade embryos have a greater chance of harbouring blastomeres with a high level of *Oct-4*. Clinical observation supports the hypothesis that a poor embryo grade does not necessarily reflect developmental potential although a high percentage of poor grade embryos are aneuploid (Munné *et al*., 1995). All of the studied embryos were discarded. Nevertheless, six embryos that developed from 2PN zygotes were graded 2.0 or better and each fit very well into the pattern of *Oct-4* expression. Thus, the foregoing description of *Oct-4* expression during early cleavage stages seems to reflect a physiological situation rather than an artificial one caused by the selection of poor quality embryos for study.

In 11 of 16 embryos one or more cell(s) were lost due to lysis during separation or had to be excluded due to negative β-*actin* mRNA expression, fragmentation or the absence of a nucleus. These lost and excluded blastomeres may have been undergoing apoptosis, and it is quite possible that they would not have contributed to the further development of the embryo. In arrested cleavage stage embryos, blastomeres undergo apoptosis and are characterized by cytosolic fragmentation, nuclear breakdown and RNA degradation (Hardy, 1999). From the above mentioned 11 embryos with lost cells, 10 were delayed or arrested in their development and all of them had fragmentation of 10–40%.

In human embryos, mosaic aneuploidies are a common finding. Between 30% (Delhanty *et al*., 1997; Harper *et al*., 1995) and 50% (Munne *et al*., 1995) of normally developing cleavage stage human embryos are thought to contain one or more aneuploid blastomeres. In our experiments mosaic aneuploidies were detected in 57% of all embryos that developed from 2PN zygotes, while 21% were totally aneuploid. One might speculate that aneuploid blastomeres are actively directed towards trophectoderm; however, they do not appear to be directed towards extraembryonic cell lineages by *Oct-4* since we found that aneuploid blastomeres are not more likely to exhibit a low *Oct-4* mRNA level.

Interestingly, high levels of *Oct-4* occurred in 70% of the blastomeres with additional chromosomes X, Y and 18 but in none of the blastomeres with a loss of one or more of these chromosomes, including binucleate blastomeres. Various homeobox genes are also differently expressed in triploid human preimplantation embryos compared with diploid (Kuliev *et al*., 1996). At the present time, there is no indication that chromosomes X, Y and 18 play critical roles in the regulation of *Oct-4* expression. However, aneuploidies in chromosomes other than X, Y and 18 may be linked to variations in *Oct-4* expression. If trisomic blastomeres are more likely to have a high level of *Oct-4* and are thus supposedly more likely to contribute to the embryo proper, this might explain in part the much higher incidence of live births with trisomies than monosomies. Clearly, further studies are needed to define the relationship between ploidy of particular chromosomes and *Oct-4* expression.

The present study demonstrates the feasibility of combining RT–PCR and FISH analyses in individual blastomeres. Similar procedures might promise new opportunities in PGD to characterize mosaic aneuploidies and to provide the means for a more complete understanding of key steps in differentiation during early embryonic development. A large variety of housekeeping genes, transcription factors, growth factors, gender determining genes and tissue specific genes are known to be expressed at the 4–8-cell stage (Pergament and Fiddler, 1998); correlating their expression with that of oct-4 in biopsied blastomeres might prove valuable in the future to select embryos with the highest potential for development. Combined with genomic PCR, one might be able to use RT–PCR and FISH appliances to monitor single gene defects, gene expression and chromosomal abnormalities in individual blastomeres biopsied for PGD.

The foregoing description of *Oct-4* expression in early human embryogenesis presents a picture comparable to that described in the mouse model in which primary function of

Oct-4 is to retain totipotency in particular embryonic cells and germ cells (Pesce *et al*., 1998). Our experiments are consistent with the hypothesis that *Oct-4* may have a similar function in human embryos. Future experiments focussing on Oct-4 protein expression and repression are necessary to establish that biological functions of *Oct-4* are the same in both species.

Acknowledgements

We would like to thank Drs Ling Chi and Caroline McCaffrey for their valuable help in collecting and assessing the embryos used in this study.

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Received on September 25, 2000; accepted on November 28, 2000